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Design and development of conformational inhibitors and activity-based probes for retaining glycosidases

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Activity-based protein profiling of lysosomal GH35 and GH59 β -galactosidases¹

3.1 Introduction

Exo- β -galactosidases catalyze the hydrolysis of terminal β -galactose residues in oligosaccharides, glycoproteins and glycosphingolipids. Two distinct β -galactosidases are present in the human lysosome, namely, acid β -galactosidase (GLB1, EC 3.2.1.23, GH35) and galactosylceramidase (GALC, EC 3.2.1.46, GH59). Whereas both enzymes recognize β -galactopyranosides as substrate, they differ in their preference for the aglycon: GALC appears to have evolved to degrade galactosphingolipid substrates, whereas GLB1 takes on β -galactopyranosides featuring a carbohydrate aglycon.² A selective readout for these enzymes in the context of complex biological mixtures would be interesting from a fundamental point of view but also from a clinical perspective. Deficiency in GLB1 is the primary cause of GM1-

gangliosidosis³⁻⁵ and Morquio B syndrome.^{2,6,7} Although both lysosomal storage disorders (LSDs) are induced by mutations in the *GLB1* gene, GM1-gangliosidosis is caused by accumulation of GM1 ganglioside in the central nervous system^{3-5,8,9}, whereas Morquio B syndrome is characterized by storage of keratan sulfate.¹⁰ Krabbe disease in turn is a LSD caused by a deficiency in *GALC*. *GALC* catalyzes the hydrolysis of galactosylceramide and psychosine in the lysosome. In the brains of Krabbe affected individuals accumulation of psychosine is observed.^{11,12} Although the molecular mechanism is not yet fully elucidated, accumulation of psychosine is considered as the main cause of the pathogenic symptoms characteristic for Krabbe disease.¹³⁻¹⁵

GLB1 and *GALC* hydrolyze their substrate β -galactopyranosides through a double-displacement mechanism following principles originally outlined by Koshland.¹⁶ Upon binding of the substrate to the enzyme active site, the exocyclic oxygen bearing the aglycon is protonated by the general acid/base catalyst (Glu-188¹⁷ in the human GH35 β -galactosidase or Glu-182¹⁸ in mice GH59 β -galactosidase on which structural studies demonstrating the mechanism of action were performed). Upon protonation and expulsion of the aglycon, the catalytic nucleophile (Glu-268¹⁷ in human GH35 β -galactosidase or Glu-258¹⁸ in mice GH59 β -galactosidase) traps the emerging oxycarbenium ion, to yield in a formal S_N2 displacement process a covalent enzyme- α -galactopyranosyl adduct (Figure 3.1A). The covalent galactosyl-enzyme intermediate is subsequently hydrolyzed to release galactopyranose with an overall retention of configuration at the anomeric center. This mechanism, specifically the involvement of a covalent intermediate in GH35/GH59 galactosidase-mediated substrate processing, invites the design of activity-based probes (ABPs) following strategies that have been reported on in recent years (Figure 3.1B).¹⁹⁻²²

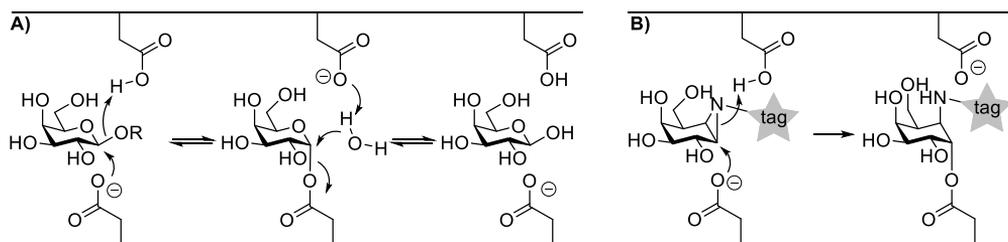


Figure 3.1 A) Double displacement mechanism of retaining β -galactosidases. B) Proposed mechanism of cyclophellitol aziridine based probes in β -galactosidases.

Cyclophellitol, a natural product and retaining β -glucosidase inhibitor, has been modified for activity-based glycosidase profiling, by featuring a reporter moiety (fluorophore, biotin) at C6 (glucose numbering, C8 in cyclophellitol numbering).²³

Cyclophellitol adopts a 4H_3 conformation and thereby emulates the transiently formed oxycarbenium half-chair intermediate that also characterizes retaining β -glucosidase-mediated substrate processing.²⁴ The presence of a large, bulky group (the reporter moiety) at C6 induced a remarkable selectivity to the probes, which out of three cellular retaining β -glucosidases selected a single species, lysosomal glucosylceramidase (GBA1) as their single target. In a follow-up study and with the aim to develop to label, besides GBA1, also the two neutral, cytosolic retaining β -glucosidases, GBA2 and GBA3 (and, in intestines, the β -glucosidase activity in the enzyme complex, lactose-phloritizin hydrolase), the reporter moiety was moved from C6 to the ring nitrogen of cyclophellitol aziridine. These studies disclosed that *N*-functionalized cyclophellitol aziridines are broad-spectrum activity-based probes for retaining β -glucosidases.²⁵ Recently Marques *et al.*²⁶ reported that β -galactose-configured cyclophellitol with a reporter group at C6 is a selective probe for GALC. This situation resembles the one encountered previously with the C6-modified cyclophellitol probe.²³ An obvious solution when aiming for profiling GALC and GLB1 in an ABPP fashion, thus both retaining β -galactosidases expressed in human cells, would be to prepare the corresponding β -galactopyranose-configured *N*-modified cyclophellitol aziridine probes. As is shown here, such β -galactopyranose-cyclophellitol aziridines bearing a reporter moiety at the aziridine nitrogen are indeed synthetically tractable, even though intermediates towards the final product are highly sensitive to hydrolysis.

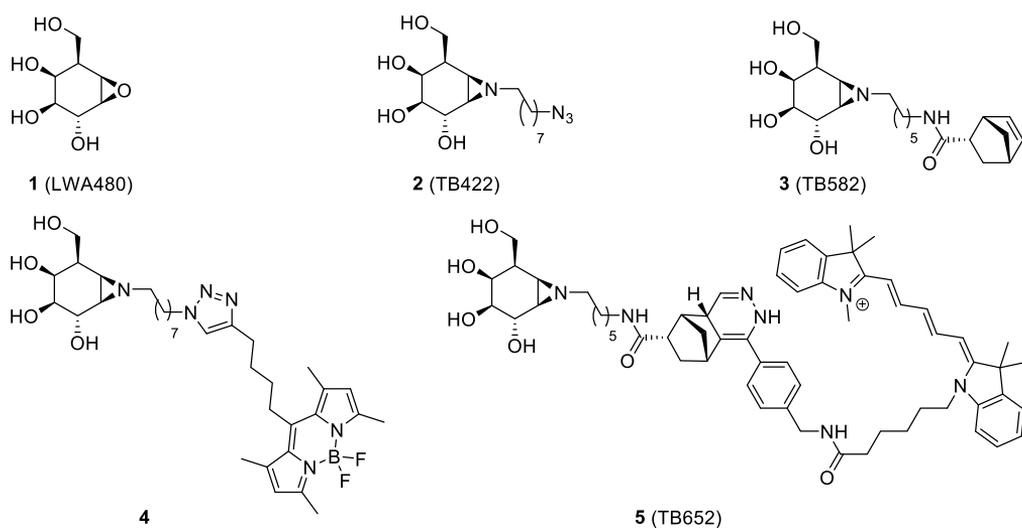


Figure 3.2 Structures of β -galactopyranose-cyclophellitol (**1**), intermediates **2** and **3** for bioorthogonal ligation, and ABPs (**4** and **5**) targeted in this study.

The free aziridine²⁷ as well as azide **2** can be obtained in analytically pure form (Figure 3.2), however ensuing copper(I)-catalyzed [2+3]-cycloaddition ‘click’ ligation to install a reporter moiety proved abortive. Altering the strategy to include inverse-electron demand Diels-Alder ligation between a norbornene and a tetrazine did yield the desired one-step broad-spectrum β -galactosidase probe, as is shown in the following.

3.2 Results and discussion

As the first research objective, a crystal of bacterial β -galactosidase of the carbohydrate family GH35 (CjGH35A) was soaked with β -galactose-configured cyclophellitol aziridine **7** (TB562)²⁷ to confirm the mechanism-based design and covalent mode of action of this class of inhibitors towards GH35. This resulted in an enzyme-**7** complex (Figure 3.3). The nucleophilic glutamate residue has reacted with compound **7** through nucleophilic addition at C1, which resulted in opening of the aziridine and concomitant conformation change of covalently bound substrate from a ⁴H₃ half chair conformation to a ⁴C₁ chair conformation. In addition, a fluorogenic substrate activity assay was performed in CjGH35A with compound **7**. After 30 min of pre-incubation with varying concentrations of the inhibitor, the residual enzyme activity was measured using the fluorogenic 4-methylumbelliferyl β -D-galactopyranoside substrate. The apparent IC₅₀ value of 21.55 \pm 2.14 nM was obtained in this assay. These results underscore that cyclophellitol aziridine **7** is a suitable starting point for the design of ABPs targeting the GH35 family of β -galactosidases.



Figure 3.3 Crystal structure of bacterial β -galactosidase of the carbohydrate family GH35 (CjGH35A) in complex with mechanism-based inhibitor **7**²⁷.

In initial studies on the use of cyclophellitol aziridine as a scaffold for ABP design, reporter moieties to the aziridine nitrogen were grafted through *N*-acylation.^{25,28-30} In a head-to-head comparative study (Chapter 2), *N*-alkyl aziridines featuring the same

carbohydrate-derived configuration were synthetically more tractable. In addition to this *N*-alkyl-cyclophellitol aziridines were equally potent in inhibiting retaining β -glucosidases as their corresponding *N*-acyl counterparts.³¹ Based on these considerations, β -galactose-configured cyclophellitol aziridine **7**, prepared as described previously²⁷, was reacted with 8-azido-1-iodooctane **10** under basic conditions (Figure 3.4A). In this way, analytically pure two-step ABP **2** was obtained in 25% yield. Cu(I)-catalyzed azide/alkyne cycloaddition (CuAAC) with Cy5-alkyne³² however proved abortive, in that no homogeneous material was obtained after reversed phase HPLC. Instead, a number of unidentified products were obtained, and it may be that the aziridine moiety is unstable under the copper(I)-catalyzed click conditions, the work-up/purification protocols used, or both.

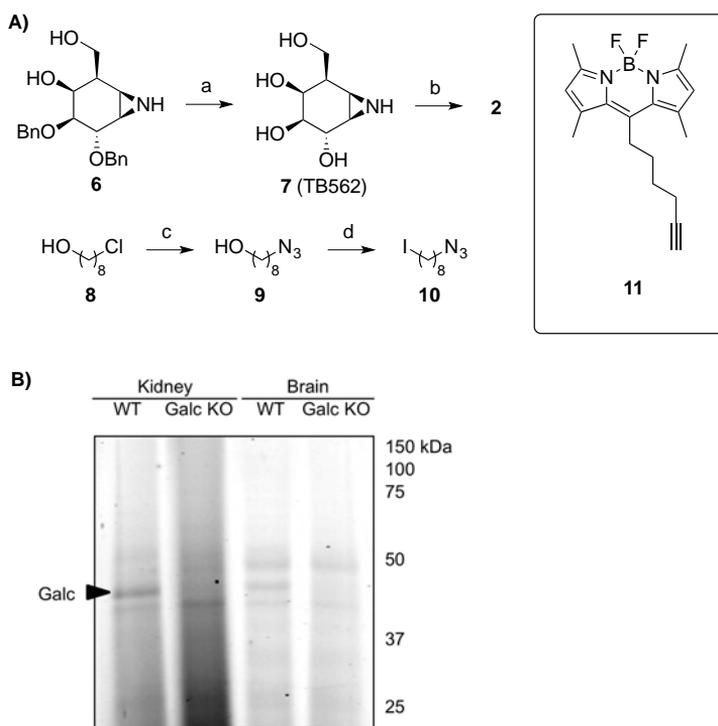
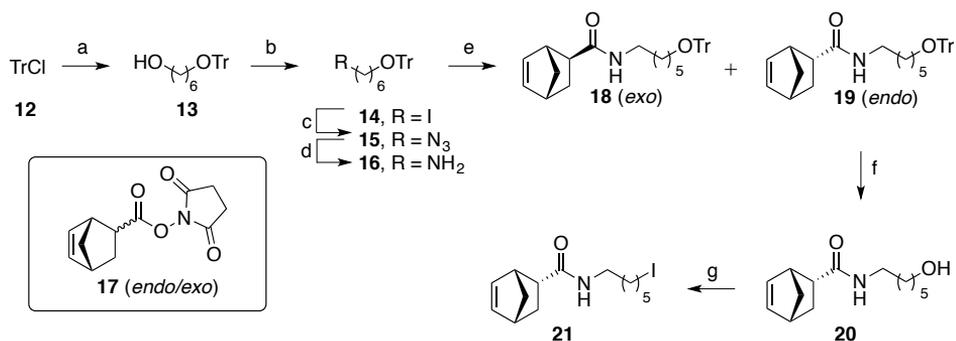


Figure 3.4 A) Synthesis of precursor probe **2**, reagents and conditions: a) Li, NH₃, -60 °C, b) **10**, K₂CO₃, DMF, 80 °C, overnight, 25%; c) NaN₃, DMF, 80 °C, quant; d) PPh₃, imidazole, I₂, THF, -20 °C to rt, 15 min, 69%. B) Labeling of GALC in kidney and brain homogenates of wt and GALC-deficient mouse tissues (Galc KO) using copper(I)-catalyzed azide/alkyne cycloaddition (CuAAC). After incubation for 1 h with compound **2** (1 μ M) at 37 °C, the mixture was diluted with a NaOAc buffer, incubated overnight at rt with BODIPY-alkyne **11** (1 μ M), TBTA, CuSO₄ and DTT and resolved by 7.5% SDS-PAGE gel with fluorescent read-out.

Efforts were next turned to two-step labeling to avoid purification of the one-step probe. This classical ABPP method was successful in labeling GBA1 in cell extracts using CuAAC.³³ Following this protocol labeling of GALC in kidney and brain mice homogenates was attempted first. After incubation of the probe for 1 h in the homogenate, a fresh mixture of fluorophore **11**³⁴, CuSO₄, tris(benzyltriazolylmethyl)amine (TBTA) and dithiothreitol (DTT) were added. The additive DTT was used to reduce Cu^{II} to active Cu^I and TBTA stabilizes the reactive Cu^I. After loading on gel a clear band in the fluorescent read-out of ABP **4** labeling GALC (~45 kDa^{35,36}) was detected in the kidney lysate (Figure 3.4B). GALC-deficient lysates of a Twitcher mice were used as a control.³⁷

In another attempt to obtain a one-step β -galactosidase ABP, the inverse-electron-demand Diels-Alder (IEDDA) ligation strategy was investigated to introduce the fluorophore at the final step of probe synthesis.³⁸⁻⁴⁰ For this purpose norbornene-modified cyclophellitol aziridine **3** was synthesized and reacted with tetrazine-Cy5 **22** to obtain ABP **5** (Figure 3.2). The synthesis of norbornene-functionalized aziridine started with monotritylation of 1,6-hexanediol to give **13** (Scheme 3.1). Subsequent hydroxyl-halogen exchange of the primary alcohol with iodine followed by substitution with sodium azide afforded **15**.⁴¹ Reduction of azide **15** using triphenylphosphine on beads gave amine **16**. Norbornene-OSu ester **17** was obtained according to the literature procedure³⁸ as a mixture of *endo*- and *exo*-isomers. This mixture was condensed with amine **16** resulting in a mixture of norbornene-trityl products (*exo/endo*, 1:2.3), which were easily separated by silica gel column chromatography. Deprotection of the major *endo*-product **19** under acidic conditions gave alcohol **20** in 86% yield. Treatment of **20** with triphenylphosphine, iodine and imidazole at elevated temperatures in tetrahydrofuran afforded iodonorbornene **21**.

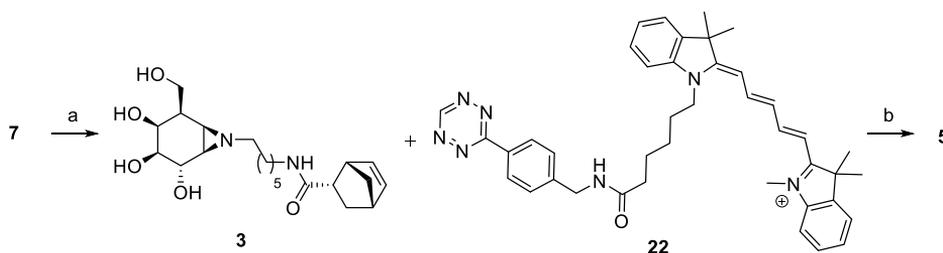
Scheme 3.1 Synthesis of probe **3** with a norbornene ligation handle.



Reagents and conditions: a) 1,6-hexanediol, pyridine, CH_2Cl_2 , rt, 90 min, 95%; b) imidazole, PPh_3 , I_2 , Et_2O , CH_3CN , rt, overnight, 80%; c) NaN_3 , DMF, 80 °C, overnight, quant; d) PPh_3 on beads, H_2O , THF, 48 h, quant; e) norbornene-OSu³⁸, DIPEA, DCE, rt, overnight, **18** 28%, **19** 68%; f) *p*-toluenesulfonic acid, CH_2Cl_2 , MeOH, rt, overnight, 86%; g) PPh_3 , I_2 , imidazole, THF, reflux, 1.5 h, 73%.

Cyclophellitol aziridine **7** was then *N*-alkylated with iodonorbornene linker **21** using potassium carbonate as the base in DMF, yielding two-step ABP **3** in poor yield but useful quantities (Scheme 3.2). Subsequent ligation⁴² with Cy5 tetrazine **22** was successful and gave ABP **5** as mixture of isomers.

Scheme 3.2 Synthesis of ABP **5** with a norbornene ligation handle.



Reagents and conditions: a) **21**, K_2CO_3 , DMF, 75 °C, overnight, 12%. b) Cy5 tetrazine **22**, MeOH, overnight, 87%.

To determine the potency of inhibitors **1-3** (Figure 3.2), a fluorogenic substrate activity assay as described for CjGH35A was performed in human embryonic kidney 293 (HEK293) cells overexpressing GALC and in human fibroblast lysates to determine the GLB1 activity. The apparent IC_{50} values are listed in Table 3.1. The obtained cyclophellitol aziridine inhibitors proved to be nanomolar inhibitors in these assays.

Table 3.1 Apparent IC_{50} values (nM) determined for compounds **1-3**, **5** and **7** for GLB1 and GALC.

Compound	GLB1	GALC
1 (LWA480)	21.7 ⁴³	39.1 ⁴³
2 (TB422)	2.55 ± 0.47	11.96 ± 2.27
3 (TB582)	57.84 ± 3.05	98.55 ± 20.84
5 (TB652)	14.85 ± 0.98	61.02 ± 6.89
7 (TB562)	2.55 ± 0.59	5.57 ± 0.36

In the next set of experiments ABP 5 probe was examined in an activity-based protein profiling setting in mouse kidney homogenates. GBA (58 - 66 kDa²³), the lysosomal glucosylceramidase, has a similar mass as GLB1 (~64 kDa⁴⁴). Therefore, lysates were pre-incubated with MDW933²³, a known selective covalent GBA inhibitor. First, the pH-dependent activity of probe 5 was determined. As depicted in Figure 3.5A, ABP 5 labels GLB1 and GALC (~45 kDa^{35,36}) in an effective manner at pH 4.0 up to pH 6.0. With this result in hand, a concentration dependent labeling was performed at pH 4.5. As shown in Figure 3.5B, the probe labels both enzymes effectively at 1 μ M concentration. Finally, the incubation time of the probe in the lysate was varied up to 120 min without pre-incubation with MDW933 (Figure 3.5C). Labeling intensity of the proteins increases over time, supporting mechanism-based action of the probe.

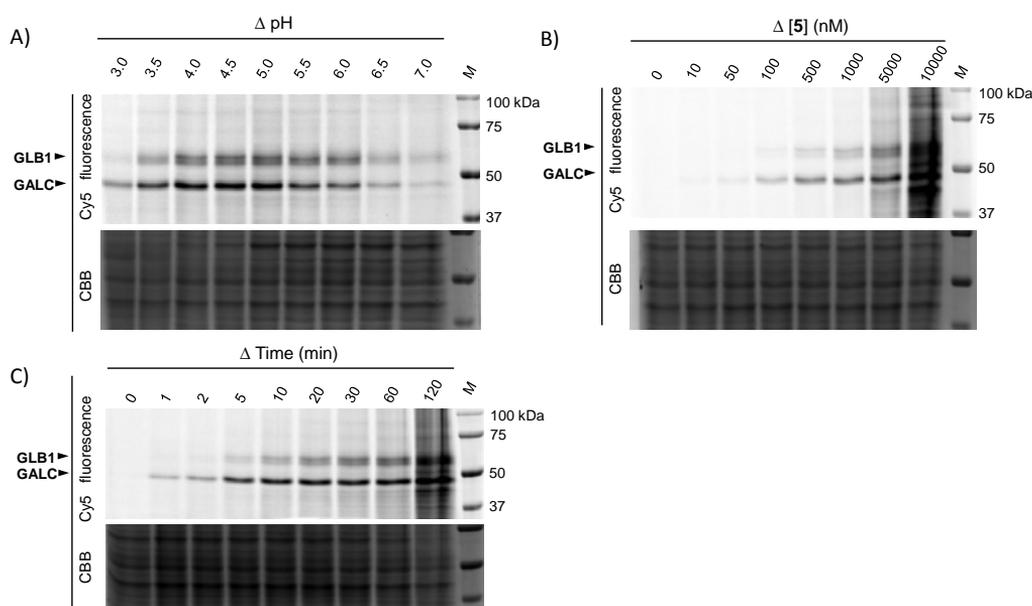


Figure 3.5 Activity-based protein profiling with probe 5 at various conditions in mouse kidney homogenates after blocking the GBA activity (A and B). A) pH-dependent labeling with 1 μ M probe 5 for 30 min. B) Concentration dependent labeling at pH 4.5 for 30 min. C) Incubation time dependent labeling with 1 μ M probe at pH 4.5.

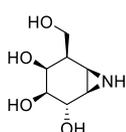
2.3 Conclusion

In conclusion, in this chapter the synthesis of cyclophellitol aziridine based ABPs for human lysosomal β -galactosidases is described. ABP 5 could be obtained even though the starting material proved to be susceptible to hydrolysis and proved, in a set of initial

experiments, to be effective in labeling the human lysosomal β -galactosidases, GLB1 and GALC. ABP **5** and differently functionalized ABPs (bearing, for instance, a biotin and potentially prepared through IEDDA as well) may be exploited to study the role of β -galactosidases in LSDs and the remarkable differences between GM1 gangliosidosis and Morquio B syndrome caused by mutations in GLB1.

Experimental

General: Chemicals were purchased from Acros, Sigma Aldrich, Biosolve, VWR, Fluka, Merck and Fisher Scientific and used as received unless stated otherwise. Dichloromethane (DCM), tetrahydrofuran (THF), *N,N*-dimethylformamide (DMF) and toluene were stored over flame-dried 4 Å molecular sieves before use. Pyridine and triethylamine were stored on KOH pellets before use. Traces of water from reagents were removed by co-evaporation with toluene in reactions that require anhydrous conditions. All reactions were performed under an argon atmosphere unless stated otherwise. TLC analysis was conducted using Merck aluminum sheets (Silica gel 60 F₂₅₄) with detection by UV absorption (254 nm), by spraying with a solution of (NH₄)₆Mo₇O₂₄·4H₂O (25 g/L) and (NH₄)₄Ce(SO₄)₄·2H₂O (10 g/L) in 10% sulfuric acid, a solution of KMnO₄ (20 g/L) and K₂CO₃ (10 g/L) in water or ninhydrin (0.75 g/L), followed by charring at ~150 °C. Size exclusion was performed on Sephadex LH-20 (eluent DCM/MeOH, 1:1). Column chromatography was performed using Screening Device b.v. Silica Gel (particle size of 40 – 63 μ m, pore diameter of 60 Å) in the indicated solvents. For reversed-phase HPLC purifications a HPLC system equipped with a C18 semi-preparative column (Gemini C18, 250x10 mm, 5 μ m particle size, Phenomenex) was used. LC/MS analysis was performed on a Surveyor HPLC system (Thermo Finnigan) equipped with a C18 column (Gemini, 4.6 mm x 50 mm, 5 μ m particle size, Phenomenex), coupled to a LCQ Advantage Max (Thermo Finnigan) ion-trap spectrometer (ESI⁺). The applied buffers were H₂O, MeCN and 1% aqueous TFA. ¹H NMR and ¹³C NMR spectra were recorded on a Brüker AV-400 (400 and 101 MHz respectively) spectrometer in the given solvent. Chemical shifts are given in ppm (δ) relative to the residual solvent peak or tetramethylsilane (0 ppm) as internal standard. Coupling constants are given in Hz. High-resolution mass spectrometry (HRMS) analysis was performed with a LTQ Orbitrap mass spectrometer (Thermo Finnigan), equipped with an electrospray ion source in positive mode (source voltage 3.5 kV, sheath gas flow 10 mL/min, capillary temperature 250 °C) with resolution R = 60000 at m/z 400 (mass range m/z = 150–2000) and dioctyl phthalate (m/z = 391.28428) as a “lock mass”. The high-resolution mass spectrometer was calibrated prior to measurements with a calibration mixture (Thermo Finnigan).



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

NH₃ gas was condensed at -60 °C and liquid NH₃ was collected (\pm 5.0 ml). To the liquid NH₃ was added lithium (35 mg) and the reaction mixture turned into a dark blue solution. The mixture was stirred until all the lithium was completely dissolved, after which a solution of benzylated aziridine **6** (70 mg, 0.20 mmol) in THF (3 ml) was added dropwise. After stirring for 45 min at -60 °C, the reaction mixture was quenched with MilliQ (2.0 ml).

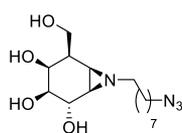
The mixture was gradually warmed to rt and concentrated *in vacuo*. The crude product was dissolved in H₂O and treated with Amberlite IR-120 NH₄⁺ for 5 min. After filtration of the resin, the filtrate was concentrated *in vacuo* and retreated with Amberlite IR-120 NH₄⁺ to obtain β-aziridine **7** (22.6 mg, 0.13 mmol, 65%) as a white solid which was used without further purification. ¹H NMR (400 MHz, D₂O) δ 3.94 (d, *J* = 8.4 Hz, 1H), 3.84 – 3.80 (m, 3H), 3.40 (dd, *J* = 8.0, 0.8 Hz, 1H), 2.41 (s, 1H), 2.30 (d, *J* = 5.6 Hz, 1H), 2.17 (s, 1H). ¹³C NMR (101 MHz, D₂O) δ 75.9, 70.3, 70.0, 61.1, 39.1, 34.3, 31.9.

 **8-azido-octan-1-ol (9)**

To a solution of 8-chloro-1-octanol (1.68 mL, 10 mmol) in DMF (25 mL) was added sodium azide (5.50 g, 100 mmol). After stirring overnight at 50 °C and an additional 4 h at 80 °C, the reaction mixture was concentrated *in vacuo*. The resulting crude product was dissolved in EtOAc (300 mL) and washed with H₂O (4 x 200 mL). The organic layer was dried over MgSO₄ and concentrated *in vacuo*, resulting in azide alcohol **9** as a clear oil in quantitative yield (1.71 g, 10.0 mmol). ¹H NMR (400 MHz, CDCl₃): δ (ppm) = 3.64 (t, *J* = 6.6 Hz, 2H), 3.26 (t, *J* = 6.9 Hz, 2H), 1.64 – 1.51 (m, 4H), 1.47 (s, 1H), 1.44 – 1.28 (m, 8H). ¹³C NMR (101 MHz, CDCl₃): δ (ppm) = 63.1, 51.6, 32.8, 29.4, 29.2, 28.9, 26.8, 25.8. HRMS: calculated for [C₈H₁₈N₃O]⁺ 172.14444, found 172.14419.

 **1-azido-8-iodooctane (10)**

To a solution of **9** (1.71 g, 10.0 mmol) in THF (50 mL) was added triphenylphosphine (3.0 g, 11.8 mmol) and imidazole (2.0 g, 29.4 mmol). The solution was cooled to -20 °C and iodine (2.61 g, 10.3 mmol) was added. After stirring the reaction mixture for 15 min at room temperature, the reaction was quenched with saturated NaHCO₃ (30 mL) at 0 °C. The reaction mixture was extracted with Et₂O (100 mL), dried over MgSO₄ and concentrated *in vacuo*. Purification over silica gel column chromatography (pentane → 5% EtOAc in pentane) gave **10** (1.95 g, 6.9 mmol, 69%) as a clear oil. ¹H NMR (400 MHz, CDCl₃): δ (ppm) = 3.26 (t, *J* = 6.9 Hz, 2H), 3.19 (t, *J* = 7.0 Hz, 2H), 1.86 – 1.77 (m, 2H), 1.66 – 1.54 (m, 2H), 1.46 – 1.27 (m, 8H). ¹³C NMR (101 MHz, CDCl₃): δ (ppm) = 51.6, 33.6, 30.5, 29.1, 28.9, 28.5, 26.7, 7.3; FT-IR: *V*_{max} (neat)/cm⁻¹ 2928, 2855, 2089, 1456, 1348, 1260, 721.



(1R,2S,3S,4S,5R,6R)-7-(8-azido-octyl)-5-(hydroxymethyl)-7-azabicyclo[4.1.0]heptane-2,3,4-triol (2)

A mixture of cyclophellitol aziridine **7** (35 mg, 0.2 mmol), **10** (0.24 mmol, 67 mg) and K₂CO₃ (119 mg, 0.86 mmol) in DMF (4 mL) was stirred at 80 °C. After stirring overnight the reaction mixture was concentrated *in vacuo* and purification over silica gel column chromatography (8% MeOH in DCM → 12% MeOH in DCM) gave azide-aziridine cyclophellitol **2** (21.5 mg, 65 μmol, 25%) as a white powder. ¹H NMR (400 MHz, D₂O): δ (ppm) = 3.93 (d, *J* = 8.0 Hz, 1H), 3.81 – 3.75 (m, 3H), 3.34 (dd, *J* = 0.2 Hz, 2.0 Hz, 1H), 3.27 (t, *J* = 6.8 Hz, 2H), 2.53 – 2.48 (m, 1H), 2.13 – 2.04 (m, 2H), 1.88 (d, *J* = 6.0 Hz, 1H), 1.85 (d, *J* = 6.0 Hz, 1H), 1.62 – 1.42 (m, 4H), 1.40 – 1.22 (m, 8H). ¹³C NMR (101 MHz, D₂O): δ (ppm) = 76.4, 70.7, 70.3, 61.0, 59.2, 51.2, 43.0, 41.0, 39.5, 29.1, 29.0, 28.9, 28.5, 27.0, 26.4. HRMS: calculated for [C₁₅H₂₉N₄O₄]⁺ 329.21818, found 329.21833.

 **6-(trityloxy)hexan-1-ol (13)**

To a solution of 1,6-hexanediol (11.8 g, 100 mmol) and pyridine (1.6 mL, 20 mmol) in DCM (100 mL) was added tritylchloride (2.78 g, 10 mmol) at room temperature. After vigorously stirring for 90 min, the solution was washed with brine (3x). The organic layer was dried with MgSO_4 , filtrated and concentrated *in vacuo*. Purification by column chromatography (30% EtOAc in pentane) gave tritylhexanol **13** (3.41 g, 9.47 mmol, 95%) as a white solid. Compound characteristics were conform with previously reported data.^{45,46}

 **(((6-iodohexyl)oxy)methanetriyl)tribenzene (14)**

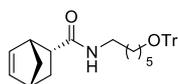
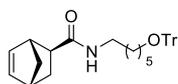
Tritylhexanol **13** (3.35 g, 9.3 mmol) was dissolved in Et_2O (35 mL) and CH_3CN (11 mL). After addition of imidazole (1.90 g, 27.9 mmol) and triphenylphosphine (13.95 mmol, 3.66 g) the reaction mixture was cooled to 0 °C and iodine (3.54 g, 13.95 mmol) was added in small portions. After stirring for 30 min, the reaction was allowed to warm to room temperature and stirred overnight. The reaction mixture was diluted with Et_2O and the precipitated was filtered off. The filtrated was washed with $\text{Na}_2\text{S}_2\text{O}_3$, dried with MgSO_4 , filtrated and concentrated *in vacuo*. Purification by column chromatography (pentane \rightarrow 3% EtOAc in pentane) yielded title compound **14** (3.51 g, 7.46 mmol, 80%) as a clear oil. Compound characteristics were conform with previously reported data.⁴⁷

 **(((6-azidohexyl)oxy)methanetriyl)tribenzene (15)**

To solution of compound **14** (0.92 g, 2.0 mmol) in DMF (5 mL) was added NaN_3 (1.30 g, 20 mmol) and stirred at 80 °C overnight. The reaction mixture was diluted with EtOAc and washed with H_2O (4x). The organic layer was dried with MgSO_4 , filtrated and concentrated *in vacuo* to obtain the azide **15** (0.787 g, 2.0 mmol) as a clear oil, which was used without further purification. ^1H NMR (400 MHz, CDCl_3) δ 7.43 (d, $J = 7.2$ Hz, 6H), 7.32 – 7.16 (m, 9H), 3.21 (t, $J = 7.0$ Hz, 2H), 3.05 (t, $J = 6.5$ Hz, 2H), 1.65 – 1.50 (m, 4H), 1.45 – 1.24 (m, 4H). ^{13}C NMR (101 MHz, CDCl_3) δ 144.5, 128.8, 127.8, 126.9, 86.4, 63.5, 51.5, 30.0, 28.9, 26.7, 26.0.

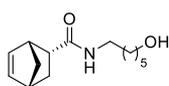
 **6-(trityloxy)hexan-1-amine (16)**

To a solution of azide **15** (193 mg, 0.5 mmol) in THF (2.3 mL) and H_2O (9 μL) was added PPh_3 on beads (200 mg, 0.60 mmol, 3 mmol/g). After stirring for 48 h at room temperature, the reaction mixture was filtered and concentrated *in vacuo*. The crude product was co-evaporated with toluene (3x) to obtain amine **16** (186 mg, 0.517 mmol) as a clear oil which was used without further purification. ^1H NMR (400 MHz, CDCl_3) δ 7.44 (d, $J = 7.6$ Hz, 6H), 7.31 – 7.18 (m, 9H), 3.05 (t, $J = 6.6$ Hz, 2H), 2.63 (t, $J = 7.0$ Hz, 2H), 1.68 – 1.54 (m, 2H), 1.43 – 1.31 (m, 4H), 1.31 – 1.22 (m, 2H), 1.21 (s, 1H). ^{13}C NMR (101 MHz, CDCl_3) δ 144.5, 128.7, 127.7, 126.9, 86.3, 63.6, 42.3, 33.8, 30.1, 26.8, 26.2. HRMS: Calculated for $[\text{C}_{25}\text{H}_{30}\text{NO}]^+$ 360.23219, found 360.23228.



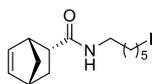
(1R,2R,4R)-N-(6-(trityloxy)hexyl)bicyclo[2.2.1]hept-5-ene-2-carboxamide (18) and (1R,2S,4R)-N-(6-(trityloxy)hexyl)bicyclo[2.2.1]hept-5-ene-2-carboxamide (19)

To a solution of amine **16** (0.90 g, 2.52 mmol) in DCE (12.6 mL) was added norbornene-OSu **17**³⁸ (0.61 g, 2.59 mmol; mixture of *endo* and *exo*, mostly *endo*) and DIPEA (1.07 mL) at room temperature. After stirring overnight the reaction mixture was concentrated *in vacuo*. Purification by column chromatography (20% EtOAc in pentane → 30% EtOAc in pentane) gave *exo*-product **18** (0.34 g, 0.70 mmol, 28%) and *endo*-product **19** (0.82 g, 1.7 mmol, 68%) as a clear oil. *Exo*-product **18** ¹H NMR (400 MHz, CDCl₃) δ 7.47 – 7.40 (m, 6H), 7.30 – 7.20 (m, 9H), 6.13 (dd, *J* = 5.5, 3.0 Hz, 1H), 6.08 (dd, *J* = 5.4, 3.2 Hz, 1H), 5.51 (s, 1H), 3.23 (d, *J* = 6.3 Hz, 1H), 3.20 (d, *J* = 6.5 Hz, 1H), 3.06 (d, *J* = 6.3 Hz, 1H), 3.03 (d, *J* = 6.5 Hz, 1H), 2.90 – 2.87 (m, 2H), 1.96 – 1.86 (m, 2H), 1.72 (d, *J* = 8.3 Hz, 1H), 1.66 – 1.57 (m, 2H), 1.52 – 1.44 (m, 2H), 1.41 – 1.34 (m, 2H), 1.33 – 1.31 (m, 1H), 1.31 – 1.25 (m, 3H). *Exo*-product **18** ¹³C NMR (101 MHz, CDCl₃) δ 175.6, 144.5, 138.3, 136.1, 128.8, 127.8, 126.9, 86.4, 63.5, 47.3, 46.5, 44.8, 41.7, 39.7, 30.6, 30.0, 29.8, 26.9, 26.1. *Exo*-product **18**: HRMS: Calculated for [C₃₃H₃₇NO₂Na]⁺ 502.27165, found 502.27059. *Endo*-product **19** ¹H NMR (400 MHz, CDCl₃) δ 7.51 – 7.39 (m, 6H), 7.32 – 7.19 (m, 9H), 6.21 (dd, *J* = 5.6, 3.1 Hz, 1H), 5.95 (dd, *J* = 5.6, 2.8 Hz, 1H), 5.39 (s, 1H), 3.17 (dd, *J* = 7.1, 2.6 Hz, 1H), 3.14 (dd, *J* = 7.2, 2.5 Hz, 1H), 3.10 (s, 1H), 3.04 (t, *J* = 6.6 Hz, 2H), 2.89 (s, 1H), 2.85 – 2.80 (m, 1H), 1.94 – 1.87 (ddd, *J* = 11.9, 9.4, 3.8 Hz, 1H), 1.65 – 1.55 (m, 2H), 1.46 – 1.40 (m, 3H), 1.40 – 1.34 (m, 2H), 1.34 – 1.30 (m, 1H), 1.30 – 1.22 (m, 3H). *Endo*-product **19** ¹³C NMR (101 MHz, CDCl₃) δ 174.2, 144.5, 137.8, 132.4, 128.7, 127.8, 126.9, 86.4, 63.5, 50.1, 46.3, 44.9, 42.8, 39.5, 30.0, 29.7, 26.9, 26.1. *Endo*-product **19** HRMS: Calculated for [C₃₃H₃₇NO₂Na]⁺ 502.27165, found 502.27141.



(1R,2R,4R)-N-(6-hydroxyhexyl)bicyclo[2.2.1]hept-5-ene-2-carboxamide (20)

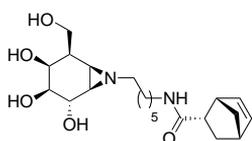
Compound **19** (0.815 g, 1.7 mmol) was dissolved in a mixture of DCM/MeOH (20 mL, 1:1) and *p*-toluenesulfonic acid was added until pH 2. After stirring overnight at room temperature, the reaction mixture was neutralized with a saturated aqueous NaHCO₃ solution. The aqueous layer was extracted with EtOAc (3x) and the combined organic layers were washed with brine, dried with MgSO₄, filtrated and concentrated *in vacuo*. Purification by column chromatography (5% Methanol in DCM → 8% Methanol in DCM) gave title compound **20** (0.349 g, 1.47 mmol, 86%) as a clear yellow oil. ¹H NMR (400 MHz, CDCl₃) δ 6.22 (dd, *J* = 5.6, 3.1 Hz, 1H), 5.96 (dd, *J* = 5.6, 2.8 Hz, 1H), 5.80 (s, 1H), 3.60 (t, *J* = 6.5 Hz, 1H), 3.23 – 3.15 (m, 2H), 3.13 (s, 1H), 2.91 (s, 1H), 2.89 – 2.80 (m, 2H), 1.92 (ddd, *J* = 11.8, 9.4, 3.7 Hz, 1H), 1.59 – 1.51 (m, 2H), 1.50 – 1.41 (m, 3H), 1.40 – 1.22 (m, 6H). ¹³C NMR (101 MHz, CDCl₃) δ 174.4, 137.7, 132.2, 62.3, 50.0, 46.2, 44.7, 42.7, 39.2, 32.5, 29.8, 29.6, 26.5, 25.3. HRMS: Calculated for [C₁₄H₂₄NO₂]⁺ 238.18016, found 238.18026.



(1R,2R,4R)-N-(6-iodohexyl)bicyclo[2.2.1]hept-5-ene-2-carboxamide (21)

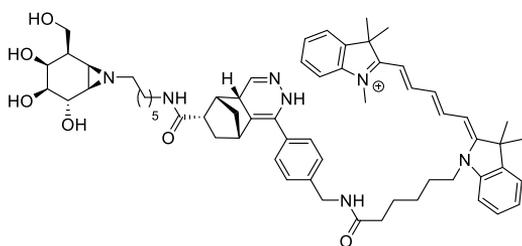
To a solution of alcohol **20** (119 mg, 0.50 mmol) in THF (2.5 mL) was added PPh₃ (197 mg, 0.75 mmol) and imidazole (64 mg, 1.0 mmol). The reaction mixture was stirred and heated to reflux temperature. Carefully a solution of I₂ (190 mg, 0.75 mmol) in THF (1.0 mL) was added. After stirring for 1.5 h, the reaction mixture was concentrated, redissolved in EtOAc and washed with Na₂S₂O₄. The organic layer was dried with MgSO₄, filtrated and concentrated *in vacuo*. Purification by column chromatography (30% EtOAc in pentane) yielded title compound **21** (126 mg, 0.361 mmol, 73%) as a clear yellow oil. ¹H NMR (400 MHz, CDCl₃) δ 6.24 (dd, *J* = 5.6, 3.1 Hz, 1H), 5.97 (dd, *J* = 5.6, 2.8 Hz, 1H), 5.39 (s, 1H), 3.25 – 3.15 (m, 4H), 3.13 (s, 1H), 2.92 (s, 1H), 2.86

(dt, $J = 9.2, 4.0$ Hz, 1H), 1.94 (ddd, $J = 11.9, 9.4, 3.8$ Hz, 1H), 1.83 (t, $J = 7.0$ Hz, 1H), 1.80 (t, $J = 7.0$ Hz, 1H), 1.54 – 1.36 (m, 5H), 1.35 – 1.27 (m, 4H). ^{13}C NMR (101 MHz, CDCl_3) δ 174.2, 137.7, 132.2, 50.0, 46.2, 44.7, 42.7, 39.2, 33.3, 30.1, 29.8, 29.5, 25.8, 7.2. HRMS: Calculated for $[\text{C}_{14}\text{H}_{23}\text{INO}]^+$ 348.08188, found 348.08177.



(1S,2S,4S)-N-(6-((1R,2S,3S,4S,5R,6R)-2,3,4-trihydroxy-5-(hydroxymethyl)-7-azabicyclo[4.1.0]heptan-7-yl)hexyl)bicyclo[2.2.1]hept-5-ene-2-carboxamide (3)

A solution of norbornene **21** (83 mg, 0.24 mmol) in DMF (2 mL) was added to aziridine **7** (0.2 mmol). K_2CO_3 (119 mg, 0.86 mmol) was added to the reaction mixture followed by heating to 75 °C. After stirring overnight, the reaction mixture was concentrated *in vacuo*. Purification by column chromatography (12% MeOH in DCM) followed by HPLC purification (A: 50 mM NH_4HCO_3 in H_2O ; B: MeCN; 20% \rightarrow 26%, A in B) and lyophilisation resulted in compound **3** (24.3 μmol , 9.6 mg, 12%). LC/MS analysis: R_t 3.81 min (linear gradient 10 \rightarrow 90% B in 12.5 min), m/z 395.27 $[\text{M} + \text{H}]^+$. ^1H NMR (400 MHz, Deuterium Oxide) δ 6.23 (dd, $J = 5.6, 3.1$ Hz, 1H), 5.90 (dd, $J = 5.6, 2.8$ Hz, 1H), 3.94 (d, $J = 8.2$ Hz, 1H), 3.79 (d, $J = 7.4$ Hz, 2H), 3.75 (s, 1H), 3.33 (dd, $J = 8.2, 2.2$ Hz, 1H), 3.16 – 3.05 (m, 3H), 2.97 – 2.92 (m, 1H), 2.90 (s, 1H), 2.50 – 2.42 (m, 1H), 2.18 – 2.04 (m, 2H), 2.02 (d, $J = 6.1$ Hz, 1H), 1.86 – 1.82 (m, 2H), 1.52 – 1.40 (m, 4H), 1.39 – 1.22 (m, 7H). ^{13}C NMR (101 MHz, D_2O) δ 177.7, 138.4, 131.8, 76.2, 70.6, 70.4, 61.1, 58.4, 49.5, 46.4, 44.1, 42.6, 42.5, 40.4, 39.2, 39.1, 29.5, 28.7, 28.3, 26.1, 25.7. HRMS: Calculated for $[\text{C}_{21}\text{H}_{35}\text{N}_2\text{O}_5]^+$ 395.25405, found 395.25336.



ABP 5

To a solution of norbornene **3** (0.94 mg, 2.38 μmol) in MeOH (4 mL) was added Cy5 tetrazine **22** (2.83 mg, 4.3 μmol). After shaking overnight, the reaction mixture was concentrated *in vacuo* and purified by HPLC under neutral conditions (A: 50 mM NH_4HCO_3 in H_2O ; B: MeCN; 49% \rightarrow 52%, A in

B) gave ABP **5** (2.12 mg, 2.07 μmol , 87%) as a mixture of regioisomers. HRMS: Calculated for $[\text{C}_{62}\text{H}_{80}\text{N}_7\text{O}_6]^+$ 1018.61646, found 1018.61611.

Crystal structure of CjGH35A complexed with TB562 (7)

The gene for CjGH35A, cloned into a pET28a vector modified for Ligation Independent Cloning, was expressed, and the protein purified and crystallized as in Larsbrink *et al.*⁴⁸, in 2.7 M sodium acetate pH 7.2 (protein at 30 mg/ml, drop 1.2:1 μl over well). A crystal was soaked in the presence of a speck of TB562 powder for 70 hours. The crystal was fished directly into liquid nitrogen without the need for additional cryoprotectant. Data were collected on beamline IO2 at the Diamond Light Source at wavelength 0.97950 Å, and were processed using DIALS⁴⁹ and scaled with AIMLESS⁵⁰ to 1.6 Å (Table 2). The space group was P1 and the unit cell dimensions, 98.9, 115.8, 116.0 Å, and angles, 90.2, 90.2, 90.4°. The structure was solved using programs from the CCP4I2 suite. Molecular replacement was

performed using *Phaser*⁵¹, with the native structure, pdb entry 4d1i, as the model. The model was built manually in *Coot*⁵², followed by repeated cycles of *REFMAC*⁵³ employing twin refinement using observed intensities.

Table 2. Data collection and refinement statistics.

CjGH35A in complex with TB562 (7) PDB code: 5JAW	
Data collection	
Space group	P1
Cell dimensions	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	98.9, 115.8, 116.0
α , β , γ (°)	90.2, 90.2, 90.4
Resolution (Å)	81.99-1.6 (1.63-1.60)
<i>R</i> _{merge}	0.080 (0.588)
<i>R</i> _{pim}	0.080 (0.588)
<i>CC</i> (1/2)	0.979 (0.482)
<i>I</i> / σ <i>I</i>	6.0 (1.2)
Completeness (%)	95.7 (94.1)
Redundancy	1.8 (1.8)
Refinement	
Resolution (Å)	1.60
No. reflections	650665
<i>R</i> _{work} / <i>R</i> _{free}	0.20/0.21
No. atoms	
Protein	33482
Ligand/ion	125
Water	1637
B-factors (Å²)	
Protein	25
Ligand/ion	20
Water	28
R.m.s. deviations	
Bond lengths (Å)	0.018
Bond angles (°)	1.927

*Values in parentheses are for highest-resolution shell.

Enzyme activity assays

Mouse GALC shares 83% identity to the human GALC homologue and was used as GALC source.⁵⁴ Therefore, in human embryonic kidney 293 (HEK293) cells recombinant murine GALC was expressed. The produced protein which was secreted to the culture medium (DMEM high glucose, Gibco) was directly used in enzyme activity studies (5 μ L volume was used). GLB1 from human fibroblast lysates (prepared in Kpi buffer (25 mM K₂HPO₄/KH₂PO₄, pH 6.5, 1% (v/v) Triton X-100, protease inhibitor cocktail (Roche, version 12)), 5.5 μ g total protein was used). CjGH79 was prepared in KPi buffer (8.9 ng protein were used). The β -D-galactosidase activities were assayed at 37 °C with 1 mM 4-methylumbelliferyl β -D-galactopyranoside as substrate in 150 mM McIlvaine buffer (pH 4.5)

with 0.2 M NaCl. To determine the apparent IC_{50} values, the enzymes were equilibrated in 12.5 μ L McIlvaine buffer pH 4.5) and incubated with a range of inhibitor dilutions (12.5 μ L) for 30 min at 37 °C in triplicates in a flat-bottomed black 96-well plate (Greiner, medium binding). The mixtures were then incubated with 100 μ L substrate. After 30 min at 37 °C, the reaction was quenched with 200 μ L 1 M NaOH-glycine (pH 10.3) and the 4-methylumbelliferyl fluorescence was measured with a fluorimeter LS-55 (PerkinElmer) using λ_{ex} 366 nm and λ_{em} 445 nm. The data were corrected for background fluorescence (without enzyme), normalized to the untreated control condition (DMSO sample) and finally the residual activity was fitted with the X-Y scatter plot with the function [inhibitor] vs response—variable slope (four parameters) using the program Prism (Graphpad, version 7.0).

Two-step labeling with BODIPY-alkyne

The 2-step labeling using compound **2** and BODIPY-alkyne **11** was performed as previously described with slight alterations.³³ Briefly, 1 μ L of kidney or brain homogenate was incubated with 9 μ L of 1.1 μ M compound **2** in McIlvaine buffer pH 4.3 (final concentration 1 μ M) for 1 hr at 37 °C. Afterwards, the mixture was diluted with NaOAc buffer (30 μ L, 50 mM pH 6.0, 0.1% SDS). A fresh mixture of TBTA (10 μ L, 2 mM in DMF), $CuSO_4$ (1 μ L, 0.1 mM in H_2O), DTT (0.5 μ L, 0.1 mM in H_2O) and **11** (0.5 μ L, 1 eq. compared to probe in ACN) was prepared, added to the enzyme solution, and the resulting mixture was incubated overnight at rt. The reaction was quenched by the addition of 4x sample buffer (15 μ L) and loaded on a 7.5% SDS-PAGE gel. The wet slab gel was scanned for fluorescence using the Typhoon Variable Mode Imager (Amersham Biosciences, Piscataway, NJ, USA), using λ_{ex} 488 nm and λ_{em} 520 nm (band pass 40).

In vitro TB652 (5) labeling in mouse kidney homogenates

Kidneys from wild type mice were homogenized in KPi buffer (25 mM K_2HO_4/KH_2PO_4 , 0.1% (v/v) Triton X-100, protease inhibitor cocktail (Roche, version 12)) with 1 mm glass beads and a FastPrep-24 homogenizer (MP Biomedicals). The basic labeling condition consists of equilibrating 25 μ g total protein from the homogenate prepared in McIlvaine buffer (150 mM, various pH) in a total volume of 10 μ L, and ABP labeling in 1 step (5 μ L of 3x concentrated ABP prepared in McIlvaine buffer) fashion. For labeling at varying pH, mouse tissue homogenates were equilibrated in McIlvaine buffer pH 3.0 – 7.0 for 15 min at 37 °C, followed by incubating with 5 μ M MDW933²³ (end concentration; prepared in McIlvaine buffer pH 3.0 - 7.0) for 30 min at 37 °C to label all GBA, then 1 μ M TB652 (prepared in McIlvaine buffer pH 3.0 -7.0) was added for 30 min at 37 °C. For labeling at varying ABP concentration, mouse kidney homogenates were equilibrated in McIlvaine buffer pH 4.5 for 5 min on ice, incubated with 5 μ M MDW933 (end concentration; pH 4.5) for 30 min at 37 °C, and then with varying concentration of TB652 (end concentration = 10nM -10 μ M; pH 4.5) for 30 min at 37 °C. For labeling at varying incubation time, mouse kidney homogenates were prepared as above, and incubated directly with 1 μ M TB652 (end concentration; pH 4.5) at 37 °C for 1 min to 2 h in absence of MDW933.

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