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**Author:** Lahav, D.
**Title:** Fluorescence polarization activity-based protein profiling on retaining glycosidases
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A Fluorescence Polarization Activity-Based Protein Profiling Assay in the Discovery of Potent, Selective Inhibitors for Human Non-Lysosomal Glucosylceramidase

2.1 Introduction
Human non-lysosomal glucosylceramidase (GBA2) hydrolyses glucosylceramide (GlcCer) into glucose and ceramide in the cytosol of human cells. GBA2 is located at the cytosolic leaflet of the endoplasmic reticulum (ER), Golgi apparatus and endosomes. The substrate, GlcCer, is synthesized at the cytosolic leaflet of the Golgi apparatus prior to translocation to the lumen of the organelle for elongation to complex glycosphingolipids. GBA2 activity is complementary to lysosomal acid glucosylceramidase (GBA1), which processes GlcCer in lysosomes. Genetic mutations in GBA1 are at the basis of the lysosomal storage disorder called Gaucher disease (GD). GD macrophages are rich in GlcCer levels and these lipid-laden macrophages are termed Gaucher cells. Other tissues in GD patients, however, appear unaffected in GlcCer levels. It is conceivable that GBA2 compensates for reduced GBA1 activity in these tissues.

While GBA2 is expressed in many tissues its activity is most abundant in the central nervous system, indicating its role in neuronal development. Loss of GBA2 function has been shown to cause hereditary spastic paraplegia (HSP) and autosomal recessive cerebellar ataxia (ARCA), underscoring the potential link between GBA2 functionality and neurodegeneration. These observations indicate that compounds able to enhance GBA2 activity could have therapeutic potential. On the other hand, compounds able to (partially) block GBA2 activity also hold therapeutic value, as it was found that lowering GBA2 activity in Niemann-Pick type C (NPC) mouse models led to elongated life-span and improved motor coordination. Mistry and co-workers demonstrated that genetic loss of GBA2 ameliorates clinical symptoms in GD mice. The latter is consistent with the hypothesis that compensatory over-activity of GBA2 during deficiency of GBA1 (in GD) has
detrimental effects. One method that was used for the reduction of neuropathology in NPC mice was via pharmacological inhibition of GBA2 using N-alkyl-deoxynojirimycin (N-alkyl-DNM) derivatives, such as N-butyl-DNM or N-(5)-adamantane-1-yl-methoxy-pentyl-DNM (respectively Zavesca (1) and AMP-DNM (2), Figure 1). These N-alkyl-DNM derivatives are reported as potent GBA2 inhibitors, with AMP-DNM (2) as the most potent of the two \((K_i = 3 \text{ nM})\). However, most potent GBA2 inhibitors have significant off-target activity, not only towards GBA1, but also the enzyme responsible for the biosynthesis of GlcCer: glucosylceramide synthase (GCS). Generally, the activity on GBA1 is significantly reduced when changing the configuration of the polyhydroxylated piperidine from \(D\)-gluco- into \(L\)-ido-DNM. For example, the potency of \(L\)-ido-biphenyl-DNM (3) on GBA1 is significantly reduced. Unfortunately, in the case of 3, the potency on GCS is also significantly increased, showing that there is no simple rule or trend to apply on the design of DNM analogues and realize GBA2 selectivity.

![Chemical structures of reported DNM analogues: Zavesca (1), AMP-DNM (2) and L-ido-biphenyl-DNM (3).](image)

\( \text{IC}_{50} \) on GCS = 50 \( \mu \text{M} \)
\( \text{IC}_{50} \) on GBA1 = 520 \( \mu \text{M} \)
\( \text{IC}_{50} \) on GBA2 = 0.23 \( \mu \text{M} \)
\( \text{IC}_{50} \) on GCS = 0.200 \( \mu \text{M} \)
\( \text{IC}_{50} \) on GBA1 = 0.100 \( \mu \text{M} \)
\( \text{IC}_{50} \) on GBA2 = 0.002 \( \mu \text{M} \)
\( \text{IC}_{50} \) on GCS = 0.008 \( \mu \text{M} \)
\( \text{IC}_{50} \) on GBA1 = 15 \( \mu \text{M} \)
\( \text{IC}_{50} \) on GBA2 = 0.001 \( \mu \text{M} \)

**Figure 1.** Chemical structures of reported DNM analogues: Zavesca (1), AMP-DNM (2) and \(L\)-ido-biphenyl-DNM (3).

Considering the pharmaceutical potential of GBA2 as a drug target, selective GBA2 inhibitors are desirable commodities. In this chapter the development of a fluorescence polarization activity-based protein-profiling (FluoPol-ABPP) assay as a tool for the fast identification of GBA2 inhibitors in extracts from cells overexpressing GBA2 is described. Screening of an (in-house composed) iminosugar library, containing 358 entries (see Appendix A for the structures of the iminosugar library), provided lead structures bearing a relatively small (compared to existing nanomolar GBA2 inhibitors, including 2 and 3) apolar head groups. Analysis of a focused library based on those leads, including the assessment of potencies against GBA2, GBA1 and GCS provides insights that may contribute in the development of future potent and selective GBA2 inhibitors.
2.2 Results and Discussion
GBA2 is part of the glycoside hydrolase family 116 (GH116) according to the Carbohydrate Active Enzyme (CAZY) database. The enzyme makes use of a Koshland two-step double displacement mechanism to process GlcCer, as discussed in the general introduction. Cyclophellitol-based probes have been designed taking into account the covalent intermediate that is formed in the first step of substrate processing: the glycosylation step. As depicted in Figure 2, cyclophellitol aziridines, with the aziridine nitrogen bearing a reporter group (a fluorophore, or biotin), are able to potently and irreversibly inhibit GBA2. Here, glutamic acid (Glu) acts as the catalytic nucleophile and aspartic acid (Asp) as the catalytic acid/base. However, these cyclophellitol aziridines are also able to target GBA1 and GBA3, as they are in the class of broad-spectrum retaining β-glucosidases. Probes grafted with a fluorophore can be used in gel-based ABPP, where both selectivity and potency of inhibitors per individual target can be assessed. As discussed in the general introduction (Chapter 1), fluorescent probes can also be used to monitor binding events in time using fluorescence polarization (FluoPol) as readout. This chapter describes how specific targeting of GBA2 is realized and the resulting FluoPol assay on this enzyme is used to identify GBA2 specific inhibitors.

2.2.1 Synthesis of a FluoPol compatible ABP
Reported FluoPol-ABPP studies make use of (red) fluorescent dyes, such as tetra-aminomethylrhodamine (TAMRA). The ABPs for glycosidases that have appeared in the literature contain a variety of fluorophores, however no rhodamine based probes were synthesized thus far. Therefore, as the first research objective, TAMRA-cyclophellitol aziridine (14) was synthesized (scheme 1). The synthesis of compound 14 commenced with a Fischer glycosylation of D-xylose giving methyl D-xylofuranoside (4), which was converted into benzyl protected furanoside (5) via
tritylation of the primary alcohol, benzylation of the secondary alcohols and subsequent detritylation. The primary alcohol in the resulting product 5 was converted to an iodide via an Appel reaction to give compound 6. Zinc-mediated reductive fragmentation of 6 afforded aldehyde 7. Subsequently indium-mediated allyl addition using bromo-crotonate in the presence of lanthanide triflate yielded diastereomerically pure 8. Cyclohexene 9 was obtained via ring-closing metathesis on diene 8 using Grubbs 2\textsuperscript{nd} generation catalyst. The ester functionality in 9 was then reduced using DIBAL-H, affording diol 10. Sodium borohydride and water were added in order to avoid aldehyde contamination. The primary alcohol in 10 was selectively converted into the corresponding trichloroacetimidate, after which iodocyclisation led to the formation of iodide 11. Acid-mediated hydrolysis of the imidate in 11 followed by exposure to alkaline conditions (sodium bicarbonate) led to nucleophilic displacement of the iodide in a stereospecific fashion, giving benzyl-protected aziridine 12. Debenzylation using Birch conditions (lithium in ammonia) afforded aziridine 13. This aziridine was alkylated using iodo-pentyne and sodium bicarbonate and the fluorophore, 5'-TAMRA-PEG\textsubscript{3}-azide 34, was subsequently attached to the crude N-alkyl aziridine via copper(I) catalyzed azide-alkyne [2+3] cycloaddition. The aziridine based cyclophellitol probe, ABP 14, was purified to homogeneity using HPLC purification.\textsuperscript{15,18,19} Synthetic details for the fluorophore are described in the experimental section (scheme 2).\textsuperscript{20,21}
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**Scheme 1:** Synthetic route towards β-glucosidase aziridine probe (14)

Reagents and conditions a) AcCl, MeOH, 98% (b) i) TrtCl, DMAP, Et3N, DMF ii) BnBr, NaH, TBAI, DMF, 4°C iii) p-TsOH, DCM/MeOH (9:1), 38% over three steps (c) I2, PPh3, imidazole, THF, reflux, 91% (d) Zn, THF/H2O (9:1), sonication, 40°C, 76% (e) ethyl 4-bromocrotonate, In, La(OTf)3, H2O, 50% (f) Grubbs 2nd generation, DCM, 91% (g) i) DIBAL-H, -20°C to ambient temperature ii) NaBH4, -20°C to ambient temperature, 90% (h) i) CCl3CN, DBU, DCM, 0°C ii) I2, NaHCO3, H2O, 65% (i) i) AcOH/dioxane/H2O (8:1:1) ii) NaHCO3, MeOH, 90% (j) Li, NH3(liq.), -60°C (k) i) 5-iodopentyne, NaHCO3, DMF ii) 34, sodium ascorbate, CuSO4, H2O, 6% over last three steps.

2.2.2 **Compound 14 is a broad spectrum retaining β-glucosidase probe**

The synthesized probe was evaluated for its labelling capability by comparing 14 with the labelling pattern on tissues of an established broad-spectrum probe, ABP 15, which contains Bodipy-FL as fluorophore. Lysates from mouse brain were treated with 1 µM probe at pH 5.0 for 30 minutes at 37°C. After incubation the proteins were denatured using Laemmli Buffer, containing an excess of sodium dodecylsulfate (SDS), and the resulting mixture was brought to 100°C for a short period of time. The protein content was then resolved by gel-electrophoresis and, as shown in Figure 3, fluorescence scanning of the wet gel slabs (λex = 488 and λem = 520 for Bodipy green; λex = 530 nm and λem = 580 nm for TAMRA), revealed bands at 102 kDa, 60 kDa and 53 kDa, corresponding to the molecular weight of...
respectively GBA2, GBA1 and GBA3. Labeling with ABPs 14 and 15 was performed as well on lysates from HEK293 cells overexpressing GBA2. Cell extracts were incubated with 14 at pH 7, and addition of the ABP was preceded by exposure of the protein mixture to Zavesca (1), AMP-DNM (2) or no inhibitor. Fluorescence scanning of the wet gel slab revealed a major band corresponding to the molecular weight of GBA2 for those samples treated with either 14 or 15 and in which no competitive inhibitor was used (Figure 3B, lanes 1, 4). Importantly, little to no endogenous GBA1 and GBA3 are seen, even though these enzymes are obviously present as well (quantification of the signals reveals that over 85% of the labeled protein correspond to GBA2). Labeling could be prevented by pretreatment with either of the two known competitive GBA2 inhibitors 1 (lanes 2, 4) or 2 (lanes 3, 6) at 100 and 10 µM final concentration, respectively. This experiment shows that relative levels of active GBA2 in the overexpressing cells are such that the preparation can be used to screen for GBA2 inhibitors, and as well that GBA2 labeling is abolished when competitive inhibitors are present.

Figure 3. Resulting image of gel-ABPP experiments on lysates of mouse brains (A) and HEK293 cells overexpressing GBA2 (HEK GBA2+) (B). In these experiments two different probes, ABPs 14 and 15, were used; gel-ABPP labelling of the three retaining glucosidases with 1 µM ABP 6 on the left panel, where ABPP labelling on right panel was performed using 1 µM ABP 7. Specific labelling of GBA2 was realized by using lysates from GBA2 overexpressing HEK cells. Complete ablation of this GBA2 signal was realized with 100 µM 1 or 10 µM 2.
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2.2.3 Optimization of FluoPol-ABPP with 14

Initial experiments aimed to establish whether ABP 14 can be used in a FluoPol-ABPP assay format to screen for GBA2 inhibitors were carried out in 96 well plates, with 0.5 mg/mL protein and a final volume ($V_{\text{final}}$) of 75 µL. Fluorescence intensities were measured at $\lambda_{\text{ex}} = 530$ nm and $\lambda_{\text{em}} = 580$ nm. The pH optimum of GBA2 is reported to be at pH 5.8. Assessment in a range from pH 4 to pH 8 (the range at which GBA2 possesses measurable activity), revealed that maximal return of polarized light occurs at pH 7 (Figure 4A). The optimal final probe concentration in the assay was found to be 25-50 nM (Figure 4B). Optimal probe concentration depends on the amount of active GBA2 present in the samples. In this setting a suitable window in FluoPol-signal ($\Delta = 180$ mPoI) was achieved between positive and negative controls, respectively samples containing an excess of AMP-DNM 2 and DMSO only, representing samples in which labelling of GBA2 is blocked completely versus samples representing 100% labelling. The $IC_{50}$ values of a set of known GBA2 inhibitors, both competitive (Zavesca 1, AMP-DNM 2, L-ido-AMP-DNM 16, DNM 17) and mechanism-based (cyclophellitol 18) were established using the FluoPol-ABPP format using 50 nM of ABP 14. Obtained potencies (Figure 4D) were in line with literature values on the same compound, both for the potent (2, 16) and less potent (1, 17, 18) inhibitors. These inhibitors are established competitors according to the literature, thereby validating the conditions of the here presented FluoPol-ABPP assay. 16
Figure 4. Optimization and validation of FluoPol-ABPP for GBA2. (A) Effect of pH. (B) Effect of probe concentration. (C) Structures of additional established inhibitors. (D) Inhibition by established inhibitors (1, 2, 16, 17 and 18). Error bars represent standard error of the mean.

2.2.4 Screen of an iminosugar based library on GBA2

The FluoPol assay was further miniaturized into a 384-well plate format ($V_{\text{final}} = 15 \mu\text{L}$), allowing to use automated liquid handling and to perform a screen of an iminosugar based compound library. The structures of all 358 compounds screened are depicted in the Appendix at the end of this Thesis. The final concentration of the iminosugars was set to be 100 nM. Figure 5A shows residual GBA2 activity plotted against a variety of iminosugar classes. Besides D-glucopyranose-configured iminosugars, the corresponding D-galacto- and L-ido-configured iminosugars are known to be potent GBA2 inhibitors and library entries adhering to this general description are picked up in this FluoPol-ABPP assay as well (Figure 5B).\(^{16}\) Alternatively configured piperidines, partially deoxygenated deoxynojirimycin derivatives and hydroxylated pyrrolidines showed up only rarely, if at all, as inhibitors in the screen.
Figure 5. (A) FluoPol-ABPP screen of an iminosugar library (358 entries) using to identify potential GBA2 inhibitors. Competition was performed at 100 nM. (B) Screen where the compounds of the library are categorized based on its sugar configuration (gluco, ido, galacto and others are classified as alternative configurations), deoxygenated variants and pyrrolizidines. (C) Chemical structures of the two identified GBA2 inhibitors, 19 and 20.

All compounds that showed more than 50% inhibition of GBA2 activity in the screen were considered as hits. The selectivity profile of these hits were established by determining the potency on GBA1, GBA2 and GCS using the conventional (fluorogenic) activity assays, which are described in the experimental section. The hits containing a D-gluco and L-ido configuration with their corresponding potencies and selectivity ratios for GBA2 offset against GBA1 and
GCS are shown in Table 1. Neopentyloxypentyl-deoxynojirimycin 19 and its L-ido-congener 20, depicted in Figure 5C, were identified as nanomolar potent GBA2 inhibitors amongst known GBA2 inhibitors. As shown in Table 1, compounds 19 and 20 are also relatively selective GBA2 inhibitors.

Table 1: Potencies (in µM) and selectivity ratios of D-gluco- and L-ido-iminosugars on GCS, GBA1 and GBA2. Calculated ratios GCS/GBA2 and GBA1/GBA2 are presented in green, for which a stronger shade of green stands for a higher ratio and thus selectivity. (* in situ = in living cells, † in vitro = on cell lysates)

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Table 2, with a selected group of the DNM collection listed, shows that the activity of 19 and 20 against GCS is significantly less than those of the previously reported N-alkyl-DNM derivatives 2 and 3. Compounds 19 and 20 contain a relatively small hydrophobic N-alkyl group compared to that of 2 and 3, which may be a determining factor for the observed enhanced GBA2 selectivity. Accordingly, a set of close analogues of compounds 19 and 20, the synthesis of which is described in the dissertation of B. Liu (2017), were assessed on their inhibitory potency against GBA2, GBA1, GBA3 and GCS (examples of 2nd generation library 21-25 in Figure 6).
Figure 6. Some examples of the 2nd generation library; 21-25, the design of which was based on inhibitors 19 and 20.22

The isobutyl derivatives 21 and 22 appeared to be more potent GBA2 inhibitors compared to inhibitors 19 and 20 and are also less potent inhibitors of GCS. L-ido configured compounds 20 and 22 are over 100,000-fold selective for GBA2 over GBA1. This selectivity window is larger than that of previously reported GBA2 inhibitors, which are at most a 10,000-fold selective with respect to GBA1.23 Compounds 19, 23 and 24 are about 1000-fold more active on GBA2 compared to their GCS inhibitory activity, while previously reported compounds are at best 150-fold more selective.24 Substitution of the neopentyl moiety for tetrahydrofuranylmethyl groups, as in 24 and 25, had a detrimental effect on inhibitory potency towards GBA1, GBA2 and GCS, and no significant effect on selectivity. In conclusion, neopentyl- and isobutyl derivatives 19-22 are showing remarkable GBA2 selectivity, allowing perhaps specific targeting of GBA2 in situ (in living cells) and even in vivo.

Table 2: Potencies & selectivity ratios of deoxynojirimycin derivatives on GCS, GBA1, GBA2 and GBA3

<table>
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<tr>
<th></th>
<th>GCSa</th>
<th>GBA1b</th>
<th>GBA2b</th>
<th>GBA3c</th>
<th>GCS/GBA2c</th>
<th>GBA1/GBA2c</th>
<th>GBA3/GBA2c</th>
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</table>

a Inhibition value for in situ assay is given as IC50 (µM)
b Inhibition value for in vitro assay is given as K_i (µM)
c Relative GBA2 offset (ratio)
2.2.5 *In situ* evaluation of neopentyl- & isobutyl-DNMs

A competitive ABPP experiment on live cells was performed in order to evaluate effective GBA2 inhibition *in situ*. To this end, cells overexpressing either GBA2 or GBA3, both of which are containing endogenous GBA1, were treated with compounds 19 - 22 at various final concentrations for 1 hour. After that the cells were lysed via snap-freeze and then treated with ABP 14 in order to determine the residual glucosidase activity. As can be seen from the images of the SDS-PAGE gels (Figure 7), all compounds are cell permeable and selectively block GBA2 over GBA1 and GBA3 at the concentrations tested.

![Figure 7. Competitive ABPP experiment of HEK293T cells overexpressing GBA2 or GBA3 (and expressing endogenous GBA1) treated with compounds 19 - 22 at various final concentrations prior to cell lysis and ABPP profiling of remaining enzyme activity.](image)

2.3 Conclusion

In the last decade several FluoPol ABPP assays have been developed for different pharmacologically interesting targets, such as serine hydrolases (RBBP9), protein arginine methyl transferases (PRMTs) and arginine deaminases (PADs).25-29 In line with these studies, this chapter describes the development of a high-throughput
compatible FluoPol-ABPP assay for the rapid screening of GBA2 inhibitors. This assay is the first of its kind combining FluoPol and ABPP on glycoconjugate processing enzymes. FluoPol assays can be conducted on enzymes in complex mixtures, as long as the probe has considerable selectivity towards the targeted enzyme or when using broad-spectrum ABPs, such as ABP 14 is, simply by bringing the targeted enzyme to overexpression. The conditions that were used in the assay resulted in a suitable difference between positive and negative controls (ca. 180 mPol) and robust response (Z’ = 0.9). The screening of an iminosugar based library using the FluoPol-ABPP assay yielded numerous potent GBA2 inhibitors. Orthogonal assays were used for hit validation and determining the selectivity profile on selected compounds. This resulted in the identification of two new GBA2 inhibitors from the 358 compound collection that possess considerable GBA2 selectivity. A small compound library around these leads was synthesized, as described in the Thesis of Dr. Bing Liu (2017). Diversification of the small (compared to the bulky adamantyl or biphenyl substituents that characterize contemporary GBA2 inhibitors) alkoxy substituent revealed that modulating the size of this hydrophobic moiety yields inhibitors more potent, and crucially also more selective for GBA2 over GCS, GBA1 and GBA3 compared to existing inhibitors. Finally, this chapter lays the foundation for the development of other FluoPol-ABPP assays on retaining glycosidases. As mentioned before, there are cyclophellitol-based probes available for ABPP on a variety of glycosidase targets. The presence of a fluorescent tag instead of the primary alcohol in the cyclophellitol allows (specific) labelling of GBA1, as shown in Chapter 3. Furthermore, α-glucose-configured cyclophellitol aziridine allows labelling of lysosomal acid α-glucosidase A (GAA), as described in Chapter 4, whereas α-mannose-configured cyclophellitol aziridine allows for screening of α-mannosidase inhibitors as described in Chapter 5.
2.4 Experimental section

Chemicals, materials and methods

All solvents and reagents were obtained commercially and used as received unless stated otherwise. Dichloromethane (DCM), dimethylformamide (DMF), tetrahydrofuran (THF) and methanol (MeOH) were dried over molecular sieves (4Å/3Å) for at least 12 hours before use. Moisture sensitive reactions were performed under argon atmosphere and carried out in oven-dried glassware. Reactions were monitored by TLC analysis using sheets with pre-coated silica with detection by UV-absorption (254 nm) wherever applicable and by spraying with 20% H$_2$SO$_4$ in MeOH, an aqueous solution containing KMnO$_4$ (5 g/L) and K$_2$CO$_3$ (95 g/L) or a solution of ninhydrin (6 g/L) in AcOH:MeOH (1:9, v/v) followed by charring at ≈ 200°C. Flash column chromatography was performed on silica gel (40-63 µm). For LC/MS analysis a HPLC-system (detection simultaneously at 213 nm, 254 nm and evaporative light detection) equipped with an analytical C-18 column (4.6 mmD x 250 mmL, 5 µ particle size) in combination with buffers A: H$_2$O, B: acetonitrile, C: 1.0% aqueous trifluoroacetic acid and coupled with an electrospray interface (ESI) was used. For RP-HPLC purifications, an automated HPLC system equipped with a semi-preparative S2 C18 column (5 µm C18, 10Å, 150 x 21.2 mm) was used. The applied buffers were A: H2O + trifluoroacetic acid (1%) and B: MeCN. HPLC-MS purification was performed on an Agilent Technologies 1200 series automated HPLC system with a Quadropole MS 6130, equipped with a semi-preparative Gemini C18 column (Phenomex, 250 x 10, 5 µm) using buffers A: H$_2$O + K$_2$CO$_3$ (1%) and B: MeCN. Compounds are characterized by $^1$H NMR-, $^{13}$C NMR-, COSY- and HSQC NMR experiments. NMR spectra were recorded on a Bruker DPX-300, AV-400 and AV-600 spectrometer in mentioned solvent. Chemical shifts are given in ppm (δ) relative to tetramethylsilane or the deuterated solvent as the internal standard. High-resolution mass spectra were recorded by direct injection (2 µL of a 2 µM solution in water/acetonitrile/tert-butanol, 1:1:1, v/v) on a mass spectrometer (Thermo Finnigan LTQ Orbitrap) equipped with an electrospray ion source with resolution R = 60000 at m/z 400 (mass range m/z = 150-2000).
**Scheme 2: Synthesis of 5’-TAMRA-PEG₃-azide 34**

Reagents and conditions: (a) MsCl, Et₃N, THF, 45% (b) NaN₃, DMF under reflux, 95% (c) PPh₃, 5% HCl (aq), 77% (d) cat. H₂SO₄, AcOH under reflux (e) BOP.PF₆, DIPEA, DMSO, 2% over two steps

**((oxybis(ethane-2,1-diyl))bis(oxy))bis(ethane-2,1-diyl) dimethanesulfonate (27)**

A mixture of triethylamine (11.2 g, 110 mmol, 2.2 eq.) in THF (10 mL) was added dropwise at 0°C into a mixture containing tetraethylene glycol (26, 9.7 g, 50 mmol) and mesylchloride (12.6 g, 110 mmol, 2.2 eq.) dissolved in dry THF (50 mL). After 30 minutes the cooling bath was removed and the reaction was stirred for 4 more hours at ambient temperature. THF was removed under reduced pressure. Subsequently a mixture H₂O (100 mL), aqueous HCl (100 mL, 1 M) and DCM (200 mL) was poured into the residue. The organic layer was washed with saturated bicarbonate (3 × 100 mL), dried with MgSO₄ and filtrated. Purification via flash column chromatography (DCM → 5% MeOH/DCM)
afforded 27 (7.943 g, 22.62 mmol, 45%) as brown oil. \(^1\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\) 4.27 - 4.14 (m, 4H), 3.66 - 3.57 (m, 4H), 3.50 (s, 8H), 2.93 (s, 6H). \(^{13}\)C NMR (75 MHz, CDCl\(_3\)) \(\delta\) 70.1, 69.3, 68.5, 37.1. HRMS: found 351.0773 [M+H]\(^+\), calculated for [C\(_{10}\)H\(_{22}\)O\(_2\)S\(_2\)+H]\(^+\) 351.0778.

**1-azido-2-(2-(2-azidoethoxy)ethoxy)ethane (28)**

A mixture of 27 (7.1 g, 20 mmol) with sodium azide (5.3 g, 82 mmol, 4 eq.) in absolute ethanol (40 mL) and DMF (10 mL) was refluxed overnight. This mixture was poured into a mixture of H\(_2\)O/DCM (200 mL, 1:1, v/v). The organic layer was washed subsequently with H\(_2\)O (3 \(\times\) 100 mL) and brine (3 \(\times\) 100 mL). The organic layer was dried over MgSO\(_4\) and filtered. Yellow oil (4.7 g, 19 mmol, 95%) was obtained after removing the solvents under reduced pressure. \(R_f = 0.85\) (MeOH:DCM, 1:9, v/v). \(^1\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\) 3.62 - 3.54 (m, 12H), 3.36 - 3.23 (m, 4H). \(^{13}\)C NMR (75 MHz, CDCl\(_3\)) \(\delta\) 70.6, 70.0, 50.6. HRMS: found 245.1358 [M+H]\(^+\), calculated for [C\(_8\)H\(_{16}\)O\(_3\)N\(_6\)+H]\(^+\) 245.1357.

**1-azido-2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethane (29)**

Triphenylphosphine (0.8 g, 3 mmol, 0.9 eq.) dissolved in ether (15 mL) was added into a solution of 28 (0.8 g, 3.3 mmol) in 5% aqueous HCl (10 mL). Addition was performed in 30 minutes at room temperature and the reaction was stirred for an additional 2.5 hours. Phases were separated using a separation funnel and the aqueous layer was washed using DCM (3 \(\times\) 25 mL). The aqueous layer was adjusted to pH 10 using KOH pellets. Product was extracted with DCM (4 \(\times\) 50 mL). Combined organic layers were dried over MgSO\(_4\) and filtered. After removal of the organic solvents under reduced pressure yellow oil (4.7 g, 19 mmol, 77%) was afforded. \(R_f = 0.2\) (MeOH:DCM, 1:9, v/v). \(^1\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\) 3.73 - 3.49 (m, 10H), 3.45 (t, \(J = 5.2\) Hz, 2H), 3.36 - 3.28 (m, 2H), 2.80 (t, \(J = 5.1\) Hz, 2H), 1.44 (s, 2H). \(^{13}\)C NMR (75 MHz, D\(_2\)O) \(\delta\) 73.5, 70.7 - 70.1, 50.8, 41.9. HRMS: found 219.1452 [M+H]\(^+\), calculated for [C\(_8\)H\(_{16}\)O\(_3\)N\(_4\)+H]\(^+\) 219.1452.
A Fluorescence Polarization Activity-Based Protein Profiling Assay in the Discovery of Potent, Selective Inhibitors for Human Non-Lysosomal Glucosylceramidase

5-carboxy-2-(6-(dimethylamino)-3-(dimethyliminio)-3H-xanthen-9-yl)benzoate (32&33)

Dimethylaminophenol (30, 6.9 g, 50 mmol) and trimellitic anhydride (31, 4.8 g, 25 mmol) were dissolved in AcOH (400 mL). After adding catalytic amounts of concentrated H₂SO₄ (ca. 0.5 mL) the mixture was refluxed overnight. Reaction mixture was concentrated under reduced pressure and pre-purified over column chromatography (DCM → 50% MeOH/DCM) to isolate 2.7 g of a mixture containing desired regio-isomers.

2-(6-(dimethylamino)-3-(dimethyliminio)-3H-xanthen-9-yl)-5-((2-(2-(2-(4-(3-((1R,2S,3S,4R,5R,6R)-2,3,4-trihydroxy-5-(hydroxymethyl)-7-azabicyclo[4.1.0]heptan-7-yl)propyl)-1H-1,2,3-triazol-1-yl)ethoxy)ethoxy)ethoxy)ethyl)carbamoyl)benzoate (34)

BOP.PF₆ (74 mg, 0.168 mmol) and DIPEA (54 µL, 0.31 mmol) were added into a mixture containing isomers 32 and 33 (60 mg) and linker 29 (30 mg, 0.14 mmol) dissolved in DMSO (2 mL). The reaction was stirred for 24 hours at ambient temperature. Desired stereoisomeric product 34 (7.41 mg, 11.7 µmol, 2% estimated yield over two steps) was isolated using HPLC purification. ¹H NMR (600 MHz, MeOD) δ 8.79 (d, J = 1.7 Hz, 1H), 8.31 - 8.25 (m, 1H), 7.54 (d, J = 7.9 Hz, 1H), 7.15 (d, J = 9.5 Hz, 2H), 7.07 (dd, J = 9.5, 2.5 Hz, 2H), 7.00 (d, J = 2.4 Hz, 2H), 3.77 - 3.60 (m, 14H), 3.35 - 3.33 (m, 2H), 3.32 (s, 12H). ¹³C NMR (150 MHz, MeOD) δ 168.6, 167.7, 161.0, 159.4, 159.3, 138.4, 138.0, 133.2, 132.7, 132.2, 131.2, 131.7, 115.9, 115.0, 97.8, 72.0 - 70.8, 52.0, 49.9, 41.5, 41.2. HRMS: found 631.2877 [M+H]^+, calculated for [C₃₃H₆₈O₇N₆+H]^+ 631.2875.

(2R,3R,4R)-methyl-2,3-D-xylofuranoside(4)

A catalytic amount of acetyl chloride (6.00 ml) was added to an ice cooled solution of D-xylose (30.0 g, 200 mmol) in MeOH (300 ml) and stirred at room temperature for 5h. The mixture was quenched with NaHCO₃ until pH 10. The salt was filtered and the filtrate was concentrated under reduced pressure. The residue was purified by flash column chromatography (EtOAc → 10% MeOH/EtOAc), which afforded pale yellow oil (32.5 g, 197 mmol, 98%) as a 1:0.8 mixture of anomers. R₂=0.1 (MeOH/EtOAc, 1:9, v/v) ¹H NMR (400 MHz, CDCl₃) δ
Chapter 2

4.99 (d, $J = 4.5$ Hz, 1H), 4.87 (s, 0.8H), 4.43 (d, $J = 5.0$ Hz, 0.8H), 4.36 – 4.30 (m, 1H), 4.25 – 4.17 (m, 2.5H), 4.10 (t, $J = 4.7$ Hz, 1H), 3.95 – 3.91 (m, 2H), 3.90 (dd, $J = 4.8$, 2.2 Hz, 1H), 3.49 (s, 3H), 3.44 (s, 2.4H), 3.16 – 2.12 (br.s., 5.6H).

13C NMR (101 MHz, CDCl$_3$) $\delta$ 108.8, 101.7, 82.1, 80.9, 79.1, 78.0, 77.8 – 77.3, 62.2, 61.9, 55.8, 55.7. HRMS: found 165.0756 [M+H]$^+$, calculated for [C$_6$H$_{12}$O$_5$+H]$^+$ 165.0757.

**Methyl-2,3-Di-O-benzyl-5-iodo-D-xylofuranoside (5)**

Methyl-xylofuranoside 4 (17.88 g, 108.9 mmol) was dissolved DMF (400 ml) followed by addition of trityl chloride (36.4 g, 131 mmol, 1.2 eq.), DMAP (0.67 g, 5.4 mmol, 0.05 eq.) and Et$_3$N (30.0 ml, 218 mmol, 2 eq.). The reaction mixture was quenched with saturated NaHCO$_3$ after 18h. Extraction proceeded with Et$_2$O (3x 250 ml) and the combined ether layers were washed with saturated NaHCO$_3$ and brine. The organic layer was dried with MgSO$_4$, filtered and concentrated *in vacuo*. This crude product was dissolved in DMF (100 ml) and slowly added to a solution of NaH (17.4 g 60% in mineral oil, 436 mmol, 4 eq.) in DMF (200 mL) at -20°C. Subsequently benzyl bromide (31.0 ml, 261 mmol, 2.5 eq.) and TBAI (0.402 g, 1.08 mmol, 0.016 eq.) were added to the cooled mixture, which was thereafter stirred at room temperature until full conversion was shown on TLC. The mixture was quenched with MeOH (20 ml) at 0°C and extracted with Et$_2$O (3x 200 ml). Ether layers were washed with H$_2$O (250 ml) and brine (2x 100ml). The organic layer was dried with MgSO$_4$, filtered and concentrated *in vacuo*. The residue was pre-purified via flash column chromatography (pentane $\rightarrow$ 10% EtOAc/pentane) resulting in yellow oil. $R_f$=0.25 (EtOAc/pentane, 1:9, v/v). The obtained oil was dissolved in MeOH/DCM (250 ml, 3:1, v/v) and the pH of this mixture was adjusted to 2-3 by addition of p-TsOH at 0°C, where an immediate change in color (from yellow into orange) was observed. The reaction mixture was neutralized using Et$_3$N after full conversion of the starting material was shown on TLC. Product was extracted using DCM (3x 150ml) and the combined DCM layers were washed with saturated NaHCO$_3$ (100 ml) and brine (2x 100ml). The organic layer was dried with MgSO$_4$ and filtered. The filtrate was concentrated *in vacuo* and the desired product was purified by column chromatography (10% EtOAc/pentane $\rightarrow$ 50% EtOAc/pentane) which afforded 5 as yellow oil (14.33 g, 41.61 mmol, 38%) and as an anomeric mixture (ratio $\alpha$:$\beta$; 0.9:1). $R_f$=0.5 (EtOAc/pentane, 1:1, v/v). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.43 – 7.22 (m, 19H), 4.89 (d, $J = 1.8$ Hz, 1H), 4.80 (d, $J = 4.2$ Hz, 0.9H), 4.75 – 4.39 (m, 8.5H), 4.34 – 4.27 (m, 1H), 4.19 (ddd, $J = 10.6$, 7.3, 3.8 Hz, 1.9H), 4.09 (dd, $J = 3.7$, 1.8 Hz, 1H), 4.04 (dd, $J = 6.4$, 4.2 Hz, 0.9H), 3.86 – 3.65 (m, 3.8H), 3.39 (s, 3H), 3.37 (s, 2.5H), 2.50 (s, 1.9H). $^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ 137.5, 128.6 – 127.6, 107.9, 100.1, 87.1, 84.5,
A Fluorescence Polarization Activity-Based Protein Profiling Assay in the Discovery of Potent, Selective Inhibitors for Human Non-Lysosomal Glucosylceramidase

82.8, 82.2, 80.6, 76.3, 72.9 – 72.2, 62.24, 62.2, 55.6, 55.1 HRMS: found 345.1700 [M+H]^+, calculated for [C_{20}H_{24}O_5+H]^+ 345.1730.

Methyl-2,3-Di-O-benzyl-5-iodo-D-xylofuranoside (6)

Compound 5 (13.8 g, 40.3 mmol) was dissolved in anhydrous THF (100 ml). Triphenylphosphine (16.05 g, 61.19 mmol, 1.5 eq.) and imidazole (5.72 g, 84.0 mmol, 2 eq.) were added to the resulting yellow mixture and heated to 75°C. A solution of iodine (15.91 g, 62.68 mmol, 1.5 eq.) in anhydrous THF (100 ml) was added to the mixture, which resulted in a dark brown mixture. This mixture was quenched after full conversion was shown on TLC with 10% potassium thiosulphate solution (100ml). The salt was filtered off and the filtrate was concentrated in vacuo. Purification was performed via flash column chromatography (10% EtOAc in pentane) and resulted in yellow oil (16.70 g, 36.75 mmol, 91%). Anomeric mixture (ratio α:β; 0.75:1). \( R_f = 0.9 \) (EtOAc/pentane, 1:1, v/v).

\( ^1H \) NMR (400 MHz, CDCl_3) \( \delta \) 7.49 – 7.01 (m, 17.5H), 4.90 (s, 1H), 4.81 (d, \( J = 4.1 \) Hz, 0.75H), 4.61 – 4.32 (m, 8.75H), 4.21 – 4.10 (m, 0.75H), 4.03 – 3.93 (m, 2.75H), 3.41 – 3.25 (m, 7.8H), 3.16 (dd, \( J = 10.1, 7.5 \) Hz, 0.75H). \( ^{13}C \) NMR (101 MHz, CDCl_3) \( \delta \) 137.2, 128.2 – 127.3, 107.9, 100.5, 86.3, 83.5, 81.8, 81.5, 81.4, 77.3, 72.5 – 71.7, 55.7, 55.1, 4.7, 3.1. HRMS found 455.0706 [M+H]^+, calculated for [C_{20}H_{23}O_4I+H]^+ 455.0714.

(2R,3S)-2,3-bis(benzyloxy)pent-4-enal (7)

Zinc dust (25.1 g, 384 mmol, 19 eq.) was activated by stirring in 2.5M HCl solution (250 ml) for 20 min at ambient temperature. Subsequently the mixture was filtered and washed with H_2O, MeOH and Et_2O. The zinc was fully dried under high vacuum at elevated temperature. Compound 6 (9.35 g, 20.6 mmol) was dissolved in THF/H_2O (250 ml, 9:1, v/v) and sonicated for 1 h at 40°C under argon flow, after which the activated zinc was added. After 3h of sonication the mixture was filtered over a pad of celite, which was rinsed with EtOAc. After removal of all solvents in vacuo, the mixture was diluted in Et_2O (200 ml) and washed with H_2O (2x 100 ml). The organic layer was dried with MgSO_4, filtered and concentrated in vacuo. Purification of the desired product was performed by column chromatography (pentane \( \rightarrow \) 25% Et_2O/pentane) which resulted in pale yellow oil (4.621 g, 15.59 mmol, 76%). \( R_f = 0.3 \) (Et_2O/pentane, 1:4, v/v). \( ^1H \) NMR (400 MHz, CDCl_3) \( \delta \) 9.66 (d, \( J = 1.5 \) Hz, 1H), 7.49 – 7.12 (m, 10H), 5.93 (ddd, \( J = 17.2, 10.5, 7.6 \) Hz, 1H), 5.38 – 5.28 (m, 2H), 4.73 (d, \( J = 12.1 \) Hz, 1H), 4.61 (dd, \( J = 12.0, 9.8 \) Hz, 2H), 4.34 (d, \( J = 12.1 \) Hz, 1H), 4.16 (dd, \( J = 7.6, 4.1 \) Hz, 1H), 3.82 (dd, \( J = 4.1, 1.5 \) Hz, 1H).
\[ ^{13}C \text{NMR (101 MHz, CDCl}_3 \delta 202.5, 137.6, 137.1, 133.9, 128.5-127.8, 119.9, 85.2, 79.9, 73.5, 70.7.} \]

**\( (2S,3R,4S,5S)-2,3-Bis(benzyloxy)-4-hydroxy-6-vinylhept-5-ethylenoate (8) \)**

Compound 4 (4.30 g, 14.5 mmol) dissolved in degassed milli-Q H\(_2\)O (125 ml) was added La(OTf)\(_3\) (17.611 g, 30.047 mmol, 2.1 eq.), indium (3.458 g, 30.12 mmol, 2.1 eq.) and ethyl-4-bromocrotonate (8.00 ml 75% pure, 43.6 mmol, 3 eq.). After 4 days, the mixture was filtered over a pad of celite, which was rinsed with Et\(_2\)O. The collected mixture was diluted with Et\(_2\)O (250 ml) and washed with H\(_2\)O (3x 50 ml). The organic layer was dried with MgSO\(_4\), filtered and concentrated in vacuo. Purification and separation of the obtained diastereomers was performed via flash column chromatography (pentane → 15% EtOAc/pentane) and resulted in clear oil (3.227 g, 7.861 mmol, 54%). \( R_f = 0.4 \) (EtOAc:pentane, 9:1, v/v). \(^1\)H NMR (400 MHz, CDCl\(_3 \) \( \delta \) 7.42 – 7.30 (m, 10H), 5.92 – 5.69 (m, 2H), 5.51 – 5.37 (m, 2H), 5.21 (d, \( J = 10.0 \) Hz, 1H), 5.06 (d, \( J = 11.3 \) Hz, 2H), 4.65 (t, \( J = 12.7 \) Hz, 2H), 4.45 (d, \( J = 11.5 \) Hz, 1H), 4.25 (d, \( J = 7.7 \) Hz, 1H), 4.15 (q, 6.7 Hz, 1H), 4.01 (s, 1H), 3.59 (d, \( J = 7.3 \) Hz, 1H), 3.34 (t, \( J = 9.1 \) Hz, 1H), 2.77 (d, \( J = 9.2 \) Hz, 1H), 1.27 (t, \( J = 6.9 \) Hz, 3H). \(^{13}\)C NMR (101 MHz, CDCl\(_3 \) \( \delta \) 172.6, 138.6, 138.5, 135.1, 133.0, 128.5-127.7, 120.1, 120.0, 83.1, 79.5, 74.7, 72.3, 70.9, 60.9, 55.3, 14.3. HRMS found 411.2162 [M+H]\(^+\), calculated for \([\text{C}_{25}\text{H}_{30}\text{O}_{5}+\text{H}]^+\) 411.2166.

**\( (2S,3R,4S,5S)-ethyl-4,5-bis(benzyloxy)-6-hydroxycyclohex-2-enecarboxylate (9) \)**

Compound 8 (2.907 g, 7.08 mmol) was dissolved in DCM (408 ml, 0.025 M) and Grubbs\(^{\text{2nd}}\) catalyst (0.157 g, 0.185 mmol, 2.61 mol\%) was added. The mixture was stirred at room temperature for 3 days in the dark after which it was concentrated in vacuo. Purification by column chromatography (10% EtOAc/pentane → 13% EtOAc/pentane) resulted in brown oil (2.46 g, 6.43 mmol, 91%). \( R_f = 0.52 \) (EtOAc:pentane, 1:3, v/v). \(^1\)H NMR (400 MHz, CDCl\(_3 \) \( \delta \) 7.38 – 7.27 (m, 10H), 5.80 (dt, \( J = 10.2 \), 2.1 Hz, 1H), 5.67 (dt, \( J = 10.2 \), 2.1 Hz, 1H), 4.96 (d, \( J = 11.3 \) Hz, 1H), 4.79 (d, \( J = 11.4 \) Hz, 1H), 4.74 – 4.62 (m, 2H), 4.25 – 4.08 (m, 4H), 3.65 (dd, \( J = 9.8 \), 7.5 Hz, 1H), 3.25 (ddd, \( J = 8.7 \), 5.7, 2.9 Hz, 1H), 2.96 (s, 1H), 1.25 (s, \( J = 7.2 \) Hz, 5H). \(^{13}\)C NMR (101 MHz, CDCl\(_3 \) \( \delta \) 172.1, 138.5, 138.1, 128.6, 128.3, 128.1 – 127.8, 124.2, 82.6, 79.4, 75.0, 72.0, 70.5, 61.4, 50.2, 14.3. HRMS found 383.1854 [H+H]\(^+\), calculated for \([\text{C}_{25}\text{H}_{26}\text{O}_{5}+\text{H}]^+\) 383.1853.
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(2S,3R,4S,5S)-2,3-Bis(benzyloxy)-4-hydroxy-5-hydroxymethyl-cyclohex-6-ene (10)

Dried compound 9 (2.04 g, 5.33 mmol) dissolved in anhydrous THF (300 ml). The mixture was cooled down to -20°C and DIBAL-H (25% in toluene) (35 ml, 53 mmol, 10 eq.) was added. The mixture was stirred 0.5h at 0°C followed by 24 h at ambient temperature. Subsequently, the mixture was quenched with EtOAc (10 ml) at 0°C, followed by addition of H2O (25 ml) and NaBH4 (1.41 g, 37.3 mmol, 7 eq.). After overnight stirring at room temperature, the reaction mixture was concentrated in vacuo. The mixture was diluted with EtOAc (50 ml) and washed with 1 M HCl (10 ml). The organic layer was dried with MgSO4, filtered and concentrated in vacuo. Purification by column chromatography (10% EtOAc/pentane → 45% EtOAc/pentane) resulted in white crystalline product (1.53 g, 4.49 mmol, 84%). Rf = 0.5 (EtOAc: pentane, 1:1, v/v).

1H NMR (400 MHz, CDCl3) δ 7.36 – 7.29 (m, 10H), 5.76 (dt, J = 10.2, 2.0 Hz, 1H), 5.50 (dt, J = 10.2, 2.0 Hz, 1H), 5.01 (d, J = 11.3 Hz, 1H), 4.77 – 4.58 (m, 3H), 4.23 – 4.15 (m, 1H), 3.81 – 3.57 (m, 4H), 2.87 (s, 2H), 2.47 (s, 1H). 13C NMR (101 MHz, CDCl3) δ 138.6, 138.2, 128.8 – 127.9, 127.6, 127.6, 83.5, 80.4, 75.0, 72.7, 71.6, 65.3, 45.4. HRMS found: 341.1750 [M+H]+, calculated for [C21H24O4]+H 341.1747.

(4aR,5R,6S,7R,8S,8aR)-6,7-bis(benzyloxy)-8-iodo-2-[(trichloromethyl)-4a,5,6,7,8,8a-hexahydro-4H-benzo[d][1,3]oxazin-5-ol (11)

Dry diol 10 (1.21 g, 3.55 mmol) dissolved in DCM (100 ml). The solution was cooled to 0°C., and trichloroacetonitrile (356 μl, 3.74 mmol) and 1,8-diazabicyclo[5.4.0]undec-7-ene (26 μl, 0.2 mmol) was added. After two hours stirring, TLC analysis revealed complete conversion of the starting material. To the solution was added H2O (15 ml), sodium hydrogen carbonate (2.99 g, 35.6 mmol, 10 eq.) and iodine (2.73 g, 10.7 mmol, 3 eq.). The resulting mixture was stirred overnight, quenched with 20% potassium thiosulfate solution (12.5 ml) and diluted with DCM. The layers were separated, after which the organic layer was dried with MgSO4 and concentrated in vacuo. Purification via flash column chromatography (pentane→10% EtOAc/pentane) afforded 11 (1.42 g, 2.33 mmol, 65%) as colorless oil. Rf=0.6 (EtOAc:pentane, 1:9, v/v). 1H NMR (400 MHz, CDCl3) δ 7.49 – 7.31 (m, 10H), 5.04 (d, J = 11.2 Hz, 1H), 4.92 – 4.81 (m, 2H), 4.77 (d, J = 11.3 Hz, 1H), 4.68 (d, J = 11.2 Hz, 1H), 4.59 (d, J = 11.3 Hz, 1H), 4.33 (dd, J = 11.1, 2.9 Hz, 1H), 4.15 – 4.08 (m, 1H), 3.79 (t, J = 9.2 Hz, 1H), 3.48 (t, J = 10.0 Hz, 1H), 2.78 – 2.62 (m, 2H), 2.52 (s, 1H). 13C NMR (101 MHz, CDCl3) δ 153.6, 138.4, 137.5, 128.9-128.2, 84.0, 77.5, 76.7, 75.7, 71.8, 68.1, 68.0, 58.6, 36.2, 33.3. HRMS found: 609.9812 [M+H]+, calculated for [C23H23Cl3INO4+H]+ 609.9810.
Chapter 2

(1R,2R,3R,4S,5S,6R)-4,5-bis(benzyloxy)-2-(hydroxymethyl)-7-azabicyclo[4.1.0]heptan-3-ol (12)

Imidate 11 (0.107 g, 0.175 mmol) was dissolved in a 7.0 ml solution of acetic acid, dioxane and water (8:1:1, v/v/v) and this mixture was stirred for three days at room temperature \([11] = 0.025 \text{M}\). After LC/MS and TLC showed full conversion of the starting material the mixture solvents were evaporated under reduced pressure. Toluene (3 ml) was used in order to azeotropically evaporate the water. The concentrated residu was redissolved in methanol, to which sodium hydrogencarbonate (0.30 g, 3.5 mmol, 20 eq.) was added and stirred for one day at elevated temperature (ca. 50°C). After LC/MS analysis showed full conversion towards the product, the reaction mixture was filtered over a small path of celite. Purification via flash column chromatography (DCM→10% MeOH/DCM) afforded compound 12 (56 mg, 0.16 mmol, 90%) as a white solid. \([R] = 0.375\) (MeOH:DCM, 1:9, v/v). \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta 7.40 – 7.27 (m, 10\text{H}), 4.96 (d, \(J = 11.3 \text{ Hz}, 1\text{H}), 4.78 (d, \(J = 11.4 \text{ Hz}, 1\text{H}), 4.66 (dd, \(J = 11.4, 3.0 \text{ Hz}, 2\text{H}), 3.94 (ddd, \(J = 15.3, 10.7, 5.3 \text{ Hz}, 2\text{H}), 3.75 (d, \(J = 8.1 \text{ Hz}, 1\text{H}), 3.53 (t, \(J = 9.9 \text{ Hz}, 1\text{H}), 3.37 (dd, \(J = 10.1, 8.1 \text{ Hz}, 1\text{H}), 3.17 – 2.51 (\text{br. s}, 2\text{H}), 2.43 (dd, \(J = 6.0, 3.2 \text{ Hz}, 1\text{H}), 2.27 (d, \(J = 6.1 \text{ Hz}, 1\text{H}), 2.10 – 2.01 (\text{m}, 1\text{H}), 1.25 (s, 1\text{H}).

\(^{13}\)C NMR (101 MHz, CDCl\(_3\)) \(\delta 138.6, 137.9, 128.7-127.8, 84.4, 81.4, 74.9, 72.4, 68.5, 64.8, 42.6, 33.1, 31.6.\) HRMS found: 356.1855 [M+H]\(^+\), calculated for [C\(_{21}\)H\(_{24}\)O\(_4\)+H]\(^+\) 356.1856.

(1R,2S,3S,4R,5R,6R)-5-(hydroxymethyl)-7-azabicyclo[4.1.0]heptane-2,3,4-triol (13)

Ammonia (10 ml) was condensed at −60°C. Lithium (50 mg) was added and the mixture was stirred until the lithium was completely dissolved. To this solution was added a solution of aziridine 12 (45 mg, 0.13 mmol) in dry THF (7 ml). The mixture was stirred for 2h at −60°C and subsequently quenched with MeOH/H\(_2\)O (5.0 ml, 8:2, v/v) The solution was allowed to get to room temperature while stirring so all ammonia could evolve. Next, the solution was concentrated \textit{in vacuo}, redissolved in H\(_2\)O (10 ml) and neutralized with Amberlite-H\(^+\). Product bound to the resin was eluted with 1M NH\(_4\)OH in MeOH (25 ml) solution. Solvents were evaporated under reduced pressure which afforded crude compound 13 (22 mg). This crude was kept under argon at -20°C and/or used directly in the acylation and N-alkylation reactions. \(^1\)H NMR (400 MHz, MeOD) \(\delta 3.96 (dd, \(J = 10.3, 4.4 \text{ Hz}, 1\text{H}), 3.72 – 3.58 (m, 1\text{H}), 3.55 (d, \(J = 8.2 \text{ Hz}, 1\text{H}), 3.18 – 3.07 (m, 1\text{H}), 2.99 (t, \(J = 9.8 \text{ Hz}, 1\text{H}), 2.52 (s, 1\text{H}), 2.21 (d, J
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= 5.5 Hz, 1H), 1.89 (d, J = 8.7 Hz, 1H). $^{13}$C NMR (101 MHz, MeOD) δ 79.5, 74.4, 69.7, 64.0, 45.7, 36.6, 34.2.

2-(6-(dimethylamino)-3-(dimethyliminio)-3H-xanthen-9-yl)-5-((2-(2-(4-(3-(1R,2S,3S,4R,5R,6R)-2,3,4-trihydroxy-5-(hydroxymethyl)-7-azabicyclo[4.1.0]hept-8-yl)propyl)-1H-1,2,3-triazol-1-yl)ethoxy)ethoxy)ethoxy)ethyl)carbamoyl) benzoate (ABP14)

Sodium bicarbonate (4.8 mg, 57 µmol) and iodo-pentyn (2.9 mg, 15 µmol) were added to a mixture of crude aziridine 13 (5 mg) dissolved in dry DMF (0.5 mL). The reaction was stirred for 24h at ca. 60°C. The volatiles were removed under reduced pressure, the residue was dissolved in water (10 mL) and washed with DCM (3 × 10 mL) and EtOAc (3 × 10 mL). The aqueous layer was concentrated under reduced pressure. The compound present in the residue containing was redissolved in H$_2$O (0.5 mL). Sodium ascorbate and CuSO$_4$ (5 µL of 1 M solution) were added into the aqueous mixture. After the mixture turned yellow-green TAMRA 34 (1.25 mg, 1.98 µmol dissolved in 1.5 mL H$_2$O) was added. The reaction was completed after 3 days according to LC/MS analysis. Solvents were evaporated and product ABP 14 (1.457 mg, 1.670 µmol, 6% yield over three steps) was isolated as a purple solid after HPLC-purification. $R_f = 0.1$ (MeOH:DCM, 1:3, v/v). $^1$H NMR (600 MHz, MeOD) δ 8.56 (s, 1H), 8.07 (d, J = 7.9 Hz, 1H), 7.81 (s, 1H), 7.38 (d, J = 7.9 Hz, 1H), 7.26 (d, J = 9.5 Hz, 2H), 7.04 (d, J = 9.5 Hz, 2H), 6.96 (s, 2H), 4.60 (s, 2H), 4.52 (t, J = 5.1 Hz, 2H), 4.00 (dd, J = 10.1, 4.4 Hz, 1H), 3.88 (t, J = 5.1 Hz, 2H), 3.74 (t, J = 5.3 Hz, 2H), 3.72 - 3.60 (m, 14H), 3.31 (d, J = 3.7 Hz, 12H), 3.12 (dd, J = 9.9, 8.2 Hz, 1H), 3.03 (t, J = 9.8 Hz, 1H), 2.81 - 2.71 (m, 2H), 2.45 - 2.37 (m, 1H), 2.24 - 2.16 (m, 1H), 2.03 - 1.99 (m, 1H), 1.96 - 1.85 (m, 3H), 1.61 (d, J = 12.0 Hz, 1H). $^{13}$C NMR (150 MHz, MeOD) δ 169.2, 169.0, 162.9, 159.0, 158.7, 157.0, 137.0, 136.9, 132.8, 132.6, 130.8, 129.5, 129.5, 124.1, 115.0, 114.8, 102.0, 97.3, 79.0, 74.0, 71.6 - 70.4, 70.14, 63.7, 61.2, 51.3, 49.8, 49.6, 45.5, 45.5, 43.1, 41.2, 40.8, 30.2, 24.1. HRMS: found 872.4184 [M+H]$^+$, calculated for [C$_{45}$H$_57$O$_{11}$N$_7$+H]$^+$ 872.4189.
Cell culture
Human Embryonic Kidney 293T (HEK293T) cells (Sigma) were cultured in DMEM high glucose (Sigma) supplemented with 10% NBS and 100 units/mL penicillin/streptomycin (Gibco) at 37°C and 5% CO₂. RAW 264.7 (American Type culture collection) were cultured in RPMI (Sigma) supplemented with 10% FCS, 1 mM Glutamax and 100 units/mL penicillin/streptomycin (Gibco) at 37°C and 5% CO₂.

Transient and stable overexpression of human GBA2 and GBA3
Primers used for transient GBA2 expressing HEK293T were designed based on NCBI reference sequence NM_020944.2. The full-length coding sequences were cloned into pcDNA3.1/Myc-His (Invitrogen, Life Technologies, Carlsbad, CA. Sub-confluent HEK293T cells were transfected with empty pcDNA3.1 or GBA2pcDNA3.1 (Plasmid:PEI ratio 1:3). Media was refreshed 24 hours later and cells collected 72 hours after transfection in PBS buffer. Cells were centrifuged at 1000 rpm for 10 minutes, after which the supernatant was removed. Cell pellets were snap-frozen with liquid nitrogen and stored at -80°C.

Stable GBA2 expressing HEK293T cells were generated as follows. The PCR-amplified human GBA2 (GBA2, acc. nr: NM_020944.2) coding sequence (using the following primers: sense 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTAACCAACCA TGGGGACCCAGGATCCAG-3' and antisense 5'- GGGGACCCTTTGTACAAGAAAGCTGGTTTCACTG GGGCCTAGGTGTTTG-3') was cloned into pDNOR-221 and sub-cloned in pLenti6.3/TO/V5-DEST using the Gateway system (Invitrogen). Correctness of the construct was verified by sequencing. HEK293T cells were transfected with pLenti6.3-GBA2 in combination with the envelope and packaging plasmids pMD2G, pRRE and pRSV. Subsequently, culture supernatant containing viral particles was collected and used for infection of HEK293T cells. Selection using blasticidin for several weeks rendered cells stably expressing human GBA2 as determined by activity assays. For stable expression of GBA3 in HEK293T cells, the PCR-amplified GBA3 (GBA3, acc. nr: NM_020973.4) coding sequence (using the following oligonucleotides: sense 5'-GAATTCGCCGCACCATGGCTTTCCCCTG GAGATTTG-3' and antisense 5'- GCGGCCGCAGATGTGCTTCAA GGCCATTG-3') was cloned into pcDNA3.1/Zeo and transfected into HEK293T cells using FuGene® 6 Transfection Reagent (Promega Benelux, Leiden, The Netherlands). Selection using Zeocin for several weeks rendered cells stably expressing human GBA3 as determined by activity assays.
Preparation of lysates
Cell pellets were suspended in lysis buffer (20 mM Hepes, 2 mM DTT, 0.25 M sucrose, 1 mM MgCl₂, 2.5 U/ml benzonase, pH 7.0). The homogenate was incubated on ice for 30 minutes after lysis and homogenization using SilentCrusher (Heidolph). Ultracentrifugation was performed at 32.000 rpm for 30 minutes at 4°C. Supernatant fractions were aliquoted in appropriate volumes, after the total protein concentration was determined via a Bradford assay, using BSA (Sigma) for standards and BioRad Quickstart Bradford Reagents. Aliquots were snap-frozen with liquid nitrogen and stored at -80°C. For the lysates of mouse brain tissue the lysis was performed in a similar lysis buffer as described above. Tissue was homogenized using sonication.

Gel-Activity Based Protein Profiling experiments
Prepared lysate was diluted in assay buffer (20 mM Hepes, 2 mM DTT at pH 7.0) until appropriate final protein concentration was reached. Samples from HEK cells were incubated with 500 nM probe at 37°C for 1 hour (V_{final} = 20 µL). Lysates from mouse tissue were treated with 1 µM of probe in McIlvaine buffer (pH 5.0). Protein content was denatured using Laemli Buffer (4x) at 100°C for 3 minutes. Reactions were resolved by 12.5% SDS-PAGE electrophoresis and wet slabs were scanned for fluorescence (Molecular Imager Gel Doc XR, Biorad). For the in situ competitive ABPP experiments stable GBA2 and GBA3 overexpressing HEK293T cells were incubated for 1 hour at 37°C with various concentrations of the selected inhibitors. Cells were washed 2 times with PBS and then harvested by scraping in 25 mM potassium phosphate buffer (pH 6.5) containing 0.1% Triton X-100 (v/v). Cells were lysed via snap-freezing in liquid nitrogen. Protein concentration was determined via BCA assay (Thermo Fischer). Labelling of the glucosidases was conducted using 500 nM ABP 6 for 30 minutes at 37°C on 20 µg protein. Protein content was denatured using Laemli Buffer (5x) at 100°C for 5 minutes. Reactions were resolved by 10% SDS-PAGE electrophoresis and wet slabs were scanned as described above.

Optimization of the FluoPol-ABPP assay
Optimal probe concentration on FluoPol signal was determined by varying probe concentrations from 25 nM to 500 nM probe at a constant lysate concentration (0.5 mg/ml) and neutral pH. FluoPol-ABPP assays were also performed at different pH values. Heps was replaced by 20 mM citric acid-citrate buffer for reactions at pH ≤ 6. The pH-experiments were performed at optimal probe concentration (50 nM). Competition experiments were conducted by 30 minutes pre-incubation of compounds in the lysates at 37°C (2.5 % DMSO). All reactions (V_{final} = 75 µL)
contained Bovine Gamma-Globulin (Sigma) and Chaps (Sigma), respectively. 0.5 mg/ml and 1 mg/ml, were carried out in 96-wells plates (flat-black bottom, Greiner). FluoPol-signals were monitored on an Infinite M1000Pro (Tecan) using $\lambda_{ex} = 530 \text{ nm}$ and $\lambda_{em} = 580 \text{ nm}$. Mock containing lysates were used as reference samples, samples without probe as blanks to correct for background polarization and GBA2 containing lysates without inhibitor as controls. All samples were corrected for background polarization and the residual enzyme activity was calculated based on the polarization signal from the controls. Polarization signals were plotted against time or inhibitor concentration and processed in GraphPad Prism 6.0. IC$_{50}$ values were calculated via non-linear regression using mentioned software (N=2, n=3).

**FluoPol-ABPP screen of the iminosugar library**

The screen, using the optimized conditions as described above, was conducted in a 384-well black-bottom plate (Greiner) with reaction volumes set at 15 µL. Concentration of the iminosugars during the screen was 100 nM. The FluoPol signal was measured on a Clariostar (BMG Labtech). Resulting polarization signals were processed as described above. Residual enzyme activities were plotted against the corresponding iminosugar.

**Analysis of selected compounds as inhibitors of enzymatic activity of GCS, GBA1, GBA2 and GBA3**

All IC$_{50}$ values were determined in triplicate and the inhibitors tested were pre-incubated for 30 minutes at 37°C. K$_i$ values were determined in duplicate using a range from 0.05 till 5 mM of 4MU-β-D-glucopyranoside in appropriate buffer containing inhibitor. Incubation time and temperature was performed as described below, but without the pre-incubation step. Observed fluorescence was curve-fitted against inhibitor or substrate concentrations using GraphPad Prism 6.0 in order to obtain IC$_{50}$ or K$_i$ values.

**GBA1**: Pure recombinant human enzyme (Cerezyme from Genzyme) was used. Activity was measured with with 3.7 mM 4-methylumbeliferone (4MU)-β-D-glucopyranoside (Sigma) in 150 mM Mcllvaine buffer pH 5.2 supplemented with 0.2% sodium taurocholate (w/v), 0.1% Triton X-100 (v/v), 0.1% bovine serum albumin (BSA) (w/v) for 30 min. The reaction was stopped with excess 1M NaOH-Glycine (pH 10.3), liberated 4MU fluorescence was measured with a fluorimeter LS55 (Perkin Elmer) using $\lambda_{ex}$ 366 nm and $\lambda_{em}$ 445 nm.

**GBA2**: For GBA2 measurements, cellular homogenates of a stable HEK293T over-expressing GBA2 cell line pre-incubated for 30 min with an inhibitor of GBA1 (1 mM
conduritol β epoxide CBE from Sigma)\textsuperscript{31} were used. Activity was measured with 3.7 mM 4MU-β-D-glucopyranoside in 150 mM McIlvaine pH 5.8, 0.1% BSA (w/v) for 1 h. Reactions were stopped as described above for GBA1.

**GBA3**: For GBA3 measurements, cellular homogenates of a stable HEK293T over-expressing GBA3 cell line also pre-incubated for 30 min with an inhibitor of GBA1 (1 mM conduritol β epoxide CBE from Sigma) were used. Reactions were conducted in 100 mM hepes pH 7.0, 0.1% BSA (w/v) for 1 h.

**GCS**: IC\textsubscript{50} values for GCS were determined in situ with 6-[N-methyl-N-(7-nitrobenz-2-oxa-1,3-diazol-4-ylaminododecanoyl]sphingosine (NBD-ceramide) as substrate. RAW 264.7 cells were grown to confluence in 6-well plates and pre-incubated for 1h with an inhibitor of GBA1 activity (300 µM CBE), followed by 1h incubation at 37°C with 1 µM C6-NBD-ceramide and in the presence of a range of inhibitor concentrations. The cells were washed 3x with PBS and harvested by scraping. After lipid extraction (described by Bligh and Dyer),\textsuperscript{30} the C6-NBD lipids were separated and detected by High Performance Liquid Chromatography (λ\textsubscript{Ex} 470 nm and λ\textsubscript{Em} 530 nm). IC\textsubscript{50} values were determined in duplicate from the titration curves of observed formed C6-NBD-glucosylceramide and data was curve-fitted via GraphPad Prism 6.0.

### 2.5 References


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