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

Bannink, S.

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Chapter 3: Identification of glucolites in GD formed through the GBA1 mediated transglucosylation reaction using GBA1 specific (ionized) substrates for LC-MS/MS analyses

3.1 Introduction

Akiyama and co-workers first discovered that GlcCer, rather than UDP-glucose, may function as the glucose donor in the formation of glucosyl cholesterol (GlcChol).[1,2] Marques *et al.* showed that, *in vitro,* GBA1 and GBA2 may both generate GlcChol from GlcCer as sugar donor via transglycosylation. Transglucosylation activity by retaining beta-glucosidases as GBA1 and GBA2 is a well-known phenomenon, and it appears that GBA2 is the prominent physiological synthetic enzyme.[2,3] Already fifty years ago, Kanfer and co-workers reported the transglucosylation activity of calf spleen beta-glucosidase.[4] A later study reported that purified placental GBA1 can catalyse the transfer of glucose to the naturally occurring lipid alcohols, such as *n*-pentanol and retinol, using GlcCer as the glucose donor.[5] The ability of recombinant human rhGBA1 to perform transglucosylation of specific metabolites is subject of further investigation at the Leiden Institute of Chemistry. This thesis aims to investigate this feature by generating glucosylated metabolites (collectively designated as glucolites) *in vitro* through the incubation of rhGBA1 with suitable acceptors and modified glucosyl donors. To facilitate detection of formed glucolites, C6-alkyl glucose donors specific for GBA1 were designed in Chapter 2 (see Figure 3.1E).

The field of lipidomics provides a powerful tool and methodology for the identification and quantification of lipid species in complex biological samples.[6–8] Lipidomics is a subsection of metabolomics in which a distinction is being made on the lipophilic and hydrophilic metabolites based on the octanol/water coefficient scale.[9] In an effort to quantify the natural occurrence of GlcChol in human plasma, Marques *et al.* developed an efficient method using LC-MS/MS which is an example of targeted lipidomics.[2] These targeted approaches are useful when the structure of the target analyte is known. To identify potential novel products of the GBA1-catalysed transglucosylation reaction, in which the structures of the target analytes are unknown, an untargeted approach is preferred. Over recent years untargeted lipidomic approaches have become more accessible[10,11] and have been applied to identify lipid metabolites relevant to diverse pathologies including Alzheimer's disease,[12] chronic obstructive pulmonary disease[13] and novel oxidized eicosanoid lysolipids.[14] These approaches use tandem MS (MS/MS) transitions measurements to analyse complex analyte mixtures, screening these against a public library of known metabolites. Reverse metabolomics, on the other hand, evaluates whether a specific MS/MS spectrum appears in public untargeted metabolomics datasets,[15] and has attracted a lot of attention due to the availability of publicly accessible

MS/MS databases.[15,16] These lipidomic approaches could also reveal potential novel metabolite acceptors for the GBA1-catalysed transglucosylation (Figure 3.1D).

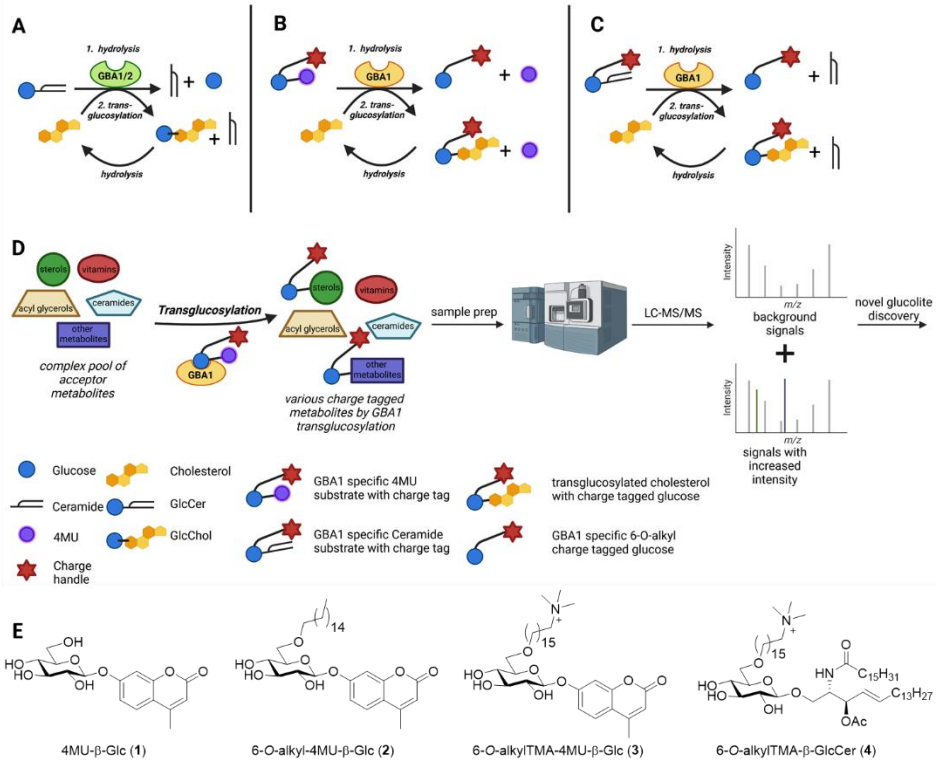


Figure 3.1: Intended strategy for the identification of novel glucolites in the GBA1 catalysed transreaction using a GBA1 specific charge-tagged glucose derived substrate. A) Hydrolysis of GlcCer and the transglucosylation of cholesterol by GBA1 and GBA2. B) Hydrolysis and transglucosylation of a charge-tagged GBA1 specific 4MU-based substrate to cholesterol. C) Hydrolysis and transglucosylation of a charge-tagged GBA1 specific ceramide-based substrate to cholesterol D) Schematic workflow for the identification of glucolites: The GBA1 specific charge-tagged substrate will label a set of suitable acceptors through GBA1 catalysed transglucosylation within a complex acceptor pool, and through the use of the introduced positively charged handle, untargeted LC-MS/MS strategies will lead to the elucidation of novel metabolite acceptors. E) Structures of 4MU-β-Glc **1** and 6-*O*-alkyl-4MU-β-Glc **2** and the trimethylammonium (TMA) functionalized analogues 6-*O*-alkylTMA-4MU-β-Glc **3** and 6-*O*-alkylTMA-β-GlcCer **4** bearing a trackable positive charge.

To detect analytes in the extremely low abundance regime it is necessary to have pre-knowledge of the molecular mass, signature transitions and chromatographic

elution properties of the unknown molecules that are connected through a metabolic network. A reverse lipidomics strategy has been employed for this purpose, as demonstrated by Liu *et al.* in the detection of oxidized arachidonoyl-lysolipids (Figure 3.2).[14] During this approach a purified enzyme (COX-2) converted a known substrate (2-Arachidonoyl-lysophosphatidylcholine, 2-AA-LPC) to novel unknown oxidized eicosanoids. These novel natural products had not been identified previously due to their low abundance in a very complex mixture of hundreds of thousands of lipids. The generated unknown analytes were used to determine elution times, fragmentation patterns and MS molecular weights of molecular and product ions. Based on this information structures were predicted for these novel unknowns which were confirmed through the synthesis and MS/MS characterization of authentic standards. The oxidized arachidonoyl-lysolipids were identified through this approach by their enzyme catalysed production through *in situ* reactions with purified enzymes and lysolipid substrates. This "reverse" approach then provided evidence that the enzymatically generated products exist as natural metabolites in biological systems, leading to the discovery of previously unknown lipid signalling pathways.[14]

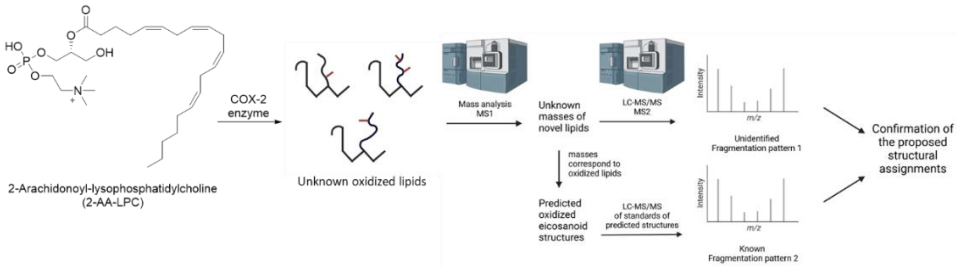


Figure 3.2: Workflow employed by Liu *et al.* to identify novel unknown oxidized eicosanoids through a reverse lipidomics approach.

The detection of low-abundant species in lipidomics is still a major challenge due to ion suppression of more abundant species.[7] Charged substrates or ionic tags (ITags) have shown signal intensities increasing 75-fold through the introduction of a permanently charged ion.[17] Noteworthy, amongst recent derivatization enhancements for targeted analysis has been the use of charge-switch derivatization with aminomethyl phenyl pyridium (AMPP) and carbodiimide developed by Gelb *et al.* which creates an amide with a permanent positive charge resulting in increases in S/N by ~50-fold for fatty acids (Figure 3.3A).[18] This approach was also used to identify the low abundant novel oxidized eicosanoids described by

Liu *et al.* (Figure 3.2). The introduction of a quaternary ammonium cation to diacyl glycerol (DAG) molecules to yield a derivatized DAG with a permanent positive charge that gives 2 orders of magnitude higher signal intensities than their underivatized sodium adducts has also been described (Figure 3.3B).[19] In another approach three glycerophospholipids (phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylserine (PS)) and the sphingolipid sphingomyelin (SM) were selectively methylated using diazomethane to generate fixed quaternary trimethylammonium charges on the corresponding lipids to produce significantly stronger signal intensities when performing MS/MS analyses, with increased signal intensity greater than 2-fold for SM, 5-fold for PC, 8-fold for PE, and 1.5 orders of magnitude for PS (Figure 3.3C).[20] Furthermore, next to the observed sensitivity enhancements also improvement in tandem mass spectrometry (MS/MS) experiments were observed due to the formation of primarily one main fragment channel and the fact that signals of the protonated ion and the sodiated adduct of the unmodified lipid merge into a single peak.[20]

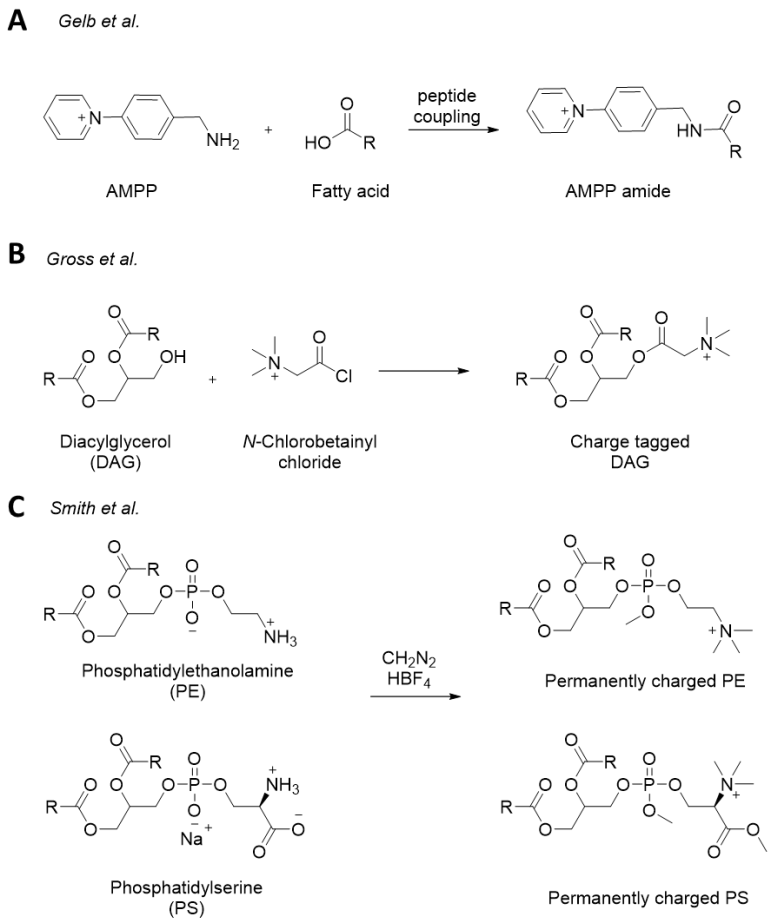


Figure 3.3: Charge derivatization of substrates performed in literature. A) charge derivatization of fatty acids with aminomethyl phenyl pyridium (AMPP) by Gelb *et al*. B) Charge derivatization of Diacylglycerol (DAG) with *N*-chlorobetainyl chloride by Gross *et al*. C) Charge derivatization of PE, PS, PC and SM to create permanently charged moieties with improved signal intensity using diazomethane by Smith *et al*.

The ionization of most steroids and sterols is also relatively low for ESI-MS compared to proteins and peptides apart from the glucuronidated and sulfated derivatives and bile acids.[21] Therefore these species have also been derivatized using a variety of reagents on their keto or hydroxy groups. The charged handles which are more commonly used in literature are the quaternary ammonium[22–25] and phosphonium groups (Figure 3.4).[26,27]

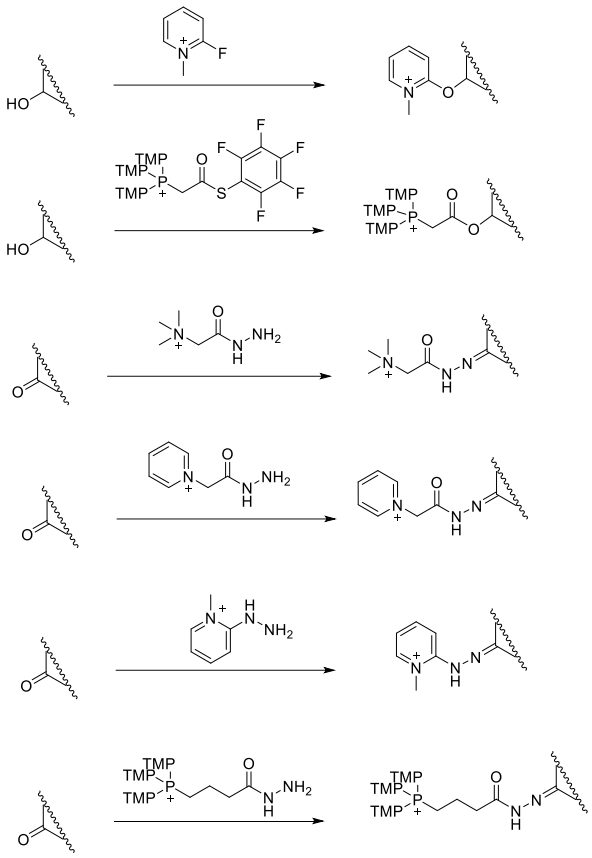


Figure 3.4: Quaternary ammonium and phosphonium reagents used to derivatize sterol hydroxyl and ketone groups to permanently charged groups for improved MS sensitivity in literature. *TMP = tris(trimethoxyphenyl)*

In glycosciences, charged handles have been used to monitor low abundant glycans,[28,29] allowing for sensitive MS analysis without radioactive or fluorescent labelling of the substrate.[17,30,31] Compounds have been modified using imidazolium tag featuring a permanent positive charge which provides the ITagged compounds with the physicochemical properties of ionic liquids (IL). These ITags can be subsequently used as purification handles but also have the benefit of enhancing the signal of the ionic species in mass spectrometry in the same manner as the permanently charged handles described above. Similar to the charged derivatization reagents described above, ITags often consist of quaternary ammonium salts[32–34] or quaternary phosphonium salts (Figure 3.5).[35] ITagged fragments exhibit a low limit of detection when compared to a non-tagged material, which allows for the

monitoring of any ITagged intermediate with high sensitivity even in complex mixtures.[36,37]

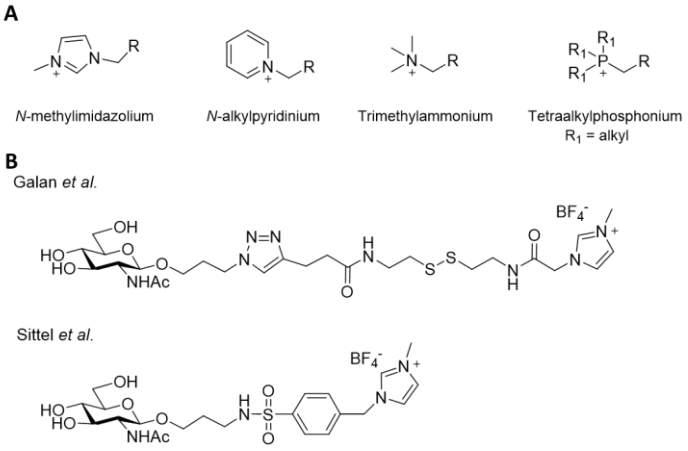


Figure 3.5: A) Commonly used charged species (quaternary ammonium and phosphonium groups) ionic tags (ITags). B) Examples of reported glycans functionalized with an ITag used as detection probes for glycans present in low concentrations.[36,37]

These permanently charged ions dominate the charge competition during the ionization process which leads to a MS signal boosting,[17] and since charge-tagged molecules are already ionized they don't require protonation, leading to enhanced sensitivity.[38] This chapter investigates the GBA1 catalysed transglucosylation of a selection of metabolite acceptors using specific GBA1 substrate 6-*O*-alkyl-4MU-β-Glc **2** (Figure 3.1B/E). With the aim of discovering novel glucolites produced by GBA1, substrate **3**, a charge-tagged 4MU substrate based on 6-*O*-alkyl-4MU-β-Glc **2**, and a physiologically more relevant GBA1-selective substrate **4** with a ceramide aglycon instead of the fluorogenic artificial 4MU aglycon were synthesized and biochemically evaluated (Figure 3.1E). The choice for a trimethylammonium (TMA) charge tag was made as it is the simplest charged moiety useful for proof of concept experiments. Although the GBA1-specific substrate **2** is useful due to its specificity towards GBA1 over GBA2 and its fluorogenic properties, it features an artificial aglycon. These rationally designed new TMA charge-tagged substrates, 6-*O*-alkylTMA-4MU-β-Glc **3** and 6-*O*-alkylTMA-β-GlcCer **4**, were assessed in the GBA1 catalysed transglucosylation of NBD-Cholesterol and cholesterol with the end goal of facilitating future untargeted lipidomics for the identification of novel glucolites.

3.2 Results and discussion

3.2.1 Transglucosylation of 6-*O*-alkyl-4MU-β-Glc 2 with NBD-Cholesterol, cholesterol, desmosterol, vitamin D_3 and retinol

To further validate that 6-*O*-alkyl-4MU-β-Glc **3** and **4** featuring an ionizable handle at C-6 would be suitable substrates in the identification of transglucosylated metabolites (glucolites), first the transferase reaction was investigated using 6-*O*-alkyl-4MU-β-Glc **2** and 4MU-β-Glc **1**. HPTLC analysis of the *in vitro* transglucosylation of NBD-Cholesterol (fluorescently labelled analogue of cholesterol) with substrates **1** and **2** was assessed over time (Figure 3.6).

The HPTLC data confirmed that GBA1 can transfer a glucose from 4MU-β-Glc to NBD-Cholesterol. The transglucosylation product, NBD-GlcChol, is formed rapidly reaching a maximum amount after 1-2 hours. Since the transglucosylation and hydrolysis reactions catalysed by rhGBA1 for 4MU-β-Glc and NBD-Chol occur simultaneously, it can be observed that after 2.5-3 hours, the 4MU-β-Glc substrate has been almost completely consumed (Figure 3.6). Furthermore, after 22 hours, the formed product NBD-GlcChol is also completely hydrolysed, indicating complete hydrolysis of the substrate and transglucosylated product after prolonged incubation times. A similar transglucosylation reaction can be observed with 6-*O*-alkyl-4MU-β-Glc substrate **2**, although the rate of transglucosylation and hydrolysis appear to be slightly slower when compared to 4MU-β-Glc **1**. These results are in agreement with the lower V_{max} values obtained for the hydrolysis of substrate **1** and **2** in Chapter 2. Complete consumption of the 6-*O*-alkyl-4MU-β-Glc substrate **2** is only observed after 22 hours, with the formed product 6-*O*-alkyl-β-GlcNBD-Chol, reaching a maximum around this time. This indicates that a longer incubation time is necessary for 6-*O*-alkyl-β-GlcNBD-Chol **2** to be completely hydrolysed and although it is a relatively poor substrate for hydrolysis, it is still transglucosylated to NBD-cholesterol.

Identification of glucolites in GD formed through the GBA1 mediated transglucosylation reaction using GBA1 specific (ionized) substrates for LC-MS/MS

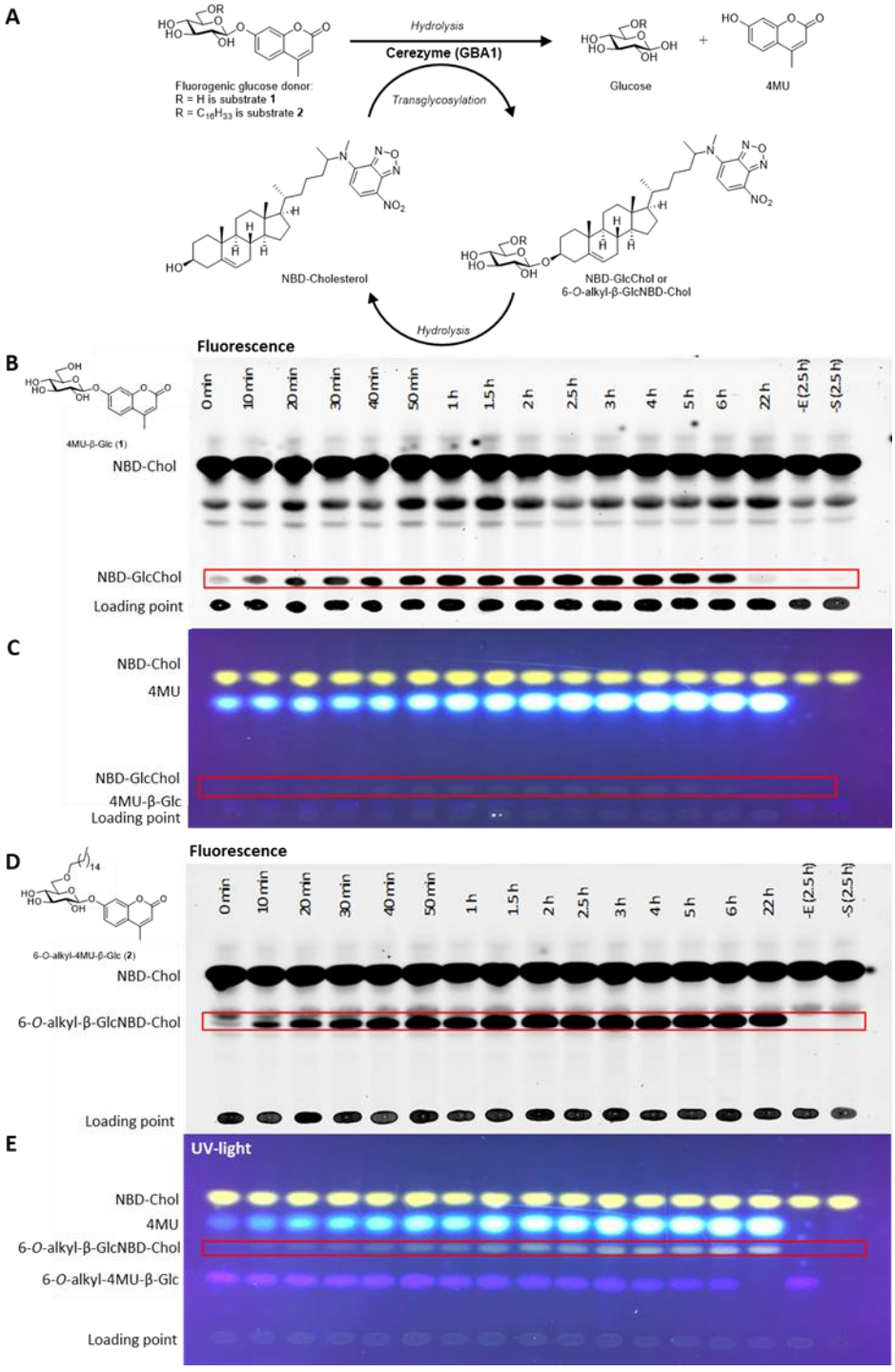


Figure 3.6. Transglucosylation of NBD-Cholesterol using 4MU-β-Glc (**1**) or 6-*O*-alkyl-4MU-β-Glc substrate **2** as a glucosyl donors, and pure recombinant human rhGBA1 (Cerezyme®). A) reaction scheme of the transglucosylation of NBD-Cholesterol using 4MU-β-Glc (**1**) or 6-*O*-alkyl-4MU-β-Glc substrate (**2**) by GBA1. B) Image of HPTLC plate visualized by fluorescence scan using Typhoon Variable Mode Imager of transglucosylation using substrate **1**. C) UV light image of B. D) fluorescence scan using Typhoon Variable Mode Imager of transglucosylation using substrate **2** E) UV light image of D.

After demonstrating that GBA1 is still capable of transglucosylating NBD-cholesterol using 6-*O*-alkyl-4MU-β-Glc as a sugar donor, the next goal was to attempt the transglucosylation with different metabolite acceptors using this substrate as donor. LC-MS/MS is a perfect technique to analyse samples and measure the amount of product formed during a transglucosylation reaction. Here, rhGBA1 is used together with different natural acceptors; cholesterol, desmosterol, vitamin D_3, and retinol. This set of metabolites was chosen based on unpublished research from the MBIOC group and collaborators, which indicated that these compounds could be transglucosylated by rhGBA1, and in particular transglucosylation of retinol by purified placental GBA1 was already shown before by Vanderjagt *et al*.[5] Here, *in vitro* transglucosylation with 6-*O*-alkyl-4MU-β-Glc **2** proved to be successful for each of the selected acceptors, with the amount of the formed products (6-*O*-alkyl-GlcChol, 6-*O*-alkyl-GlcDesm, 6-*O*-alkyl-GlcReti and 6-*O*-alkyl-GlcVitD) increasing over time (Figure 3.7).

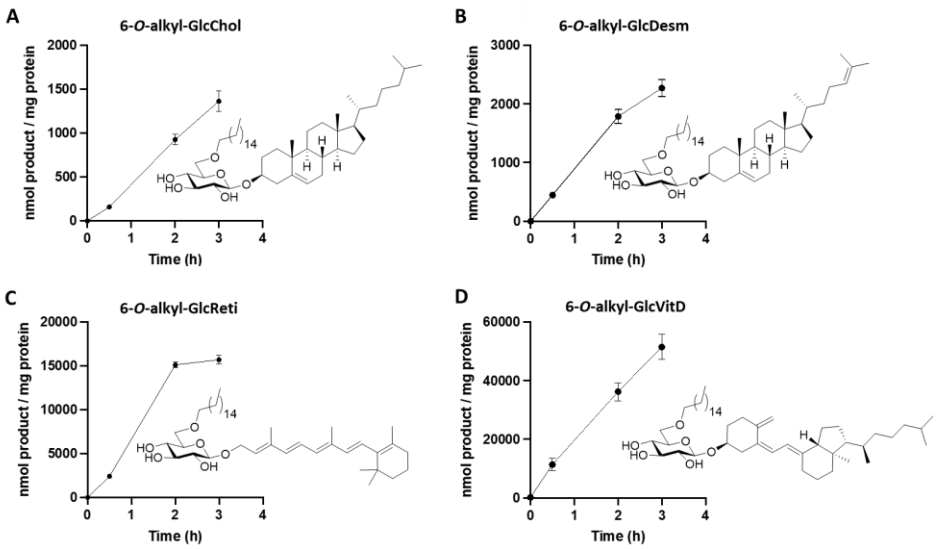


Figure 3.7: Formation of glycosylated product by rhGBA1 over time. Reaction mixtures containing 1 mM 6-*O*-alkyl-4MU-β-Glc (**2**) and 136 ng rhGBA1 (Cerezyme®) were incubated with different acceptors: A) cholesterol B) desmosterol C) retinol and D) vitamin D_3. Samples were incubated at 37 °C for 0 h, 0.5 h, 2 h and 3 h, and product formation was measured using LC-MS/MS, using $^{13}C_6$-GlcChol as an internal standard.

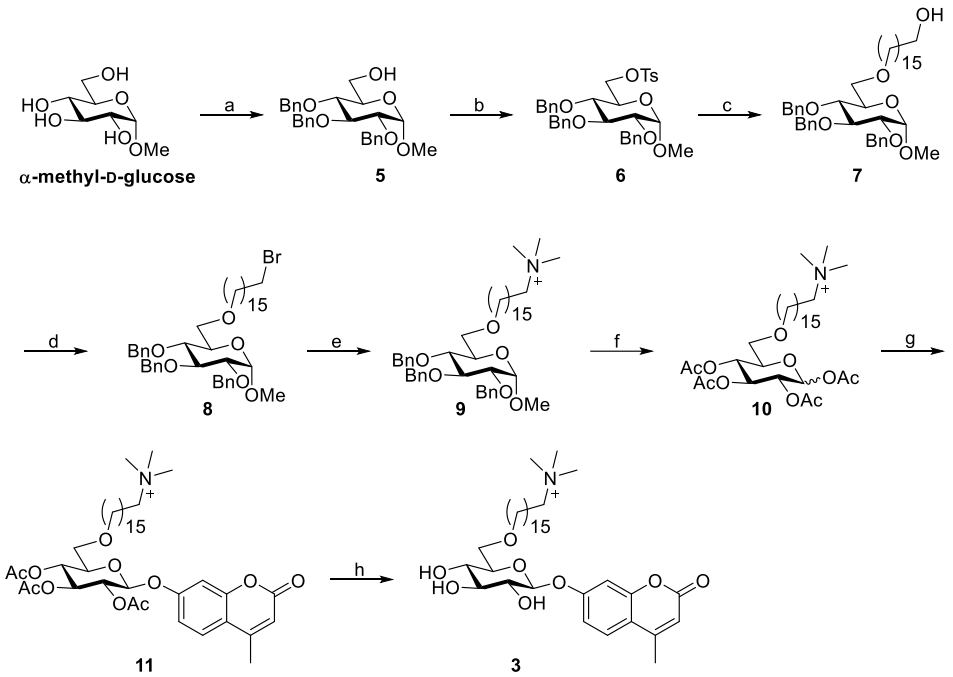
After three hours, 6-*O*-alkyl-GlcVitD showed the highest formation among the tested acceptors. Retinol also yielded a substantial amount of 6-*O*-alkyl-GlcReti per mg of enzyme, although its formation plateaued after two hours. In contrast, cholesterol and desmosterol produced significantly lower amounts of glycosylated products, approximately ten-fold less than vitamin D_3 and retinol. It is important to note that these *in vitro* results do not reflect the physiological abundance and compartmentalization of GBA1 and the selected metabolites, and future experiments on more complex systems might yield different outcomes. Nonetheless, this experiment demonstrates the broad acceptor and substrate pool and the promiscuity of the GBA1 enzyme *in vitro*.

Building on the strategy of developing GBA1-specific substrates by incorporating 6-*O*-alkyl moieties at the sugar moiety and demonstrating the enzyme's ability to glucosylate metabolite acceptors using these modified substrates, the next step involved the introduction of a suitable handle to the alkyl chain with the ultimate objective of elucidating novel glucolites in future untargeted LC-MS/MS studies. By introducing a trimethylammonium (TMA) handle at the terminal end of the alkyl

chain, a permanent positive charge is conferred, enhancing the substrate's MS ionization sensitivity.[17,31,39–41] For this purpose, the 6-*O*-alkylTMA-4MU-β-Glc **3** was designed (Figure 3.1E). Since the fluorogenic 4MU aglycon of this substrate is not the physiological substrate of GBA1, but rather a mimic of GlcCer, it was also deemed worthwhile to synthesize the 6-*O*-alkylTMA-β-GlcCer **4** substrate. Testing this substrate alongside the fluorogenic 4MU derivative will allow the evaluation of the importance of the aglycon moiety on GBA's transglucosylation activity. In all, the increased MS sensitivity of these substrates and their potential transglucosylated metabolites may facilitate the detection of novel, low-abundant metabolite acceptors for GBA1 transglucosylation.[17,31,39–41]

3.2.2 Synthesis of substrates with permanent charge

The synthesis of 6-*O*-alkylTMA-4MU-β-Glc **3** started from the same intermediate as for substrate **2** in Chapter 2, methyl α-D-glucoside (Scheme 3.1). Following a series of protection and deprotection manipulations, building block **5** was obtained as previously described in literature.[42] Tosylation of this intermediate, followed by alkylation with 1,16-hexadecanediol in a refluxing mixture of DMF and THF, led to the alkylated intermediate **7**. The positive charged handle was introduced through a two-step process: first, bromination of the primary hydroxyl using standard Appel conditions, followed by a S_N2 reaction with trimethylamine in a water/ACN mixture at 70 °C overnight. The resulting quaternary amine intermediate **9** was then subjected to Pd/C hydrogenation, and subsequent anomeric hydrolysis and acetylation of the free hydroxyls using H_2SO_4 in acetic anhydride and acetic acid. For the introduction of the 4MU fluorogenic handle, the anomeric carbon was brominated using hydrogen bromide in acetic acid, and after a simple work up, the crude bromide intermediate was coupled to 4MU using phase transfer conditions. Finally, deprotection of the acetyl groups using sodium methoxide yielded the final compound 6-*O*-alkylTMA-4MU-β-Glc **3**.

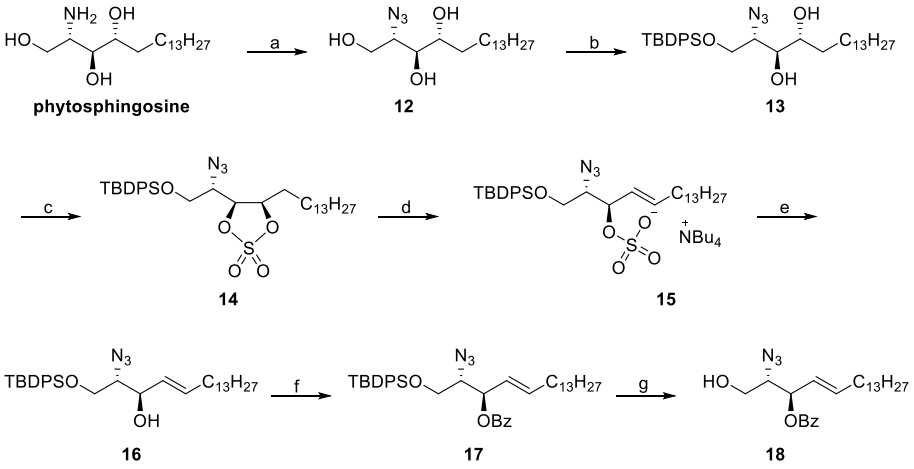


Scheme 3.1: Synthesis of 6-*O*-alkylTMA-4MU-β-Glc substrate **3**. Reagent and conditions: a) i. TBSCl, pyridine, 0 °C - r.t., 2 h; ii. BnBr, NaH, DMF, 70 °C, 3 h; iii. HCOOH/H_2O (4:1), THF, 0 °C to r.t., 3 h, 62% (3 steps). b) *p*-TsCl, pyridine, 0 °C - rt., 16 h, 84%. c) 1,16-hexadecadiol, NaH, DMF, 90 °C, 72 h, 51%. d) CBr_4, PPh_3, CH_2Cl_2, r.t., 16 h, 70%. e) NMe_3 (40% in H_2O), MeCN/H_2O, 70° C, 16 h, 94%. f) i. Pd/C, H_2, HCl, EtOAc/EtOH (1:1), r.t., 4 h; ii. H_2SO_4, Ac_2O/AcOH, 0 °C – r.t., 24 h, 74% (2 steps). g) i. HBr (33% in AcOH), CH_2Cl_2, 0 °C to r.t., 3 h; ii. 4MU, NaOH, Acetone/H_2O (1:1), r.t., dark, 18 h, 10% (2 steps). h) NaOMe, MeOH/CH_2Cl_2, r.t., 4 h, 63%.

For the synthesis of the GlcCer analogue substrate 6-*O*-alkylTMA-β-GlcCer **4**, a palmityl fatty acid was introduced at the amide position of the ceramide since this, alongside stearic acid, is the physiologically most abundant glycosphingolipid.[43] The designed synthetic route enables easy differentiation of this amide functionality in the one-pot Staudinger reduction and amide coupling reaction. This approach also provides potential for diversification, allowing the incorporation of other fatty acids and functional groups at this position in future experiments with relative ease.

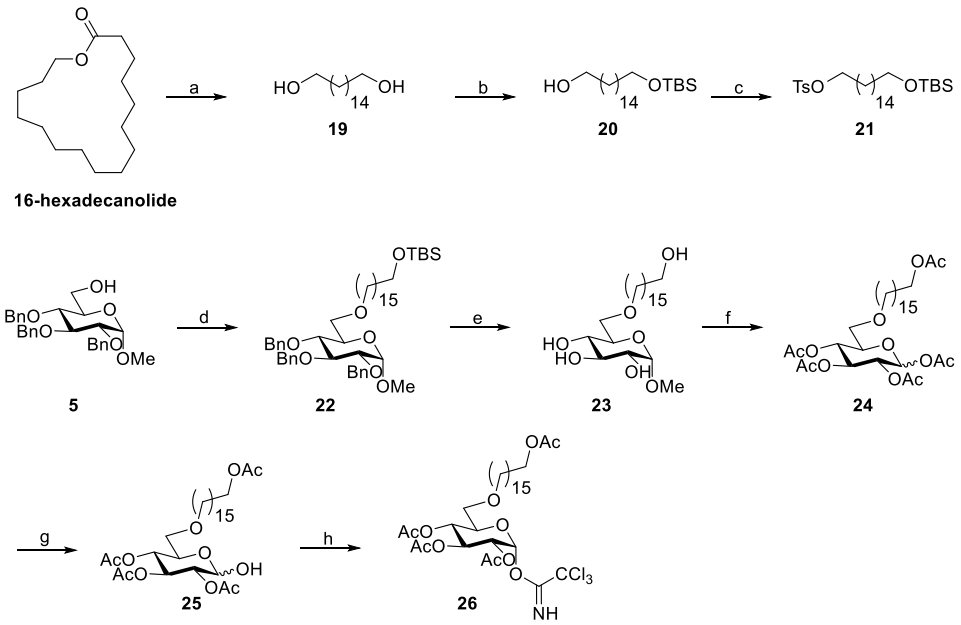
Sphingosine acceptor **18** was synthetized following similar synthetic conditions as described by Kim *et al.* (Scheme 3.2).[44] The amino group in phytosphingosine was masked into an azido group via a diazo-transfer reaction using Stick reagent, and the

primary hydroxyl was selectively protected with *tert*-butyldiphenylsilyl (TBDPS) in relatively high yield (73% over two steps) to give compound **13**. The vicinal diol was then reacted with thionyl chloride and the resulting cyclic sulfite was subsequently oxidized into cyclic sulfate **14** using sodium periodate ($NaIO_4$) and ruthenium(III) chloride ($RuCl_3$) in 65% yield. As described earlier, when attempting the direct one-pot cyclic sulfate ring opening and dehydrohalogenation using 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) and tetrabutylammonium iodide (TBAI), only the sulfate-tetrabutylammonium salt **15** was formed. In contrast to the described synthetic procedure, subsequent addition of 1 M H_2SO_4 did not result in the desired hydrolysis of the sulfate ester to give compound **16**, as evidenced by the presence of proton peaks in the ^{1}H-NMR corresponding to tetrabutylammonium in a 1:1 ratio to the sphingosine protons. An additional step was therefore required to remove the sulfate ester, which was achieved by acid catalysed hydrolysis using 5 M HCl to give **16** in 84% over two steps. The resulting free secondary hydroxyl of **16** was then protected with a benzoyl group to give compound **17**. To prevent the migration of the benzoyl group to the primary hydroxyl group, the silyl group on the primary hydroxyl of **17** was removed using boron trifluoride acetonitrile complex at -25 °C and sphingosine acceptor **18** was obtained in 51% yield over two steps.



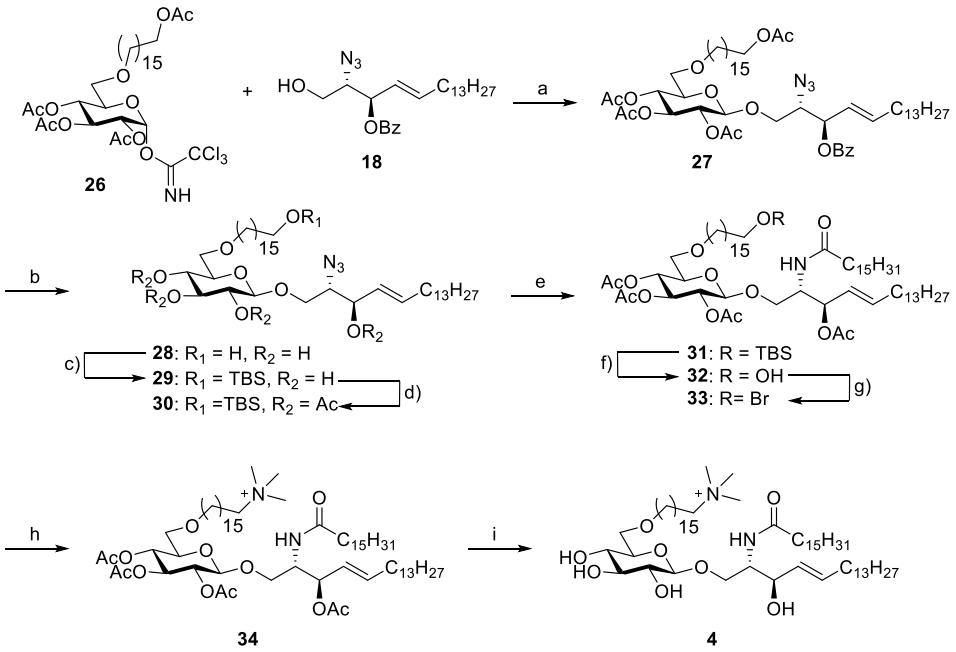
Scheme 3.2: Synthesis of sphingosine acceptor **18**. Reagent and conditions: a) Stick reagent, K_2CO_3, $CuSO_4{\cdot}5H_2O$, MeOH, rt, 5 h, 93%; b) TBDPSCl, DMAP, pyridine, rt, 16 h, 79%; c) i. $SOCl_2$, Et_3N, CH_2Cl_2, 0 °C 30 min; ii. $NaIO_4$, $RuCl_3{\cdot}H_2O$, EtOAc/MeCN/H_2O 1:1:1, rt. 2 h, 65%; d) i. TBAI, DBU, toluene, reflux, 1.5 h; ii. H_2SO_4/H_2O/THF 1:1.2:15.5, rt, 30 min; e) HCl, THF, 40 °C, 5 h, 84% (two steps); f) BzCl, pyridine, rt, 2 h; g) $BF_3{\cdot}MeCN$, CH_2Cl_2, rt, 4 h, 51% (two steps).

For the synthesis of 6-*O*-alkylTMA-β-GlcCer **4** sugar donor **26** was needed (Scheme 3.3). Due to the high cost of commercially available 1,16-hexadecanediol (**19**), an alternative starting material was considered. 1,16-Hexadecanediol **19** was prepared in-house from 16-hexadecanolide via reduction with lithium aluminum hydride ($LiAlH_4$), offering a more economical alternative. As 1,16-hexadecanediol showed poor solubility, and the alkylation of intermediate **5** with this diol was hard to reproduce at large scale, another synthetic route was devised. To improve the diol solubility, one of the hydroxyls was protected with a TBS group while the other was tosylated using standard conditions. In contrast to the synthetic strategy described in Scheme 3.1, intermediate **21** was used as the electrophile and the sugar was employed as the nucleophilic counterpart, thereby improving the alkylation yield significantly due to the induced electronic effect of the endocyclic oxygen in the sugar. Intermediate **22** was then subjected to Pd/C-catalysed hydrogenation, followed by peracetylation to give **24** in 51% yield over two steps. During the hydrogenation step, a drop of concentrated HCl was added to remove the TBS protecting group in one pot. Peracetylation using acetic anhydride, acetic acid and sulfuric acid resulted in peracetylated glucose **24** as an α/β mixture (2:1). The anomeric hydroxyl group was deacetylated using dimethylaminopropylamine to give **25**, which was subsequently reacted with trichloroacetonitrile yielding glucosyl imidate donor **26** in 43% yield over two steps. Only the α-anomer was isolated here as the β-fraction contained an impurity which was difficult to separate from the product.



Scheme 3.3: Synthesis of alkylated imidate sugar donor **26**. Reagent and conditions: a) $LiAlH_4$ (2 eq), THF, 0 °C to reflux, 3 h; b) TBSCl, imidazole, THF, rt, 16 h, 48% over two steps; c) *p*-TsCl, pyridine, rt, 16 h, 95%; d) **21**, NaH (60% in mineral oil), DMF, rt, 16 h, 58%; e) Pd/C (10%), H_2, EtOAc/EtOH 1:1, rt, 4 h; f) H_2SO_4, Ac_2O/AcOH 1:1, rt, 20 h, 43% (two steps); g) DMAPA, THF, rt, 6 h; h) CCl_3CN, Cs_2CO_3, CH_2Cl_2, rt, 4 h, 43% (two steps).

Imidate donor **26** was then coupled to the sphingosine acceptor using standard glycosylation conditions with TMSOTf at -50 °C in 74% yield. The acetyl groups and the benzoyl group were subsequently removed to give **28** in 94% yield. The primary hydroxyl was selectively protected using *tert*-butyldimethylsilyl chloride resulting in the mono-silylation of **28** to give **29** in 89% yield. The ceramide moiety was then synthesized after peracetylation of **29** by reacting the azide in **30** with palmitic acid in a one-pot Staudinger reduction and amide coupling in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and hydroxybenzotriazole (HOBt) as catalysts, yielding **31** in 63% over two steps. Subsequently, the TBS group was removed using TBAF in THF to afford hydroxyl **32**, which was then brominated via an Appel reaction to give bromide **33** in 62% yield over two steps. Finally, the bromide **33** was reacted with trimethylamine (NMe_3) at 50 °C resulting in **34**, which was subsequently deacetylated to afford the final product 6-*O*-alkylTMA-β-GlcCer **3** in 68% over two final steps.



Scheme 3.4: Synthesis of 6-*O*-alkylTMA-β-GlcCer **4**. Reagent and conditions a) TMSOTf, CH_2Cl_2, -50 °C, 2 h, 74%; b) NaOMe, MeOH/CH_2Cl_2 1:1, rt, 6.5 h, 94%; c) TBSCl, pyridine, rt, 3 h, 89%; d) Ac_2O, pyridine, rt, 22 h, 99%; e) Palmitic acid, HOBt, EDC, P(*n*-Bu)$_3$, MeCN, rt, 16 h, 63%; f) TBAF, THF, rt, 22 h, 74%; g) CBr_4, PPh_3, CH_2Cl_2, rt, 16 h, 84%; h) NMe_3, MeCN, 50 °C, 16 h, 81%; i) NaOMe, MeOH/CH_2Cl_2 1:1, rt, 2.5 h, 80%.

3.2.3 Biochemical characterization of 6-*O*-alkylTMA-4MU-β-Glc 3

The hydrolysis of 6-*O*-alkylTMA-4MU-β-Glc **3** by rhGBA1 (Cerezyme®) was then assessed and compared to its non-charged analogue 6-*O*-alkyl-4MU-β-Glc substrate **2** (Figure 3.8). Similar kinetics were observed as for substrate **2** at lower concentrations of substrate. However, at higher substrate concentrations a decrease in enzymatic activity was observed. This decrease may be due to the fact that GBA1 benefits from having negatively charged lipids, such as sodium taurocholate, in the assay. The introduction of a positively charged TMA group on substrate **3** may counteract this effect, leading to reduced enzymatic activity at elevated substrate concentrations. Considering this, the maximum rate of hydrolysis (V_{max} = 2.41 x 10^5 nmol/h/mg protein) and the Michaelis constant (K_m = 0.14 mM) were calculated using a maximum substrate concentration of 1.5 mM.

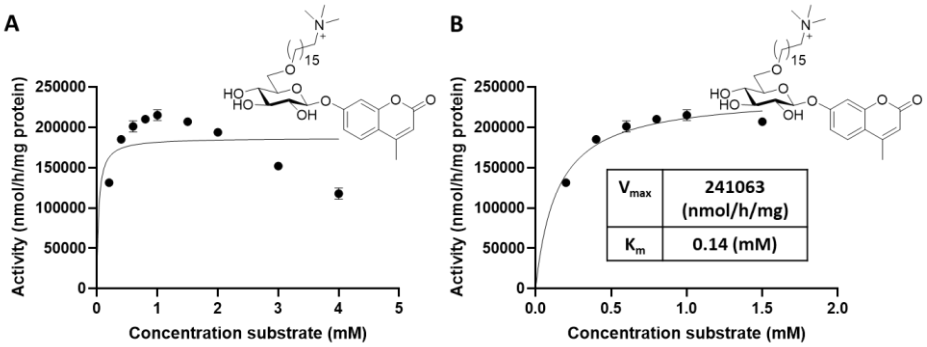


Figure 3.8: Maximum rate of hydrolysis (V_{max}) and the Michaelis constant (K_m) for fluorogenic substrate 6-*O*-alkylTMA-4MU-β-Glc **3** by rhGBA1 (Cerezyme®). A) rhGBA1 kinetics across the complete substrate concentration range up to 4 mM. B) rhGBA1 Kinetics with substrate concentrations above 1.5 mM excluded for more accurate kinetic constants calculations.

3.2.4 Transglucosylation of NBD-Cholesterol with TMA charge-tagged substrates using HPTLC

Next, HPTLC experiments were performed for both TMA charge-tagged substrates (6-*O*-alkylTMA-4MU-β-Glc (**3**) and 6-*O*-alkylTMA-β-GlcCer (**4**)) to assess their activity as sugar donors in the GBA1 catalysed transglucosylation reaction. Although more polar eluents were required to properly visualize the 6-*O*-alkylTMA-β-GlcNBD-Chol on HPTLC plates (70-30 $CHCl_3$:MeOH), both substrates **3** and **4** generated the glucosylated product (Figure 3.9). For comparison, the 6-*O*-alkyl-4MU-β-Glc (**2**) and its corresponding product were visualized as before using 92:8 $CHCl_3$:MeOH (Figure S3.1).

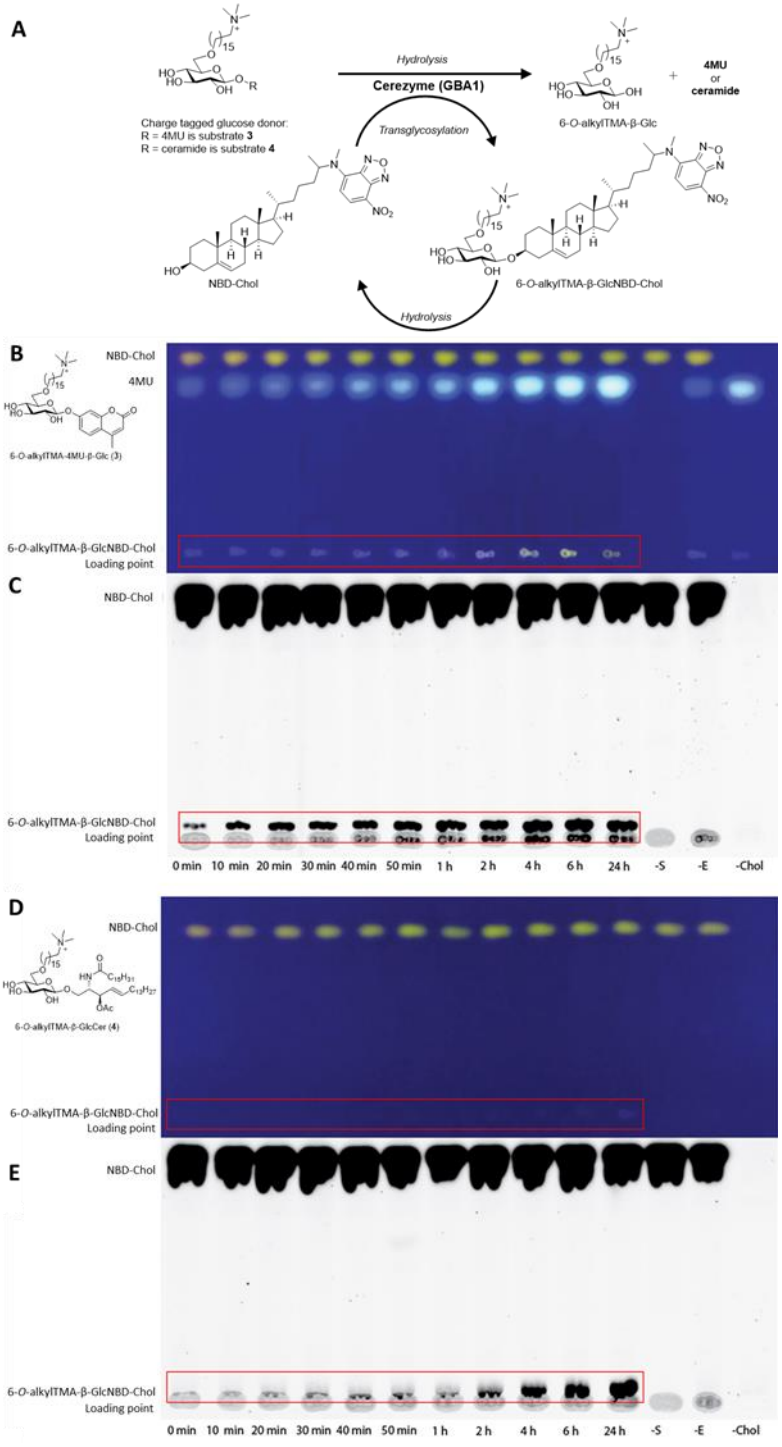

A
Hydrolysis
Cerezyme (GBA1)
Transglycosylation
Hydrolysis
Charge tagged glucose donor:
R = 4MU is substrate 3
R = ceramide is substrate 4
4MU
or
ceramide
6-O-alkylTMA-β-Glc
NBD-Chol
6-O-alkylTMA-β-GlcNBD-Chol
B
NBD-Chol
4MU
6-O-alkylTMA-4MU-β-Glc (3)
6-O-alkylTMA-β-GlcNBD-Chol
Loading point
C
NBD-Chol
6-O-alkylTMA-β-GlcNBD-Chol
Loading point
0 min 10 min 20 min 30 min 40 min 50 min 1 h 2 h 4 h 6 h 24 h -S -E -Chol
D
NBD-Chol
6-O-alkylTMA-β-GlcCer (4)
6-O-alkylTMA-β-GlcNBD-Chol
Loading point
E
NBD-Chol
6-O-alkylTMA-β-GlcNBD-Chol
Loading point
0 min 10 min 20 min 30 min 40 min 50 min 1 h 2 h 4 h 6 h 24 h -S -E -Chol

Figure 3.9: Transglucosylation of NBD-Cholesterol using 6-*O*-alkylTMA-4MU-β-Glc (**3**) or 6-*O*-alkylTMA-β-GlcCer (**4**) as a glucosyl donor and pure recombinant rhGBA1 (Cerezyme®). A) reaction scheme of the transglucosylation NBD-Cholesterol using 6-*O*-alkylTMA-4MU-β-Glc (**3**) or 6-*O*-alkylTMA-β-GlcCer (**4**), and the hydrolysis of 6-*O*-alkylTMA-4MU-β-GlcNBD-Chol by rhGBA1. HPTLC plate images: B) fluorescence scan using Typhoon Variable Mode Imager of transglucosylation using substrate **3** or C) UV light image of B. D) fluorescence scan using Typhoon Variable Mode Imager of transglucosylation using substrate **4** or E) UV light image of D.

In this experiment, transglucosylation of NBD-Chol occurs faster for compound **2** and **3** compared to ceramide analogue **4**, suggesting that the natural substrate analogue is a less efficient transglucosylation donor under the tested conditions. Literature reports have indicated that replacing the 16:0 ceramide amide tail with a 18:1 or 8:0 carbon tails increases the rate of transglucosylation approximately 2-2,5 fold *in vitro* using purified recombinant GBA1.[1]

3.2.4 Detection of transglucosylated cholesterol by LC-MS/MS

To determine the increase in signal intensity due to the TMA handle, 0.1 pmol of the three substrates **2**-**4** was injected into the LC-MS system, and the peak areas and signal intensity were compared. During the optimisation of the LC-MS/MS parameters (Table 3.1 and 3.2), it was found that the charged substrates required significantly higher collision energies. This increase in collision energy led to the formation of multiple molecular fragments, resulting in a decrease in signal detected by MS/MS compared to LC-MS. Based on this observation, the signal intensity of the ionised substrates was analysed before and after fragmentation (Table 3.1 and Table 3.2).

Table 3.1: Integral of LC-MS peak intensity of 0.1 pmol of substrate **2**-**4**

Substrate	Run 1	Run 2	Run 3	Average	Ratio
6-*O*-alkyl-4MU-β-Glc (**2**)	26388784	24663932	25818164	25623627	1
6-*O*-alkylTMA-4MU-β-Glc (**3**)	69833176	85683008	94837888	83451357	3.26
6-*O*-alkylTMA-β-GlcCer (**4**)	30289762	37003036	45218324	37503707	1.46

Ratio of 6-*O*-alkyl-4MU-β-Glc (**2**) : 6-*O*-alkylTMA-4MU-β-Glc (**3**) : 6-*O*-alkylTMA-β-GlcCer (**4**) = 1 : 3.26 : 1.46

Table 3.2: Integral of LC-MS/MS peak intensity of 0.1 pmol of substrate **2**-**4**

Substrate	Run 1	Run 2	Run 3	Average	Ratio
6-*O*-alkyl-4MU-β-Glc (**2**)	403502	413721	413925	410383	1
6-*O*-alkylTMA-4MU-β-Glc (**3**)	486413	523649	566323	525462	1.28
6-*O*-alkylTMA-β-GlcCer (**4**)	113602	122763	121964	119443	0.29

Ratio of 6-*O*-alkyl-4MU-β-Glc (**2**) : 6-*O*-alkylTMA-4MU-β-Glc (**3**) : 6-*O*-alkylTMA-β-GlcCer (**4**) = 1 : 1.28 : 0.29

When comparing the LC-MS signal intensities of compounds **2** and **3**, the introduction of the TMA handle results in a threefold increase in signal. In contrast, the ceramide based substrate **4** shows only a modest 1.5-fold enhancement compared to the non-charged substrate **2**. While this improvement is lower than initially anticipated, further experiments are required to determine whether it is

sufficient to facilitate the identification of novel glycolites in future LC-MS/MS analyses using more complex metabolite acceptor pools. In literature, charged substrates have been reported to exhibit signal intensity increases of up to 75-fold through the incorporation of a permanently charged ion.[17] The observed differences in ionization potential between substrates **2** and **4** are unsurprising, given their distinct aglycon structures. A comparison of the LC-MS and LC-MS/MS signal intensities (Table 3.2) reveals the signal enhancement of substrate **3** relative to **2** decreases from 3.26 to 1.28, indicating significant signal loss upon fragmentation. As mentioned above, the higher collision energies required to generate distinct fragments for the charged substrates resulted in the formation of multiple fragments, leading to a reduction in signal intensity for the measured mass transition. A similar trend is observed for substrate **4**, whose signal intensity also drops significantly compared to substrate **2**. While this loss of signal intensity upon compound fragmentation is undesirable, further research is needed to determine if the introduction of ionized handles will be an effective strategy for metabolite identification.

Next, the transglucosylation of cholesterol using the three sugar donors **2**-**4** was assessed through an LC-MS/MS lipidomics approach. The *in vitro* transglucosylation of cholesterol was performed following the same procedure described for the HPTLC transglucosylation of NBD-cholesterol, using cholesterol instead. A master mixture containing the substrate, cholesterol and rhGBA1 was incubated and small aliquots were taken and frozen at multiple different time points. The LC-MS/MS eluent system, mass transitions, cone voltages and collision energies for the substrates, products and internal standards (Table 3.4) were optimized using the 24-hour sample to achieve optimal separation and signal intensities. Based on this optimization, the samples were diluted 1000-fold before the lipid extraction, and only the lower extraction layer was analysed, as the upper layer contained no significant compound signals. The uncharged substrate **2** showed a gradual decrease in substrate concentration over time, accompanied by an increase in the amount of 6-*O*-alkyl-β-GlcChol (Figure 3.10).

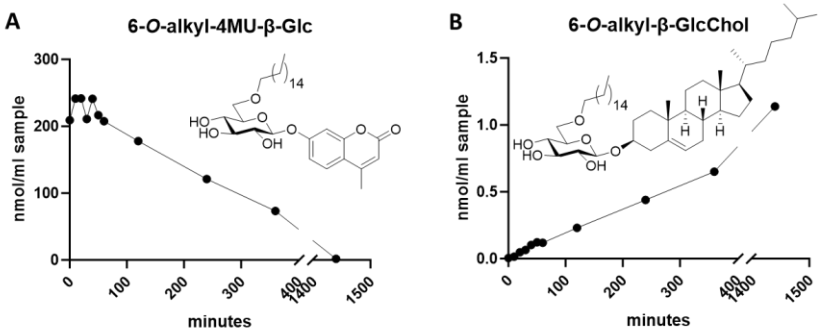


Figure 3.10: LC-MS/MS analysis of A) the consumption of 6-*O*-alkyl-4MU-β-Glc (**2**) over time and B) the formation of the transglucosylated 6-*O*-alkyl-β-GlcChol over time. Data was analysed using $^{13}C_6$-AcylGlcChol 16:0 as internal standard.

The consumption of substrate **2** occurs significantly faster than the formation of the transglucosylated 6-*O*-alkyl-β-GlcChol product. This is because the substrate undergoes not only transglucosylation but also hydrolysis. After 24 hours (1440 minutes), the substrate is fully consumed, resulting in the formation of approximately 1.1 nmol of product. These results further confirm that GBA1 is capable of transglucosylating these 6-alkylated fluorogenic substrates to cholesterol, and that their concentrations can be reliably quantified using LC-MS/MS analysis. However, despite clear product formation over time observed in the HPTLC analysis, LC-MS/MS did not yield quantifiable data when using the charged substrates **3** and **4** (Figure S3.3). While substrate levels initially appear to decrease over time, they return to the original level of substrate after 24 hours. Additionally, the charged 6-*O*-alkylTMA-β-GlcChol product is already significantly present in the 0-minute starting samples (Figure S3.3B and D). Further optimisation of the LC-MS/MS detection and analysis is required to establish a reliable and sensitive workflow for the quantification of ionized-tagged glucolites.

3.3 Conclusion

This chapter focuses on the rational design of biochemical tools for the identification of glucolites formed through the GBA1-catalysed transglucosylation reaction. Here, the GBA1 specific substrate 6-*O*-alkyl-4MU-β-Glc **2**, synthesized in Chapter 2, is described to transglucosylate NBD-Cholesterol, a fluorescent analogue of cholesterol. This substrate proved to be an efficient glucose donor although at a slower rate than 4MU-β-Glc **1**. Here, substrate **2** proved to be a suitable modified glucose donor for the *in vitro* transglucosylation reaction of a variety of metabolite acceptors, including cholesterol, desmosterol, retinol and vitamin D_3 and, upon LC-MS/MS analysis, formation of the respective transglucosylated products was shown to increase over time. This initial proof of concept experiment indicates the potential of this substrate for identifying other GBA1 related glucolites, and prompted further identification of glucolites using more advanced LC-MS/MS based experiments.

To improve the sensitivity for detecting potentially low-abundant metabolite acceptors, an analogue of substrate **2** equipped with a high-ionizing handle (trimethylammonium (TMA)), was synthesized. This handle is known to enhance signal intensities on MS systems and may aid in the identification of novel metabolite acceptor. Substrate 6-*O*-alkylTMA-4MU-β-Glc **3** and the natural ceramide analogue, 6-*O*-alkylTMA-β-GlcCer **4**, were synthesized to investigate the relevance of the natural ceramide aglycon versus the artificial 4MU aglycon. Since the fluorogenic 4MU handle is necessary only for determining kinetics through the 4MU activity assays and not for LC-MS/MS-based strategies, returning to a more physiological ceramide might lead to a more physiologically relevant transglucosylation substrate.

The kinetics of the fluorogenic TMA substrate **3** were assessed using a 4MU-β-Glc activity assay, and the results showed similar kinetics as the ones observed with alkyl substrate **2**. At higher substrate concentrations, a decrease in maximum hydrolysis was observed for the charged substrate **3**, suggesting that the positive charge might counteract the effect of sodium taurocholate, a negatively charged lipid that enhances GBA1 activity. Next, all three substrates were transglucosylated to NBD-Cholesterol *in vitro*, and product formation and degradation was visualized over time using HPTLC. This revealed that the natural ceramide substrate analogue transglucosylates at a slower rate compared to the 4MU-based substrates. This

reduced transglucosylation rate for 6-*O*-alkylTMA-β-GlcCer **3** could potentially be enhanced by equipping the ceramide amide tail with a C8:0 or C18:1 fatty acid.[1]

LC-MS analysis also revealed a three-fold increase in signal intensity due to the incorporation of the positively charged TMA handle however, this signal increase was reduced to 1.5-fold approximately upon fragmentation by LC-MS/MS analysis due to the fact that higher collision energies are required to generate detectable fragments for the charged compounds. Future experiments will determine if this increase in signal intensities is sufficient to identify novel low-abundant glucolites using LC-MS/MS based strategies. Initial MS/MS analysis of the transglucosylation of the three substrates to cholesterol and their respective products focused on determining and optimizing parameters such as the fragmentation patterns of the substrates and product molecules, cone voltages, collision energies and solvent systems for LC separation. While more research and optimization is required to generate a suitable workflow to reliably detect and quantify the ionized-tagged glucolites (Figure S3.3), the detection and quantification of the formation of 6-*O*-alkyl-β-GlcChol over time due to the GBA1 catalysed transglucosylation of cholesterol with the 6-*O*-alkyl-4MU-β-Glc (**2**) substrate shows promise for further research (Figure 3.10). Future untargeted lipidomic experiments will assess whether the TMA handle can be used to identify novel GBA1 related glucolites.

3.4 Acknowledgements

Figure 3.1 was created using Biorender. Bart Imthorn is kindly acknowledged for synthesizing the 6-*O*-alkylTMA-β-GlcCer substrate during his M.Sc. internship. Joosje van Weperen is kindly acknowledged for the first HPTLC experiments using 6-*O*-alkyl-4MU-β-Glc. Joosje van Weperen and Kate Bila are also acknowledged for the LC-MS/MS experiments on the transglucosylation of cholesterol, desmosterol, retinol and vitamin D_3. Maria Ferraz is kindly acknowledged for her assistance with lipid extraction and the transglucosylation of cholesterol and the accompanied LC-MS/MS experiments of the charged compounds.

3.5 Experimental methods

3.5.1 Biochemical and Biological Methods

Cerezyme® (rhGBA, 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). Substrates **2**-**4** and internal heavy isotope standard 6-*O*-palmitoyl-$^{13}C_6$-GlcChol were synthesized as described here and in Chapter 2. 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).

Fluorogenic assays for 4MU substrate 3.

The *in vitro* enzyme activity of rhGBA1 (Cerezyme®) using substrate **3** was determined by measuring the release of the fluorescent 4-methylumbelliferyl.[45] Substrate mixtures were made in 150 mM McIlvaine buffer pH 5.2 containing 0.2% (w/v) sodium taurocholate. 34.1 ng Cerezyme® was diluted in 25 mM KPI pH 5.2 and 0.1% (v/v) Triton X-100. Per well 12.5 µL of enzyme mixture, 12.5 µL of 150 mM McIlvaine buffer and 100 µL of substrate mixture were incubated for 30 minutes at 37 °C. The reaction was stopped by adding 200 µL of the stop buffer 1 M glycine-NaOH pH 10.3. As a standard and for quantification of the obtained signals, 1 nmol of 4MU was added. GraphPad Prism 9 was used to analyse 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. Substrate mixtures with 0.2, 0.4, 0.6, 0.8, 1.0, 1.5 2.0, 3.0 and 4.0 mM substrate **3** were prepared with additional 0.5% (v/v) Triton X-100 and a maximum of 10% (v/v) DMSO.

HPTLC analysis of rhGBA1-catalysed transglycosylation with substrate 2-4

55 µL of substrate stock solution (40 mM in DMSO) was added to a 10 mL plastic centrifuge tube and 2145 µL of 150 mM McIlvain buffer pH 5.2 containing 0.5% Triton X-100 and 0.2% sodium taurocholate was added. 44 µL of 25-NBD-cholesterol (2 mM in EtOH), 110 µL H_2O (Milli-Q) and 440 µL Cerezyme® (1.363 mg/mL diluted 400x with 25 mM KPi buffer 6.5 pH yielding 0.34 µg/mL) were added to the tube and the mixture was incubated at 37 °C for 24 h in a shaking water bath. At time intervals 0 min, 10 min, 20 min, 30 min, 40 min, 50 min, 1 h, 2 h, 4 h, 6 h and 24 h, 240 µL was

taken from the tube and transferred to a 2 mL safe lock Eppendorf tube, which was subsequently frozen with liquid nitrogen and stored in the freezer at -20 °C.

For the -S control sample (without substrate), the following were added to a 2 mL safe lock Eppendorf tube: 200 µL of 150 mM McIlvain buffer pH 5.2 containing 0.5% Triton X-100 and 0.2% sodium taurocholate, 4 µL of 2 mM 25-NBD-cholesterol in EtOH, 10 µL of H_2O (Milli-Q) and 136 ng of Cerezyme® (diluted in 25 mM KPi buffer 6.5 pH). For the -E control sample (without enzyme) the following were added to a 2 mL safe lock Eppendorf tube: 5 µL of substrate stock solution (40 mM in DMSO), 195 µL of 150 mM McIlvain buffer pH 5.2 containing 0.5% Triton X-100 and 0.2% sodium taurocholate, 4 µL of 2 mM 25-NBD-cholesterol in EtOH, 10 µL of H_2O (Milli-Q) and 40 µL of 25 mM KPi buffer 6.5 pH. For the -Chol control sample (without cholesterol) the following were added to a 2 mL safe lock Eppendorf tube: 5 µL of substrate stock solution (40 mM in DMSO), 195 µL of 150 mM McIlvain buffer pH 5.2 containing 0.5% Triton X-100 and 0.2% sodium taurocholate, 4 µL of 25 mM KPi buffer 6.5 pH, 10 µL of H_2O (Milli-Q) and 136 ng of Cerezyme® (diluted in 25 mM KPi buffer 6.5 pH). All control sample tubes were incubated at 37 °C for 2 h in a shaking water bath after which 240 µL was transferred to a 2 mL Eppendorf tube and subsequently frozen with liquid nitrogen and stored in the freezer at -20 °C.

Lipid extraction was performed on all samples following the procedure described by Bligh and Dyer.[46] The lower phase was transferred to a new 2 mL Eppendorf tube, concentrated and re-dissolved in 50 µL of MeOH. 2 µL was applied to a prewarmed (80 °C) HPTLC plate, which was placed in a CAMAG horizontal development chamber. The HPTLC was run using 8% MeOH in $CHCl_3$ for glucose donor 6-*O*-alkyl-4MU-β-Glc **2** and 30% MeOH in $CHCl_3$ for glucose donors 6-*O*-alkylTMA-4MU-β-Glc **3** and 6-*O*-alkylTMA-β-GlcCer **4** as eluent. Pictures were taken with a Typhoon Variable Mode Imager (GE Healthcare Bio-science Corp., Piscataway, NJ, USA) and with a camera under UV light.

Transglucosylation of cholesterol, desmosterol, retinol and vitamin D_3 with 6-*O*-alkyl-4MU-β-Glc 2 by Cerezyme® over time as analysed by LC-MS/MS

Transglucosylation activity was determined using 6-*O*-alkyl-4MU-β-Glc **2** as donor and natural cholesterol, desmosterol, vitamin D_3 and retinol as acceptors. For this, 136 ng of enzyme (Cerezyme®, diluted in KPi buffer 6.5 pH), 10 µL of MS-H_2O, 200 µL of substrate (1 mM) and 12.5 µL of cholesterol (2 mM) / desmosterol (2 mM) / vitamin D_3 (2 mM) and retinol (4 mM), all dissolved in 150 mM McIlvain buffer pH 5.2 containing 0.5% Triton X-100 and 0.2% sodium taurocholate, were incubated for different periods of time (0 h, 0.5 h, 2 h and 3 h) in a 37 °C shaking water bath. $^{13}C_6$-GlcChol, with a concentration of 0.1 pmol/ µL, was used as internal standard and was added after incubation. Lipids were extracted according to Bligh&Dyer[46] (MeOH:$CHCl_3$:H_2O, 1:1:0.9, v/v/v) followed by a ^{t}BuOH/H_2O (1:1, v/v) extraction. Samples were dried under nitrogen stream, resuspended in 100 µL of MS-MeOH and sonicated 30 seconds and 90 µL of this mixture was pipetted to the MS vial with insert. LC-MS/MS analysis was performed as described by Marques *et al.*[2] with a Waters Acquity™ TQD instrument. The run duration was 12 minutes and the instrument parameters and chromatograms are displayed in appendix Figure S3.2.

Mass spectrometry peak intensity of 0.1 pmol of substrate 2-4

A Waters Acquity TM TQD instrument was used in all experiments. The instrument consisted of a UPLC system combined with a tandem quadrupole mass spectrometer as mass analyser. Data were analysed with Masslynx 4.1 software (Waters Corporation, Milford, MA). 0.1 pmol samples were separated using BEH C18 reversed-phase column (2.1 × 50 mm, particle size 1.7 µm; Waters Corporation), by applying a gradient elution of mobile phases, 2-propanol:acetonitrile:methanol 70:20:10 (v/v/v) containing 10 mM ammonium formate (eluent A) and methanol:water 50:50 (v/v) containing 10 mM ammonium formate (eluent B). The UPLC program was run for 10 minutes using first 100% eluent B for 1 minute, followed by a gradient of 100 to 0% eluent B over the next 6 minutes. The 100% A eluent was kept for the next minute after which the last 2 minutes a gradient of 0 to 100% eluent B was applied. The divert valve of the mass spectrometer was programmed to discard the UPLC effluent before (0–1.5 min) and after (8–10 min) the elution of the analytes to prevent system contamination. The flow rate was 0.50 mL/min and the retention time of substrates **2-4** are given in Table 3.4. The column temperature and the temperature of the autosampler were kept at 40 °C and 4 °C, respectively, during the run. Solutions of the substrates 6-*O*-alkyl-4MU-β-Glc **2**, 6-

O-alkylTMA-4MU-β-Glc **3**, and 6-*O*-alkylTMA-β-GlcCer **4** were prepared with concentrations of 0.1 pmol/µL in methanol.

LC-MS/MS of the transglucosylation of cholesterol by rhGBA1 with substrates 2-4

55 µL of substrate stock solution (40 mM in DMSO) was added to a 10 mL plastic centrifuge tube and 2145 µL of 150 mM McIlvain buffer pH 5.2 containing 0.5% Triton X-100 and 0.2% sodium taurocholate was added. 44 µL of Cholesterol (2 mM in EtOH), 110 µL H_2O (Milli-Q) and 440 µL Cerezyme® (1.363 mg/ml diluted 400x with 25 mM KPi buffer 6.5 pH yielding 0.34 µg/ml) were added to the tube and the mixture was incubated at 37 °C for 24 h in a shaking water bath. At time intervals 0 min, 10 min, 20 min, 30 min, 40 min, 50 min, 1 h, 2 h, 4 h, 6 h and 24 h, 240 µl was taken from the tube and transferred to a 2 mL safe lock Eppendorf tube, which was subsequently frozen with liquid nitrogen and stored in the freezer at -20 °C.

For the -S control sample (without substrate), the following were added to a 2 mL safe lock Eppendorf tube: 200 µL of 150 mM McIlvain buffer pH 5.2 containing 0.5% Triton X-100 and 0.2% sodium taurocholate, 4 µL of 2 mM Cholesterol in EtOH, 10 µL of H_2O (Milli-Q) and 136 ng of Cerezyme® (diluted in 25 mM KPi buffer 6.5 pH). For the -E control sample (without enzyme) the following were added to a 2 mL safe lock Eppendorf tube: 5 µL of substrate stock solution (40 mM in DMSO), 195 µL of 150 mM McIlvain buffer pH 5.2 containing 0.5% Triton X-100 and 0.2% sodium taurocholate, 4 µL of 2 mM Cholesterol in EtOH, 10 µL of H_2O (Milli-Q) and 40 µL of 25 mM KPi buffer 6.5 pH. For the -Chol control sample (without cholesterol) the following were added to a 2 mL safe lock Eppendorf tube: 5 µL of substrate stock solution (40 mM in DMSO), 195 µL of 150 mM McIlvain buffer pH 5.2 containing 0.5% Triton X-100 and 0.2% sodium taurocholate, 4 µL of 25 mM KPi buffer 6.5 pH, 10 µL of H_2O (Milli-Q) and 136 ng of Cerezyme® (diluted in 25 mM KPi buffer 6.5 pH). All control sample tubes were incubated at 37 °C for 2 h in a shaking water bath after which 240 µL was transferred to a 2 mL Eppendorf tube and subsequently frozen with liquid nitrogen and stored in the freezer at -20 °C.

Prior to sample injection into the LC-MS/MS lipids were extracted from the samples above which were diluted 1000 times. $^{13}C_6$-GlcChol and $^{13}C_6$-AcylGlcChol 16:0, with a concentration of 0.1 pmol/ µL, were used as internal standard and were added after dilution of the incubated samples. after which 100 µl of the samples was used for sample preparation. Lipids were extracted according to Bligh&Dyer[46] (MeOH:$CHCl_3$:H_2O, 1:1:0.9, v/v/v) followed by a ^{t}BuOH/H_2O (1:1, v/v) extraction.

Identification of glucolites in GD formed through the GBA1 mediated transglucosylation reaction using GBA1 specific (ionized) substrates for LC-MS/MS

Samples were dried under nitrogen stream, resuspended in 100 µL of MS-MeOH and sonicated 30 seconds and 90 µL of this mixture was pipetted to the MS vial with insert. LC-MS/MS analysis was performed with a Waters Acquity TM TQD instrument was used in all experiments. The instrument consisted of a UPLC system combined with a tandem quadrupole mass spectrometer as mass analyser. Data were analysed with Masslynx 4.1 software (Waters Corporation, Milford, MA). $^{13}C_6$-GlcChol and 6-*O*-palmitoyl-$^{13}C_6$-GlcChol were used as internal standards. Samples were separated using BEH C18 reversed-phase column (2.1 × 50 mm, particle size 1.7 µm; Waters Corporation), by applying a gradient elution of mobile phases, 2-propanol:ACN:water 70:20:10 (v/v/v) containing 10 mM ammonium formate (eluent A) and methanol:water 50:50 (v/v) containing 10 mM ammonium formate (eluent B). The UPLC program (Table 3.2) was applied for 10 minutes consisting of first 1 minute 100% eluent B following a gradient of 100→0% over the next 6 minutes. The 100% A eluent was kept for the next minute after which the last 2 minutes a gradient of 0→100% eluent B was applied. The divert valve of the mass spectrometer was programmed to discard the UPLC effluent before (0–1.5 min) and after (8–10 min) the elution of the analytes to prevent system contamination. The flow rate was 0.45 ml/min and the retention time of substrates, products and the internal standards are given in Table 3.4. The column temperature and the temperature of the autosampler were kept at 40 °C and 10 °C, respectively, during the run. Solutions of $^{13}C_6$-GlcChol and 6-*O*-palmitoyl-$^{13}C_6$-GlcChol and a mixture of both compounds were prepared with concentrations of 0.1 pmol/µL in methanol. The gradient and solvents used for LC-MS and LC-MS/MS compound separation can be found in Table 3.3 while the molecular species, transitions, retention times, cone voltages and collision energies used to measure the internal standards, substrates and transglucosylated products can be found in Table 3.4.

Table 3.3: gradient used for LC-MS/MS

Minute	Gradient
0-1	100% B
1-7	→ 100% A
7-8	100% A
9-10	→100% B

A = 2-propanol:ACN:MeOH 70:20:10 (v/v/v) + 10 mM ammonium formate
B = methanol:water 50:50 (v/v) + 10 mM ammonium formate

Table 3.4: Molecular species, transitions and retention times of the internal standards, substrates and products of the LC-MS/MS experiment

Molecular Species	**Transition** ***m/z***	**Retention (min)**	**Cone Voltage**	**Collision Energy**
$^{13}C_6$-GlcChol $[M+NH_4]^+$	572.6 > 369.4	6.01	20	15
$^{13}C_6$-AcylGlcChol 16:0 $[M+NH_4]^+$	810.7 > 369.4	7.12	20	20
6-O-alkylTMA-β-GlcCer $[M+]^+$	981.9 > 264.0	6.34	25	60
6-O-alkylTMA-4MU-β-Glc $[M+]^+$	620.4 > 444.4	2.93	25	40
6-O-alkyl-4MU-β-Glc $[M+H]^+$	563.3 > 177.1	4.88	25	28
6-O-alkylTMA-β-GlcChol $[M+]^+$	830.7 > 255.0	6.04	25	12
6-O-alkyl-β-GlcChol $[M+NH_4]^+$	791.0 > 369.4	7.14	25	45

3.6 Chemical Synthesis

3.6.1 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 $(NH_4)_6Mo_7O_{24}{\cdot}H_2O$ (25 g/L) and $(NH_4)_4Ce(SO_4)_4{\cdot}H_2O$ (10 g/L) in 10% sulfuric acid followed by charring at ~150 ˚C or by spraying with an aqueous solution of $KMnO_4$ (7%) and K_2CO_3 (2%) followed by charring at ~150 °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, 60Å) in the indicated solvents. ^{1}H-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-methylumbeliferone, TMA (trimethylammonium)). 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.

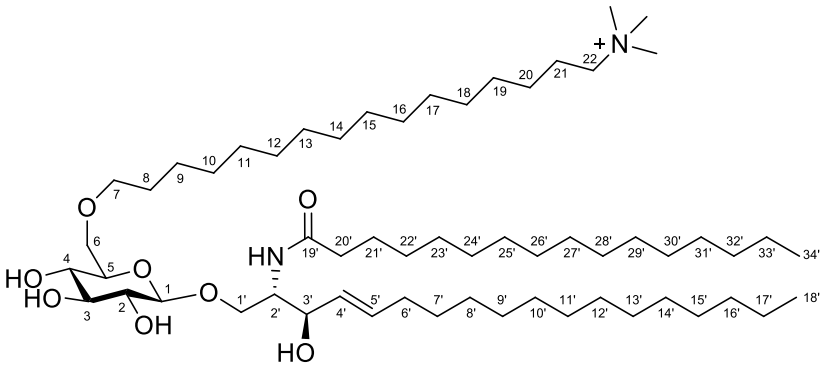
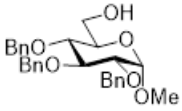


Figure 3.10: Numbering of protons and carbons for NMR assignment.

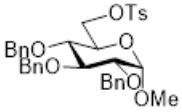
3.6.2 Synthesis and characterization data

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



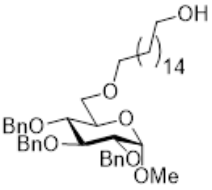
2,3,4-Tri-*O*-benzyl-6-hydroxyl-1-methoxyl-α-D-glucopyranoside was synthesized starting from *a*-methyl-D-glucose as described in the literature and its spectroscopic data are in accordance with published data.[42]

((2*R*,3*R*,4*S*,5*R*,6*R*)-3,4,5-Tris(benzyloxy)-6-methoxytetrahydro-2*H*-pyran-2-yl)methyl 4-methylbenzenesulfonate (6)



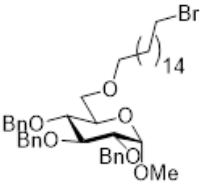
2,3,4-Tri-*O*-benzyl-6-hydroxyl-1-methoxyl-α-D-glucopyranoside **5** (1.29 g, 2.76 mmol) was dissolved in dry pyridine (19 mL) under protected atmosphere and cooled to 0 °C. PTSCl (684 mg, 3.59 mmol) was added and the reaction mixture was stirred overnight allowing to reach room temperature. The mixture was diluted with EtOAc and washed with 1 M HCl, $NaHCO_3$ and brine. All water layers were extracted with EtOAc and the combined organic layers were dried ($MgSO_4$), filtered and concentrated under reduced pressure. The crude residue was purified with column chromatography to yield the tosylated product **6** (1.44 g, 2.33 mmol, 84% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76 (d, *J* = 8.3 Hz, 2H, 2xCH-OTs), 7.36 – 7.26 (m, 15H, CH-OBn), 7.17 – 7.12 (m, 2H, 2xCH-OTs), 4.97 (d, *J* = 10.9 Hz, 1H, CH_2-OBn), 4.86 – 4.74 (m, 3H, 3xCH_2-OBn), 4.63 (d, *J* = 12.2 Hz, 1H, CH_2-OBn), 4.52 (d, *J* = 3.5 Hz, 1H, H-1), 4.42 (d, *J* = 10.7 Hz, 1H, CH_2-OBn), 4.21 (dd, *J* = 10.5, 4.2 Hz, 1H, H-6a), 4.16 (dd, *J* = 10.5, 2.3 Hz, 1H, H-6b), 3.95 (t, *J* = 9.3 Hz, 1H, H-3), 3.75 (ddd, *J* = 10.0, 4.2, 2.2 Hz, 1H, H-5), 3.50 – 3.40 (m, 2H, H-2 and H-4), 3.31 (s, 3H, CH_3-OMe), 2.39 (s, 3H, CH_3-OTs); ^{13}C NMR (101 MHz, $CDCl_3$) δ 145.0, 138.7, 138.1, 137.9, 132.9 (3xC_q-OBn and 2xC_q-OTs), 129.9, 128.62, 128.55, 128.5, 128.2, 128.13, 128.11, 128.05, 128.02, 127.98, 127.8 (15xCH-OBn and 4xCH-OTs), 98.1 (C-1), 81.9 (C-3), 79.8 (C-2), 77.0 (C-4), 75.9, 75.1, 73.6 (3xCH_2-OBn), 68.7 (C-6), 68.6 (C-5), 55.5 (CH_3-OMe), 21.8 (CH_3-OTs). HRMS: calcd. for $C_{35}H_{38}O_8S$ $[M+Na]^+$ 641,21796, found: 641,21761.

16-(((2*R*,3*R*,4*S*,5*R*,6*S*)-3,4,5-Tris(benzyloxy)-6-methoxytetrahydro-2*H*-pyran-2-yl)methoxy)hexadecan-1-ol (7)



The tosylated sugar **6** (1.60 g, 2.59 mmol) was dissolved in dry DMF (25 mL) under argon atmosphere and cooled to 0 °C. NaH (311 mg, 7.77 mmol) and hexadecane-1,16-diol (2.68 g, 10.4 mmol) were added and the reaction mixture was stirred 72 hours at 90 °C. The mixture was diluted with EtOAc and washed with 1 M HCl, $NaHCO_3$ and brine. All water layers were extracted with EtOAc and the combined organic layers were dried ($MgSO_4$), filtered and concentrated under reduced pressure. The crude residue was purified with column chromatography to yield the product **7** (1.09 g, 2.59 mmol, 59% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.38 – 7.26 (m, 15H, CH-OBn), 4.97 (d, J = 10.9 Hz, 1H, CH_2-OBn), 4.88 (d, J = 10.9 Hz, 1H, CH_2-OBn), 4.85 – 4.77 (m, 2H, CH_2-OBn), 4.68 – 4.57 (m, 3H, H-1 and 2xCH_2-OBn), 3.98 (t, J = 9.26 Hz, 1H, H-3), 3.74 – 3.68 (m, 1H, H-5), 3.68 – 3.38 (m, 8H, H-2, H-4, H-6a, H6-b and 2xCH_2-alkyl), 3.36 (s, 3H, CH_3-OMe), 1.69 – 1.50 (m, 6H, 3xCH_2-alkyl), 1.23 (br s, 22H, 11xCH_2-alkyl). ^{13}C NMR (101 MHz, $CDCl_3$) δ 138.9, 138.6, 138.3 (3xC_q-OBn), 128.6, 128.53, 128.50, 128.3, 128.1, 128.0, 127.9, 127.8, 127.7 (15xCH-OBn), 98.3 (C-1), 82.2 (C-3), 79.9 (C-2), 77.8 (C-4), 75.9, 75.2, 73.5 (3xCH_2-OBn), 71.8 (CH_2-alkyl), 70.1 (C-5), 69.2 (C-6), 63.2 (CH_2-alkyl), 55.2 (CH_3-OMe), 32.9, 29.8, 29.73, 29.70, 29.62, 29.55, 26.3, 25.9 (14xCH_2-alkyl). HRMS: calcd. for $C_{44}H_{64}O_7$ $[M+NH_4]^+$ 722,49903, found: 722,49840.

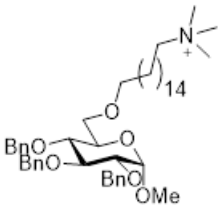
(2*R*,3*R*,4*S*,5*R*,6*S*)-3,4,5-Tris(benzyloxy)-2-(((16-bromohexadecyl)oxy)methyl)-6-methoxytetrahydro-2*H*-pyran (8)



Triphenylphosphine (162 mg, 0.62 mmol) and carbon tetrabromide (205 mg, 0.62 mmol) were successively added to a solution of the hydroxyl sugar **7** (290 mg, 0.41 mmol) in THF (2 mL) at 0 °C. The mixture was stirred for 18 h and then filtered. The filtrate was concentrated *in vacuo* and purified by column chromatography on silica gel to yield the brominated sugar **8** (220 mg, 0.29 mmol, 70%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.38 – 7.24 (m, 15H, CH-OBn), 4.97 (d, J = 10.8 Hz, 1H, CH_2-OBn), 4.88 (d, J = 10.9 Hz, 1H, CH_2-OBn), 4.86 – 4.76 (m, 2H, CH_2-OBn), 4.69 – 4.56 (m, 3H, H-1 and 2xCH_2-OBn), 3.98 (t, J = 9.23 Hz, 1H, H-3), 3.71 (ddd, J = 9.8, 3.6, 2.0 Hz, 1H, H-5), 3.68 – 3.45 (m, 5H, H-2, H-4, H-6a, H-6b and CH_2-alkyl), 3.40 (t, J = 6.9 Hz, 2H, CH_2-alkyl), 3.37 (s, 3H, CH_3-OMe), 3.33 (dd, J = 9.3, 7.0 Hz, 1H, CH_2-alkyl), 1.90 – 1.80 (m, 2H, CH_2-alkyl), 1.57 (m, 2H, CH_2-alkyl), 1.46 – 1.37 (m, 2H, CH_2-alkyl), 1.23 (br s, 22H, 11xCH_2-alkyl). ^{13}C NMR (101 MHz, $CDCl_3$) δ 139.0, 138.6, 138.3 (3xC_q-OBn) 128.57, 128.55, 128.5, 128.3, 128.1, 128.03, 127.92, 127.8, 127.7 (15xCH-OBn), 97.8 (C-1), 82.3 (C-3), 80.0 (C-2), 77.8 (C-4), 75.9, 75.2, 73.5 (3xCH_2-OBn), 71.9

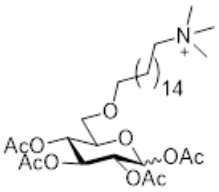
(CH_2-alkyl), 70.1 (C-5), 69.3 (C-6), 55.3 (CH_3-OMe), 34.2 (CH_2Br-alkyl), 33.0, 29.79, 29.77, 29.75, 29.73, 29.68, 29.65, 29.6, 28.9, 28.3, 26.3 (14xCH_2-alkyl). HRMS: calcd. for $C_{44}H_{63}BrO_6$ $[M+NH_4]^+$ 784,41463, found: 784,41400.

_N_,_N_,_N_-Trimethyl-16-(((2_R_,3_R_,4_S_,5_R_,6_S_)-3,4,5-tris(benzyloxy)-6-methoxytetrahydro-2_H_-pyran-2-yl)methoxy)hexadecan-1-aminium (9)



To a solution of the alkyl halide **8** (786 mg, 1.02 mmol) in acetonitrile (10 mL), 2.5 equivalents of trimethylamine (0.6 mL, 2.56 mmol, 40% in water) was added. After 18 h at 70 °C, solvents were removed under vacuum and the crude residue was purified using column chromatography to yield the desired product **9** (845 mg, 1.02 mmol, quantitative yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.38 – 7.27 (m, 15H, CH-OBn), 4.97 (d, J = 10.9 Hz, 1H, CH_2-OBn), 4.88 (d, J = 10.9 Hz, 1H, CH_2-OBn), 4.84 – 4.77 (m, 2H, CH_2-OBn), 4.68 – 4.57 (m, 3H, H-1 and CH_2-OBn), 3.98 (t, J = 9.2 Hz, 1H, H-3), 3.73 – 3.68 (m, 1H, H-5), 3.67 – 3.48 (m, 7H, H-2, H-4, H-6a, H-6b, 2xCH_2-alkyl), 3.46 (s, 9H, 3xCH_3-TMA), 3.37 (s, 3H, CH_3-OMe), 3.36 – 3.31 (m, 1H, CH_2-alkyl), 1.73 (m, 2H, CH_2-alkyl), 1.57 (m, 2H, CH_2-alkyl), 1.37 (m, 2H, CH_2-alkyl), 1.32 – 1.20 (m, 22H, 11xCH_2-alkyl). ^{13}C NMR (101 MHz, $CDCl_3$) δ 138.9, 138.6, 138.3 (3xC_q-OBn), 128.58, 128.56, 128.5, 128.3, 128.1, 128.0, 127.9, 127.8, 127.7 (15xCH-OBn), 98.3 (C-1), 82.3 (C-3), 80.0 (C-2), 77.8 (C-4), 75.9, 75.2, 73.5 (3xCH_2-OBn), 71.9 (CH_2-alkyl), 70.1 (C-5), 69.3 (C-6), 67.3 (CH_2-alkyl), 55.3 (CH_3-OMe), 53.5 (3xCH_3-TMA), 29.8, 29.73, 29.65, 29.6, 29.5, 29.4, 26.33, 26.28, 23.3, 22.4 (14xCH_2-alkyl). HRMS: calcd. for $C_{47}H_{72}NO_6$ $[M]^+$ 746,53542, found: 746,53446.

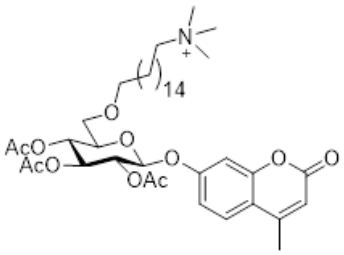
_N_,_N_,_N_-Trimethyl-16-(((2_R_,3_R_,4_S_,5_R_,6_S_)-3,4,5,6-tetraacetoxytetrahydro-2_H_-pyran-2-yl)methoxy)hexadecan-1-aminium (10)



Palladium on carbon (Pd/C) 10% (361 mg, 0.34 mmol) was added to a stirred solution of the protected sugar **9** (845 mg, 1.13 mmol) and concentrated aq. HCl (100 µL) in ethanol/ethyl acetate (1/1, v/v, 16 mL). The reaction mixture was then purged with H_2 and stirred for 18 hours at rt. Upon completion, the solid was filtered off and the filtrate was concentrated *in vacuo*. The crude was used in the next step without further purification. The crude sugar (539 mg, 1.13 mmol) was dissolved in 1:1 AcOH/Ac_2O (22 mL), and cooled down to 0 °C in an ice bath. Concentrated H_2SO_4 (244 µL, 4.35 mmol) was added dropwise into the reaction. The reaction was then removed from ice bath and stirred at room temperature overnight. After 18 h, the reaction was cooled to 0 °C in an ice bath, and saturated $NaHCO_3$ was added dropwise until the reaction was neutralized. The aqueous phase of the reaction

was extracted with EtOAc, and the organic layer was recovered, dried over $MgSO_4$, filtered, and evaporated to dryness. The resulting crude residue was purified with column chromatography to yield the desired product **10** (531 mg, 0.84 mmol, 74% yield over 2 steps as an α/β mixture 1:0.15). α-anomer: ^{1}H NMR (400 MHz, MeOD) *δ* 6.29 (d, *J* = 3.6 Hz, 1H, H-1), 5.43 (t, *J* = 9.92, 1H, H-3), 5.17 (t, *J* = 10.1, 1H, H-4), 5.02 (dd, *J* = 10.3, 3.7 Hz, 1H, H-2), 4.06 (dt, *J* = 10.2, 3.4 Hz, 1H, H-5), 3.54 – 3.32 (m, 6H, H-6a, H-6b and 2xCH_2-alkyl), 3.14 (s, 9H, 3xCH_3-TMA), 2.18 (s, 3H, CH_3-OAc), 2.03 (s, 3H, CH_3-OAc), 2.01 (s, 3H, CH_3-OAc), 2.00 (s, 3H, CH_3-OAc), 1.79 (m, 2H, CH_2-alkyl), 1.53 (m, 2H, CH_2-alkyl), 1.39 (m, 2H, CH_2-alkyl), 1.30 (br s, 22H, 11xCH_2-alkyl). ^{13}C NMR (101 MHz, MeOD) *δ* 171.7, 171.3, 171.0, 170.6 (4xC_q-OAc), 90.2 (C-1), 72.6 (C-6), 72.4 (C-5), 71.4 (C-3), 70.8 (C-2), 69.9 (CH_2-alkyl), 69.7(C-4), 67.9 (CH_2-alkyl), 53.6, 53.52, 53.48 (3xCH_3-TMA), 30.8, 30.70, 30.68, 30.61, 30.57, 30.5, 30.2, 27.3, 27.1, 23.9 (14xCH_2-alkyl), 20.68, 20.66, 20.6, 20.4 (4xCH_3-OAc). β-anomer: ^{1}H NMR (400 MHz, MeOD) *δ* 5.79 (d, *J* = 8.3 Hz, 1H, H-1), 5.32 (t, *J* = 9.5 Hz, 1H, H-3), 5.11 (t, *J* = 9.38 Hz, 1H, H-4), 5.02 (dd, *J* = 10.3, 3.7 Hz, 1H), 3.89 (ddd, *J* = 10.0, 4.4, 2.7 Hz, 1H, H-5), 3.54 – 3.32 (m, 6H, H-6a, H-6b and 2xCH_2-alkyl), 3.14 (s, 9H, 3xCH_3-TMA), 2.08 (s, 3H), 2.02 (m, 6H, 2xCH_3-OAc), 1.98 (s, 3H, CH_3-OAc), 1.79 (m, 2H, CH_2-alkyl), 1.53 (m, 2H, CH_2-alkyl), 1.39 (m, 2H, CH_2-alkyl), 1.30 (br s, 22H, 11xCH_2-alkyl). ^{13}C NMR (101 MHz, MeOD) *δ* 171.6, 171.04, 170.95, 170.6 (4xC_q-OAc), 93.0 (C-1), 74.9 (C-5), 74.4 (C-3), 72.8 (C-6), 71.8 (C-2), 69.91 (CH_2-alkyl), 69.86 (C-4), 67.9 (CH_2-alkyl), 53.6, 53.52, 53.48 (3xCH_3-TMA), 30.8, 30.70, 30.68, 30.61, 30.57, 30.5, 30.2, 27.3, 27.1, 23.9 (14xCH_2-alkyl), 20.68, 20.66, 20.6, 20.4 (4xCH_3-OAc). HRMS: calcd. for $C_{33}H_{60}NO_{10}$ $[M]^+$ 630,42117, found: 630,42064.

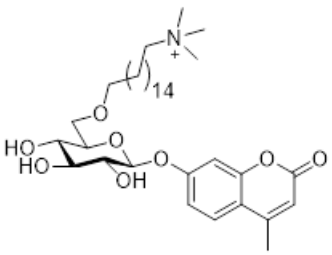
_N_,_N_,_N_-Trimethyl-16-(((2_R_,3_R_,4_S_,5_R_,6_S_)-3,4,5-triacetoxy-6-((4-methyl-2-oxo-2_H_-chromen-7-yl)oxy)tetrahydro-2_H_-pyran-2-yl)methoxy)hexadecan-1-aminium (11).



HBr (33% wt in acetic acid, 2.31 mL, 14.0 mmol) was added dropwise to a cooled solution (0 °C) of an anomeric mixture of the sugar **10** (385 mg, 0.61 mmol) in dichloromethane (2.5 mL) and stirred at rt for 3 h. Ice water was added to the mixture and the aqueous phase was extracted with CH_2Cl_2 (3 x 50 mL). The combined organic layers were washed with 0.5% $Na_2S_2O_3$ (1 x 75 mL), sat. aq. $NaHCO_3$ (3 x 75 mL), brine (1 x 75 mL) and dried over $MgSO_4$. The solvent was evaporated under reduced pressure and the crude product was used in the next step without further purification. 4MU (196 mg, 1.14 mmol) was added to a solution of NaOH (42 mg, 1.06 mmol) in H_2O (5.6 mL). The brominated sugar (363 mg, 0.557 mmol) was added to this solution dissolved in acetone (5.6 mL). The mixture was stirred in the dark for 18 h at room temperature. After the reaction was completed, the

mixture 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 crude was purified by silica column chromatography to afford the product **11** (43 mg, 58 µmol, 10% yield over 2 steps) as a white solid. ^{1}H NMR (400 MHz, MeOD) δ 7.72 – 7.69 (m, 1H, CH-Um), 7.06 – 7.02 (m, 2H, 2x CH-Um), 6.22 (d, *J* = 1.3 Hz, 1H, CH-Um), 5.49 (d, *J* = 7.9 Hz, 1H, H-1), 5.40 (t, *J* = 9.5 Hz, 1H, H-3), 5.24 – 5.19 (m, 1H, H-2), 5.12 (t, *J* = 9.77 Hz, 1H, H-4), 4.08 (ddd, *J* = 10.1, 5.8, 2.5 Hz, 1H, H-5), 3.62 (dd, *J* = 11.2, 2.5 Hz, 1H, H-6a), 3.57 – 3.33 (m, 5H, H-6b and 2xCH_2-alkyl), 3.14 (s, 9H, 3xCH_3-TMA), 2.45 (d, J = 1.3 Hz, 3H, CH_3-Um), 2.05 (m, 6H, 2xCH_3-OAc), 2.00 (s, 3H, CH_3-OAc), 1.85 – 1.74 (m, 2H, CH_2-alkyl), 1.54 (m, 2H, CH_2-alkyl), 1.41 – 1.38 (m, 4H, 2xCH_2-alkyl), 1.33 – 1.18 (m, 20H, 10xCH_2-alkyl). ^{13}C NMR (101 MHz, MeOD) δ 171.6, 171.2, 171.1 (3xC_q-OAc), 162.9, 161.0, 156.0, 155.2 (4xC_q-Um), 127.5 (CH-Um), 116.5 (C_q-Um), 114.9, 113.4, 105.0 (3xCH-Um), 98.9 (C-1), 74.6 (C-5), 74.3 (C-3), 72.8 (CH_2-alkyl), 72.6 (C-2), 70.3 (C-6), 70.1 (C-4), 67.9 (CH_2-alkyl), 53.6, 53.50, 53.46 (3xCH_3-TMA), 30.73, 30.70, 30.6, 30.53, 30.51, 30.2, 27.4, 27.1, 23.9 (14xCH_2-alkyl), 20.7, 20.60, 20.58 (3xCH_3-OAc), 18.7 (CH_3-Um). HRMS: calcd. for $C_{41}H_{64}NO_{11}$ $[M]^+$ 746,44739, found: 746,44652.

_N_,_N_,_N_-trimethyl-16-(((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)methoxy)hexadecan-1-aminium (3)

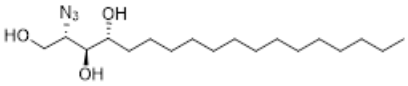


The protected sugar **11** (40 mg, 54 µmol) was dissolved in MeOH/CH_2Cl_2 (1:1, v/v, 1.2 mL) followed by the addition of a catalytic amount of sodium methoxide (30% in MeOH, 3 drops). The mixture was stirred for 4 h at room temperature. After reaching completion the reaction was quenched with Amberlite™ IRC-120(H), which was subsequently filtered off and washed with MeOH. The solution was concentrated yielding the crude product which was purified by silica column chromatography yielding the final product 6-*O*-alkylTMA-4MU-β-Glc **3** (21 mg, 34 µmol, 63% yield) as a white solid. ^{1}H NMR (400 MHz, MeOD) δ 7.70 (d, *J* = 8.8 Hz, 1H, CH-Um), 7.11 (dd, *J* = 8.8, 2.4 Hz, 1H, CH-Um), 7.07 (d, *J* = 2.4 Hz, 1H, CH-Um), 6.20 (d, *J* = 1.2 Hz, 1H, CH-Um), 5.04 – 5.01 (m, 1H, H-1), 3.84 (dd, J = 11.0, 1.9 Hz, 1H, H-4), 3.68 – 3.62 (m, 1H, H-3), 3.60 – 3.33 (m, 8H, H-2, H-5, H-6a, H-6b and 2xCH_2-alkyl), 3.12 (s, 9H, 3xCH_3-TMA), 2.46 (d, *J* = 1.2 Hz, 3H, CH_3-Um), 1.84 – 1.73 (m, 2H, CH_2-alkyl), 1.56 (p, *J* = 6.7 Hz, 2H, CH_2-alkyl), 1.44 – 1.15 (m, 24H, 12xCH_2-alkyl). ^{13}C NMR (101 MHz, MeOD) δ 163.2, 162.0, 156.1, 155.4 (4xC_q-Um), 127.2 (CH-Um), 116.0 (C_q-Um), 115.1, 113.0, 105.0 (CH-Um), 101.7 (C-1), 77.9 (C-5), 77.3 (C-3), 74.7 (C-2), 72.7 (CH_2-alkyl), 71.7 (C-4), 71.2 (C-6), 67.9 (CH_2-alkyl), 53.51, 53.47, 53.4 (3xCH_3-

TMA), 30.8, 30.72, 30.68, 30.6, 30.54, 30.51, 30.2, 27.4, 27.2, 23.9 (14xCH_2-alkyl), 18.7 (CH_3-Um). HRMS: calcd. for $C_{35}H_{58}NO_8$ $[M]^+$ 620,41569, found: 620,41517

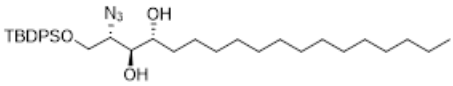
Synthesis of sphingosine acceptor

(2*S*,3*S*,4*R*)-2-Azidooctadecane-1,3,4-triol (12)



Phytosphingosine (7.21 g, 22.7 mmol) was dissolved in MeOH (113.5 mL) and cooled to 0 °C. Freshly prepared Stick reagent[47] (7.39 g, 27.2 mmol, 1.2 eq), $CuSO_4 \cdot 5H_2O$ (0.34 g, 1.4 mmol, 0.06 eq) and K_2CO_3 (6.59 g, 47.7 mmol, 2.1 eq) were added and the reaction was stirred for 5 h at rt. The reaction mixture was filtered and the resulting filtrate was acidified to pH 2 using 1 M HCl. The resulting solution was extracted with EtOAc (3x) and the combined organic layers were washed with saturated aqueous $NaHCO_3$ (2x) and brine (1x), dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude product **12** (7.26 g, 21.2 mmol, 93%) was used in the next step without any further purification. ^{1}H NMR (400 MHz, MeOD/$CDCl_3$) δ 3.90 (dd, J = 11.7, 4.0 Hz, 1H, H1A), 3.76 (dd, J = 11.7, 6.1 Hz, 1H, H1B), 3.62 – 3.55 (m, 2H, H3', H4'), 3.53 (m, 1H, H2), 1.66 – 1.46 (m, 2H, H-5'), 1.22 (m, 24H, 12xCH_2-alkyl), 0.87 – 0.81 (t, J = 8 Hz, 3H, H18). ^{13}C NMR (101 MHz, MeOD/$CDCl_3$) δ 74.9, 72.4 (C3, C4), 64.4 (C2), 61.8 (C1), 32.6 (CH_2), 32.2, 29.99, 29.96, 29.7, 26.1, 23.0 (12xCH_2-alkyl), 14.2 (C18). HRMS: not found

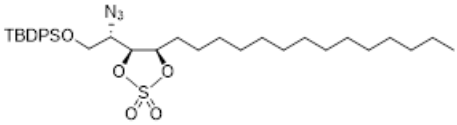
(2*S*,3*S*,4*R*)-2-Azido-1-((*tert*-butyldiphenylsilyl)oxy)octadecane-3,4-diol (13)



Compound **12** (7.26 g, 21.1 mmol) was dissolved in pyridine (106 mL) and cooled to 0 °C. DMAP (129 mg, 1.06 mmol, 0.05 eq) and TBDPSCl (6.05 mL, 23.3 mmol, 1.1 eq) were added and the reaction was stirred for 16 h at rt. The reaction mixture was diluted with EtOAc and washed with 1 M HCl (3x) and brine (1x). The organic layer was dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude product was purified by column chromatography (0% to 10% EtOAc in pentane) to give compound **13** (9.76 g, 16.8 mmol, 79%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.69 (d, J = 6.6 Hz, 4H, 4xCH-OTBDPS), 7.48 – 7.37 (m, 6H, 6xCH-OTBDPS), 4.03 (dd, J = 10.9, 4.1 Hz, 1H, H1A), 3.91 (dd, J = 10.9, 5.8 Hz, 1H, H1B), 3.67 (m, 2H, H3+H4), 3.56 (m, 1H, H2), 2.63 (s, 1H, OH), 2.10 (s, 1H, OH), 1.58-1.38 (m, 3H, H5 + H6A), 1.26 (m, 25H, H6B + 12xCH_2-alkyl), 1.08 (s, 9H, CH-OTBDPS), 0.88 (t, J = 8 Hz, 3H, H-18). ^{13}C NMR (101 MHz, $CDCl_3$) δ 135.8, 135.7, 132.7, 132.6, 130.2, 128.0 (10xCH-OTBDPS, 2xC_q-OTBDPS), 74.2, 72.5 (C3, C4), 64.3 (C1), 63.5 (C2), 31.9 (C5), 32.1, 29.83, 29.80, 29.76, 29.7, 29.5,

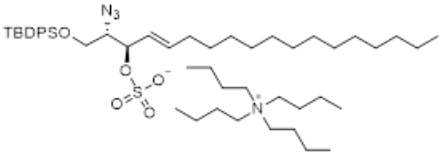
25.8, 22.8 (12xCH_2), 26.9 (3xCH_3-OTBDPS), 19.2 (C_q-OTBDPS), 14.3 (C18). HRMS: calcd. for $C_{34}H_{55}N_3O_3Si$ $[M+Na]^+$: 604.39049, found: 604.39045.

(4*S*,5*R*)-4-((*S*)-1-Azido-2-((*tert*-butyldiphenylsilyl)oxy)ethyl)-5-tetradecyl-1,3,2-dioxathiolane 2,2-dioxide (14)



Compound **13** (9.54 g, 16.4 mmol) was dissolved in CH_2Cl_2 (82 mL) and cooled to 0 °C. Et_3N (20.6 mL, 148 mmol, 9 eq) was added and subsequently $SOCl_2$ (1.43 mL, 19.7 mmol, 1.2 eq) was added dropwise whilst the flask was purged with N_2. The reaction was stirred for 30 min under N_2 atmosphere at 0 °C, after which the reaction mixture was poured into cooled brine and extracted with EtOAc (3x). The combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude intermediate was dissolved in a 1:1:1 mixture of EtOAc, MeCN and H_2O (110 mL) and cooled to 0 °C. $NaIO_4$ (10.5 g, 49.2 mmol, 3 eq) and $RuCl_3 \cdot H_2O$ (185 mg, 0.82 mmol, 0.05 eq) were added and the reaction was stirred for 2 h at rt. The reaction mixture was diluted with EtOAc and washed with saturated aqueous $Na_2S_2O_3$ (1x) and brine (1x). The organic layer was dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude product was purified by column chromatography (0% to 30% toluene in *n*-pentane) to give compound **14** (6.86 g, 10.7 mmol, 65%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.68 (m, 4H, 4xCH-OTBDPS), 7.50 – 7.39 (m, 6H, 6xCH-OTBDPS), 4.98 (ddd, *J* = 11.2, 5.2, 2.8 Hz, 1H, H4), 4.92 (dd, *J* = 10.1, 5.1 Hz, 1H, H3), 4.03 (dd, *J* = 11.3, 2.4 Hz, 1H, H1A), 3.89 (dd, *J* = 11.3, 5.2 Hz, 1H, H1B), 3.69 (ddd, *J* = 10.2, 5.2, 2.5 Hz, 1H, H2), 2.08 – 1.86 (m, 1H, H5A), 1.74 (m, 1H, H5B), 1.56 (m, 1H, H6A), 1.29-1.24 (m, 23H, H6B + 11xCH_2-alkyl), 1.09 (s, 9H, 3xCH_3-OTBDPS) 0.88 (t, *J* = 6.6 Hz, 3H, H18). ^{13}C NMR (101 MHz, $CDCl_3$) δ 135.73, 135.70, 132.3, 132.0, 130.31, 130.30, 128.14, 128.12 (12xCH and C_q-OTBDPS), 86.6 (C4), 80.0 (C3), 63.7 (C1), 59.3 (C2), 32.1, 30.3, 29.9, 29.8, 29.7, 29.6, 29.5, 29.4, 29.1, 22.9 (11x CH_2-alkyl), 28.3 (C5), 26.9 (3xCH_3-OTBDPS), 25.3 (C6), 19.3 (C_q-OTBDPS), 14.3 (C18). HRMS: calcd. for $C_{34}H_{53}N_3O_5SSi$ $[M+NH_4]^+$: 661.38134, Found: 661.38173

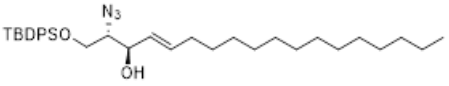
Tetrabutylammonium (2*S*,3*R*,*E*)-2-azido-1-((*tert*-butyldiphenylsilyl)oxy)octadec-4-en-3-yl sulfate (15)



Compound **14** (6.86 g, 10.7 mmol) was dissolved in toluene (107 mL). TBAI (4.33 g, 11.7 mmol, 1.1 eq) and DBU (2.4 mL, 16 mmol, 1.5 eq) were added and the reaction was refluxed for 90 min after which the reaction mixture was cooled to rt and

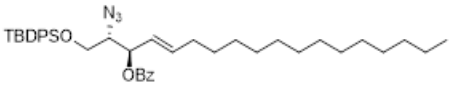
subsequently H_2O (0.2 mL), THF (2.8 mL) and concentrated H_2SO_4 (0.2 mL) were added and the reaction was stirred for 1 h at rt. The reaction mixture was diluted with EtOAc and washed with saturated aqueous $NaHCO_3$ (1x) and brine (1x). The organic layer was dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude product was purified by column chromatography (0% to 5% MeOH in CH_2Cl_2) to give compound **15** (8.92 g, 10.1 mmol, 95%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.74 – 7.63 (m, 4H, 4xCHAr-OTBDPS), 7.45 – 7.33 (m, 6H, 6xCHAr-OTBDPS), 5.66 (dt, *J* = 15.6, 6.7 Hz, 1H, H5), 5.43 (dd, *J* = 15.6, 7.8 Hz, 1H, H4), 4.78 (dd, *J* = 7.8, 3.3 Hz, 1H, H3), 4.33 (dt, *J* = 7.7, 3.8 Hz, 1H, H2), 3.72 (dd, *J* = 10.6, 4.1 Hz, 1H, H1A), 3.53 (dd, *J* = 10.6, 8.8 Hz, 1H, H1B), 3.30 – 3.21 (m, 8H, 4xCH_2-NBu_4^+), 1.92 (m, 2H, H6), 1.71 – 1.57 (m, 8H, 4xCH_2-NBu_4^+), 1.43 (s, *J* = 7.3 Hz, 8H, 4xCH_2-NBu_4^+) 1.22 (m, 22H, 11xCH_2-alkyl), 1.04 (s, 9H, 3xCH_3-OTBDPS), 1.00 (t, *J* = 7.3 Hz, 12H, 4x$CH_3NBu_4^+$), 0.88 (t, *J* = 6.6 Hz, 3H, H18). ^{13}C NMR (101 MHz, $CDCl_3$) δ 135.8, 133.4, 133.3, 129.73, 129.68, 127.80, 127.76 (12xCH and C_q-OTBDPS), 135.7 (C11), 125.1 (C10), 77.6 (C9), 67.1 (C8), 64.5 (C7), 58.8 (4xCH_2-NBu_4^+), 32.5 (C12), 32.0, 29.83, 29.80, 29.78, 29.7, 29.6, 29.5, 29.4, 29.0, 22.8, 19.8, 19.2 (11xCH_2-alkyl + CH_3-OTBDPS), 26.8 (3xCH_3-OTBDPS), 24.1 (4xCH_2-NBu_4^+), 19.2 (4xCH_2-NBu_4^+), 14.3 (C18'), 13.8 (4xCH_3-NBu_4^+). HRMS not found

(2*S*,3*R*,*E*)-2-Azido-1-((*tert*-butyldiphenylsilyl)oxy)octadec-4-en-3-ol (16)



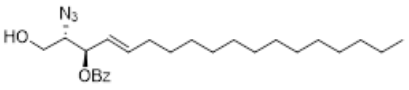
Compound **15** (8.93 g, 10.1 mmol) was dissolved in THF and cooled to 0 °C. 5 M HCl (17.5 mL) was added to the mixture and the reaction was stirred for 3 h at 40 °C. Another 8 mL of 5 M HCl was added and the reaction was stirred for another 1 h at 40 °C. The reaction was diluted with EtOAc and slowly quenched with saturated aqueous $NaHCO_3$ at 0 °C. The mixture was extracted with EtOAc (3x) and the combined organic layers were washed with brine (1x), dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude product was purified by column chromatography (0% to 5% EtOAc in pentane) to give compound **16** (5.04 g, 8.93 mmol, 89%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.73 – 7.65 (m, 4H, 4xCH-OTBDPS), 7.48 – 7.35 (m, 6H, 6xCH-OTBDPS), 5.78 – 5.69 (m, 1H, H5), 5.47 – 5.39 (m, 1H, H4), 4.25 – 4.19 (m, 1H, H3), 3,83 – 3.74 (m, 2H, H1), 3.53 – 3.48 (dt, 1H, H2), 2.14 – 2.10 (d, 1H, OH), 2.05 – 1.97 (m, 2H, H6), 1.37 – 1.20 (m, 22H, 11xCH_2-alkyl), 1.08 – 1.06 (s, 9H, 3xCH_3-OTBDPS), 0.90 – 0.85 (t, 3H, H18). ^{13}C NMR (101 MHz, $CDCl_3$) δ 135.73, 135.72, 135.6, 134.9, 132.9, 130.1, 129.8, 127.97, 127.95, 127.90 (12xCH-OTBDPS + C4 + C5), 73.0 (C3), 67.0 (C2), 64.3 (C1), 32.4, 32.1 (C6, C7), 29.8, 29.7, 29.6, 29.5, 29.3, 29.1, 22.9 (10xCH_2-alkyl), 26.9, 26.7 (3xCH_3-OTBDPS), 19.3 (C_q-OTBDPS), 14.3 (C18). HRMS: calcd. for $C_{34}H_{53}N_3O_2Si$ $[M+Na]^+$: 586.37993, Found: 586.37994

(2*S*,3*R*,*E*)-2-Azido-1-((*tert*-butyldiphenylsilyl)oxy)octadec-4-en-3-yl benzoate (17)



Compound **16** (5.04 g, 8.93 mmol) was dissolved in pyridine (90 mL) and cooled to 0 °C. BzCl (2.1 mL, 18 mmol, 2 eq) was added and the reaction was stirred for 2 h at rt. The reaction was quenched with MeOH and diluted with EtOAc. The reaction mixture was washed with 1 M HCl (3x) and brine (1x) and the organic layer was dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude product was purified by column chromatography (0% to 5% EtOAc in *n*-pentane) to give compound **17** (5.52 g, 8.26 mmol, 93%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.04 – 7.97 (m, 2H, 2xCH-OBz), 7.66 (m, 4H, 4xCH-OTBDPS), 7.60 – 7.53 (m, 1H, CH-OBz), 7.49 – 7.36 (m, 6H, 4xCH-OTBDPS + 2xCH-OBz), 7.36 – 7.28 (m, 2H, 2xCH-OTBDPS), 5.89 (dt, *J* = 15.4, 6.7 Hz, 1H, H5), 5.68 (dd, *J* = 8.0, 4.8 Hz, 1H, H3'), 5.50 (m, 1H, H4), 3.83 (dt, *J* = 6.7, 5.1 Hz, 1H, H2), 3.74 (d, *J* = 5.4 Hz, 1H, H1), 2.08 – 1.95 (m, 2H, H6), 1.42 – 1.16 (m, 22H, 11xCH_2-alkyl), 1.07 (s, 9H, 3xCH_3-OTBDPS), 0.95 – 0.81 (m, 3H, H18). ^{13}C NMR (101 MHz, $CDCl_3$) δ 165.3 (C_q-OBz), 138.7 (C5), 135.7, 133.2, 132.9, 132.8, 130.2, 130.0, 129.95, 129.9, 128.5, 128.0, 127.9 (12xC_q-OTBDPS + 6xCH-OBz), 123.3 (C4), 74.4 (C3), 65.9 (C2), 63.5 (C1), 32.5, 32.1, 29.83, 29.81, 29.7, 29.6, 29.5, 29.3, 28.83, 22.84 (11xCH_2-alkyl), 26.8, 19.2 (C_q-OTBDPS), 14.3 (C18). HRMS: calcd. for $C_{41}H_{57}N_3O_3Si$ $[M+NH_4]^+$: 685.45074, Found: 685.45082

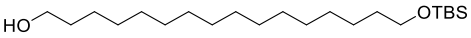
(2*S*,3*R*,*E*)-2-Azido-1-hydroxyoctadec-4-en-3-yl benzoate (18)



Compound **17** (4.85 g, 7.26 mmol) was dissolved in CH_2Cl_2 (73 mL) and cooled to -25 °C. $BF_3 \cdot$MeCN (15 mL, 18 mmol, 2.5 eq) was added and the reaction mixture was slowly warmed to 0 °C and stirred for 2 h. The reaction was then cooled again to -25 °C and another equivalent of $BF_3 \cdot$MeCN (6 mL, 7.2, 1 eq) was added. The reaction was again slowly warmed to 0 °C and stirred for 2 h. The reaction mixture was diluted with EtOAc and quenched with phosphate buffer (0.1 M, 7.4 pH). The layers were separated and the organic layer was washed with brine, dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude product was purified by column chromatography (0% to 10% EtOAc in *n*-pentane) to give sphingosine acceptor **18** (1.83 g, 4.26 mmol, 59%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.07 – 8.04 (m, 2H, 2xCH-OBz), 7.59 – 7.54 (m, 1H, CH-OBz), 7.47 – 7.42 (m, 2H, 2xCH-OBz), 6.01 – 5.89 (m, 1H, H5), 5.66 – 5.56 (m, 2H, H4 + H3), 3.85 – 3.80 (m, 1H, H2), 3.78 – 3.71 (dd, 1H, H1'A), 3.66 – 3.59 (dd, 1H, H1'B), 2.85 – 2.40 (br s, 1H, OH), 2.12 – 2.03 (m, 2H, H6), 1.46 – 1.34 (m, 2H, H7), 1.34 – 1.16 (m, 20H, 10xCH_2-alkyl), 0.91 – 0.82 (t, 3H, H18). ^{13}C NMR (101 MHz, $CDCl_3$) δ 165.6 (C_q-OBz), 138.9 (C5), 133.4, 129.9, 129.8, 128.6 (6xCH + C_q-OBz), 123.2

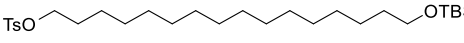
(C4), 74.8 (C3), 66.3 (C2), 62.0 (C1), 32.46 (C6), 32.0, 29.77, 29.75, 29.73, 29.66, 29.5, 29.4, 29.2, 28.8, 22.8 (11xCH_2-alkyl), 14.2 (C18). HRMS: calcd. for $C_{25}H_{39}N_3O_3$ $[M+Na]^+$: 452.28836, Found: 452.28823

16-((*Tert*-butyldimethylsilyl)oxy)hexadecan-1-ol (20)



$LiAlH_4$ (2 M in THF, 19.7 mL, 39.3 mmol, 2 eq) was dissolved in dry THF and cooled to 0 °C. 16-hexadecanolide (5.00 g, 19.7 mmol) dissolved in dry THF (40 mL) was added dropwise to the mixture over 30 min and the reaction was stirred for 1 h at 0 °C. The reaction was then refluxed at 72 °C for 2 h and quenched with H_2O (3 mL) and 3 M NaOH (3 mL) at 0 °C. $MgSO_4$ and Celite® were added and the suspension was filtered. The filtrate was concentrated *in vacuo* and the crude hexadecanediol was used in the next step without any further purification. Hexadecane-1,16-diol (10.8 g, 41.7 mmol) was dissolved in dry THF (300 mL) under inert atmosphere. Imidazole (5.68 g, 83.4 mmol, 2 eq) and TBSCl (6.29 g, 41.7 mmol, 1 eq) were added and the reaction was stirred for 16 h at rt. The reaction was quenched with saturated aqueous $NaHCO_3$ and the mixture was extracted with EtOAc (3x). The combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude product was purified by column chromatography (from 0% to 30 % Et_2O in *n*-pentane and 100% Et_2O to recover starting material) to give **20** (7.49 g, 20.1 mmol, 48%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.68 – 3.57 (m, 4H, H7 + H22), 1.65 – 1.45 (m, 4H, H8 + H21), 1.39 – 1.20 (m, 24H, 12xCH_2-alkyl), 0.89 (s, 9H, CH-OTBS), 0.05 (s, 6H, 2xCH_3-OTBS). ^{13}C NMR (101 MHz, $CDCl_3$) δ 63.5, 63.3 (C7 + C22), 33.04, 32.96 (C8 + C21), 29.81, 29.77, 29.75, 29.60, 29.58, , 26.0, 25.9 (12xCH_2-alkyl), 26.1 (3xCH_3-OTBS), 18.5 (C_q-OTBS), -5.1 (2xCH_3-OTBS). HRMS: calcd. for $C_{22}H_{48}O_2Si$ $[M+H]^+$: 373.34963, Found: 373.34963

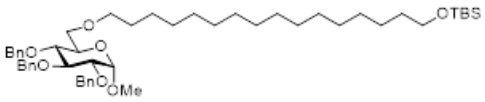
16-((*Tert*-butyldimethylsilyl)oxy)hexadecyl 4-methylbenzenesulfonate (21)



Compound **20** (16.4 g, 44.1 mmol) was dissolved in CH_2Cl_2 (300 mL). Et_3N (14.7 mL, 106 mmol, 2.4 eq), *p*-TsCl (16.80 g, 88.1 mmol, 2 eq) and DMAP (2.69 g, 22.0 mmol, 0.5 eq) were added and the reaction was stirred for 16 h at rt. All volatile materials were evaporated and the crude product was purified by column chromatography (0% to 5% Et_2O in *n*-pentane) to give **21** (15.9 g, 30.1 mmol, 68%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.82 – 7.76 (m, 2H, 2xCH-OTs), 7.38 – 7.31 (m, 2H, 2xCH-OTs), 4.02 (t, J = 6.5 Hz, 2H, H7), 3.60 (t, J = 6.7 Hz, 2H. H22), 2.45 (s, 3H, CH_3-OTs), 1.68 – 1.58 (m, 2H, H8), 1.56 – 1.45 (m, 2H, H21), 1.41 – 1.13 (m, 24H, 12xCH_2-alkyl), 0.89 (s, 9H, 3xCH_3-OTBS), 0.05 (s, 6H, 2xCH_3-OTBS). ^{13}C NMR (101 MHz, $CDCl_3$) δ 144.7, 133.4 (2xC_q-OTs), 129.9, 128.0 (4xCH-OTs), 70.9 (C7), 63.5 (C22), 33.0 (C21), 29.82, 29.80, 29.76, 29.7, 29.6, 29.5, 29.1, 26.0, 25.5 (12xCH_2-alkyl), 29.0 (C8), 26.1

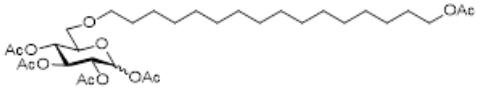
(3xCH_3-OTBS), 21.9 (CH_3-OTs), 18.5 (C_q-OTBS), -5.1 (2xCH_3-OTBS). HRMS: calcd. for $C_{29}H_{54}O_4SSi$ $[M+H]^+$: 527.35848, Found: 527.35846

Tert-butyldimethyl((16-(((2*R*,3*R*,4*S*,5*R*,6*S*)-3,4,5-tris(benzyloxy)-6-methoxytetrahydro-2*H*-pyran-2-yl)methoxy)hexadecyl)oxy)silane (22)



Protected sugar **5** (11.37 g, 24.5 mmol) was dissolved in dry DMF (200 mL) under inert atmosphere and cooled to 0 °C. NaH (60% dispersion in mineral oil, 1.96 g, 48.9 mmol, 2 eq) was added and the reaction stirred for 10 min. The compound **21** (15.5 g, 29.4 mmol, 1.2 eq) was added at 0 °C in dry DMF (50 mL) and the reaction was stirred for 45 min. The reaction was then slowly warmed to rt and stirred for 18 h at this temperature. The reaction was quenched with MeOH at 0 °C and diluted with Et_2O. The mixture was washed with H_2O (3x) and brine (1x) and the combined aqueous layers were extracted with Et_2O (1x). The combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude product was purified by column chromatography (10% to 40% Et_2O in *n*-pentane) to give **22** (11.6 g, 14.2 mmol, 58%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.40 – 7.26 (m, 15H, 15xCH-OBn), 4.97 (d, *J* = 10.8 Hz, 1H, CH_2-OBn), 4.91 – 4.76 (m, 3H, CH_2-OBn), 4.70 – 4.56 (m, 3H, CH_2-OBn + H1), 3.98 (t, *J* = 8.0 Hz, 1H, H3), 3.75 – 3.44 (m, 8H, H2 + H4 + H5 + H6 + CH_2-alkyl), 3.36 (s, 3H, CH_3-OMe), 3.36 – 3.30 (m, 1H, CH_2-alkyl), 1.65 – 1.46 (m, 4H, 2xCH_2-alkyl), 1.39 – 1.17 (m, 24H, 12xCH_2-alkyl), 0.89 (s, 9H, 3xCH_3-OTBS), 0.05 (s, 6H, 2xCH_3-OTBS). ^{13}C NMR (101 MHz, $CDCl_3$) δ 138.9, 138.6, 138.3, 128.57, 128.55, 128.5, 128.3, 128.2, 128.0, 127.9, 127.8, 127.7 (18xCH + C_q-OBn), 98.3 (C1), 82.3 (C3), 79.9 (C2), 77.8 (C4), 75.9, 75.2, 73.5 (3xCH_2-OBn), 70.1 (C5), 71.9, 69.3, 63.5 (C6 + C7 + C22), 55.3 (CH_3-OMe), 33.0, 29.82, 29.79, 29.8, 29.7, 29.6, 26.3, 25.9 (14xCH_2-alkyl), 26.1 (3xCH_3-OTBS), 18.5 (C_q-OTBS), -5.1 (2xCH_3-OTBS). HRMS: calcd. for $C_{50}H_{78}O_7Si$ $[M+NH_4]^+$: 836.58551, Found: 836.58547

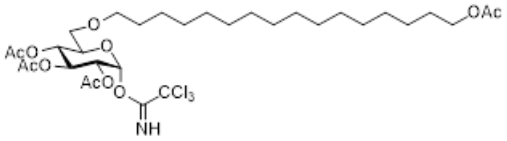
(3*R*,4*S*,5*R*,6*R*)-6-(((16-Acetoxyhexadecyl)oxy)methyl)tetrahydro-2*H*-pyran-2,3,4,5-tetrayl tetraacetate (24)



Alkylated sugar **22** (362 mg, 0.44 mmol) was dissolved in a 1:1 mixture (14 mL) of EtOAc and EtOH and a drop of concentrated HCl was added. The mixture purged with argon gas for 10 min whilst stirring. Pd/C (10 wt%) (94 mg, 9 µmol, 0.2 eq) was added and the mixture was purged with argon gas for an additional 10 min. H_2 gas was then introduced and the reaction was stirred for 4 h at rt. The reaction mixture was filtered and the filtrate was concentrated *in vacuo*. The crude product was used in the next step

without any further purification. Crude compound **23** (120 mg, 277 µmol) was dissolved in a 1:1 mixture (5.4 mL) of Ac_2O and AcOH and cooled to 0 °C. Concentrated H_2SO_4 (60 µL, 1.1 mmol, 4 eq) was added dropwise to the solution and the reaction was stirred for 20 h at rt. The reaction was cooled to 0 °C and slowly quenched with saturated aqueous $NaHCO_3$ and the mixture was extracted with CH_2Cl_2 (3x). The combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude product was purified by column chromatography (0% to 35% EtOAc in *n*-pentane) to give the peracetylated sugar **24** (121 mg, 191 µmol, 43% over two steps as an α:β mixture 2:1). α-anomer: ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.33 (d, J = 3.7 Hz, 1H, H1), 5.55 – 5.39 (m, 1H, H3), 5.33 – 4.99 (m, 2H, H2 + H4), 4.17 – 3.95 (m, 3H, H5 + H22), 3.64 – 3.25 (m, 4H, H6 + H7), 2.25 – 1.94 (m, 15H, 5xCH_3-OAc), 1.68 – 1.46 (m, 4H, H8 + H21), 1.37 – 1.16 (m, 24H, 12xCH_2-alkyl). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.5, 169.8, 169.5, 169.0 (5xC_q-OAc), 89.3 (C1), 72.3 (C7), 71.3 (C5), 70.1 (C3), 69.4 (C2), 69.2 (C6), 68.9 (C4), 64.8 (C22), 29.78, 29.75, 29.7, 29.63, 29.58, 29.4, 28.7, 26.04, 26.02 (14xCH_2-alkyl), 21.1, 21.03, 20.83, 20.79, 20.6 (5xCH_3-OAc). β-anomer: ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.70 (d, J = 8.2 Hz, 1H, H1), 5.32 – 4.99 (m, 3H, H2 + H3 + H4), 4.13 – 3.94 (m, 2H, H22), 3.83 – 3.70 (m, 1H, H5), 3.63 – 3.28 (m, 4H, H6 + H7), 2.28 – 1.93 (m, 15H, 5xCH_3-OAc), 1.67 – 1.46 (m, 4H, H8 + H21), 1.26 (d, J = 4.5 Hz, 24H, 12xCH_2-alkyl). ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.4, 170.4, 169.5, 169.4, 169.1 (5xC_q-OAc), 91.9 (C1), 74.1 (C5), 73.1 (C3), 72.3 (C7), 70.50 (C2), 69.15 (C6), 68.87 (C4), 64.8 (C22), 29.78, 29.75, 29.7, 29.63, 29.58, 29.4, 28.7, 26.04, 26.02 (14xCH_2-alkyl), 20.9, 20.83, 20.79, 20.73, 20.69 (5xCH_3-OAc). HRMS: calcd. for $C_{32}H_{54}O_{12}$ $[M+NH_4]^+$: 648.39535, Found: 648.39506

(2*R*,3*R*,4*S*,5*R*,6*S*)-2-(((16-Acetoxyhexadecyl)oxy)methyl)-6-(2,2,2-trifluoro-1-iminoethoxy)tetrahydro-2*H*-pyran-3,4,5-triyl triacetate (26)

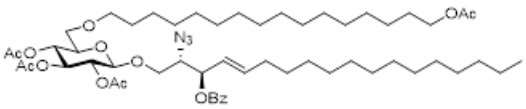


Peracetylated sugar **24** (3.34 g, 5.29 mmol) **25** was dissolved in dry THF (50 mL) and cooled to 0 °C. DMAPA was added (1.3 mL, 10.6 mmol, 2 eq) and the reaction was stirred for 6 h at rt. The reaction was diluted with EtOAc and washed with 1 M HCl (2x) and brine (1x) and the aqueous layers were extracted with EtOAc (1x). The combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude product was used in the next step without any further purification. Compound **25** (3.11 g, 5.29 mmol) was dissolved in dry CH_2Cl_2 (20 mL) under inert atmosphere and cooled to 0 °C. CCl_3CN (1.6 mL, 16 mmol, 3 eq) and Cs_2CO_3 (689 mg, 2.12 mmol, 0.4 eq) was added and the reaction was stirred for 3 h at rt. The reaction mixture was diluted with CH_2Cl_2 and washed with saturated aqueous $NaHCO_3$. The organic layer was dried

over $MgSO_4$, filtered and concentrated *in vacuo*. The crude product was purified by column chromatography (0% to 35% EtOAc in *n*-pentane) to give imidate donor **3** (1.67 g, 2.28 mmol, 43% over two steps). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.65 (s, 1H, NH), 6.56 (d, *J* = 3.7 Hz, 1H, H1), 5.56 (t, *J* = 9.8 Hz, 1H, H3), 5.22 (dd, *J* = 10.2, 9.5 Hz, 1H, H4), 5.12 (dd, *J* = 10.2, 3.7 Hz, 1H, H2), 4.18 – 4.10 (m, 1H, H5), 4.05 (t, *J* = 6.8 Hz, 2H, H22), 3.61 – 3.47 (m, 2H, H6), 3.47 – 3.31 (m, 2H, H7), 2.07 – 1.96 (m, 12H, 4xCH_3-OAc), 1.66 – 1.58 (m, 2H, H21), 1.58 – 1.49 (m, 2H, H8), 1.40 – 1.18 (m, 24H, 12xCH_2-alkyl). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.3, 170.0, 169.6 (4xC_q-OAc), 161.0 (C=NH), 93.2 (C1), 72.2 (C7), 71.5 (C5), 70.2 (C3), 70.0 (C2), 68.9 (C6), 68.7 (C4), 64.8 (C22), 29.82, 29.79, 29.73, 29.67, 29.6, 29.4, 28.7, 26.10, 26.06 (C8 + C21 + 12xCH_2-alkyl), 21.2, 20.89, 20.85, 20.6 (4xCH_3-OAc). HRMS: calcd. for $C_{32}H_{52}Cl_3NO_{11}$ $[M+Na]^+$: 754.24982, Found: 754.24919

Synthesis of glucosylceramide

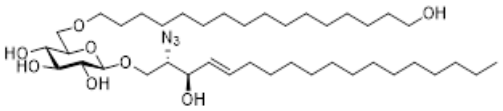
(2*R*,3*R*,4*S*,5*R*,6*R*)-2-(((16-Acetoxyhexadecyl)oxy)methyl)-6-(((2*S*,3*R*,*E*)-2-azido-3-(benzoyloxy)octadec-4-en-1-yl)oxy)tetrahydro-2*H*-pyran-3,4,5-triyl triacetate (27)



Imidate donor **26** (1.64 g, 2.24 mmol) was dried azeotropically with toluene and dissolved in dry CH_2Cl_2 (25 mL) in a flame dried flask containing activated mol sieves (4 Å). Azeotropically dried sphingosine acceptor **18** (1.48 g, 3.45 mmol, 1.5 eq) was added and the solution was cooled to -50 °C. TMSOTf (80 µL, 44 µmol, 0.2 eq) was added and the reaction was stirred for 2 h at –50 °C and closely monitored by TLC. The reaction was quenched on ice with saturated aqueous $NaHCO_3$ and diluted with CH_2Cl_2. The mixture was washed with saturated aqueous $NaHCO_3$ (3x) and brine (1x) and the combined aqueous layers were extracted with CH_2Cl_2 (1x). The combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude product was purified by column chromatography (10% to 20% EtOAc in *n*-pentane) to give glucosylsphingosine **27** (1.67 g, 1.67 mmol, 74%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.09 – 8.02 (m, 2H, 2xCH-OBz), 7.61 – 7.54 (m, 1H, CH-OBz), 7.48 – 7.43 (m, 2H, 2xCH-OBz), 5.92 (dt, *J* = 14.6, 6.7 Hz, 1H, H5'), 5.65 – 5.50 (m, 2H, H3' + H4'), 5.20 (t, *J* = 9.5 Hz, 1H, H3), 5.09 – 4.96 (m, 2H, H2 + H4), 4.52 (d, *J* = 7.9 Hz, 1H, H1), 4.05 (t, *J* = 6.8 Hz, 2H, H22), 3.99 – 3.88 (m, 2H, H1'A + H2'), 3.63 (m, 1H, H5), 3.57 (dd, J = 10.0, 5.7 Hz, 1H, H1'B), 3.55 – 3.29 (m, 4H, H6 + H7), 2.15 – 1.97 (m, 14H, H6' + 4xCH_3-OAc), 1.67 – 1.57 (m, 2H, H21), 1.56 – 1.45 (m, 2H, H8), 1.45 – 1.15 (m, 46H, 23xCH_2-alkyl), 0.90 – 0.85 (m, 3H, H18'). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.5, 169.6, 169.5 (4xC_q-OAc), 165.2 (C_q-OBz), 139.2 (C5'), 133.3, 130.1, 129.9, 128.6 (5xCH-OBz), 122.8 (C4'), 100.6 (C1), 74.9 (C3'), 73.7 (C5), 73.1 (C3), 72.3 (C7), 71.3 (C2), 70.0 (C6),

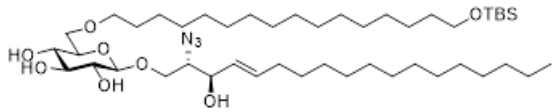
69.5 (C4), 68.2 (C1'), 64.8 (C22), 63.6 (C2'), 32.1 (C6'), 32.5, 29.84, 29.81, 29.8, 29.73, 29.71, 29.67, 29.64, 29.57, 29.5, 29.4, 29.3, 28.9, 28.7, 26.11, 26.05, 22.8 (C21 + C8 + 23xCH_2-alkyl), 21.2, 20.84, 20.80 (4xCH_3-OAc), 14.3 (C18'). HRMS: calcd. for $C_{55}H_{89}N_3O_{13}$ $[M+Na]^+$: 1022.62876, Found: 1022.62866

(2*R*,3*R*,4*S*,5*S*,6*R*)-2-(((2*S*,3*R*,*E*)-2-Azido-3-hydroxyoctadec-4-en-1-yl)oxy)-6-(((16-hydroxyhexadecyl)oxy)methyl)tetrahydro-2*H*-pyran-3,4,5-triol (28)



Compound **27** (1.69 g, 1.69 mmol) was dissolved in a 1:1 mixture (30 mL) of MeOH and CH_2Cl_2. A few catalytic drops of NaOMe (4.3 M in MeOH) were added and the reaction was stirred for 6.5 h at rt. The reaction was quenched with Amberlite™ IRC-120(H) until neutral pH, filtered and concentrated *in vacuo*. The crude product was purified by column chromatography (0% to 5% MeOH in CH_2Cl_2) to give **28** (1.16 g, 1.59 mmol, 94%). ^{1}H NMR (400 MHz, MeOD/$CDCl_3$) δ 5.90 – 5.69 (m, 1H, H5'), 5.53 (ddt, *J* = 15.3, 7.5, 1.4 Hz, 1H, H4'), 4.28 (d, *J* = 7.7 Hz, 1H, H1), 4.21 (t, *J* = 6.9 Hz, 1H, H3'), 3.98 (dd, *J* = 10.8, 6.0 Hz, 1H, H1'A), 3.83 – 3.74 (m, 2H, H1'B + H6A), 3.68 – 3.46 (m, 6H, H6B + H7 + H22 + H2'), 3.46 – 3.25 (m, 4H, H2 + H3 + H4 + H5), 2.08 (q, *J* = 6.8 Hz, 2H, H6'), 1.68 – 1.48 (m, 4H, H8 + H21), 1.45 – 1.16 (m, 46H, 23xCH_2-alkyl), 0.94 – 0.81 (m, 3H, H18'). ^{13}C NMR (101 MHz, MeOD/$CDCl_3$) δ 134.9 (C5'), 127.9 (C4'), 102.8 (C1), 76.3, 74.9, 70.4, 65.3 (C3 + C4 + C5 + C2'), 73.1 (C2), 71.8 (C7), 71.5 (C3'), 70.1 (C6), 68.8 (C1'), 62.0 (C22), 32.2, 32.1, 31.6, 29.39, 29.36, 29.33, 29.27, 29.22, 29.20, 29.1, 29.0, 28.8, 25.7, 25.5, 22.4 (C6' + C8 + C21 + 23xCH_2-alkyl), 13.6 (C18'). HRMS: calcd. for $C_{40}H_{77}N_3O_8$ $[M+NH_4]^+$: 745.60489, Found: 745.60454

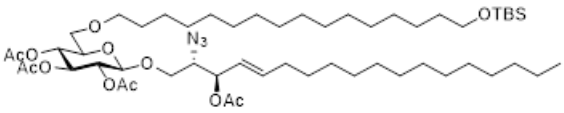
(2*R*,3*R*,4*S*,5*S*,6*R*)-2-(((2*S*,3*R*,*E*)-2-Azido-3-hydroxyoctadec-4-en-1-yl)oxy)-6-(((16-((*tert*-butyldimethylsilyl)oxy)hexadecyl)oxy)methyl)tetrahydro-2*H*-pyran-3,4,5-triol (29)



Compound **28** (1.08 g, 1.48 mmol) was dissolved in dry pyridine (15 mL) and cooled to 0 °C. TBSCl (245 mg, 1.63 mmol, 1.1 eq) was added and the reaction was stirred for 3 h at rt. The reaction was diluted with EtOAc, quenched with saturated aqueous $NaHCO_3$ on ice and washed with 1 M HCl (3x) and brine (1x). The combined aqueous layers were extracted with EtOAc (1x) and the combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude product was purified by column chromatography (0% to 5% MeOH in CH_2Cl_2) to give TBS-protected glucosylsphingosine **29** (1.11 g, 1.32 mmol, 89%). ^{1}H NMR (400 MHz, MeOD/$CDCl_3$) δ 5.85 – 5.72 (m, 1H, H5'), 5.57 – 5.44 (m, 1H,

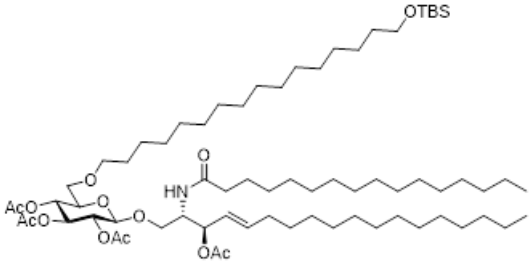
H4'), 4.28 (d, *J* = 7.7 Hz, 1H, H1), 4.23 – 4.17 (m, 1H, H3'), 3.95 (dd, *J* = 10.7, 6.5 Hz, 1H, H1'A), 3.84 – 3.73 (m, 2H, H1'B + H6A), 3.67 – 3.47 (m, 5H, H6B + H22 + H5 + H2'), 3.46 – 3.23 (m, 5H, H2 + H3 + H4 + H7), 2.10 – 2.03 (m, 2H, H6'), 1.67 – 1.47 (m, 4H, H8 + H21), 1.47 – 1.21 (m, 46H, 23xCH_2-alkyl), 0.95 – 0.83 (m, 12H, H18' + 3xCH_3-OTBS), 0.07 (s, 6H, 2xCH_3-OTBS). ^{13}C NMR (101 MHz, MeOD/$CDCl_3$) δ 135.6 (C5'), 128.7 (C4'), 103.6 (C1), 77.2, 76.0, 71.2 (C3 + C4 + C5), 74.0 (C2), 72.6 (C7), 72.5 (C3'), 70.9 (C6), 69.6 (C1'), 66.3 (C2'), 64.1 (C22), 33.3, 32.9, 32.5, 30.22, 30.19, 30.16, 30.13, 30.06, 29.94, 29.89, 29.8, 29.60, 26.56, 26.29 (C6 + C8 + C21 + 23xCH_2-alkyl), 26.26 (3xCH_3-OTBS), 23.2 (C_q-OTBS), 14.3 (C18'), -5.1 (2xCH_3-OTBS). HRMS: calcd. for $C_{46}H_{91}N_3O_8Si$ $[M+Na]^+$: 864.64676, Found: 864.64674

(2*R*,3*R*,4*S*,5*R*,6*R*)-2-(((2*S*,3*R*,*E*)-3-Acetoxy-2-azidooctadec-4-en-1-yl)oxy)-6-(((16-((*tert*-butyldimethylsilyl)oxy)hexadecyl)oxy)methyl)tetrahydro-2*H*-pyran-3,4,5-triyl triacetate (30)



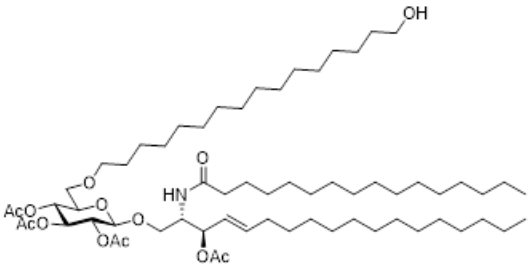
Compound **29** (0.95 g, 1.12 mmol) was dissolved in dry pyridine (15 mL) and cooled to 0 °C. Ac_2O (1.25 mL, 13.5 mmol, 12 eq) was added and the reaction was stirred for 22 h at rt. The reaction was quenched on ice with saturated aqueous $NaHCO_3$ and diluted with EtOAc. The mixture was washed with 1 M HCl (3x) and brine (1x) and the organic layer was dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude product was purified by column chromatography (5% to 20% EtOAc in *n*-pentane) to give intermediate **30** (1.20 g, 1.12 mmol, quantitative yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.82 (dt, J = 15.4, 6.7 Hz, 1H, H5'), 5.48 – 5.37 (m, 1H, H4'), 5.31 (dd, *J* = 8.2, 4.2 Hz, 1H, H3'), 5.19 (t, *J* = 9.5 Hz, 1H, H3), 5.07 – 4.93 (m, 2H, H2 + H4), 4.50 (d, *J* = 8.0 Hz, 1H, H1), 3.85 (dd, *J* = 10.2, 6.7 Hz, 1H, H1'A), 3.80 – 3.73 (m, 1H, H2'), 3.67 – 3.34 (m, 8H, H1'B + H5 + H6 + H7 + H22), 2.14 – 1.92 (m, 14H, 4xCH_3-OAC + H6'), 1.64 – 1.45 (m, 4H, H8 + H21), 1.42 – 1.19 (m, 46H, 23xCH_2-alkyl), 0.93 – 0.78 (m, 12H, 3xCH_3-OTBS + H18'), 0.05 (s, 6H, 2xCH_3-OTBS). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.5, 169.62, 169.58, 169.4 (4xC_q-OAc), 138.9 (C5'), 122.9 (C4'), 100.4 (C1), 74.2 (C3'), 73.7 (C5), 73.0 (C3), 72.3 (C7), 71.3 (C2), 70.0 (C6), 69.5 (C4), 68.0 (C1'), 63.5 (C22), 63.2 (C2'), 33.0, 32.5, 32.1, 29.83, 29.81, 29.78, 29.75, 29.7, 29.63, 29.58, 29.56, 29.5, 29.3, 28.9, 26.13, 25.9, 22.8 (C6' + C8 + C21 + 23xCH_2-alkyl), 26.11 (3xCH_3-OTBS), 21.2, 20.81, 20.77 (4xCH_3-OAc), 18.5 (C_q-OTBS), 14.3 (C18'), -5.1 (2xCH_3-OTBS). HRMS: calcd. for $C_{54}H_{99}N_3O_{12}Si$ $[M+Na]^+$: 1032.68902, Found: 1032.69029

(2*R*,3*R*,4*S*,5*R*,6*R*)-2-(((2*S*,3*R*,*E*)-3-Acetoxy-2-palmitamidooctadec-4-en-1-yl)oxy)-6-(((16-((*tert*-butyldimethylsilyl)oxy)hexadecyl)oxy)methyl)tetrahydro-2*H*-pyran-3,4,5-triyl triacetate (31)



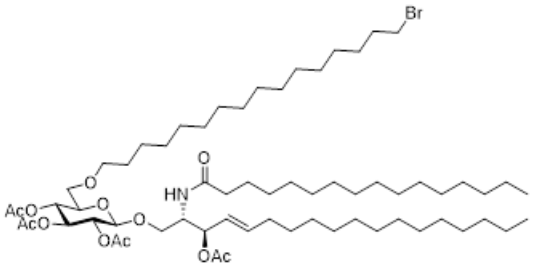
Palmitic acid (358 mg, 1.39 mmol, 1.2 eq) was suspended in dry MeCN (12 mL) under inert atmosphere and cooled to 0 °C. HOBt (314 mg, 2.23 mmol, 2 eq) and EDC (445 mg, 2.23 mmol, 2 eq) were added and stirred for 10 min at 0 °C. Intermediate **30** (1.17 g, 1.16 mmol) in dry CH_2Cl_2 (15 mL) and $P(Bu)_3$ (0.4 mL, 1.7 mmol, 1.5 eq) were added and the reaction was stirred for 16 h at rt in the dark. All volatile materials were evaporated and the crude product was purified by column chromatography (10% to 30% EtOAc in *n*-pentane) to give protected glucosylceramide **31** (897 mg, 734 µmol, 63%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.86 – 5.68 (m, 2H, H5' + NH), 5.41 – 5.31 (m, 1H, H4'), 5.26 (t, *J* = 7.0 Hz, 1H, H3'), 5.19 (t, *J* = 9.5 Hz, 1H, H3), 5.04 – 4.89 (m, 2H, H2 + H4), 4.45 (d, *J* = 7.9 Hz, 1H, H1), 4.33 – 4.24 (m, 1H, H2'), 3.94 (dd, *J* = 10.1, 4.2 Hz, 1H, H1'A), 3.68 – 3.31 (m, 8H, H1'B + H5 + H6 + H7 + H22), 2.21 – 2.07 (m, 2H, H20'), 2.07 – 1.97 (m, 14H, 4xCH_3-OAc + H6'), 1.69 – 1.44 (m, 6H, H21' + H8 + H21), 1.40 – 1.17 (m, 70H, 35xCH_2-alkyl), 0.94 – 0.82 (m, 15H, H18' + H34' + 3xCH_3-OTBS), 0.05 (s, 6H, 2xCH_3-OTBS). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.9 (C_q-CONH), 170.4, 170.1, 169.6 (4xC_q-OAc), 137.2 (C5'), 124.8 (C4'), 100.6 (C1), 73.9 (C3'), 73.4 (C5), 72.9 (C3), 72.2 (C7), 71.5 (C2), 70.0 (C6), 69.5 (C4), 67.3 (C1'), 63.5 (C22), 50.8 (C2'), 37.0, 33.0, 32.5, 32.1, 29.9, 29.83, 29.80, 29.78, 29.74, 29.71, 29.67, 29.6, 29.51, 29.49, 29.4, 29.3, 29.1, 26.2, 25.9, 25.8, 22.8 (C6' + C20' + C21' + C8 + C21 + 35xCH_2-alkyl), 26.1 (3xCH_3-OTBS), 21.3, 20.9, 20.83, 20.78 (4xCH_3-OAc), 14.3 (C18' + C34'), -5.1 (2xCH_3-OTBS). HRMS not found

(2*R*,3*R*,4*S*,5*R*,6*R*)-2-(((2*S*,3*R*,*E*)-3-Acetoxy-2-palmitamidooctadec-4-en-1-yl)oxy)-6-(((16-hydroxyhexadecyl)oxy)methyl)tetrahydro-2*H*-pyran-3,4,5-triyl triacetate (32)



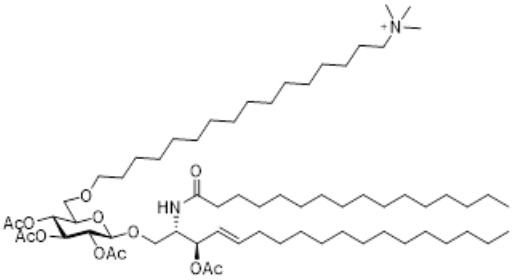
Protected glucosylceramide **31** (897 mg, 730 µmol) was dissolved in dry THF and cooled to 0 °C. TBAF (1 M in THF, 1.5 mL, 1.5 mmol, 2 eq) was added and the reaction was stirred for 22 h at rt. The reaction mixture was diluted with EtOAc and washed with saturated aqueous $NaHCO_3$ (3x) and brine (1x) and the combined aqueous layers were extracted with EtOAc (1x). The combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude product was purified by column chromatography (10% to 40% EtOAc in *n*-pentane) to give glucosylceramide **32** (605 mg, 550 µmol, 74%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.93 (d, *J* = 8.9 Hz, 1H, NH), 5.77 (dt, *J* = 15.3, 6.8 Hz, 1H, H5'), 5.43 – 5.31 (m, 1H, H4'), 5.26 (t, *J* = 7.0 Hz, 1H, H3'), 5.20 (t, *J* = 9.5 Hz, 1H, H3), 5.01 (t, *J* = 9.6 Hz, 1H, H4), 4.94 (dd, *J* = 9.7, 7.9 Hz, 1H, H2), 4.46 (d, *J* = 7.9 Hz, 1H, H1), 4.35 – 4.21 (m, 1H, H2'), 3.93 (dd, *J* = 10.2, 4.3 Hz, 1H, H1'A), 3.68 – 3.55 (m, 4H, H1'B + H5 + H22), 3.55 – 3.33 (m, 4H, H6 + H7), 2.25 – 2.07 (m, 2H, H20'), 2.07 – 1.96 (m, 14H, 4xCH_3-OAc + H6'), 1.66 – 1.44 (m, 6H, H21' + H8 + H21), 1.43 – 1.12 (m, 70H, 35xCH_2-alkyl), 0.96 – 0.82 (m, 6H, H18'+ H34'). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.9 (C_q-CONH), 170.3, 170.0, 169.54, 169.50 (4xC_q-OAc), 137.0 (C5'), 124.7 (C4'), 100.4 (C1), 73.7 (C3'), 73.3 (C5), 72.8 (C3), 72.1 (C7), 71.4 (C2), 69.8 (C6), 69.4 (C4), 67.2 (C1'), 62.9 (C22), 50.7 (C2'), 36.8, 32.8, 32.4, 32.0, 29.73, 29.68, 29.63, 29.61, 29.59, 29.54, 29.50, 29.47, 29.39, 29.36, 29.3, 29.0, 26.0, 25.8, 25.7, 22.7 (C6' + C20' + C21'+ C8 + C21 + 35xCH_2-alky), 21.1, 20.74, 20.68, 20.6 (4xCH_3-OAc), 14.1 (H18'+ H34'). HRMS: calcd. for $C_{64}H_{117}NO_{13}$ $[M+H]^+$: 1108.85977, Found: 1108.86109

(2*R*,3*R*,4*S*,5*R*,6*R*)-2-(((2*S*,3*R*,*E*)-3-Acetoxy-2-palmitamidooctadec-4-en-1-yl)oxy)-6-(((16-bromohexadecyl)oxy)methyl)tetrahydro-2*H*-pyran-3,4,5-triyl triacetate (33)



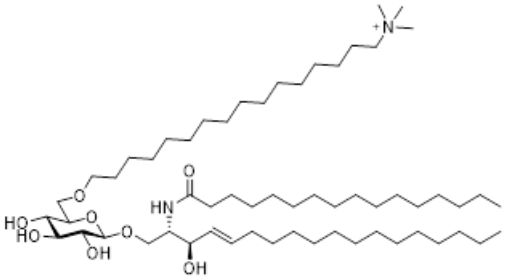
Glucosylceramide **32** (214 mg, 190 µmol) was dissolved in dry CH_2Cl_2 (2 mL). PPh_3 (61 mg, 230 µmol, 1.2 eq) and CBr_4 (70 mg, 210 µmol, 1.1 eq) were added and the reaction was stirred for 16 h at rt. The reaction mixture was diluted with CH_2Cl_2 and washed with H_2O (1x) and brine (1x). The aqueous layers were extracted with CH_2Cl_2 (1x) and the combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude product was purified by silica column chromatography (10% to 25% EtOAc in *n*-pentane) to give bromide **33** (190 mg, 160 µmol, 84%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.84 – 5.71 (m, 2H, H5' + NH), 5.41 – 5.32 (m, 1H, H4'), 5.26 (t, *J* = 7.0 Hz, 1H, H3'), 5.19 (t, *J* = 9.5 Hz, 1H, H3), 5.01 (t, *J* = 9.7 Hz, 1H, H4), 4.94 (dd, *J* = 9.7, 7.9 Hz, 1H, H2), 4.45 (d, *J* = 7.9 Hz, 1H, H1), 4.33 – 4.24 (m, 1H, H2'), 3.93 (dd, *J* = 10.2, 4.2 Hz, 1H, H1'A), 3.67 – 3.56 (m, 2H, H1'B + H5), 3.55 – 3.32 (m, 6H, H6 + H7 + H22), 2.19 – 2.07 (m, 2H, H20'), 2.06 – 1.94 (m, 14H, 4xCH_3-OAc + H6'), 1.85 (dt, *J* = 14.5, 6.9 Hz, 2H, H21), 1.66 – 1.48 (m, 4H, H21' + H8), 1.41 (q, *J* = 7.1 Hz, 2H, H20), 1.37 – 1.10 (m, 68H, 34xCH_2-alkyl), 0.88 (t, *J* = 6.9 Hz, 6H, H18' + H34'). ^{13}C NMR (126 MHz, $CDCl_3$) δ 172.9 (C_q-CONH), 170.4, 170.1, 169.63, 169.61 (4xC_q-OAc), 137.2 (C5'), 124.8 (C4'), 100.6 (C1), 73.9 (C3'), 73.5 (C5), 72.9 (C3), 72.3 (C7), 71.6 (C2), 70.0 (C6), 69.5 (C4), 67.3 (C1'), 50.9 (C2'), 37.0 (C20'), 34.2 (C22), 33.0 (C21), 32.5 (C6'), 32.1, 29.9, 29.84, 29.82, 29.78, 29.76, 29.72, 29.70, 29.68, 29.6, 29.5, 29.4, 29.2, 28.9, 28.3, 26.2, 25.7, 22.8 (C21' + C8 + C20 + 34xCH_2-alkyl), 21.3, 20.9, 20.84, 20.79 (4xCH_3-OAc), 14.3 (C18'+ C34'). HRMS not found

_N_,_N_,_N_-Trimethyl-16-(((2_R_,3_R_,4_S_,5_R_,6_R_)-3,4,5-triacetoxy-6-(((2_S_,3_R_,_E_)-3-acetoxy-2-palmitamidooctadec-4-en-1-yl)oxy)tetrahydro-2_H_-pyran-2-yl)methoxy)hexadecan-1-aminium (34)



Bromide **33** (170 mg, 145 µmol) was dissolved in dry MeCN and NMe_3 (40% w/w in H_2O, 50 µL, 290 µmol, 2 eq) was added. The reaction was stirred for 16 h at 50 °C, after which the reaction mixture was concentrated *in vacuo* and the crude product was purified by column chromatography (0% to 7% MeOH in CH_2Cl_2) to give compound **34** (139 mg, 117 µmol, 81%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.85 – 5.71 (m, 2H, H5'+ NH), 5.37 (dd, *J* = 15.4, 7.5 Hz, 1H, H4'), 5.26 (t, *J* = 7.0 Hz, 1H, H3'), 5.19 (t, *J* = 9.5 Hz, 1H, H3), 5.01 (t, *J* = 9.6 Hz, 1H, H4), 4.93 (dd, *J* = 9.6, 7.9 Hz, 1H, H2), 4.45 (d, *J* = 7.9 Hz, 1H, H1), 4.33 – 4.23 (m, 1H, H2'), 3.94 (dd, *J* = 10.1, 4.3 Hz, 1H, H1'A), 3.68 – 3.28 (m, 17H, H1'B + H5 + H6 + H7 + H22 + 3xCH_3-TMA), 2.18 – 2.07 (m, 2H, H20'), 2.07 – 1.97 (m, 14H, 4xCH_3-OAc + H6'), 1.73 (d, *J* = 21.0 Hz, 2H, H21), 1.64 – 1.49 (m, 4H, H8 + H21'), 1.44 – 1.09 (m, 70H, 35xCH_2-alkyl), 0.88 (t, *J* = 6.9 Hz, 6H, H18' + H34'). ^{13}C NMR (126 MHz, $CDCl_3$) δ 172.9 (C_q-CONH), 170.4, 170.1, 169.6 (4xC_q-OAc), 137.1 (C5'), 124.8 (C4'), 100.6 (C1), 73.9 (C3'), 73.4 (C5), 72.9 (C3), 72.2 (C7), 71.5 (C2), 70.0 (C6), 69.5 (C4), 67.4 (C1' + C22), 53.6 (3xCH_3-TMA), 50.8 (C2'), 37.0 (C20'), 32.5, 32.1, 29.9, 29.83, 29.80, 29.77, 29.73, 29.70, 29.68, 29.65, 29.59, 29.56, 29.50, 29.47, 29.41, 29.3, 29.1, 26.3, 26.1, 25.9, 23.3, 22.8 (C6' + C21 + C8 + C21' + 35xCH_2-alkyl), 21.3, 20.9, 20.83, 20.77 (4xCH_3-OAc), 14.3 (C18' + C34'). HRMS: calcd. for $C_{67}H_{125}N_2O_{12}$ $[M+]^+$: 1149.92270, Found: 1149.92235

_N_,_N_,_N_-Trimethyl-16-(((2_R_,3_S_,4_S_,5_R_,6_R_)-3,4,5-trihydroxy-6-(((2_S_,3_R_,_E_)-3-hydroxy-2-palmitamidooctadec-4-en-1-yl)oxy)tetrahydro-2_H_-pyran-2-yl)methoxy)hexadecan-1-aminium (4)



Compound **34** (108 mg, 91 µmol) was dissolved in a 1:1 mixture (4 mL) of MeOH and CH_2Cl_2. A catalytic drop of NaOMe (4.3 M in MeOH) was added and the reaction was stirred for 2.5 h at rt. The reaction was quenched with Amberlite™ IRC-120(H) until neutral pH, filtered and concentrated *in vacuo*. The crude product was purified by column chromatography (1% to 10% MeOH in CH_2Cl_2) to give 6-*O*-alkylTMA-β-GlcCer **4** (74 mg, 73 µmol, 80%). ^{1}H NMR (400 MHz, MeOD/$CDCl_3$) δ 5.77 – 5.64 (m, 1H, H5'), 5.50 – 5.41 (m, 1H, H4'), 4.26 (d, *J* = 7.8 Hz, 1H, H1), 4.17 – 4.06 (m, 2H, H1'A + H3'), 4.02 – 3.95 (m, 1H, H2'), 3.78 (dd, *J* = 10.9, 2.2 Hz, 1H, H6A), 3.68 – 3.58 (m, 2H, H6B + H1'B), 3.51 (td, *J* = 6.9, 2.6 Hz, 2H, H7), 3.45 – 3.22 (m, 6H, H2 + H4 + H3 + H5 + H22), 3.17 (s, 9H, 3xCH_3-TMA), 2.19 (dd, *J* = 8.5, 6.8 Hz, 2H, H20'), 2.03 (q, *J* = 7.1 Hz, 2H, H6'), 1.84 – 1.72 (m, 2H, H21), 1.65 – 1.54 (m, 4H, H8 + H21'), 1.44 – 1.19 (m, 70H, 35xCH_2-alkyl), 0.93 – 0.86 (m, 6H, H18' + H34'). ^{13}C NMR (101 MHz, MeOD/$CDCl_3$) δ 174.41 (C_q-CONH), 133.6 (C5'), 129.0 (C4'), 103.0 (C1), 76.1(C5), 74.8 (C3), 73.1 (C2), 71.53 (C3'), 71.47 (C7), 70.1 (C4), 69.7 (C6), 68.6 (C1), 66.5 (C22), 53.1 (C2'), 52.4 (3xCH_3-TMA), 36.0, 31.9, 31.5, 29.3, 29.23, 29.18, 29.13, 29.11, 29.06, 29.0, 28.94, 28.89, 28.8, 28.7, 25.7, 25.5, 22.5, 22.2 (C8 + C21 + C6' + C20' + C21' + 35xCH_2-alkyl), 13.3 (C18' + C34'). HRMS: calcd. for $C_{59}H_{117}N_2O_8$ $[M+]^+$: 981.88044, Found: 981.88017

3.7 References

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3.8 Appendix

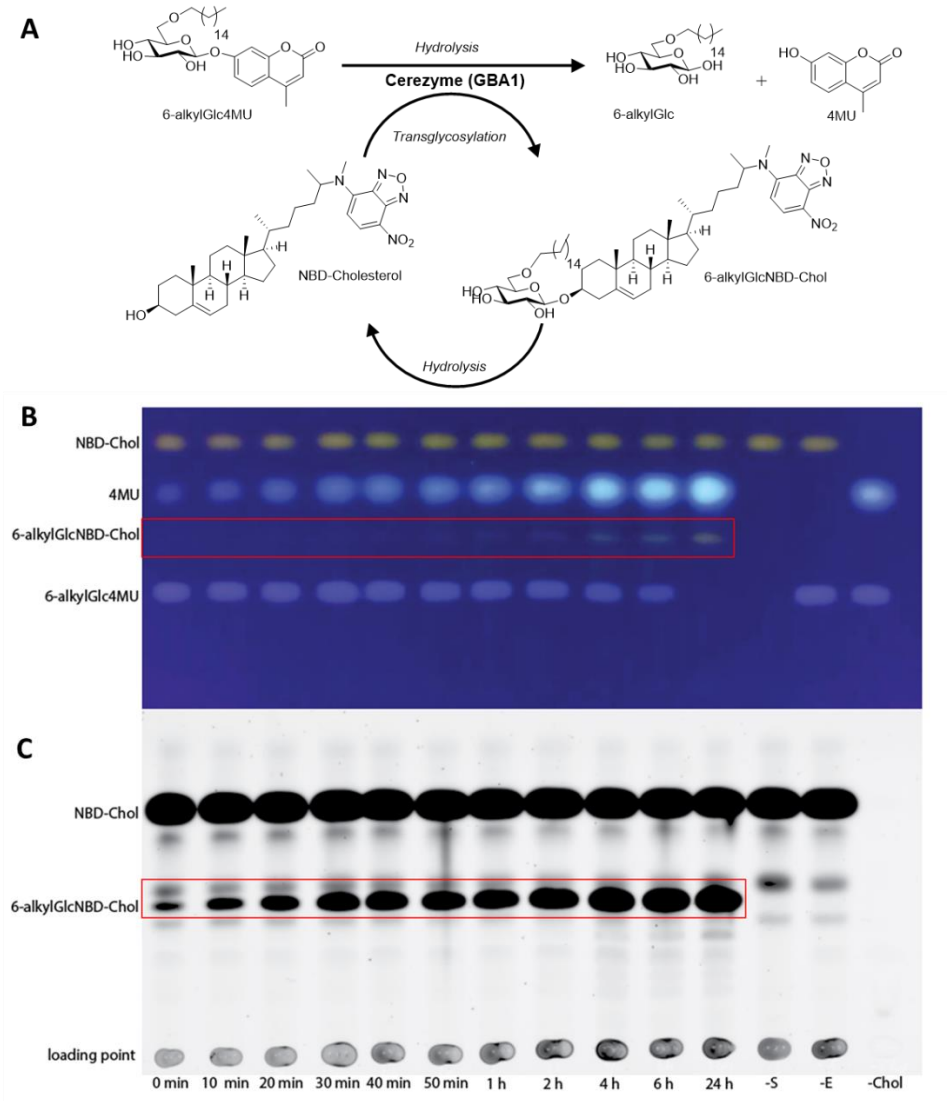


Figure S3.1: Transglucosylation of NBD-Cholesterol using 6-*O*-alkyl-4MU-β-Glc (**2**) as a glucosyl donor and pure recombinant rhGBA1 (Cerezyme®). A) reaction scheme of the transglucosylation NBD-Cholesterol using 6-*O*-alky-4MU-β-Glc (**2**) and the hydrolysis of 6-*O*-alkyl-β-GlcNBD-Chol by rhGBA1. Image of HPTLC plate visualized by B) fluorescence scan using Typhoon Variable Mode Imager or C) UV light image of B.

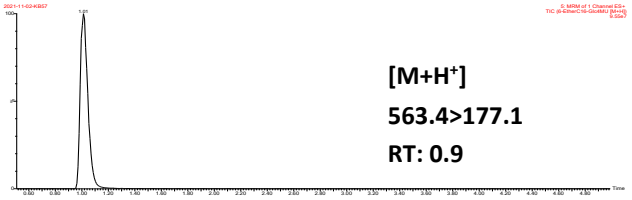
Table S3.1: MS/MS instrumental parameters.

Capillary voltage	**3.50 KV**
Cone voltage	20 V
Source temperature	120 °C
Desolvation temperature	450 °C
Cone gas	50 L/h
Desolvation gas	950 L/h
Collision gas	0.20 mL/min
Collision voltage	20 V
Type	Multiple reaction monitoring
Ion mode	ES^+
Dwell time	0.250 s
Interchannel delay	0.005 s
Interscan delay	0.005 s
Transitions	
$^{13}C_6$-GlcChol	RT: 1.4 min
6-*O*-alkyl-4MU-β-Glc	RT: 0.9 min
6-*O*-alkyl-β-GlcCholesterol	RT: 10.45 min
6-*O*-alkyl-β-GlcRetinol	RT: 2.75 min
6-*O*-alkyl-β-GlcDesmosterol	RT: 8.3 min
6-*O*-alkyl-β-GlcVitaminD_3	RT: 7.3 min
Fit weight	RT: None
Smooth method	RT: Mean
Smooth width	RT: 2 min

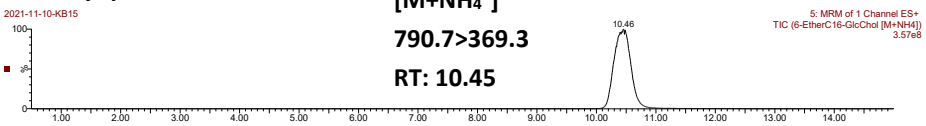
Table S3.2: The retention time (RT) in minutes and m/z of the different compounds detected by LC-MS/MS.

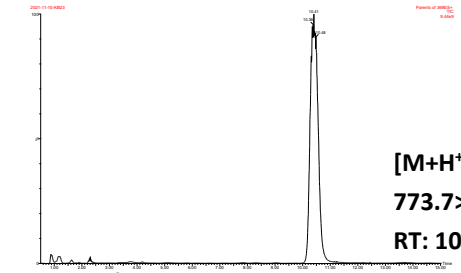
Compound	Transition	RT (min)
$^{13}C_6$-GlcChol [M+NH_4^+]	572.6 > 369.4	1.40
6-*O*-alkyl-4MU-β-Glc [M+H^+]	563.3 > 177.1	0.90
6-*O*-alkyl-β-GlcCholesterol [M+H^+]	773.7 > 369.3	10.40
6-*O*-alkyl-β-GlcCholesterol [M+NH_4^+]	790.7 > 369.3	10.45
6-*O*-alkyl-β-GlcRetinol [M+H^+]	673.5 > 269.2	2.75
6-*O*-alkyl-β-GlcDesmosterol [M+H^+]	771.6 > 367.4	8.30
6-*O*-alkyl-β-GlcVitaminD_3 [M+H^+]	771.6 > 367.4	7.30

6-*O*-alkyl-4MU-β-Glc

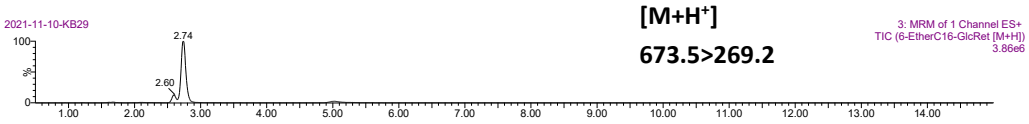


6-*O*-alkyl-β-GlcCholesterol

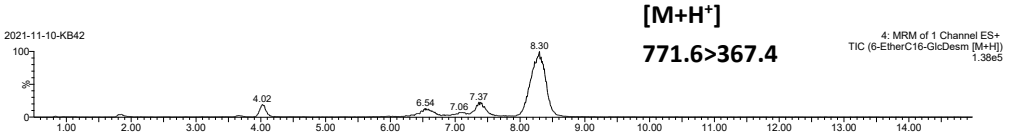




6-*O*-alkyl-β-GlcRetinol



6-*O*-alkyl-β-GlcDesmosterol



6-*O*-alkyl-β-GlcVitaminD$_3$

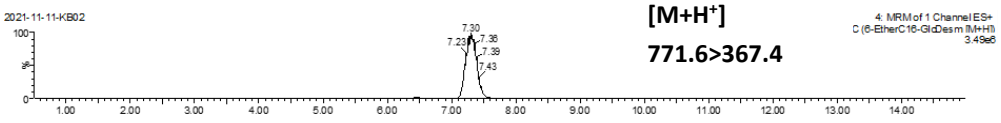


Figure S3.2: LC-MS/MS chromatograms of the transitions of the substrate 6-*O*-alkyl-4MU-β-Glc, and the transglucosylated products of cholesterol, desmosterol, retinol and vitamin D_3.

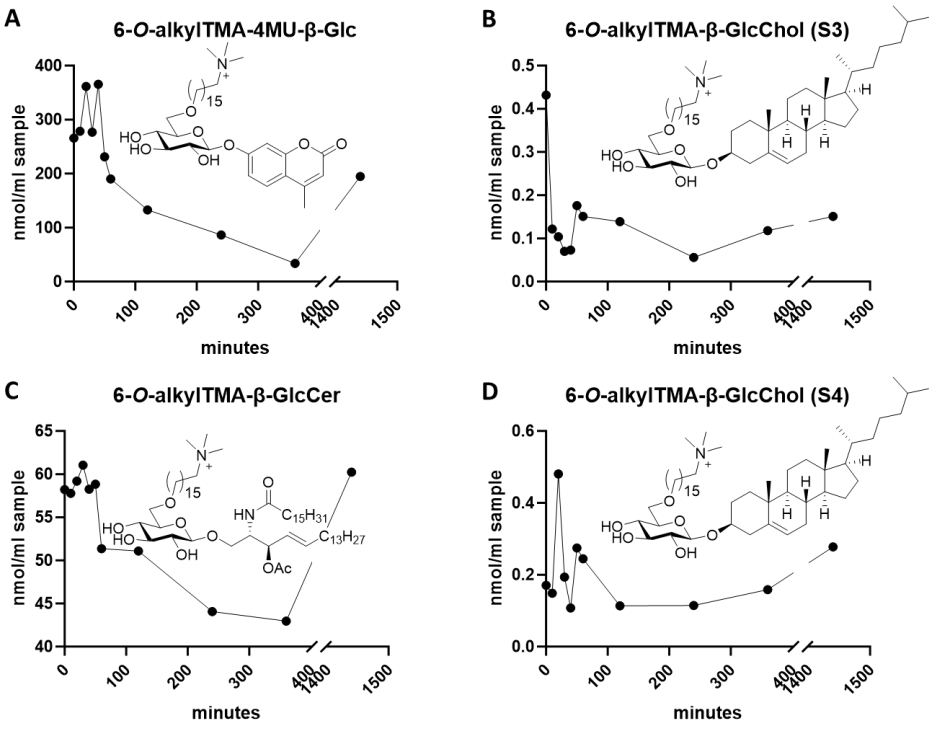


Figure S3.3: LC-MS/MS analysis of A) the consumption of 6-*O*-alkylTMA-4MU-β-Glc (**3**) over time. B) The formation of the transglucosylated 6-*O*-alkylTMA-β-GlcChol using substrate **3** over time. C) The consumption of 6-*O*-alkylTMA-β-GlcCer (**4**) over time. D) The formation of the transglucosylated 6-*O*-alkylTMA-β-GlcChol using substrate **4** over time. Data was analysed using $^{13}C_6$-AcylGlcChol 16:0 as internal standard.