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

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

PROEFSCHRIFT

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Thomas Johannes Maria Beenakker

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List of abbreviations

2,4-DNPG	2,4-dinitrophenyl- β -D-glucoside	DCI	4,5-dicyanoimidazole
4-mu	4-methylumbelliferyl	DCM	dichloromethane
ABP	activity-based probe	dd	double doublet
ABPP	activity-based protein profiling	ddd	double double doublet
Ac	acetyl	DFT	density functional theory
acac	acetylacetonate	dGMII	drosophila Golgi α -mannosidase II
ACN	acetonitrile	DHP	3,4-dihydro-2H-pyran
α Gal A	α -galactosidase A	DIBAL	diisobutylaluminium hydride
α -NAGAL	α -N-acetylgalactosaminidase	DIC	<i>N,N'</i> -diisopropylcarbodiimide
AMP-DNM	<i>N</i> -(5'-adamantane-1'-yl-methoxy)- pentyl-1-deoxynojirimycin	DIPEA	<i>N,N</i> -diisopropylethylamine
Asn	asparagine	DMAP	4-(<i>N,N</i> -dimethylamino)pyridine
Asp	aspartic acid	DMF	<i>N,N</i> -dimethylformamide
B	boat	DMSO	dimethylsulfoxide
BCl ₃	boron trichloride	DNJ	deoxynojirimycin
Bn	benzyl	DTT	dithiothreitol
Boc	<i>tert</i> -butoxycarbonyl	EDA	ethyl diazoacetate
Boc ₂ O	di- <i>tert</i> -butyldicarbonate	EEDQ	2-ethoxy-1-ethoxycarbonyl-1,2- dihydroquinoline
Bodipy	boron-dipyrrromethene	Em	emission
BSA	bovine serum albumin	eq.	molar equivalents
Bz	benzoyl	ESI	electro-spray ionization
C	chair	Et	ethyl
calc.	calculated	EtOAc	ethyl acetate
CAZy	Carbohydrate Active Enzyme	Ex	excitation
CBB	Coomassie Brilliant Blue	exp.	experimental
CBE	conduritol B epoxide	FEL	free energy landscape
C _q	quarternary carbon atom	GAA	lysosomal α -glucosidase
Ctrl	control experiment	GALC	gene encoding galactosylceramidase
CuAAC	copper(I)-catalyzed azide/alkyne cycloaddition	GalT	galactosyltransferase
Cy	cyanine dye	GANAB	neutral alpha-glucosidase AB
d	doublet	GBA1	glucosylceramidase
δ	chemical shift	GBA2	non-lysosomal glucosylceramidase
Da	Dalton	GBA3	cytosolic β -glucosidase
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene	GCS	glucosylceramide synthase
DCE	dichloroethane	GH	glycosidase hydrolase

GLA	gene encoding α Gal A	PAGE	polyacrylamide gel electrophoresis
GLB1	gene encoding lysosomal acid β -galactosidase	PBS	phosphate-buffered saline
Gln	glutamine	Pd/C	palladium on carbon
Glu	glutamic acid	PDB	protein data bank
GT	glycosyltransferase	PEG	polyethylene glycol
GTA	α -1,3- <i>N</i> -acetylgalactosaminyltransferase	Ph	phenyl
GTB	α -1,3-galactosyltransferase	Piv	pivaloyl
h	hour(s)	<i>p</i> -NPG	<i>para</i> -nitrophenyl β -D-glucopyranoside
H	half-chair	ppm	parts per million
<i>Helix p.</i>	<i>Helix pomatia</i>	<i>p</i> TsOH	<i>para</i> -toluenesulfonic acid
HEK293	human embryonic kidney 293	q	quartet
HPLC	high-performance liquid chromatography	rGLA	recombinant α -galactosidase A
HRMS	high resolution mass spectrometry	r.m.s.	root mean square
IC ₅₀	half maximal inhibitory concentration	rt	room temperature
IEDDA	inverse-electron-demand Diels Alder	R _i	retention time
<i>J</i>	coupling constant	s	singlet
KO	Knockout	S	skew-boat
KHMDs	potassium hexamethyldisilazane	SDS	sodium dodecyl sulfate
LC/MS	liquid chromatography mass spectrometry	sp.	species
LDA	lithium diisopropylamide	t	triplet
LSD	lysosomal storage disorder	TBA	tertbutylammonium
m	multiplet	TBAF	tetrabutylammonium fluoride
M	marker	TBAI	tetrabutylammonium iodide
M	molar	TBDMS	<i>tert</i> -butyldimethylsilyl
m/z	mass to charge ratio	TBTA	tris[(1-benzyl-1 <i>H</i> -1,2,3-triazol-4-yl)methyl]amine
MANBA	β -mannosidase	^t Bu	<i>tert</i> -butyl
<i>m</i> CPBA	<i>meta</i> -chloroperoxybenzoic acid	TFA	trifluoroacetic acid
MD	molecular dynamics	THF	tetrahydrofuran
Me	methyl	THP	tetrahydropyran
MeOH	methanol	TLC	thin layer chromatography
min	minute(s)	<i>Tm</i>	<i>Thermotoga maritima</i>
MS	mass spectrometry	Tr	trityl
n.d.	not determined	Tris	tris(hydroxymethyl)aminomethane
NMR	nuclear magnetic resonance	Trp	tryptophan
NOESY	nuclear Overhauser effect spectroscopy	Ts	<i>para</i> -toluenesulfonyl
		UDP	uridine diphosphate
		UV	ultraviolet
		Val	valine
		WT	wild-type

1

General introduction

Glycosidases are essential in fundamental biological processes and are responsible for the degradation of most (oligo)saccharides, glycolipids and glycoproteins. Glycosidases are able to hydrolyze glycosidic linkages – acetal linkages that are very stable otherwise in physiological surroundings – with high efficiency.¹ They are moreover often highly selective towards specific glycosidic linkages, in terms of the number (monosaccharide, oligosaccharide) and nature (configuration) of the carbohydrate moieties that make up these linkages. Malfunction of glycosidases is the cause of a variety of human inherited diseases referred to as lysosomal storage disorders (LSD).²⁻⁶ Fabry disease, for example, is caused by genetic deficiency of the lysosomal α -galactosidase, α Gal A^{7,8}, and results in accumulation of the α Gal A substrate, globotriaosylceramide, in several tissues.^{9,10} Malfunction of the lysosomal β -galactosidase, GALC, in turn may cause Krabbe disease, which is characterized by the storage of psychosine in the brain.¹¹⁻¹³ To study glycosidases in their physiological surroundings, activity-based protein profiling (ABPP) has come to the fore as a powerful technique in recent years.¹⁴⁻¹⁸ The research described in this Thesis entails the development and application of a set of activity-based probes (ABPs) for α -galactosidases, β -galactosidases, α -mannosidases and β -mannosidases. Furthermore, the development and evaluation of a new class of competitive inhibitors is presented.

1.1 Activity-based protein profiling

Differentiation between functional and non-functional proteins in biological systems is a challenging objective. In the past decades, ABPP has emerged as a powerful strategy to obtain qualitative and quantitative information on active enzymes as present in complex proteomes.¹⁴⁻¹⁸ A requirement for the development of an ABP for ABPP experiments is the transient emergence of a covalent enzyme-substrate intermediate during the action of a given enzyme on its substrate. Mechanism-based covalent inhibitors can be developed based on this catalytic mechanism, and introduction of a reporter entity (a fluorophore, biotin or a bioorthogonal tag) onto an effective mechanism-based inhibitor may yield an effective ABP (Figure 1.1).

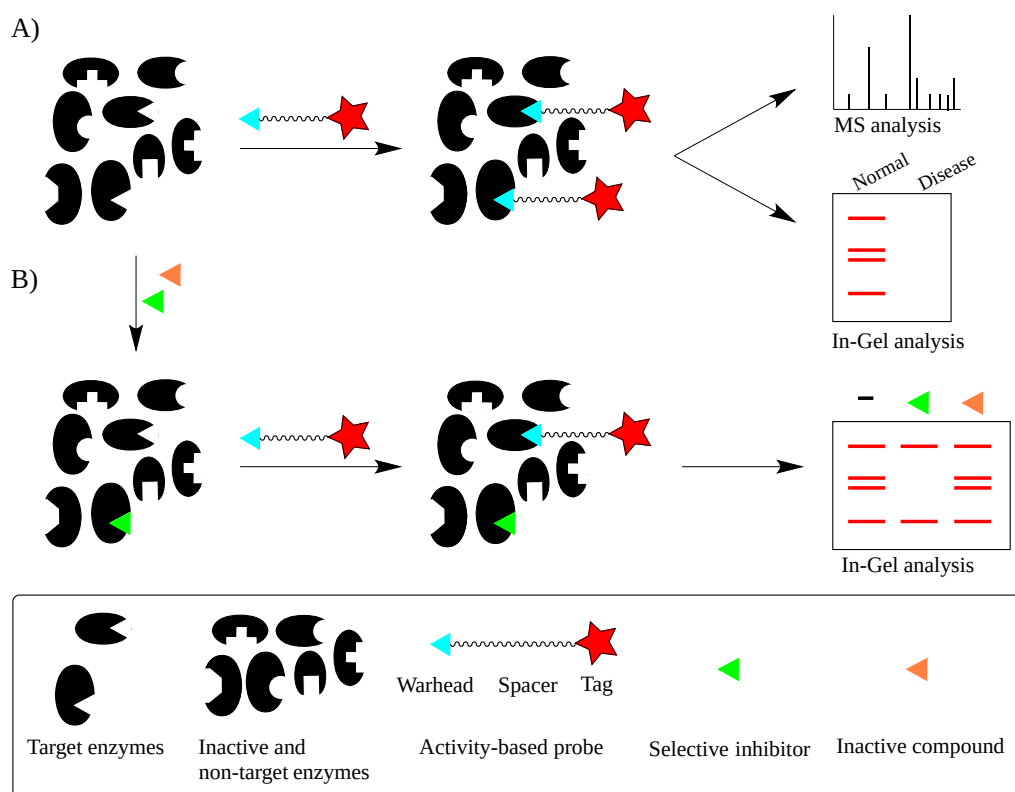


Figure 1.1 Activity-based protein profiling (ABPP). A) Identification and quantification of the tagged enzymes. B) Determination of the inhibition potency by ABPs. MS: mass spectrometry.

Biotin-containing ABPs can be used for qualitative ABPP as follows (Figure 1.1A). After incubation of a tissue culture or cell extract with a biotin-ABP and pull-down of all biotinylated proteins (both those that have reacted irreversibly with the ABP and endogenously biotinylated proteins) with streptavidin-coated magnetic beads, the

tagged enzymes are subjected to proteolysis (generally trypsin digestion) and the resulting oligopeptides are analysed by mass spectrometry. The thus obtained protein sequences are matched against protein sequence databases and in this way the ABP-reactive enzymes are identified.¹⁹⁻²³ Fluorescent ABPs can be used to obtain quantitative information on enzyme expression levels, provided that the identity of the modified enzymes is known. After incubation of a biological sample with a fluorescent ABP, denaturation of the protein contents and resolving the polypeptides by SDS-PAGE, ABP-modified proteins are detected and visualized by in-gel fluorescence scanning.²³⁻²⁷ This reveals bands of the labelled proteins depending on the concentration of active enzyme, excluding precursor enzymes (zymogens) or malfunctioning enzymes.²⁸⁻³⁰ In this way, the level of active enzymes between different tissues can be analysed in a straightforward manner and the methodology can be applied in a diagnostic setting by linking enzyme activity levels with disease states. Fluorescent ABPs can moreover be used in competitive ABPP.³¹⁻³⁴ For instance, a biological sample is incubated with a prospective inhibitor prior to incubation with the probe. After denaturation and resolving by SDS-PAGE, the inhibitor potency can be visualized by in-gel fluorescence scanning, in which inhibitor potency is directly related to the decrease in fluorescence intensity. Important advantages of competitive ABPP over other methodology is that information can be gleaned, next to inhibitor potency, also on inhibitor selectivity (in case the ABP used for ABPP readout has a broad enzyme specificity, and only one or a few of these targets are competed for) and cell permeability (for which purpose living cells are incubated with the prospective inhibitor prior to ABPP).³⁵

1.2 Classification of glycosidases

Glycosidases, present in all kingdoms of life, are encoded by approximately 1% of the genome.³⁶ This large variety of enzymes is able to hydrolyse a large selection of substrates with high efficiency. Classification of glycosidases according to the amino acid sequence similarities³⁷ resulted into the Carbohydrate Active Enzyme (CAZy) database.³⁸ Herein, 129 different families of glycosidase hydrolases (GHs) are classified to date. Although the substrate specificity of glycosidases in a specific family can be diverse, the catalytic mechanistic features are in general conserved within each GH family.^{39,40} These catalytic mechanisms can be classified as either inverting or retaining, based on the stereochemical outcome of the glycosidic bond (Figure 1.2).⁴¹ A pair of carboxylic acids is present in inverting glycosidases that act as general acid/base catalysts to deprotonate the incoming water molecule and to protonate the expelled

aglycon (Figure 1.2A). In retaining glycosidases, one carboxylic acid residue acts as a nucleophile and forms a covalent enzyme-substrate adduct while the other carboxylic acid acts as a general acid/base catalyst to protonate the expelled aglycon (Figure 1.2B). Subsequently, an incoming water molecule is deprotonated by this general acid/base catalyst and substitutes the anomeric carboxylate to release the enzyme.⁴²⁻⁴⁴ The covalent enzyme-substrate formation step in the itinerary of retaining glycosidases makes these GH families suitable for ABPP.

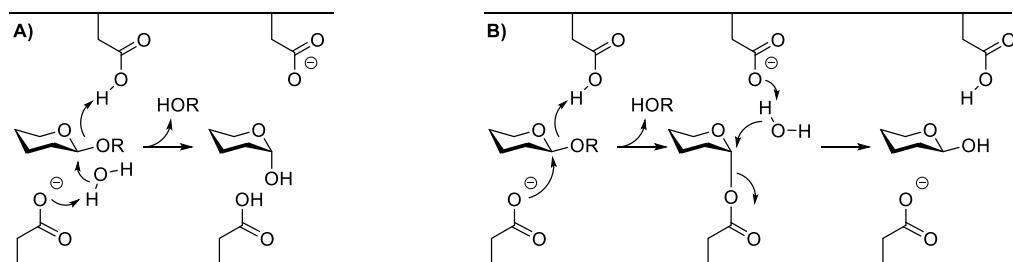


Figure 1.2 General mechanism of inverting glycosidases (A) and retaining glycosidases (B).

1.3 ABPs for retaining glycosidases

(+)-Cyclophellitol (**1**), isolated from mushroom *Phellinus* sp. in 1990⁴⁵, adopts a ⁴H₃ conformation (Figure 1.3A) and thereby mimics the transition state conformation that emerges during retaining β -glucosidase-mediated hydrolysis of β -glucosidic linkages (Figure 1.3B). Cyclophellitol turned out to be a potent⁴⁵, covalent and irreversible inhibitor of retaining β -glucosidases from various origin^{28,30,45}, including the human lysosomal glucosylceramidase, GBA. Inspired by the mode of action of cyclophellitol³⁰, cyclophellitol aziridine based probes (e.g. compound **2**, Figure 1.3A) were synthesized and shown to be broad spectrum retaining β -glucosidase ABPs that could be used in both comparative and competitive ABPP experiments.²³ The reaction itinerary of GH27 α -galactosidases follow the same ⁴H₃ transition state (Figure 1.3C)⁴⁶, which allowed the development of a related, cyclophellitol-inspired, ABPP methodology.⁴⁷ Thus α -galactopyranose-configured cyclophellitol and the corresponding cyclophellitol aziridine were synthesized, after which the corresponding ABPs were developed from these and applied in ABPP experiments. Finally, α -glucopyranose-configured cyclophellitol and cyclophellitol aziridine based probes have been synthesized and applied to monitor GH31 α -glucosidase⁴⁸ by again mimicking the substrate ⁴H₃ transition state conformation.⁴⁹

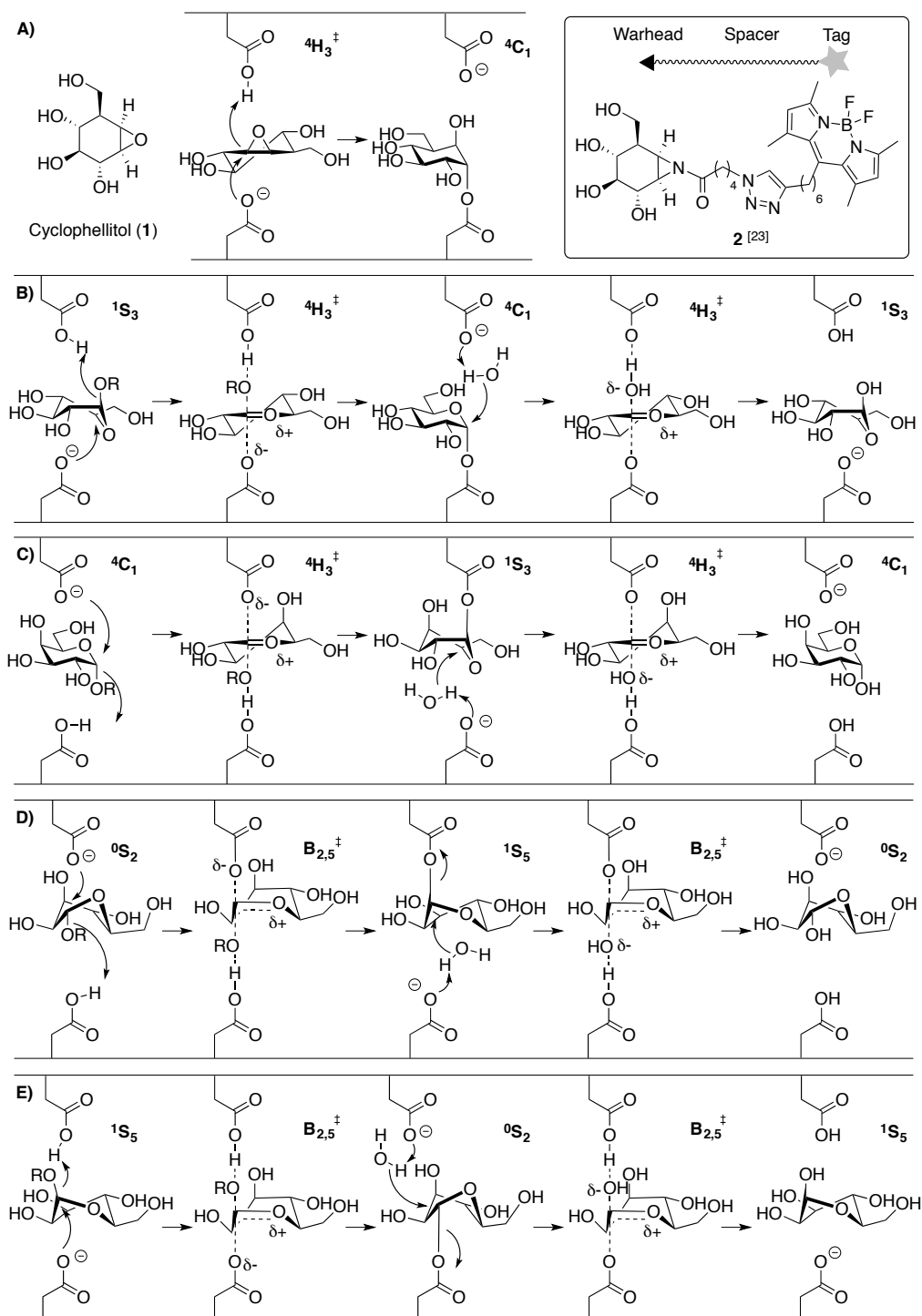


Figure 1.3 A) Mechanism-based inhibition by cyclophellitol (**1**) of retaining β -glucosidases and the structure of cyclophellitol aziridine **2**. Proposed mechanisms⁴⁹ of β -glucosidases (B), GH27 α -galactosidases⁴⁶ (C), α -mannosidases^{50,51} (D) and β -mannosidases⁵⁶⁻⁵⁸ (E).

These results raised the question whether cyclophellitol-based ABPs are also applicable for retaining exoglycosidases for which a $^4H_3$ conformation is not part of the reaction itinerary. In the proposed reaction itinerary of GH38 α -mannosidases, for example, the substrate following protonation and expulsion of the aglycon, adopts a boat conformation ($B_{2,5}$) in the transition state (Figure 1.3D).^{50,51} Interestingly, although α -mannopyranose-configured cyclophellitol is expected to be in a $^4H_3$ conformation, this compound has been shown in the past to be an effective jack bean α -mannosidase inhibitor.^{52,53} This suggests that retaining α -mannosidases, which are connected with various pathologies^{54,55}, may be targeted by cyclophellitol aziridine based ABPs as well. The same holds true for retaining β -mannosidases (both from GH2 and GH113 families), which process their substrate through a similar $B_{2,5}$ transition state⁵⁶⁻⁵⁸ (Figure 1.3E), and for which selective ABPs are not reported either. Such probes would be attractive to analyse β -mannosidosis, a lysosomal storage disorder that is caused by a deficiency of the retaining β -mannosidase, MANBA.⁵⁹⁻⁶¹

1.4 Reversible inhibitors based on transition state mimicry

Several reversible, competitive glycosidase inhibitors have been developed and are employed for therapeutic applications.⁶² For example, the reversible α -glucosidase inhibitors acarbose (Glucobay, **3**) and miglitol (Glyset, **4**) are employed for clinical treatment of diabetes type II (Figure 1.4).⁶³ Glycosidases enhance the reaction rate with an estimated 10^{17} fold compared to non-catalyzed hydrolysis of interglycosidic linkages.¹ Therefore, the binding affinity of the substrate in transition state conformation with the enzyme is expected to be exceptionally strong ($K_d < 10^{-22}$ M).⁶³ Based on this observation, transition state mimics are interesting motifs for the development of inhibitors. Zanamivir (Relenza, **5**) and oseltamivir (Tamiflu, **6**)⁴⁹ employ the transition state conformation and inhibit virus sialidases to treat influenza (Figure 1.4).⁶⁴

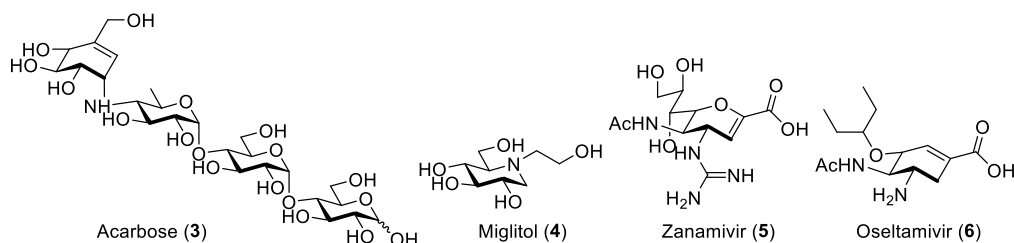


Figure 1.4 Clinically used competitive glycosidases inhibitors. Acarbose (Glucobay, **3**) and miglitol (Glyset, **4**) for the treatment of diabetes type II. Zanamivir (Relenza, **5**) and oseltamivir (Tamiflu, **6**) for the treatment of influenza.

Besides these examples of potent reversible inhibitors with an unsaturated structure (**3**, **5** and **6**), bicyclic structures can be employed to alter the conformation to emulate the transition state conformation (Figure 1.5). Nojiritetrazole **7**, for example, adopts a $^4H_3$ conformation⁶⁵ and is a potent retaining glucosidase inhibitor.⁶⁶ Based on the $^4H_3$ conformation adopted by cyclophellitol (**1**), carba-cyclophellitol (**8**), in which the cyclophellitol epoxide-oxygen is substituted for carbon, is expected to be a potent reversible glucosidase inhibitor. Prior to the research described in this Thesis, the synthesis of two carba-cyclophellitols (**8** and **9**) has been reported but no data on their glycosidase-inhibitory properties has been given.⁶⁷ In this Thesis, the synthesis of a library of carba-cyclophellitols based compounds and their inhibitory property towards several glycosidases is described (Chapter 6, 7 and 8).

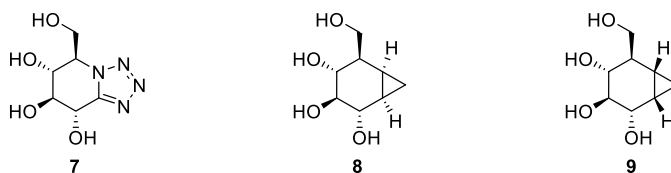


Figure 1.5 Bicyclic structures that employ⁶⁵ (**7**) or may employ⁶⁷ (**8** and **9**) the $^4H_3$ conformation to mimic the substrate conformation at the transition state of retaining glucosidases.

1.5 Aim and outline of this thesis

The research described in the first part of this Thesis aimed to develop and validate a set of selective ABPs for retaining α -galactosidases, β -galactosidases, α -mannosidases and β -mannosidases. The second part of the Thesis describes research efforts directed at the design of new competitive inhibitors for α -galactosidases, β -galactosidases, α -glucosidases, β -glucosidases, galactosyltransferases and glucosyltransferases.

In **Chapter 2** α -galactosidase selective probes are developed and examined in biological settings to target lysosomal retaining α -galactosidases. **Chapter 3** discusses the synthesis of a β -galactose-configured cyclophellitol aziridine and ABPs derived thereof. As part of the research described, the inverse-electron-demand Diels-Alder (IEDDA) ligation strategy was investigated, both in two-step labeling and as a means to derive an appropriately functionalised one-step ABP. The strategy of cyclophellitol aziridine based probes is extended to α -mannosidases and β -mannosidases in **Chapter 4** and **Chapter 5**, respectively. Interestingly, during the reaction itinerary of α -mannosidases and β -mannosidases in their processing of their substrates a B_{2,5} conformation emerges instead of the ⁴H₃ conformation as in galactosidases or glucosidases. In both cases, however, mannosidase-configured cyclophellitol aziridine probes proved to be effective ABPs and thus the research described in **Chapter 4** and **5** entails expansion of the ABP toolset by means of which retaining exoglycosidases can be monitored in complex biological samples.

Chapter 6 describes the synthesis of a library of carba-cyclophellitols as potential inhibitors for α -galactosidases, β -galactosidases, α -glucosidases and β -glucosidases. The β -glucopyranose-configured carba-cyclophellitols are evaluated as inhibitors of the *Thermotoga maritima* TmGH1 retaining β -glucosidase, human lysosomal retaining β -glucosidase GBA1 and human lysosomal α -glucosidase GAA as is described in **Chapter 7**. Furthermore, NMR analysis, quantum mechanical analysis, DFT calculations and crystallography studies are performed to determine the conformation of the synthesised carba-cyclophellitols. **Chapter 8** reports on the synthesis and evaluation of carba-cyclophellitol based inhibitors of galactosyltransferases and glucosyltransferases. **Chapter 9** gives a summary of this Thesis and includes some future prospects, based on the here presented results.

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2

A new generation of fluorescent ABPs to target retaining α -galactosidases¹

2.1 Introduction

The human lysosomal α -galactosidase A (α Gal A, GLA, EC. 3.2.1.22) is responsible for the hydrolysis of terminal α -galactosides in glycosphingolipids, glycoproteins and oligosaccharides. Several mutations in the GLA gene^{2,3} have been reported which results in a deficiency of α Gal A. As a consequence, accumulation of the globotriaosylceramide substrate in various tissues is observed^{4,5} and detected in plasma or urine samples.⁶ Affected patients are diagnosed with Fabry disease, which is an X-linked recessive lysosomal storage disorder (LSD). Classically affected Fabry patients demonstrate symptoms such as corneal opacities, angiokeratomata, intolerance to heat, hypohidrosis, neuropathic pain and micro-albuminuria.^{7,8} Besides these diverse clinical phenotypes, a variety in age of onset is reported, demonstrating the complexity of the disease.

α -N-acetylgalactosaminidase (α -NAGAL, EC 3.2.1.49) is a lysosomal retaining α -exo-galactosidase closely related to α Gal A. Both enzymes have 46% amino acid sequence identity, are within the CAZY GH27 family and are the same in 11 of the 13 active site residues.⁹ Compared to α Gal A, α -NAGAL selectively cleaves terminal α -GalNAc residues from glycosphingolipids and is less efficient in processing galactose monosaccharides.¹⁰ Various mutations in the NAGA gene have been reported that cause a deficiency of α -NAGAL^{11,12}, resulting into accumulation of glycoconjugates constituted with a terminal sialic-acid- or galactose-terminal saccharides.¹³ Patients of this rare lysosomal storage disorder, termed Schindler disease¹⁴, suffer from a large spectrum of symptoms such as seizures, autistic disorders and angiokeratoma.¹⁵ However, these clinical observations are not completely understood in correlation with the genetic malfunction.^{16,17}

Glycosyl hydrolases of the family GH27 process their substrates via a double displacement reaction mechanism (Figure 2.1A).^{18,19} Following protonation by the active site acid/base catalyst of the exocyclic oxygen of the scissile acetal linkage, the aglycon is displaced in a formal S_N2 reaction by the catalytic nucleophile residue. In the next step, the deprotonated aspartic residue acts as a base and deprotonates a water molecule, after which the resulting hydroxide substitutes the anomeric carboxylate, thereby releasing galactose and reforming the enzyme active site. This mechanism, first proposed by Koshland¹⁸, results in retention of configuration at the anomeric carbon.

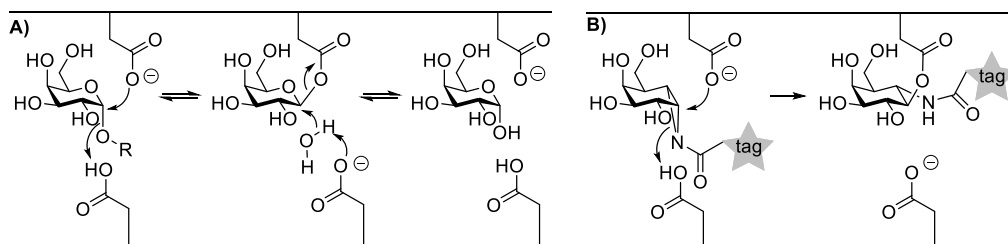


Figure 2.1 A) Double displacement mechanism of retaining α -galactosidases. B) Proposed mechanism of α -galactopyranose-configured cyclophellitol aziridines as mechanism-based α -galactosidase inhibitors.

Based on this mechanism, which includes a covalent bonded enzyme-substrate adduct after nucleophilic attack at the anomeric center, a covalent binding inhibitor (Figure 2.1B) of α -galactosidases has been synthesized²⁰ and tested *in vitro*.²¹ This α -galactopyranose-configured cyclophellitol aziridine bearing a fluorescent *N*-acyl substituent could be used in activity-based protein profiling (ABPP) of α Gal A and α -NAGAL activities. ABPP is a powerful technique to study active enzymes in various

complex systems, such as cell cultures and living organisms.²²⁻²⁴ A caveat in the α -galactosidase-directed probes proved to be the hydrolytic lability of the *N*-acyl substituent, rendering their synthesis, purification and usage somewhat complicated. This chapter describes the synthesis and biological characterization of *N*-alkyl activity-based probe (ABP) **3**. Head-to-head comparison with previously described *N*-acyl ABP **2** (Figure 2.2) reveals that, despite this caveat, *N*-acyl ABP **2** is more effective compared to its *N*-alkyl counterpart because it appeared to have better fluorescence properties. Following these observations, expansion of the α -galactosidase probe set was undertaken by the synthesis and biological evaluation of BodipyGreen and Cy5 modified probes (**4** and **5**, respectively), results on which are presented in this chapter as well.

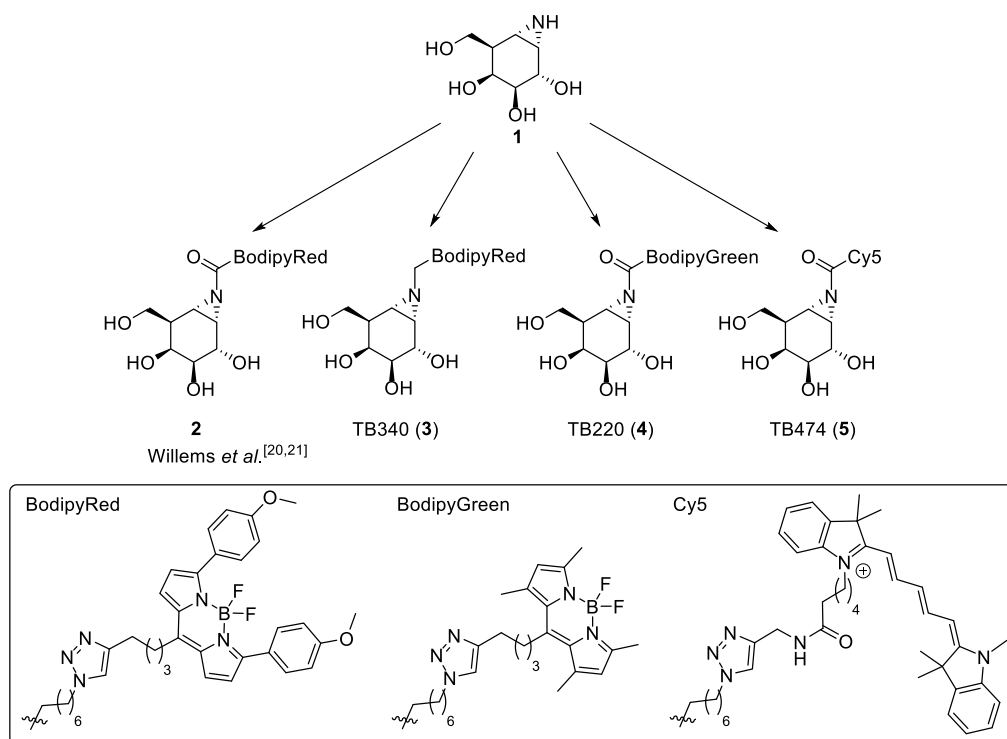


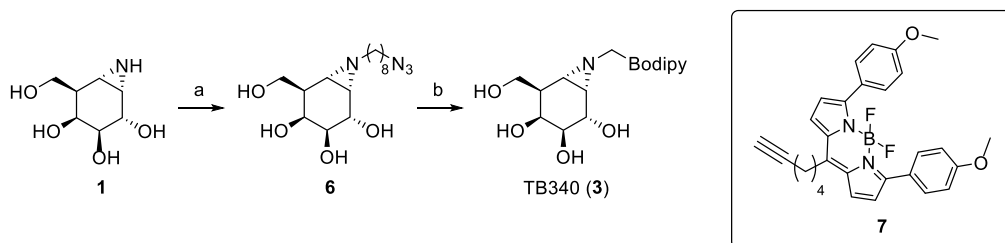
Figure 2.2 Structures of α -galactopyranose-configured cyclophellitol aziridine (**1**) and the *N*-acylated or *N*-alkylated ABPs (**2** - **5**) which are examined in this study.

2.2 Results and discussion

2.2.1 *N*-alkyl Bodipy-probe TB340 (3).

Cyclophellitol aziridine ABP **1** was synthesized following the strategy depicted in Scheme 2.1. Alkylation of the aziridine nitrogen of cyclophellitol aziridine **1** with 1-azido-8-iodooctane²⁵ in the presence of K₂CO₃ at elevated temperatures in DMF, gave azide **6**. In the final stage Bodipy alkyne **7**²⁶ was reacted in a Huisgen copper(I)-catalyzed [2+3]-cycloaddition with azide **6**, yielding 2.3 mg of TB340 (**3**) in 2% over 2 steps. In comparison with *N*-acyl ABP **2** (7.4 mg, 2%)²⁰ the efficiency of both synthetic strategies appears to be similar. However, *N*-acyl cyclophellitol aziridines are prone to hydrolysis during lyophilization and purification has to be performed via HPLC purification at neutral (H₂O, MeCN) or basic conditions (25 mM to 50 mM NH₄HCO₃ in H₂O/MeCN). This is in contrast to the successful purification by standard silica gel chromatography of *N*-alkyl aziridine **6**. Finally, opening of the aziridine moiety after concentration under reduced pressure or after lyophilization was not encountered.

Scheme 2.1 Synthesis of *N*-alkyl Bodipy probe TB340 (**3**).



Reagent and conditions: a) 1-azido-8-iodooctane²⁵, K₂CO₃, DMF, 80 °C, 18 h, 24%; b) **7**²⁶, CuSO₄·5H₂O, sodium ascorbate, DMF, rt, 18 h, 8%.

In a next set of head-to-head comparison experiments, *N*-alkyl probe **3** and *N*-acyl probe **2** were examined *in vitro* against recombinant α -galactosidase (rGLA) and *in situ* against wild-type GLA fibroblasts. First, the inhibition potential of both probes was tested *in vitro* and *in situ* (Figure 2.3A). Both probes are very potent inhibitors with apparent IC₅₀ values in the low nanomolar range *in vitro* and *in situ*. As depicted in Figure 2.3B and 2.3C, both probes are pH dependent and labeling of GLA was also effective in a slightly more basic medium. However, from a kinetic point of view, *N*-acyl probe **2** is more efficient and labeling of rGLA was immediately detected at 4 °C in a time-course-dependent labeling. *N*-alkyl probe **3** in contrast labels significantly slower (Figure 2.3D). Additionally, the observed fluorescence output was lower for *N*-alkyl probe **3** (Figure 2.3E). In a competition experiment by pre-incubation with known

selective α -galactosidase inhibitors cyclophellitol aziridine **8**²⁰ and cyclophellitol **9**²¹, labeling of both probes was prevented (Figure 2.3F). Finally, hourly washing of the samples did not affect the labeling, which proves the covalent mode of binding of both probes (Figure 2.3G).

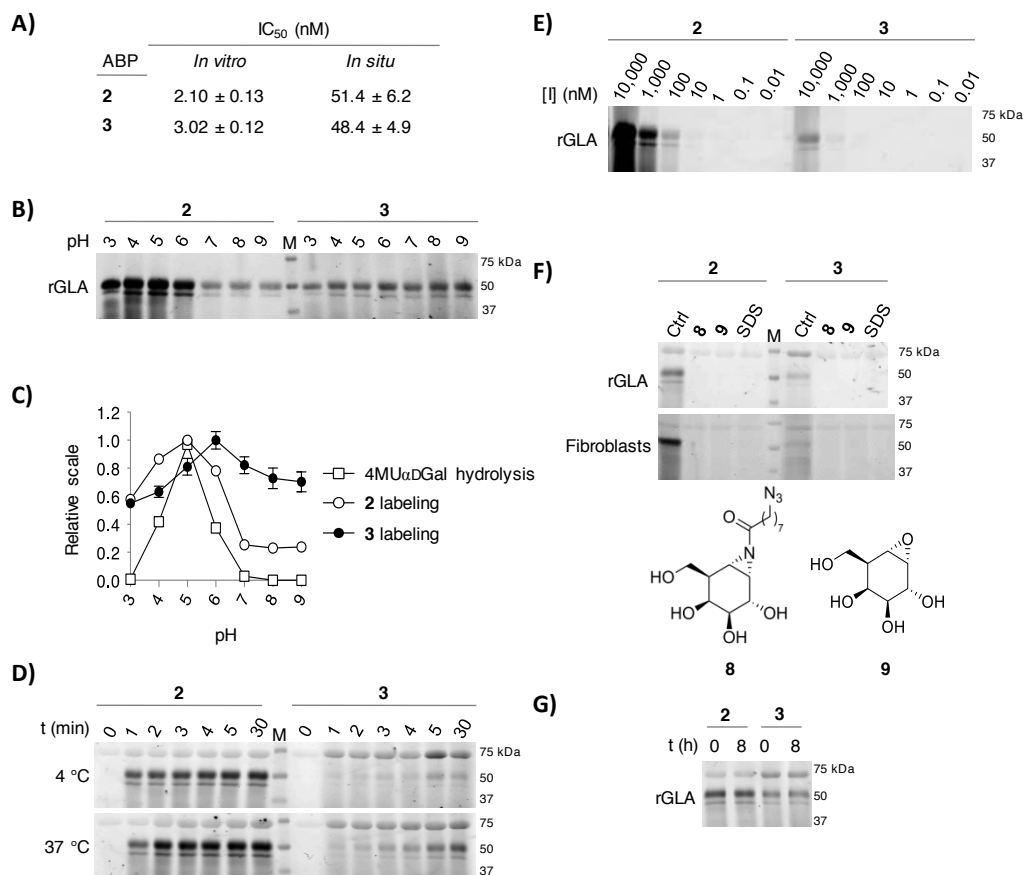
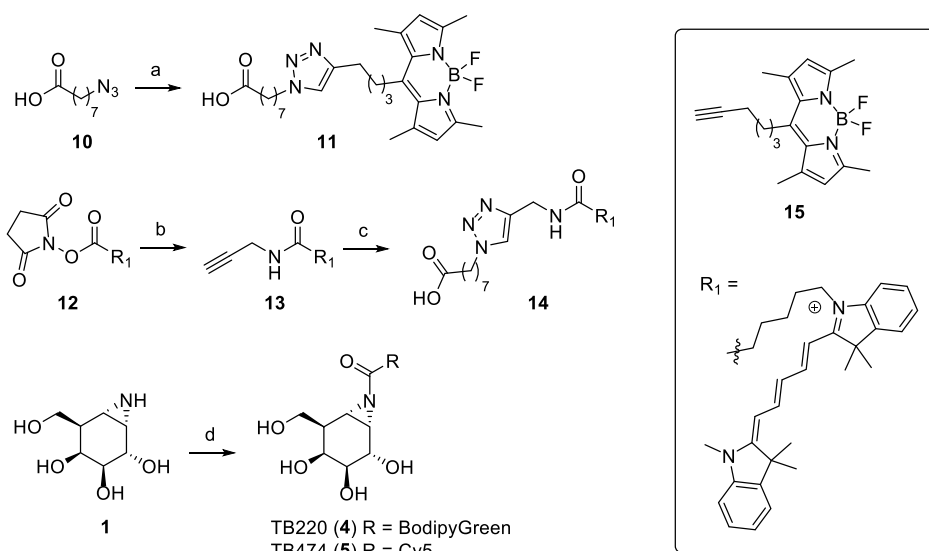


Figure 2.3 A) The inhibition potential of ABPs **2** and **3** were determined *in vitro* (rGLA) and *in situ* (wild-type GLA fibroblast); B) In-gel labeling of rGLA at various pH values; C) Quantification of the fluorescent readout of ABP **2** and **3** (B) compared to 4-methylumbelliferone production from 4-methylumbelliferyl- α -galactopyranoside at various pH values; D) Time and temperature depended labeling of 100 fmol rGLA with 100 fmol ABP at 48 °C (top) and 37 °C (bottom); E) The detection limit of ABP-emitted fluorescence was determined using rGLA (10 pmol) with varying concentrations of ABP; F) Competition assay using known selective inhibitor **8**²⁰, **9**²¹, SDS with ABP **2** and **3** in rGLA (top) and in wild-type GLA fibroblast lysate (bottom); G) The samples were hourly extensive washed in the dark before analyzing on SDS-PAGE analysis.

2.2.2 *N*-acyl BodipyGreen (TB220, **4**) and Cy5 (TB474, **5**).

The synthesis of ABPs **4** and **5** is depicted in Scheme 2.2. The required BodipyGreen-acid **11** was obtained after copper(I)-catalyzed click reaction of 8-azidooctanoic acid **10**²⁷ with BodipyGreen **15**²⁶. Cy5 derivative **14** in turn was synthesized in two steps. First, Cy5-OSu ester **12**²⁸ was reacted with propargylamine to obtain compound **13**. Subsequently coupling with 8-azidooctanoic acid **10**²⁷ in the presence of copper(II) sulfate pentahydrate and sodium ascorbate resulted in compound **14**. In the final stage of the synthetic route, cyclophellitol aziridine **1**²⁰ was coupled with **11** or **14** in the presence of 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) to obtain target compounds **4** and **5**.

Scheme 2.2 Synthesis of TB220 (**4**) and TB474 (**5**).



Reagent and conditions: a) **15**²⁶, CuSO₄·5H₂O, sodium ascorbate, DMF, 80 °C, overnight, 94%; b) propargylamine, Et₃N, CH₂Cl₂, rt, 2.5 h, 25%; c) **10**²⁷, CuSO₄·5H₂O, sodium ascorbate, DMF, 80 °C, overnight, 86%; d) **11** or **14**, EEDQ, 0 °C, DMF, 18% (**4**), 5% (**5**).

After having TB220 (**4**) and TB474 (**5**) in hand, the inhibitory potential towards recombinant GLA (rGLA) was first assessed. Various concentrations of the probes were pre-incubated for 30 min in rGLA, after which the residual enzyme activity was measured using the fluorogenic substrate, 4-methylumbelliferyl α-D-galactoside (4- μ α-gal), and residual enzyme activity towards this substrate in the presence of varying inhibitor concentrations was used to obtain IC₅₀ values. Both TB220 (**4**) and TB474 (**5**) proved to inhibit rGLA in the nanomolar range (2.0 nM and 11.39 nM, respectively),

which corresponds to *N*-acyl aziridine **2** (2.0 nM^{21}) for rGLA. In the next experiment 0.5 equivalent of TB220 (**4**) was used to label 1.0 equivalent Fabrazyme (Figure 2.4A) and incubated for 1h. The residual α -galactosidase in the sample was either directly labeled with ABP **2** or the sample was first washed for 8h before addition of ABP **2**. Labeling of GLA due to TB220 (**4**) was clearly visual in the green channel despite the washing protocol, demonstrating the covalent mode of labeling. In order to confirm the irreversible mode of binding of probe TB474 (**5**), the pre-incubation time of the probe and rGLA was varied. As depicted in Figure 2.4B, longer incubation time of the probe and rGLA increases the apparent inhibition potency, supporting the non-reversal mode of binding of this probe. Finally, effective fluorescent labeling of rGLA with TB474 (**5**) was demonstrated (Figure 2.4C).

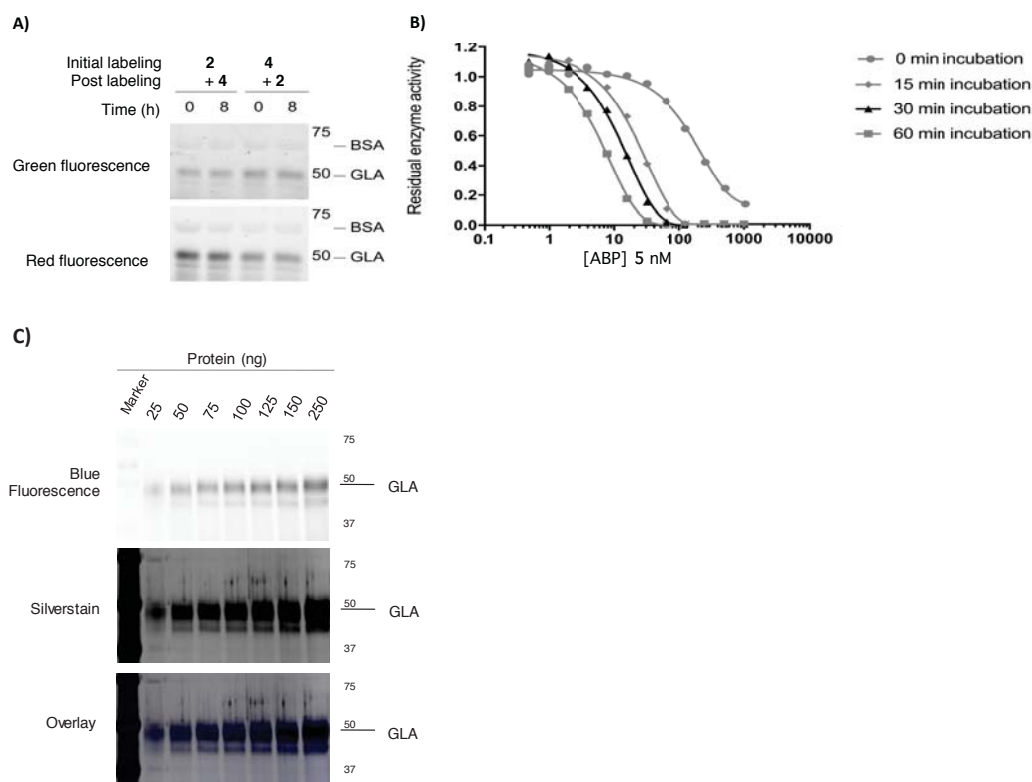


Figure 2.4 A) Fabrazyme (GLA) was labeled with **2** or TB220 (**4**), washed for 0 or 8 hours and post-labeled with TB220 (**4**) and **2**, respectively; B) The inhibitory potential of TB474 (**5**) was determined towards GLA with various incubation times; C) *In vitro* labeling of rGLA at different protein concentrations with TB474 (**5**).

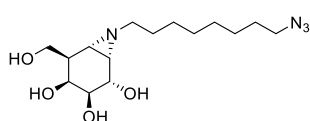
2.3 Conclusion

This chapter describes the synthesis of the *N*-alkylated version (**3**) of the *N*-acyl-cyclophellitol aziridine **2** which proved to be a highly potent α -galactosidase inhibitor and ABP. From a synthetic perspective, efficiencies towards both compounds are comparable but *N*-acyl aziridine **2** is prone to hydrolysis during lyophilization and purification. The biological properties were examined in head-to-head experiments, demonstrating equal potency towards recombinant α -galactosidase. *N*-acyl probe **2**, however, outcompetes *N*-alkyl probe **3** in labeling efficiency. Therefore, *N*-acylated probes are preferable for α -galactosidase detection in an activity-based protein profiling setting. In the second part of this chapter, BodipyGreen and Cy5 modified probes (**4** and **5**, respectively) were synthesized and demonstrated to be covalent inhibitors of GLA in the low nanomolar range. Selective labeling of GLA was visualized by gel electrophoresis on SDS-PAGE of the protein-ABP complex followed by fluorescence detection.

Experimental

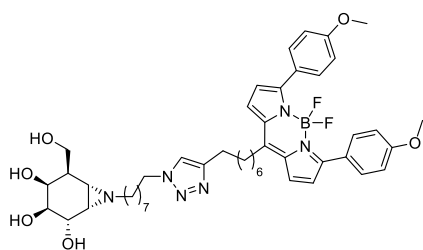
General: Chemicals were purchased from Acros, Sigma Aldrich, Biosolve, VWR, Fluka, Merck and Fisher Scientific and used as received unless stated otherwise. Dichloromethane (DCM), *N,N*-dimethylformamide (DMF) and toluene were stored over flame-dried 4 Å molecular sieves before use. Traces of water from 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. Column chromatography was performed using Screening Device b.v. Silica Gel (particle size of 40 – 63 µm, pore diameter of 60 Å) in the indicated solvents. For reversed-phase HPLC purifications an Agilent Technologies 1200 series instrument equipped with a semi-preparative column (Gemini C18, 250x10 mm, 5 µm 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 µm 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) or a Bruker AV-850 (850 and 214 MHz respectively) spectrometer in the given solvent. Chemical shifts are given in ppm (δ) relative to the residual solvent peak 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).



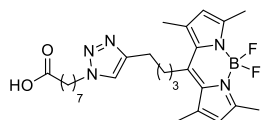
(1S,2S,3S,4S,5R,6S)-7-(8-azido-octyl)-5-(hydroxymethyl)-7-azabicyclo[4.1.0]heptane-2,3,4-triol (6)

Unprotected cyclophellitol aziridine **1**²⁰ (87.6 mg, 0.50 mmol) was dissolved in DMF (4 mL). 1-Azido-8-iodooctane²⁵ (224 mg, 0.80 mmol) and K_2CO_3 (297 mg, 2.15 mmol) were added to the solution and the mixture was stirred at 80 °C for 18 h. After the full conversion of the starting material, the resulting solution was concentrated under reduced pressure. Then the crude product was purified by silica gel column chromatography (3% MeOH in DCM $\rightarrow$ 20% MeOH in DCM) resulting as a colorless oil **6** (40 mg, 0.121 mmol, 24%). TLC: $R_f = 0.3$ (10%, v/v, MeOH in DCM). LC/MS analysis: R_t : 5.47 min (linear gradient 10% $\rightarrow$ 50% B in 12.5 min), m/z 329.20 $[M+H]^+$. ^1H NMR (850 MHz, CD_3OD): δ 4.02 (dd, $J = 4.3, 8.5$ Hz, 1H), 3.78 (t, $J = 1.7$ Hz, 1H), 3.70 (ddd, $J = 44, 8.5, 6.8$ Hz, 2H), 3.33 (dd, $J = 8.5, 1.7$ Hz 1H), 3.27 (t, $J = 4.0$ Hz, 2H), 2.38 – 2.34 (m, 1H), 2.20 – 2.16 (m, 1H), 1.94 (dd, $J = 6.8, 4.3$ Hz, 1H), 1.91 – 1.88 (m, 1H), 1.61 – 1.57 (m, 4H), 1.55 (d, $J = 6.8$ Hz, 1H), 1.41 – 1.31 (m, 6H). ^{13}C NMR (214 MHz, CD_3OD): δ 74.9, 73.2, 70.4, 63.4, 62.4, 52.5, 46.2, 44.9, 42.2, 30.6, 30.5, 30.2, 29.9, 28.4, 27.8. HRMS: calculated for $[C_{15}H_{28}N_4O_4]^+$ 329.21833, found 329.21815.



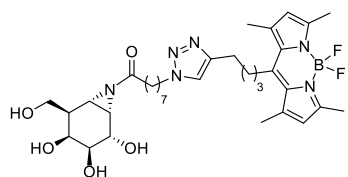
ABP 3

Azide compound **6** (12.0 mg, 0.036 mmol) was dissolved in DMF (2 mL), Bodipy **7**²⁶ (20.9 mg, 0.043 mmol), $CuSO_4$ (1 M, 13 μ L, 13 μ mol) and sodium ascorbate (1 M, 14 μ L, 14 μ mol) were added to the solution under argon atmosphere. After stirring at room temperature for 18 h, the resulting mixture was concentrated under reduced pressure. Then the crude product was purified by semi-preparative reversed HPLC (linear gradient: 52% $\rightarrow$ 60% B in A, 12 min, solutions used A: 50 mM NH_4HCO_3 in H_2O , B: MeCN) and lyophilized resulting as a purple powder product **3** (2.3 mg, 2.8 μ mol, 8%). LC/MS analysis: R_t 6.83 min (linear gradient 10% $\rightarrow$ 90% B in 12.5 min), m/z 813.33 $[M+H]^+$. ^1H NMR (850 MHz, CD_3OD): δ 7.85 (d, $J = 8.5$ Hz, 4H), 7.70 (s, 1H), 7.44 (d, $J = 7.44$ Hz, 2H), 6.97 (d, $J = 7.7$ Hz, 4H), 6.69 (d, $J = 4.3$ Hz, 2H), 4.33 (t, $J = 6.8$ Hz, 2H), 4.00 (dd, $J = 8.5, 4.3$ Hz, 1H), 3.85 (s, 3H), 3.78 – 3.76 (m, 1H), 3.73 (ddd, $J = 48, 7.7, 6.8$ Hz, 2H), 3.33 (t, $J = 1.7$ Hz, 1H), 3.07 (t, $J = 7.7$ Hz, 2H), 3.30 (s, 3H), 2.79 (t, $J = 6.8$ Hz, 2H), 2.30 – 2.27 (m, 1H), 2.14 – 2.10 (m, 1H), 1.90 – 1.82 (m, 8H), 1.55 – 1.49 (m, 3H), 1.31 – 1.21 (m, 8H). ^{13}C NMR (214 MHz, CD_3OD): δ 162.2, 158.8, 146.8, 137.5, 132.2, 128.4, 126.5, 121.0, 114.6, 74.9, 73.2, 70.4, 63.3, 62.3, 55.9, 55.8, 52.3, 49.4, 46.2, 44.8, 42.1, 34.1, 31.2, 31.0, 30.4, 30.4, 30.3, 29.9, 28.2, 27.4, 25.8. HRMS: calculated for $[C_{44}H_{55}BF_2N_6O_6]^+$ 813.43245, found 813.43237.



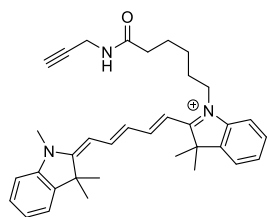
8-(BodipyGreen)-triazole-octanoic acid (**11**)

8-Azido-octanoic acid **10**²⁷ (0.50 mmol, 93 mg) and BodipyGreen-alkyne **15**²⁶ (0.50 mmol, 0.16 g, 1.0 eq.) were dissolved in DMF under argon atmosphere. After addition of copper(II)sulphate pentahydrate (100 μ mol, 100 μ L 1 M in H₂O, 0.2 eq.) and sodium ascorbate (200 μ mol, 200 μ L 1 M in H₂O, 0.4 eq.) the reaction mixture was heated to 80 °C and stirred overnight. The mixture was then evaporated *in vacuo* and purified by column chromatography (DCM $\rightarrow$ 2% MeOH in DCM) to yield BodipyGreen-acid **11** as an orange solid (0.24 g, 0.47 mmol, 94%). ¹H NMR (400 MHz, MeOD): δ (ppm) 7.68 (d, J = 8.4 Hz, 1H), 6.06 (s, 2H), 4.29 (t, J = 7.2 Hz, 2H), 2.84 – 2.78 (m, 2H), 2.70 (t, J = 6.8 Hz, 2H), 2.41 (s, 6H), 2.26 (s, 6H), 2.26 – 2.21 (m, 2H), 1.86 – 1.77 (m, 4H), 1.56 – 1.49 (m, 4H), 1.40 – 1.17 (m, 6H). ¹³C NMR (100 MHz, MeOD): δ (ppm) 177.5, 154.8, 147.8, 142.2, 136.4, 132.5, 122.6, 51.2, 32.0, 31.2, 30.8, 29.9, 29.7, 28.9, 27.6, 27.2, 25.9, 25.8, 16.4, 14.5.



ABP 4

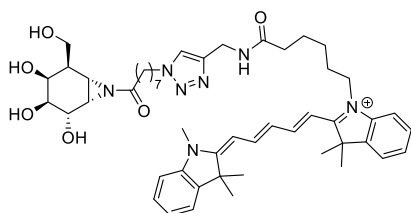
BodipyGreen-acid **11** (0.10 mmol, 51 mg) was preactivated with EEDQ (0.10 mmol, 25 mg, 1.0 eq.) in DMF (0.6 mL) under argon for 2 hours at room temperature. Deprotected aziridine **1** (0.10 mmol) was dissolved in DMF (0.5 mL) under argon and cooled to 0 °C, after which 0.25 mL of the activated ester solution was added (50 μ mol, 0.5 eq.) and the mixture was stirred at 0 °C for 30 min. Then another 0.5 eq. of the activated ester was added and the reaction mixture was stirred at 0 °C for another 60 min. After quenching with MeOH, the mixture was evaporated *in vacuo*, purified by HPLC under basic conditions using a gradient of 44 $\rightarrow$ 50% ACN in 25 mM aqueous NH₄HCO₃ in 12 min, and lyophilized to yield aziridine **4** as an orange powder (12 mg, 18 μ mol, 18%). ¹H NMR (600 MHz, MeOD): δ 7.69 (s, 1H), 6.11 (s, 2H), 4.35 (t, J = 7.0 Hz, 2H), 4.07 (dd, J = 8.6, 4.0 Hz, 1H), 3.8 – 3.84 (m, 1H), 3.81 – 3.74 (m, 2H), 3.36 (dd, J = 1.7, 8.6 Hz, 1H), 3.04 – 3.00 (m, 2H), 2.96 (dd, J = 6.0, 4.0 Hz, 1H), 2.78 (t, J = 12.6 Hz, 2H), 2.59 (d, J = 6.0 Hz, 1H), 2.54 – 2.42 (m, 2H), 2.44 (s, 6H), 2.38 (s, 6H), 2.01 (td, J = 7.8, 7.3, 3.6 Hz, 1H), 1.96 – 1.83 (m, 4H), 1.69 – 1.63 (m, 2H), 1.61 – 1.55 (m, 2H), 1.36 – 1.23 (m, 6H). ¹³C NMR (100 MHz, CDCl₃): δ 188.6, 154.9, 148.5, 147.9, 142.2, 132.6, 123.4, 122.6, 74.3, 74.2, 69.5, 62.8, 51.2, 44.5, 43.3, 39.4, 37.0, 32.2, 31.2, 30.8, 30.0, 29.6, 29.1, 27.4, 25.9, 25.8, 16.5, 14.4. HRMS: calculated for [C₃₄H₅₀BF₂N₆O₅]⁺ 671.39028, found 671.39043.



2-[5-(1,3,3-Trimethyl-2,3-dihydro-1H-indol-2-ylidene)-1,3-pentadienyl]-3,3-dimethyl-1-(6-oxo-6-(prop-2-yn-1-ylamino)hexy)-3H-indolium (**13**)

Cy5 OSu ester **12**²⁸ (3.10 g, 5.0 mmol) and propargylamine (0.35 mL, 5.5 mmol) were dissolved in CH₂Cl₂ (25 mL). After addition of Et₃N (6.0 mmol, 0.84 mL), the reaction mixture was stirred for 2.5 h at room temperature. The reaction mixture was concentrated *in vacuo* and purification by column chromatography (1% MeOH in CH₂Cl₂ $\rightarrow$ 4% MeOH in CH₂Cl₂) gave Cy5-alkyne **13** (690 mg, 1.24 mmol, 25%) as a dark-blue foam. ¹H NMR (400 MHz, MeOD): δ 8.26 (t, J = 13.1 Hz, 2H), 7.48 (d, J = 7.4 Hz, 2H), 7.44 – 7.35 (m, 2H), 7.29 (d, J = 7.9 Hz, 2H), 7.24 (t, J = 7.4 Hz,

2H), 6.71 (t, $J = 12.4$ Hz, 1H), 6.31 (t, $J = 13.5$ Hz, 2H), 4.12 (t, $J = 7.4$ Hz, 2H), 3.92 (d, $J = 2.5$ Hz, 2H), 3.63 (s, 3H), 2.57 (t, $J = 2.6$ Hz, 1H), 2.23 (t, $J = 7.3$ Hz, 2H), 1.84 (dt, $J = 14.9, 6.3$ Hz, 2H), 1.75 – 1.66 (m, 14H), 1.51 – 1.46 (m, 2H). ^{13}C NMR (101 MHz, MeOD): δ 175.4, 175.2, 174.5, 155.4, 144.2, 143.5, 142.6, 142.5, 129.7, 129.6, 126.8, 126.1, 123.4, 123.3, 112.0, 111.8, 104.4, 55.9, 50.5, 44.8, 36.4, 31.8, 29.4, 28.2, 28.0, 27.9, 27.3, 26.4, 26.3. HRMS: calculated for $[\text{C}_{35}\text{H}_{42}\text{N}_3\text{O}]^+$ 520.33224, found 520.33172.



ABP 5

To a solution of 8-azido-octanoic acid²⁷ (94 mg, 0.50 mmol) in DMF (4 mL) was added Cy5-alkyne **13** (282 mg, 0.50 mmol) at room temperature. After addition of copper(II)sulfate pentahydrate (500 μL , 1 M in H_2O) and sodium ascorbate (700 μL , 1 M in H_2O) the reaction mixture was heated up to 80 $^\circ\text{C}$ and stirred overnight.

The reaction mixture was concentrated *in vacuo*, redissolved in CH_2Cl_2 and washed with HCl (0.1 M, 3 x) and brine (1 x). The organic layer was dried over MgSO_4 and concentrated *in vacuo* to obtain Cy5 acid **14** (0.43 mmol, 0.32 g, 86%) which was used without further purification. LC/MS analysis: R_t 7.27 min linear gradient 10% $\rightarrow$ 90% B in 12.5 min, m/z 705.33 $[\text{M}]^+$. HRMS: calculated for $[\text{C}_{43}\text{H}_{57}\text{N}_6\text{O}_3]^+$ 705.44867, found 705.44892. To a solution of crude Cy5 acid **14** (111 mg, 0.12 mmol) in DMF (0.8 mL) was added EEDQ (30 mg, 0.12 mmol) and stirred for 2 h at room temperature to obtain a pre-activated mixed anhydride solution. Aziridine **1**²⁰ (17.4 mg, 0.1 mmol) was dissolved in DMF (0.4 mL) and pre-activated mixed anhydride solution (0.5 eq.) was added at 0 $^\circ\text{C}$. After stirring for 30 min additional pre-activated mixed anhydride solution (0.5 eq.) was added. The reaction mixture was stirred for 30 min after which additional EEDQ (25 mg, 0.10 mmol) was added. After stirring overnight at 4 $^\circ\text{C}$, the reaction mixture was quenched with MeOH at 0 $^\circ\text{C}$ and concentrated *in vacuo*. Purification by semi preparative reversed phase HPLC at neutral conditions (A: 50 mM NH_4HCO_3 in H_2O , B: MeCN; linear gradient: 44% $\rightarrow$ 50% B in 12 min) gave compound **5** (4.39 mg, 4.88 μmol , 5%) as a blue powder. LC/MS analysis: R_t 6.13 min and 6.37 min, linear gradient 10% $\rightarrow$ 90% B in 12.5 min, m/z 862.40 $[\text{M}]^+$. ^1H NMR (850 MHz, MeOD): δ 8.24 (td, $J = 13.2, 3.5$ Hz, 2H), 7.84 (d, $J = 4.7$ Hz, 1H), 7.49 (d, $J = 7.3$ Hz, 2H), 7.44 – 7.39 (m, 2H), 7.32 – 7.24 (m, 4H), 6.62 (t, $J = 12.3$ Hz, 1H), 6.30 – 6.25 (m, 2H), 4.41 (d, $J = 7.2$ Hz, 2H), 4.38 – 4.34 (m, 2H), 4.11 – 4.06 (m, 3H), 3.87 – 3.84 (m, 1H), 3.63 (s, 3H), 3.59 – 3.55 (m, 2H), 3.53 (dd, $J = 10.7, 6.3$ Hz, 1H), 3.44 (dd, $J = 10.7, 6.0$ Hz, 1H), 3.36 (dd, $J = 8.9, 1.9$ Hz, 1H), 2.95 (dd, $J = 6.0, 4.0$ Hz, 1H), 2.60 (d, $J = 6.0$ Hz, 1H), 2.25 (t, $J = 7.3$ Hz, 2H), 2.01 (td, $J = 7.4, 3.8$ Hz, 1H), 1.88 – 1.86 (m, 2H), 1.84 – 1.79 (m, 2H), 1.74 – 1.71 (m, 13H), 1.62 – 1.55 (m, 2H), 1.48 – 1.45 (m, 2H), 1.36 – 1.27 (m, 6H). ^{13}C NMR (214 MHz, MeOD): δ 187.2, 174.3, 174.0, 173.2, 154.1, 154.0, 144.7, 142.8, 142.1, 141.2, 141.1, 128.3, 128.3, 125.2, 124.9, 124.8, 122.7, 122.0, 121.8, 110.6, 110.4, 103.0, 102.8, 72.9, 71.7, 68.1, 64.2, 61.4, 61.4, 49.7, 43.3, 43.1, 41.8, 38.0, 35.0, 34.2, 29.7, 28.7, 28.5, 28.2, 26.7, 26.5, 26.4, 25.9, 25.8, 24.9. HRMS: calculated for $[\text{C}_{50}\text{H}_{68}\text{N}_7\text{O}_6]^+$ 862.52256, found 862.52229.

Materials of biological assays

Recombinant GLA (Fabrazyme) was obtained from Genzyme (Cambridge, MA, USA).

Enzyme activity assay

To determine the apparent *in vitro* IC₅₀ value, GLA was diluted in 150 mM McIlvaine buffer (pH 4.6, supplemented with 0.1% (w/v) BSA and 0.1% (w/v) Triton X-100 (in case of ABP 5)) and pre-incubated with a range of inhibitor dilutions. After 30 min at 37 °C, the substrate mix (100 µL, 1.0 mg/mL 4-methylumbelliferyl- α -D-galactopyranoside in 150 mM McIlvaine pH 4.6 with 0.1% (w/v) BSA) was added. After 20 min of incubation at 37 °C, the reaction was quenched by adding an excess of NaOH-glycine buffer (pH 10.6 or 10.3 (in case of ABP 5)). The fluorescence of the formed 4-methylumbelliferyl was measured with a fluorimeter LS55 (PerkinElmer) using $\lambda_{\text{EX}} = 366$ nm and $\lambda_{\text{EM}} = 445$ nm. To determine the influence of pH on the enzymatic activity, enzyme mixtures were firstly pre-incubated for 30 min on ice with McIlvaine buffers of pH 3–9 whereafter substrate was added, dissolved in Nanopure H₂O. The influence of length of pre-incubation time on apparent IC₅₀ was observed to determine the mode of binding of ABP 5. The pre-incubation time of ABP 5 and rGLA was varied from 0 min (no pre-incubation) to 60 min whereafter the substrate mix was added. After incubation for 20 min at 37 °C, the reaction mixture was quenched and analyzed as described above. The *in situ* IC₅₀ value was determined by incubating fibroblast cell lines, grown to confluency, with a range of inhibitor dilutions for 2 h. Hereafter, cells were washed three times with PBS and subsequently harvested by scraping in potassium phosphate buffer (25 mM K₂HPO₄-KH₂PO₄, pH 6.5, supplemented with 0.1% (v/v) Triton X-100 and protease inhibitor cocktail (Roche)). After determination of the protein concentration (BCA kit, Pierce), lysates were aliquoted and frozen at -80 °C. The IC₅₀ values of 2 and 3 were determined by replicating each assay twice in duplo in two separate cell lines. The IC₅₀ values of 4 and 5 were measured in duplo. All IC₅₀ values were corrected for background fluorescence (samples without enzyme), followed by normalization to the control sample (incubation without ABP). Curve-fitting via one phase exponential decay function was used to calculate the apparent IC₅₀ values (Graphpad Prism 5.0 software (2, 3 and 4) and Graphpad Prism 6 software (5)).²¹

In vitro labeling and SDS-PAGE analysis of ABP 2 and ABP 3

All pre-incubations and ABP labeling reactions occurred for 30 min at 37 °C, unless stated otherwise. The detection limit of was analyzed by labeling rGLA (10 pmol) with 10 000 – 0.01 nM ABP (2 and 3) for 30 min at 37 °C. The influence of pH on ABP labeling involved pre-incubation of the aforementioned enzyme at pH 3–10 for 30 min on ice, prior to addition of 1 mM ABP 2 and 3), dissolved in Nanopure H₂O and incubation for 30 min at 37 °C. Assessment of ABP labeling kinetics involved precooling of rGLA (10 pmol) in 150 mM McIlvaine buffer (pH 4.6, supplemented with 0.1% (w/v) BSA) on ice for 15 min, followed by addition of similarly cooled 10 pmol ABP solution in NanopureH₂O. After mixing, ABP labeling was chased for 0–30 min, afterwhich labeling was stopped by denaturation. For ABPP, rGLA (10 pmol) or 100 mg total protein in lysate of human, wild-type GLA fibroblasts, was pre-incubated with 100 mM 8 (LWA481)²⁰ or 9 (LWA468)²¹ or boiled for 4 min in 2% (w/v) SDS, prior to labeling with 1 mM ABP 2 or 3 (all dissolved in Nanopure H₂O) for 30 min at 37 °C. Stability of the ABP–nucleophile adduct was analyzed by firstly labeling the various enzyme mixtures with the corresponding ABPs in the McIlvaine buffer after which the mixture was washed over Zeba spin desalting columns with 40K MWCO resin, according to the manufacturer's instructions (Thermo Scientific). The eluted sample was separated, with 50% being snap-frozen in

liquid nitrogen and stored at -20 °C, whereas the remaining 50% was chased for 8 h, including hourly washing with the McIlvaine buffer, over a new Zeba column. Samples were denatured with 5 x Laemmli buffer (50% (v/v) 1M Tris-HCl, pH 6.8, 50% (v/v) 100% glycerol, 10% (w/v) DTT, 10% (w/v) SDS, 0.01% (w/v) bromophenol blue), boiled for 4 min at 100 °C, and separated by gel electrophoresis on 10% (w/v) SDS-PAGE gels running continuously at 90 V.²¹

Wet slab-gels were then scanned for ABP-emitted fluorescence using a Typhoon TRIO variable mode imager (Amersham Biosciences) using λ_{EX} = 532 nm and λ_{EM} = 610 nm (band pass filter 30 nm) for the red fluorescent ABPs.

Stability of ABP labeling of ABP 4

Fabrazyme (5 nmol) in 150 mM McIlvaine buffer, pH 4.6, supplemented with 0.1% (w/v) BSA was labeled with 2.5 nmol ABP 2 or 4, solubilized in Nanopure H₂O, during 1 h at 37 °C. Next, the mixture was washed over Zeba Spin Desalting Columns with 40K MWCO resin according to manufacturer's instructions (Thermo Scientific). After elution, half the sample was snap frozen in liquid nitrogen and stored at -20 °C. The remaining sample was chased and washed for 8 h by loading on a new Zeba column, thorough washing with 150 mM McIlvaine buffer, pH 4.6, and recollection (repeated every 2 h). Residual unlabeled α -galactosidase was post-labeled with 10 pmol ABP 4 (in case of pre-labeling with ABP 2) or with ABP 2 (in case of pre-labeling with ABP 4). Sample denaturation, SDS-PAGE electrophoresis and fluorescence detection was according to methods published earlier.²⁹

In vitro labeling and SDS-PAGE analysis of ABP 5

Recombinant GLA was dissolved in 150 mM McIlvaine buffer (pH 4.6) and 5 μ L protein was mixed with 5 μ L ABP 4 (20 mM, in H₂O) at 37 °C. After incubation of 30 min, the proteins were denatured by adding Laemmli buffer (2.5 μ L, 50% (v/v) 1 M Tris-HCl, pH 6.8, 50% (v/v) glycerol, 10% (w/v) DTT, 10% (w/v) SDS and 0.01% (w/v) bromophenol blue). The reaction mixture was briefly vortexed and boiled for 4 min at 100 °C. The protein-ABP complex was loaded and separated by gel electrophoresis on 10% (w/v) SDS-PAGE, running at 90 V.²¹ The wet slab gel was scanned for fluorescence using the ChemiDoc imager (Bio-Rad) in the Cy5 channel (λ_{EX} = 647 nm; λ_{EM} = 695 nm; 55 nm band pass filter). After fluorescence scanning, the SDS-PAGE gel was subjected to silver staining (Invitrogen Silverquest Silver Staining Kit; basic staining protocol) and scanned using the ChemiDoc imager (Bio-Rad) in transparency mode.

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3

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

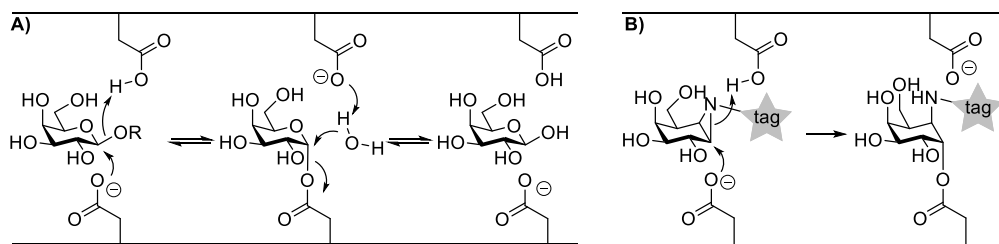


Figure 3.1 A) Double displacement mechanism of retaining β -galactosidases. B) Proposed mechanism of cyclophellitol aziridine based probes in β -galactosidases.

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.

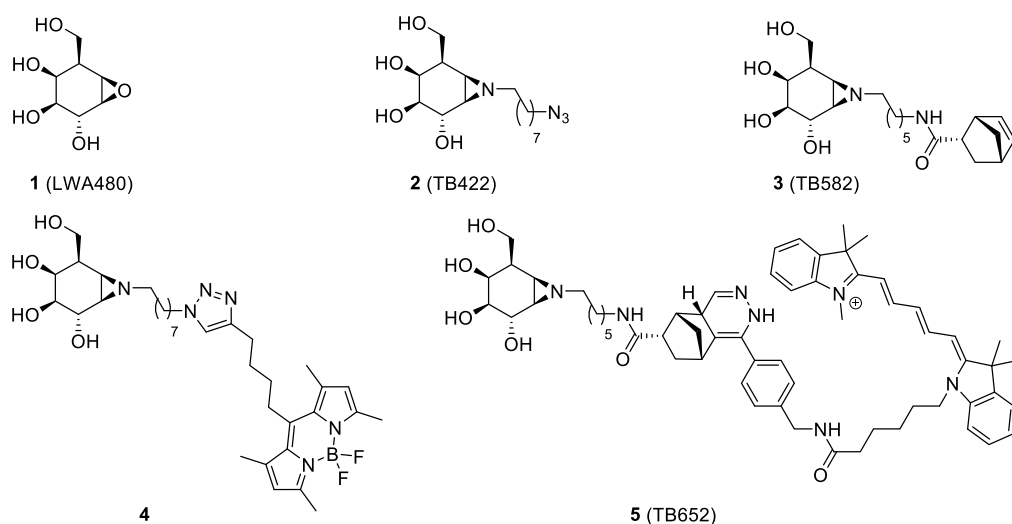


Figure 3.2 Structures of β -galactopyranose-cyclophellitol (1), intermediates 2 and 3 for bioorthogonal ligation, and ABPs (4 and 5) targeted in this study.

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 ± 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.

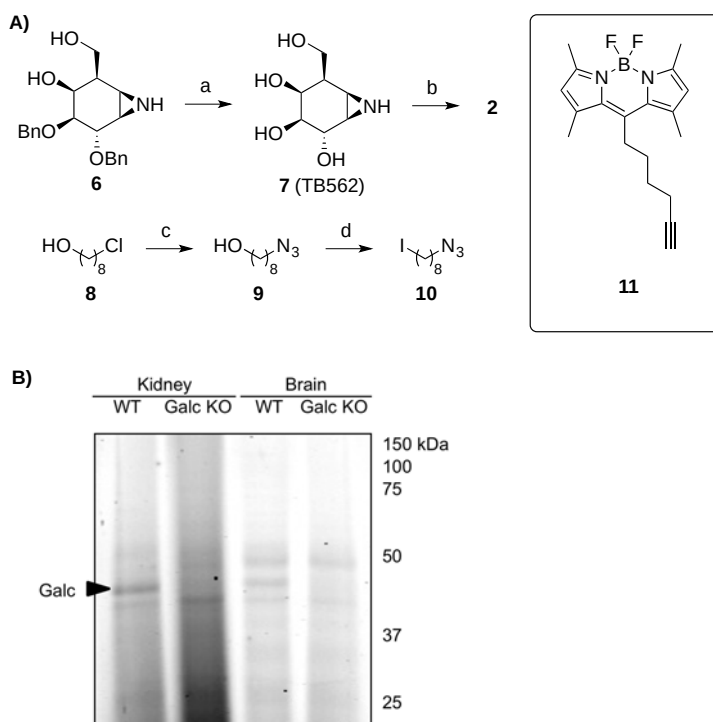
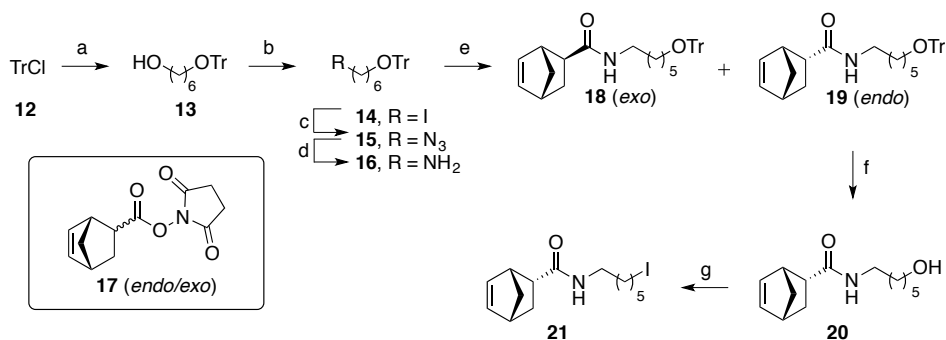


Figure 3.4 A) Synthesis of precursor probe **2**, reagents and conditions: a) Li, NH₃, -60 °C, b) **10**, K₂CO₃, DMF, 80 °C, overnight, 25%; c) NaN₃, DMF, 80 °C, quant; d) PPh₃, imidazole, I₂, THF, -20 °C to rt, 15 min, 69%. B) Labeling of GALC in kidney and brain homogenates of wt and GALC-deficient 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 idonorborene **21**.

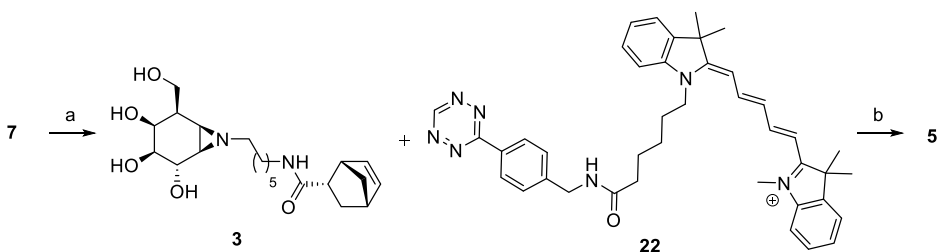
Scheme 3.1 Synthesis of probe **3** with a norbornene ligation handle.



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 $\pm$ 0.47	11.96 $\pm$ 2.27
3 (TB582)	57.84 $\pm$ 3.05	98.55 $\pm$ 20.84
5 (TB652)	14.85 $\pm$ 0.98	61.02 $\pm$ 6.89
7 (TB562)	2.55 $\pm$ 0.59	5.57 $\pm$ 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.

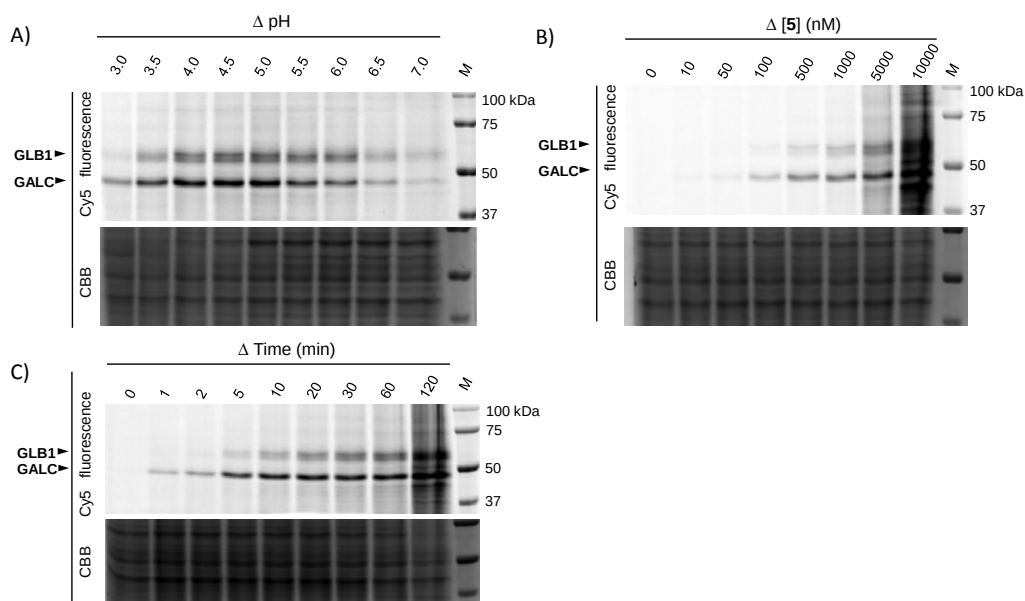


Figure 3.5 Activity-based protein profiling with probe **5** at various conditions in mouse kidney homogenates after blocking the GBA activity (A and B). A) pH-dependent labeling with 1 μ M probe **5** for 30 min. B) Concentration dependent labeling at pH 4.5 for 30 min. C) Incubation time dependent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

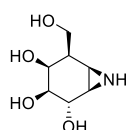
2.3 Conclusion

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 µm, 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 µm 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 µm 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 condensated 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).

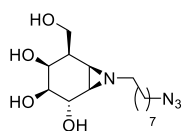
The mixture was gradually warmed to rt and concentrated *in vacuo*. The crude product was dissolved in H₂O and treated with Amberlite IR-120 NH₄⁺ for 5 min. After filtration of the resin, the filtrate was concentrated *in vacuo* and retreated with Amberlite IR-120 NH₄⁺ to obtain β -aziridine **7** (22.6 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 $\rightarrow$ 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 $\rightarrow$ 12% MeOH in DCM) gave azide-aziridine cyclophellitol **2** (21.5 mg, 65 μ mol, 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)

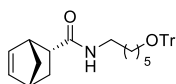
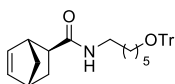
Tritylhexanol **13** (3.35 g, 9.3 mmol) was dissolved in Et_2O (35 mL) and CH_3CN (11 mL). After addition of imidazole (1.90 g, 27.9 mmol) and triphenylphosphine (13.95 mmol, 3.66 g) the reaction mixture was cooled to 0 °C and iodine (3.54 g, 13.95 mmol) was added in small portions. After stirring for 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.

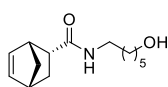
 6-(trityloxy)hexan-1-amine (16)

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.



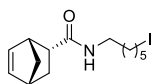
(1R,2R,4R)-N-(6-(trityloxy)hexyl)bicyclo[2.2.1]hept-5-ene-2-carboxamide (18) and (1R,2S,4R)-N-(6-(trityloxy)hexyl)bicyclo[2.2.1]hept-5-ene-2-carboxamide (19)

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)

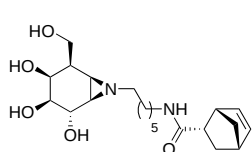
Compound **19** (0.815 g, 1.7 mmol) was dissolved in a mixture of DCM/MeOH (20 mL, 1:1) and *p*-toluenesulfonic acid was added until pH 2. After stirring overnight at room temperature, the reaction mixture was neutralized with a saturated aqueous NaHCO₃ solution. The aqueous layer was extracted with 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)

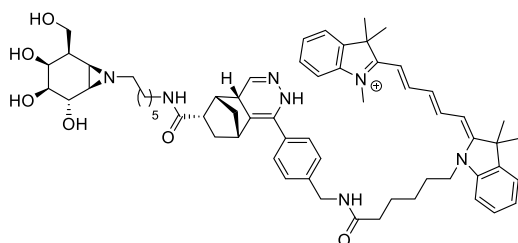
To a solution of alcohol **20** (119 mg, 0.50 mmol) in THF (2.5 mL) was added PPh₃ (197 mg, 0.75 mmol) and imidazole (64 mg, 1.0 mmol). The reaction mixture was stirred and heated to reflux temperature. Carefully a solution of I₂ (190 mg, 0.75 mmol) in THF (1.0 mL) was added. After stirring for 1.5 h, 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), CuSO₄ (1 μ L, 0.1 mM in H₂O), DTT (0.5 μ L, 0.1 mM in H₂O) 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 K₂HO₄/KH₂PO₄, 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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4

Cyclophellitol based ABPs for broad-spectrum profiling of retaining exo- α -mannosidases¹

4.1 Introduction

Retaining exo- α -mannosidases are found in numerous organisms in nature. In humans, five different forms are present: MA2B1, MA2B2, MA2C1, MA2A1 and MA2A2, which are all part of the GH38 family. These glycosidases are capable of hydrolyzing α 1-2, α 1-3 or α 1-6 linkages of high-, hybrid- or complex mannose *N*-linked glycans.² Diverse biological functions have been ascribed to these glycosidases. Some are involved in the biosynthesis assembly of *N*-linked glycans in the Golgi system (MA2A1 and MA2A2)³, whereas others are present in the lysosome (MA2B1, MA2B2)^{4,5} or in the cytosol (MA2C1)^{6,7} as part of the catabolic system.⁸ Retaining exo- α -mannosidases have been associated with various pathologies. For instance, increased

expression of lysosomal α -mannosidases in larynx cancer cells was observed.⁹ α -Mannosidosis, a lysosomal storage disorder^{10,11}, results from mutations in the MAN2B1 gen. Lack of hydrolysis results in swelling of lysosomes and loss of cellular functions.¹² The enzyme reaction itinerary of retaining exo- α -mannosidases takes place via a double-displacement mechanism, i.e. with retention of the configuration of the anomeric center. In general, the substrate proceeds according to the ${}^0S_2 \rightarrow B_{2,5} \rightarrow {}^1S_5 \rightarrow B_{2,5} \rightarrow {}^0S_2$ catalytic itinerary (Figure 4.1).^{13,14} After the substrates adapts to a ${}^0S_2$ skew boat conformation in the active site, an aspartic acid residue of the enzyme attacks the anomeric center and forms a covalent intermediate with the substrate after expulsion of the aglycon which is protonated by the acid/base residue. The glycosidic bond of the formed enzyme-substrate complex is then hydrolyzed, resulting in the enzyme and the α -mannose product. The catalytic nucleophile has been trapped with a fluoruous substrate and analyzed by the group of Withers, who identified an aspartic acid as the catalytic nucleophile, which supports this mechanism.^{15,16}

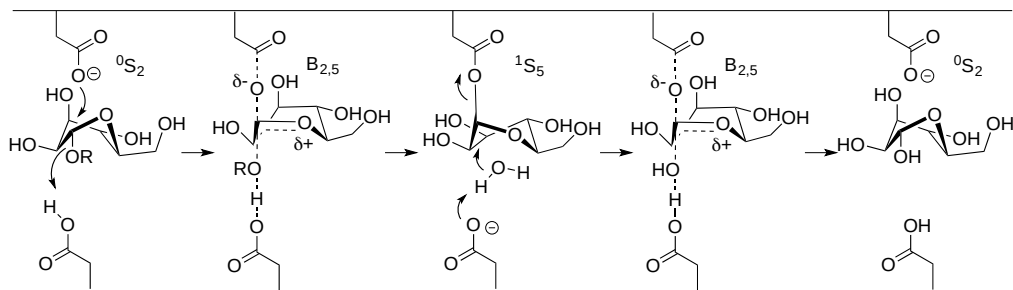


Figure 4.1 Proposed mechanism of substrate hydrolysis by exo- α -mannosidases.^{13,14}

(+)-Cyclophellitol is a covalent inhibitor of retaining β -glucosidases.^{17,18} Several configurational isomers have been synthesized and resulted in selective and potent inhibitors of different glycosidases.¹⁹⁻²¹ Based on these precedents, in this chapter the synthesis of α -mannopyranose-configured cyclophellitol and cyclophellitol aziridine is described (Figure 4.2). Cyclophellitol aziridine **2** was used as a starting point in the synthesis of activity-based probes (ABPs) that specifically tag retaining exo- α -mannosidases, which could help to elucidate the biological processes connected with the complex pathophysiology of α -mannosidase linked diseases.²²

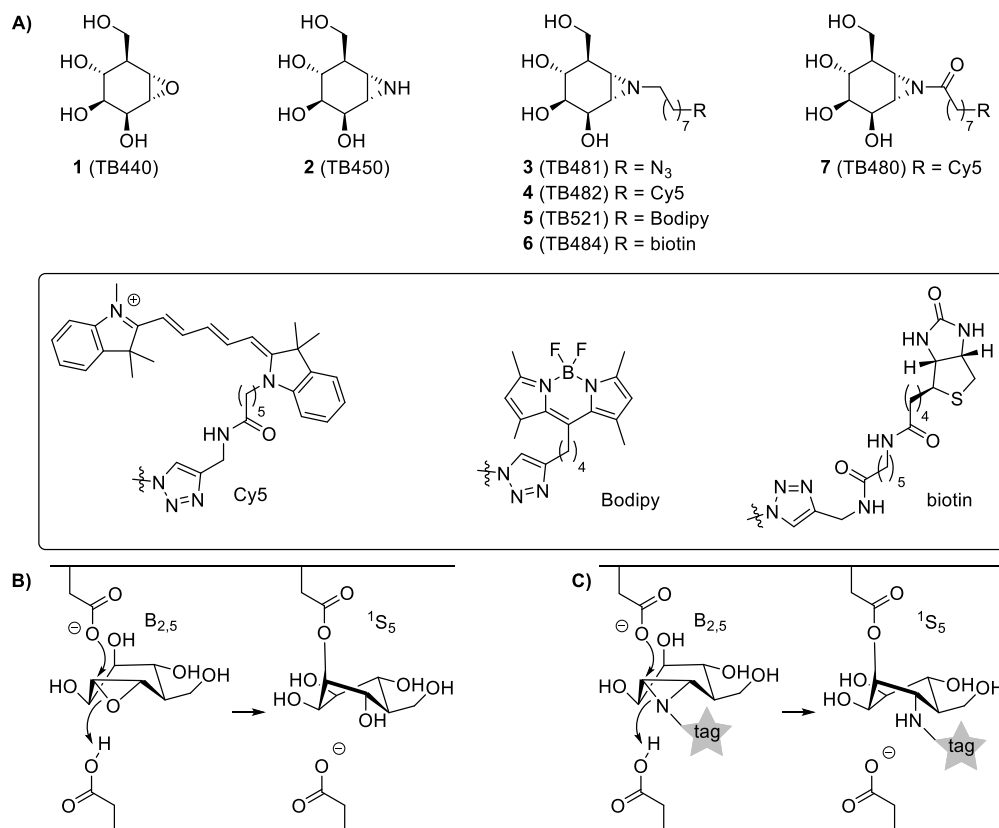


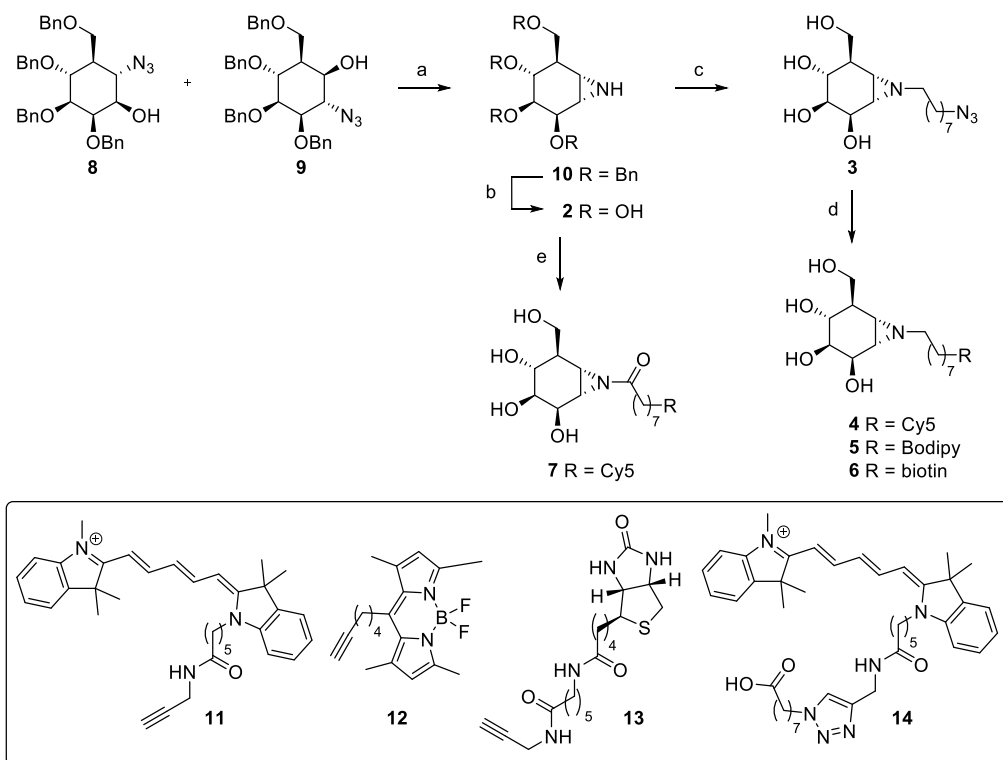
Figure 4.2 A) Structures of α -mannopyranose-configured cyclophellitol and cyclophellitol aziridine analogues examined in this study. B) Proposed mechanism of α -mannopyranose-configured cyclophellitol and α -mannopyranose-configured cyclophellitol aziridine (C) as mechanism-based α -mannosidase inhibitors.

4.2 Results and discussion

The synthesis of α -mannose-configured cyclophellitol **1** and α -mannose-configured cyclophellitol aziridine **2** was previously described.²³ However, a one-step synthesis of aziridine **10** was investigated²⁴ with the aim to improve on this synthesis, which comprised mesylation of alcohols **8** and **9**, followed by α -aziridine formation by treatment with LiAlH₄, (Scheme 4.1). Staudinger reaction with compound **8** and **9** using triphenylphosphine on beads gave aziridine **10** which was easily purified by filtration. Compared to the earlier described method (22%), the yield could be considerably improved in this manner (54%). Debenzylation using Birch reduction conditions gave aziridine **2**.²³ ABPs **4** - **6** were obtained by *N*-alkylation of aziridine **2** with 1-azido-8-iodooctane followed by copper(I)-catalyzed [2+3]-cycloaddition ‘click’

ligation with fluorophore **11** (see Chapter 2), **12**²⁵ or biotin-alkyne **13**²⁶. As described in Chapter 2, *N*-alkyl aziridines are more easily synthesized compared to *N*-acyl aziridines. However, in the case of GH29 α -fucosidases, the appropriately configured *N*-alkyl probes proved to be considerably less effective than the corresponding *N*-acyl probes.²⁷ Therefore and to be on the safe side, *N*-acyl probe **7** was synthesized as well.

Scheme 4.1 Synthesis of α -mannopyranose-configured aziridine inhibitors and ABPs.



Reagents and conditions: a) PPh_3 polymer-bound on styrene-divinylbenzene copolymer, CH_3CN , reflux, overnight, 54%; b) Li, THF, NH_3 (l), -60 °C, 75 min; c) 1-azido-8-iodooctane (see Chapter 3), K_2CO_3 , DMF, 80 °C, overnight, 38% over 2 steps; d) **11** (see Chapter 2), **12**²⁵ or **13**²⁶, $\text{Cu}\cdot\text{SO}_4\cdot 5\text{H}_2\text{O}$, sodium ascorbate, DMF, rt, **4** 35%, **5** 8.5%, **6** 16%; e) **14** (see Chapter 2), EEDQ, DMF, 0 °C, 2.5 h, 8% over 2 steps.

Inhibitory potency of α -mannopyranose-configured cyclophellitol 1 – 7.

Having compounds **1** – **7** in hand, their inhibition potential was assessed towards GH38 jack bean (*Canavalia ensiformis*) α -mannosidase. The inhibitor was pre-incubated for 30 min in varying concentrations after which the residual enzymatic

activity was measured using the fluorogenic substrate 4-methylumbelliferyl α -D-mannoside. The resulting apparent IC_{50} values are listed in Table 4.1.

Table 4.1 Apparent IC_{50} values of inhibitors **1** - **7** towards α -mannosidase from GH38 jack bean.

Compound	IC_{50} (μM)
1 (TB440)	35.36 ± 6.7
2 (TB450)	7.19 ± 0.1
3 (TB481)	3.54 ± 0.5
4 (TB482)	3.43 ± 0.7
5 (TB521)	38.69 ± 4.5
6 (TB484)	2.22 ± 0.2
7 (TB480)	3.36 ± 1.3

The potency of α -mannosidase configured cyclophellitol **1** is in agreement with previous published data.²¹ As observed in Table 4.1, the cyclophellitol aziridine isomers are in general more potent compared to the cyclophellitol isomer. The introduction of a lipophilic tail increases in general the potency even more.

The next research object was to determine whether α -mannosidase configured cyclophellitols have an irreversible mode of binding – a prerequisite to allow their use in ABPP experiments. Therefore, Golgi α -mannosidase II from drosophila (GH38) was soaked with aziridine **2**. Indeed, the catalytic nucleophile (Asp 204¹³) opened the aziridine moiety and a covalent enzyme-**2** complex was formed. As depicted in Figure 4.3, the cyclohexane adopts a skew boat (¹S₅) as expected according to the proposed itinerary of this class of α -mannosidases.^{13,14}

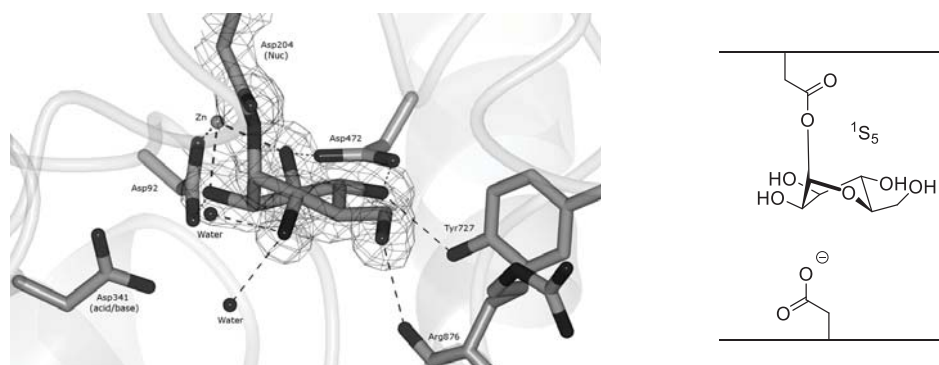


Figure 4.3 Crystal structure of Golgi α -mannosidase II from drosophila (GH38) in complex with aziridine **2** (left). Proposed conformation of the substrate in GH38 retaining exo- α -mannosidases (right).

Finally, ABPP was performed in commercial available GH38 jack bean α -mannosidase with compound **4** (Figure 4.4). The pH dependent labeling efficiency was investigated by treatment of **4** with the enzyme at various pH followed by analyzing the samples by SDS-PAGE and subsequently fluorescence scanning of the gels. Indeed, labeling of both subunits was observed (66 kDa and 44 kDa).²⁸ The pH dependent labeling corresponds with the pH dependent activity of jack bean α -mannosidase.²⁹

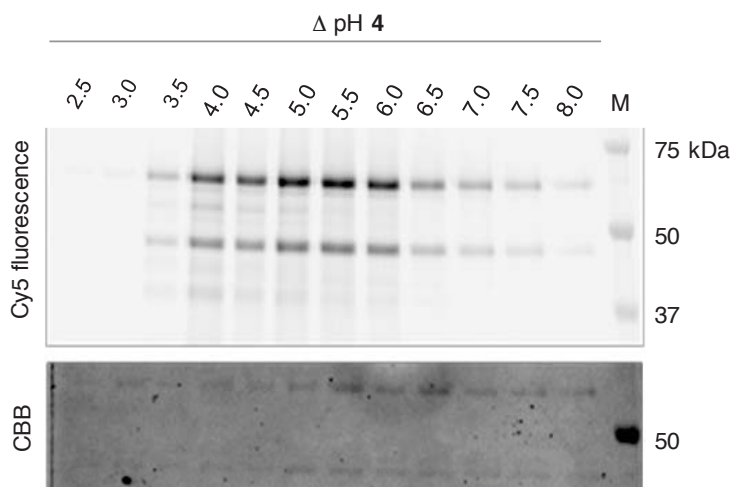


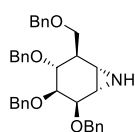
Figure 4.4 ABPP of GH38 jack bean α -mannosidase with 10 μ M compound **4** at various pH at 37 $^{\circ}$ C. After incubation for 2 h, the samples were denatured, resolved by SDS-PAGE and scanned for Cy5 fluorescence followed by Coomassie Brilliant Blue (CBB) staining.

4.3 Conclusion

In this chapter α -mannopyranose-configured cyclophellitol aziridine based inhibitors and probes (**4** – **7**) were synthesized in order to specifically target retaining exo- α -mannosidases. A fluorogenic substrate activity assay was performed to evaluate the inhibition potency of these probes, which revealed apparent IC_{50} values in the low micromolar range towards α -mannosidase from jack bean. Next, a covalent enzyme-aziridine **2** complex was obtained, supporting the irreversible mode of binding of this class of compounds in GH38 glycosidases. Finally, ABPP with **4** in jack bean α -mannosidase successfully labelled both subunits in a pH dependent fashion. These results set the stage for the synthesized probes (**4** – **7**) to perform additional ABPP experiments *in vitro* and *in vivo* and to use these ABPs as tools to further examine α -mannosidosis and other α -mannosidase connected diseases such as larynx cancer.

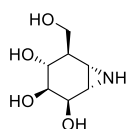
Experimental

General: Chemicals were purchased from Acros, Sigma Aldrich, Biosolve, VWR, Fluka, Merck and Fisher Scientific and used as received unless stated otherwise. Tetrahydrofuran (THF), *N,N*-dimethylformamide (DMF) and toluene were stored over flame-dried 4 Å molecular sieves 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 or a solution of KMnO₄ (20 g/L) and K₂CO₃ (10 g/L) in water, followed by charring at ~150 °C. Column chromatography was performed using Screening Device b.v. Silica Gel (particle size of 40 – 63 µm, pore diameter of 60 Å) in the indicated solvents. For reversed-phase HPLC purifications an Agilent Technologies 1200 series instrument equipped with a semi-preparative column (Gemini C18, 250 x 10 mm, 5 µm 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 µm 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) or a Brüker DMX-600 (600 and 151 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).



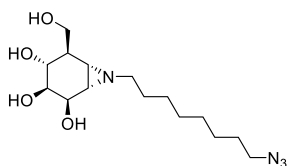
(1S,2R,3S,4R,5R,6S)-2,3,4-tris(benzyloxy)-5-((benzyloxy)methyl)-7-azabicyclo[4.1.0]heptane (10)

To a mixture of **8** and **9** (0.193 g, 0.332 mol) in MeCN (3.3 mL) was added PPh₃ polymer-bound on styrene-divinylbenzene copolymer (0.188 g, 0.564 mmol, 3 mmol/g). After stirring at reflux conditions overnight, the reaction mixture was concentrated *in vacuo*. Purification over silica gel column chromatography (1% MeOH in DCM) gave cyclophellitol aziridine **10** (95.6 mg, 0.178 mmol, 54%) as a yellow oil. ¹H NMR (400 MHz, CDCl₃) δ 7.42 – 7.17 (m, 20H), 4.93 (d, *J* = 12.3 Hz, 1H), 4.84 (d, *J* = 11.3 Hz, 1H), 4.76 – 4.57 (m, 3H), 4.55 – 4.34 (m, 3H), 4.21 (t, *J* = 2.2 Hz, 1H), 3.80 – 3.65 (m, 2H), 3.61 (dd, *J* = 9.1, 4.0 Hz, 1H), 3.50 (t, *J* = 8.7 Hz, 1H), 2.42 (dd, *J* = 5.8, 2.3 Hz, 1H), 2.33 (d, *J* = 5.8 Hz, 1H), 2.30 – 2.22 (m, 1H). ¹³C NMR (101 MHz, CDCl₃) δ 139.2, 139.0, 139.0, 138.5, 128.4, 128.4, 128.3, 128.1, 127.8, 127.7, 127.6, 127.5, 127.5, 80.8, 75.6, 75.2, 74.7, 73.3, 73.0, 73.0, 70.9, 43.4, 34.5, 31.8. HRMS: calculated for [C₃₅H₃₈NO₄]⁺ 536.27954, found 536.27923.



(1S,2R,3S,4R,5R,6S)-5-(hydroxymethyl)-7-azabicyclo[4.1.0]heptane-2,3,4-triol (2, TB450)

Liquid NH_3 (30 mL) was obtained by condensation of gas NH_3 at -60°C . Lithium (140 mg) was added and the mixture was stirred for 30 min, resulting in a dark blue solution. Aziridine **10** (149 mg, 278 mmol) was dissolved in THF (7 mL) and slowly added to the reaction mixture. After stirring for 75 min, the reaction was quenched with milliQ- H_2O (4 mL). The solution was allowed to come to room temperature followed by concentrating *in vacuo*. The crude mixture was taken up in milliQ- H_2O and neutralized with Amberlite IR H^+ . Product bound on the resin was washed with milliQ- H_2O followed by elution with NH_4OH (1M). The basic solution was concentrated *in vacuo* and dissolved again in milliQ- H_2O . Addition of Amberlite- NH_4^+ , followed by elution with milliQ- H_2O and concentration *in vacuo* gave cyclophellitol aziridine **2** as a clear oil which was used without further purification. ^1H NMR (400 MHz, D_2O) δ 4.34 (s, 2H), 3.89 (dd, J = 11.1, 3.4 Hz, 1H), 3.72 (dd, J = 10.9, 7.6 Hz, 1H), 3.51 – 3.42 (m, 2H), 2.50 (d, J = 5.5 Hz, 1H), 2.31 (d, J = 5.7 Hz, 1H), 1.87 (s, 1H). ^{13}C NMR (101 MHz, D_2O) δ 70.5, 68.3, 66.5, 62.0, 45.0, 35.2, 31.0.

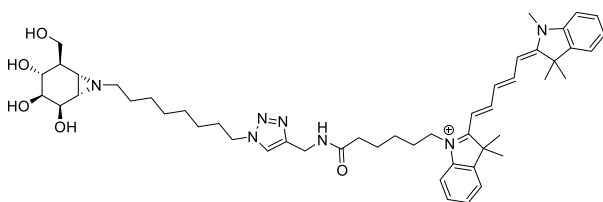


(1S,2R,3S,4R,5R,6S)-7-(8-azido-octyl)-5-(hydroxymethyl)-7-azabicyclo[4.1.0]heptane-2,3,4-triol (3, TB481)

Cyclophellitol aziridine **2** (0.2 mmol), 1-azido-8-iodooctane (see Chapter 3, 0.24 mmol, 67 mg) and K_2CO_3 (118 mg, 0.86 mmol) in DMF (2 mL) was stirred at 80°C . After stirring overnight the reaction mixture was concentrated *in vacuo* and purification over silica gel column chromatography (12% MeOH in DCM) gave azide-cyclophellitol aziridine **3** (25.2 mg, 76 μmol , 38% over two steps) as a clear oil. ^1H NMR (400 MHz, MeOD) δ 4.27 – 4.20 (m, 1H), 3.92 (dd, J = 10.6, 4.2 Hz, 1H), 3.62 (dd, J = 10.6, 8.4 Hz, 1H), 3.44 (dd, J = 10.2, 3.4 Hz, 4H), 3.38 (d, J = 9.2 Hz, 1H), 3.30 (t, J = 3.4 Hz, 2H), 2.38 – 2.23 (m, 2H), 1.89 – 1.81 (m, 2H), 1.73 (d, J = 6.1 Hz, 1H), 1.66 – 1.57 (m, 4H), 1.44 – 1.35 (m, 8H). ^{13}C NMR (101 MHz, MeOD) δ 72.8, 69.7, 68.2, 63.9, 61.8, 52.4, 47.0, 45.9, 41.7, 30.6, 30.2, 29.9, 28.3, 27.8. HRMS: calculated for $[\text{C}_{15}\text{H}_{29}\text{N}_4\text{O}_4]^+$ 329.21833, found 329.21822.

General click procedure

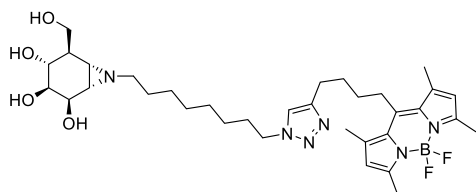
To a solution of aziridine-azide (1 equiv.) and desired alkyne (1 equiv.) in DMF (2.0 mL) were added copper(II)-sulfate pentahydrate (0.66 equiv., 1 M in H_2O) and sodium ascorbate (0.76 equiv., 1 M in H_2O). After stirring overnight at room temperature, the reaction mixture was concentrated *in vacuo*, purified with HPLC (linear gradient, A: 50 mM NH_4HCO_3 in H_2O , B: MeCN, in 12 min) and lyophilized.



ABP 4 (TB482)

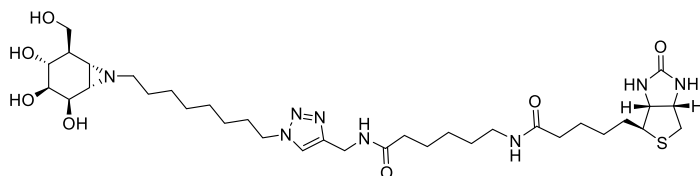
Aziridine-azide **3** (7.0 mg, 21 μmol) and Cy5-alkyne **11** (see Chapter 2, 11.7 mg, 21 μmol) were treated according to General Click Procedure. Purification by HPLC (44% $\rightarrow$ 50%, A

in B) followed by lyophilizing gave ABP **4** (6.44 mg, 7.3 μ mol, 35%) as a blue powder. ^1H NMR (500 MHz, MeOD) δ 8.29 – 8.21 (m, 2H), 7.84 (s, 1H), 7.50 (d, J = 7.2 Hz, 2H), 7.46 – 7.37 (m, 2H), 7.33 – 7.23 (m, 4H), 6.62 (s, J = 12 Hz, 1H), 6.28 (d, J = 13.6 Hz, 2H), 4.41 (s, 2H), 4.37 (t, J = 7.0 Hz, 2H), 4.22 – 4.16 (m, 1H), 4.09 (t, J = 7.4 Hz, 2H), 3.89 (dd, J = 10.6, 4.1 Hz, 1H), 3.63 (s, 3H), 3.59 (dd, J = 10.6, 8.4 Hz, 1H), 3.40 (dd, J = 10.2, 3.5 Hz, 1H), 3.35 (t, J = 5.0 Hz, 1H), 2.30 – 2.19 (m, 4H), 1.90 (s, 2H), 1.89 – 1.84 (m, 2H), 1.84 – 1.78 (m, 4H), 1.74 – 1.70 (m, 12H), 1.68 (d, J = 6.5 Hz, 1H), 1.55 – 1.49 (m, 2H), 1.49 – 1.43 (m, 2H), 1.36 – 1.26 (m, 8H). ^{13}C NMR (126 MHz, MeOD) δ 175.7, 175.4, 174.6, 155.6, 146.2, 144.3, 143.5, 142.6, 142.5, 129.8, 129.7, 126.6, 126.3, 126.2, 124.1, 123.4, 123.3, 112.0, 111.9, 104.4, 104.2, 72.8, 69.7, 68.2, 63.9, 61.8, 51.3, 50.6, 49.5, 49.3, 49.2, 49.0, 48.8, 48.7, 48.5, 47.0, 45.9, 44.8, 41.7, 36.5, 35.6, 31.5, 31.3, 30.4, 29.9, 28.2, 28.1, 28.0, 27.8, 27.4, 27.3, 26.4. LC/MS analysis: R_t 6.10 min (linear gradient 10 $\rightarrow$ 90% B in 12.5 min), m/z 848.33 $[\text{M}]^+$ HRMS: calculated for $[\text{C}_{50}\text{H}_{70}\text{N}_7\text{O}_5]^+$ 848.54329, found 848.54294.



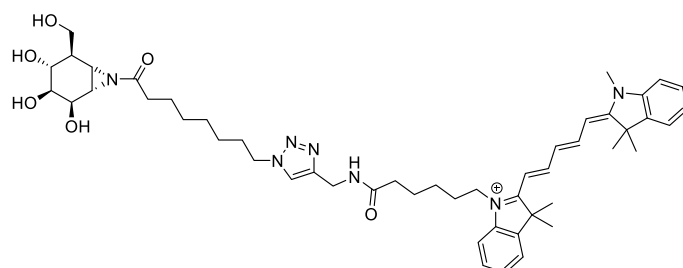
ABP 5 (TB582)

Azidirene-azide **3** (8.0 mg, 24 μ mol) and Bodipy **12**²⁵ (8.0 mg, 24 μ mol) were treated according to General Click Procedure. Purification by HPLC (42% $\rightarrow$ 48%, A in B) followed by lyophilizing gave ABP **5** (1.34 mg, 2.05 μ mol, 8.5%) as an orange powder. ^1H NMR (600 MHz, MeOD) δ 7.74 (s, 1H), 6.12 (s, 2H), 4.35 (t, J = 6.9 Hz, 2H), 4.20 (dd, J = 3.3, 1.9 Hz, 1H), 3.62 – 3.59 (m, 1H), 3.58 – 3.51 (m, 1H), 3.49 – 3.44 (m, 1H), 3.41 (dd, J = 10.2, 3.5 Hz, 1H), 3.07 – 3.00 (m, 2H), 2.79 (t, J = 7.2 Hz, 2H), 2.44 (s, 6H), 2.39 (s, 6H), 2.31 – 2.19 (m, 2H), 1.95 – 1.79 (m, 6H), 1.70 – 1.63 (m, 3H), 1.55 – 1.49 (m, 2H), 1.35 – 1.26 (m, 8H). LC/MS analysis: R_t 6.13 min (linear gradient 10 $\rightarrow$ 90% B in 12.5 min), m/z 657.00 $[\text{M} + \text{H}]^+$. HRMS: calculated for $[\text{C}_{34}\text{H}_{52}\text{BF}_2\text{N}_6\text{O}_4]^+$ 657.41117, found 657.41122.



ABP 6 (TB484)

Azidirene-azide **3** (7.2 mg, 22 μ mol) and biotin **13** (9.1 mg, 25 μ mol) were treated according to General Click Procedure. Purification by HPLC (18% $\rightarrow$ 24%, A in B) followed by lyophilizing gave ABP **6** (2.6 mg, 3.6 μ mol, 16%, purity > 80%) as a white powder. LC/MS analysis: R_t 3.91 min (linear gradient 10 $\rightarrow$ 90% B in 12.5 min), m/z 723.13 $[\text{M} + \text{H}]^+$. ^1H NMR (600 MHz, MeOD) δ 7.84 (s, 1H), 4.49 (dd, J = 7.8, 4.8 Hz, 1H), 4.42 (s, 2H), 4.38 (t, J = 7.0 Hz, 2H), 4.30 (dd, J = 7.8, 4.5 Hz, 1H), 4.20 (dd, J = 3.2, 1.9 Hz, 1H), 3.90 (dd, J = 10.7, 4.1 Hz, 1H), 3.70 – 3.66 (m, 1H), 3.58 – 3.55 (m, 3H), 3.53 (dd, J = 10.7, 6.3 Hz, 1H), 3.46 – 3.39 (m, 1H), 3.37 – 3.34 (m, 1H), 3.21 (dd, J = 8.8, 5.0 Hz, 1H), 3.16 (td, J = 6.9, 2.2 Hz, 2H), 2.92 (dd, J = 12.7, 5.0 Hz, 1H), 2.70 (d, J = 12.7 Hz, 1H), 2.33 – 2.28 (m, 1H), 2.25 – 2.17 (m, 5H), 1.89 (s, 2H), 1.86 – 1.81 (m, 2H), 1.71 – 1.69 (m, 1H), 1.66 – 1.55 (m, 4H), 1.53 – 1.46 (m, 2H), 1.46 – 1.40 (m, 2H), 1.39 – 1.26 (m, 9H). HRMS: calculated for $[\text{C}_{34}\text{H}_{59}\text{N}_8\text{O}_7\text{S}]^+$ 723.42219, found 723.42225.

**ABP 7 (TB480)**

Cy5-acid **14** (see Chapter 2, 63 mg, 85 μmol) was pre-activated with EEDQ (21 mg, 85 μmol) in DMF (2 mL) for 3 h at room temperature. Deprotected aziridine **2** (0.10 mmol) was dissolved in DMF (0.4 mL) and cooled to 0 $^{\circ}\text{C}$,

after which the activated ester solution (1.0 mL, 30 μmol , 0.5 equiv.) was added. After stirring at 0 $^{\circ}\text{C}$ for 30 min, another 0.5 equiv. of the activated ester was added. The reaction mixture was stirred for another 2 h at 0 $^{\circ}\text{C}$ before the reaction mixture was quenched with MeOH and concentration *in vacuo*. Purification by HPLC under neutral conditions with use of a gradient of 43 $\rightarrow$ 49% MeCN in H_2O in 12 min followed by lyophilizing yielded ABP 7 (7.0 mg, 7.8 μmol , 8% over 2 steps) as a blue powder. ^1H NMR (600 MHz, MeOD) δ 8.26 (t, J = 13.0 Hz, 2H), 7.82 (s, 1H), 7.51 (d, J = 7.4 Hz, 2H), 7.49 – 7.42 (m, 2H), 7.34 – 7.25 (m, 4H), 6.64 (t, J = 12.5 Hz, 1H), 6.30 (d, J = 13.7 Hz, 2H), 4.42 (s, 2H), 4.39 (t, J = 7.0 Hz, 2H), 4.31 – 4.27 (m, 1H), 4.11 (t, J = 7.5 Hz, 2H), 3.95 – 3.89 (m, 1H), 3.64 – 3.62 (m, 4H), 3.50 – 3.42 (m, 2H), 2.89 (dd, J = 5.6, 2.1 Hz, 1H), 2.78 (d, J = 5.4 Hz, 1H), 2.49 – 2.34 (m, 2H), 2.26 (t, J = 7.3 Hz, 2H), 2.07 – 1.94 (m, 3H), 1.90 – 1.87 (m, 2H), 1.85 – 1.82 (m, 2H), 1.74 (s, 12H), 1.65 – 1.58 (m, 2H), 1.50 – 1.45 (m, 2H), 1.36 – 1.29 (m, 6H). ^{13}C NMR (151 MHz, MeOD) δ 187.9, 180.2, 175.7, 175.4, 174.6, 155.5, 155.4, 146.1, 144.2, 143.5, 142.6, 142.5, 129.7, 129.7, 126.6, 126.3, 126.2, 124.1, 123.4, 123.2, 112.0, 111.8, 104.4, 104.2, 72.3, 69.1, 68.0, 63.3, 51.3, 50.5, 46.9, 44.7, 42.6, 39.0, 37.2, 36.4, 35.5, 31.5, 31.2, 29.9, 29.6, 28.1, 27.9, 27.8, 27.3, 27.2, 26.4, 25.9. LC/MS analysis: R_t 6.44 min (linear gradient 10 $\rightarrow$ 90% B in 12.5 min), m/z 862.33 $[\text{M} + \text{H}]^+$. HRMS: calculated for $[\text{C}_{50}\text{H}_{68}\text{N}_7\text{O}_6]^+$ 862.52256, found 862.52240.

Determination of the apparent IC_{50} values

For the determination of apparent IC_{50} values of the inhibitors, α -mannosidase from Jack bean, (*Canavalia ensiformis*, Sigma Aldrich) 4 ng total protein was equilibrated in 12.5 μL McIlvaine buffer (150 mM, pH 4.5) for 5 min on ice. After which it was incubated with 12.5 μL inhibitor dilutions prepared in McIlvaine buffer for 30 min at 37 $^{\circ}\text{C}$. After addition of 100 μL of 4-methylumbelliferyl- β -D-mannopyranoside substrate mixture (10 mM 4-mu- α -D-mannopyranoside in McIlvaine buffer) and incubation for 30 min at 37 $^{\circ}\text{C}$, the reaction was quenched by adding 200 μL 1M Glycine-NaOH (pH 10.3) to the reaction mixture. The 4-mu fluorescence was measured in 96-well plates using a LS55 fluorometer (PerkinElmer) at λ_{Ex} = 366 nm and λ_{Em} = 445 nm. Measured activity values were subtracted with background values, normalized against control values (no inhibitor), and plotted against the inhibitor concentrations. IC_{50} values were calculated with Prism 7.0 (Graphpad), using one-phase-exponential decay function. Standard deviations were obtained from 2 sets of calculated IC_{50} values, each derived from 3 technical repeats at each inhibitor concentrations.

Cloning and expression of dGMII

A plasmid containing cDNA encoding for the dGMII gene was obtained with kind permission from Dr. Sean Sweeny (University of York). From this plasmid, cDNA encoding for amino acids 76-1108 of dGMII (to remove the *N*-terminal cytosolic, transmembrane, and stalk domains) was subcloned into the pOMNIBac vector (Geneva Biotech), behind a honeybee mellitin secretion peptide, 6xHis tag, and TEV cleavage site.

Recombinant bacmid was produced using the Tn7 transposition method in DH10EMBacY (Geneva Biotech)³⁰, and purified using the PureLink miniprep kit (Invitrogen) following standard protocols. V1 baculovirus was produced by transfection of bacmid into low passage adherent Sf21 cells (Invitrogen) using FuGENE HD transfection reagent (ProMega), at a ratio of 2 μ g DNA to 4.5 μ L FuGENE. V1 $\rightarrow$ V2 virus amplification was carried out using suspension Sf21 cells, using the YFP marker present in EMBacY baculovirus to determine optimum amplification prior to harvesting (typically ~60% cells fluorescent). For expression, *T. Ni* cells (Invitrogen) were infected with V2 baculovirus at a MOI>1, and infection followed using the EMBacY YFP marker to determine optimum timepoint for harvesting (typically 72 h, with > 80 % cells fluorescent). All insect cells used tested negative for mycoplasma contamination.

Purification of dGMII

Harvested High Five cultures were spun for 15 minutes at 200 g to remove cells and spun again at 4000 g for 1 hour to remove insoluble cellular components. AEBSF (to a final concentration of 0.1 mM) and DDT (to a final concentration of 1 mM) were added to the supernatant. Clarified supernatant was loaded on to 2 x 5mL HiTrap Blue HP columns (GE Healthcare) pre-equilibrated in blue agarose buffer A (20 mM HEPES pH 7.4, 100 mM NaCl, 1 mM DTT). Loaded columns were washed with 5 column volumes of blue agarose buffer A and eluted using a linear gradient of blue agarose buffer B (20 mM HEPES pH 7.4, 2 M NaCl, 1 mM DTT) over 20 column volumes.

HiTrap Blue fractions containing dGMII (as determined by SDS-PAGE) were pooled and diluted approximately 5-fold in HisTrap buffer A (50 mM HEPES pH 8.0, 0.5 M NaCl, 30 mM Imidazole, 1 mM DTT) and loaded on to a 1 mL HisTrap FF Crude column (GE Healthcare). The loaded histrap column was washed with 10 column volumes of HisTrap buffer A and dGMII eluted using a linear gradient of HisTrap buffer B (50 mM HEPES pH 8.0, 0.5 M NaCl, 0.5 M imidazole, 1 mM DTT) over 20 column volumes, followed by 10 column volumes of 100 % Buffer B.

HisTrap fractions containing dGMII were pooled and concentrated to less than 2 ml by centrifugation using VivaSpin 30,000 MW concentrator (GE Healthcare). Pooled dGMII was rediluted to ~2 mL in 1x AcTEV (Invitrogen) reaction buffer before addition of 5 μ L AcTEV protease to remove the N-His-TEV tag. Digests were typically carried out overnight at ambient temperature, and reaction progress assessed by comparison to positive and negative controls using SDS-PAGE.

Upon completion of the AcTEV digest, as indicated by SDS-PAGE, dGMII was purified using a S200 16/600 size exclusion chromatography (SEC) column equilibrated in SEC buffer (50 mM HEPES pH 7.4, 200 mM NaCl, 1 mM DTT), at 1 ml min⁻¹. SEC fractions containing dGMII were pooled, concentrated to 10 mg ml⁻¹ and stored in 20 μ L aliquots at -80 °C.

3D Crystallography of dGMII

Initial crystallization conditions were screened using JCSG+ HT-96, PACT *premier* HT-96 (both Molecular Dimensions), Index HT and PEG/Ion HT (both Hampton Research) commercial screens. Hits were optimized, scaled up to maxi 48-well plates and a dGMII seed stock generated from crystals grown in 0.1 M imidazole pH 7.0 and 10 % (w/v) PEG 3350 using the Seed Bead protocol (Hampton Research). The above screens were repeated with seeding using an Oryx8 (Douglas Instruments) and additional hits optimised further. Diffraction quality dGMII crystals were grown in maxi 48-well plates using sitting drop vapour diffusion in 0.1 M sodium succinate pH 7.0 and 10 % (w/v) PEG 3350. Crystals were cryoprotected using cryoprotectant solution (mother liquor supplemented with 25% v/v ethylene glycol) prior to flash freezing in liquid N₂ for data collection.

Datasets were collected using beamline I04-1 at the Diamond Light Source, processed via with the xia2 pipeline³¹ of the CCP4 software suite, using XDS³² and Aimless³³ for data processing. Apo dGMII was solved by molecular replacement with PDB model 1HWW³⁴ using MolRep³⁵, followed by alternating rounds of manual model building and refinement using Coot and REFMAC5 respectively^{36,37}. CCP4mg was used to produce figures of the crystal structure.

For ligand complexes, dGMII crystals were soaked in solutions of TB450 (1 mM) or TB482 (1 mM) in dGMII cryoprotectant solution for ~3 hours before flash freezing in liquid N₂ for data collection. Complexes were solved by molecular replacement with the apo dGMII structure, followed by rounds of manual model building and refinement using Coot and REFMAC5. Ligand coordinates were built using jLigand.³⁸ Diagrams of dGMII-ligand complexes were generated using CCP4mg³⁹.

Labeling of jack bean α -mannosidase with TB482 (4) at varying pH

Jack bean α -mannosidase (Sigma Aldrich, 1 pmol) was equilibrated in 10 μ L McIlvaine buffer at varying pH (2.5 – 8.0) at 37 °C for 15 min. After incubation with 10 μ M TB482 (4, end concentration; prepared in McIlvaine buffer at pH 2.5 – 8.0) at 37 °C for 2 h, the sample was denatured, resolved by SDS-PAGE and scanned for Cy5 fluorescence with a Typhoon FLA 9500 imager (GE Healthcare).

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5

Activity-based protein profiling of lysosomal GH2 exo- β -mannosidase¹

5.1 Introduction

GH2 exo- β -mannosidase is the lysosomal exoglycosidase responsible for the cleavage of the single β -mannoside linkage in the lysosomal degradation pathway of *N*-linked glycoproteins.² Specifically, this glycosidase (EC 3.2.1.25), found in eukaryotes³ and bacteria⁴, hydrolyses the β -glycosidic linkage of the Man(β 1 $\rightarrow$ 4)GlcNAc moiety. Therefore, a deficiency of this enzyme (MANBA gene) results in accumulation of Man(β 1 $\rightarrow$ 4)GlcNAc glycans including sialylated versions thereof in various tissues. This causes β -mannosidosis, a lysosomal storage disorder, which is observed in mammals and a few examples in humans have been described.⁵⁻⁷

GH2 α -mannosidases are retaining glycosidases that catalyse the hydrolysis of their substrate via a double-displacement mechanism (Figure 1A). As depicted in Figure 1B, the proposed mechanism for α -mannosidases proceeds via the ${}^1S_5 \rightarrow B_{2,5} \rightarrow {}^0S_2$ catalytic itinerary. First, the conformation of the carbohydrate substrate changes into a ${}^1S_5$ skew boat and forms a Michaelis complex with the enzyme.⁸ The aglycon is protonated by the acid/base catalyst and subsequently expelled. The emerging

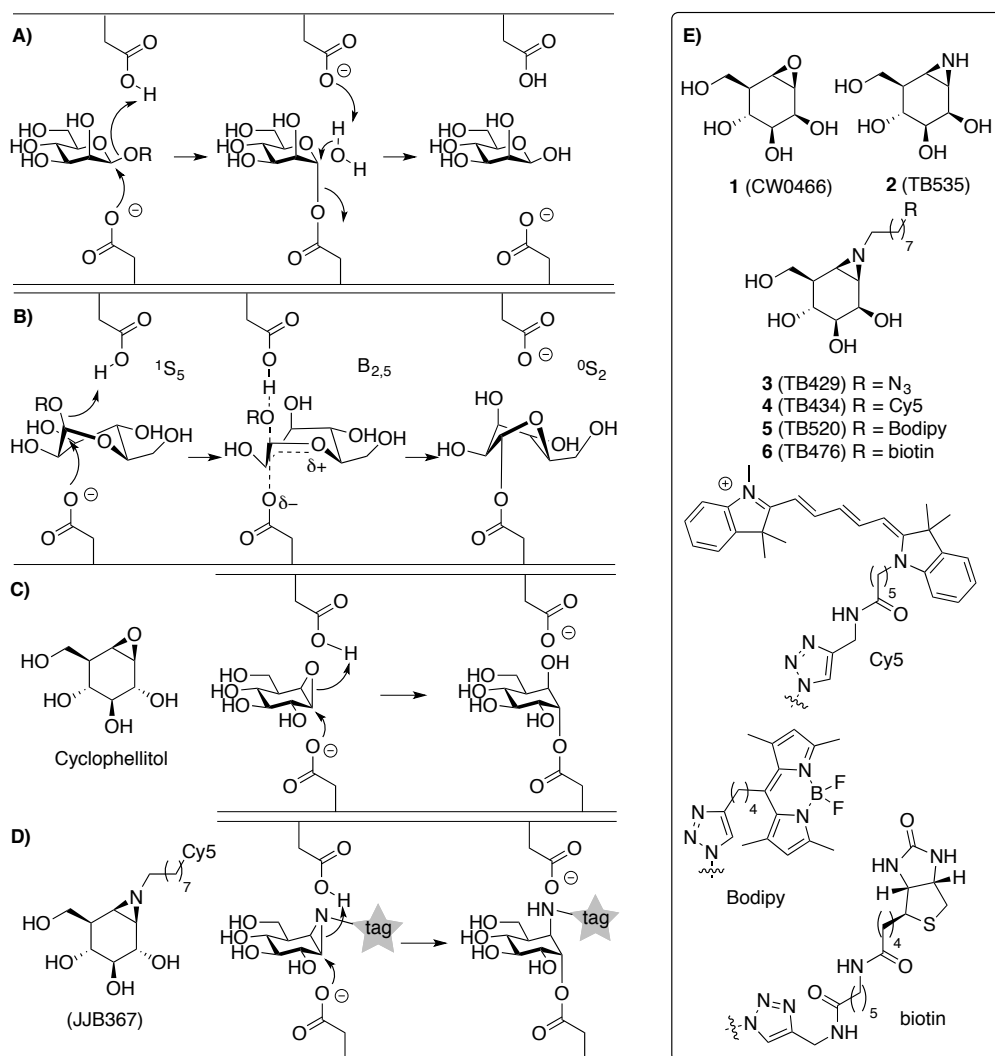


Figure 5.1 A) General retaining mechanism of α -mannosidase. B) The proposed conformations of the catalytic itinerary of α -mannosidase.^{9,22,23} Structure of cyclophellitol (C) and cyclophellitol aziridine (D) as mechanism-based β -glucosidase inhibitors. E) Structures of β -mannopyranose-configured cyclophellitol and β -mannopyranose-configured cyclophellitol aziridine derivatives used in this study.

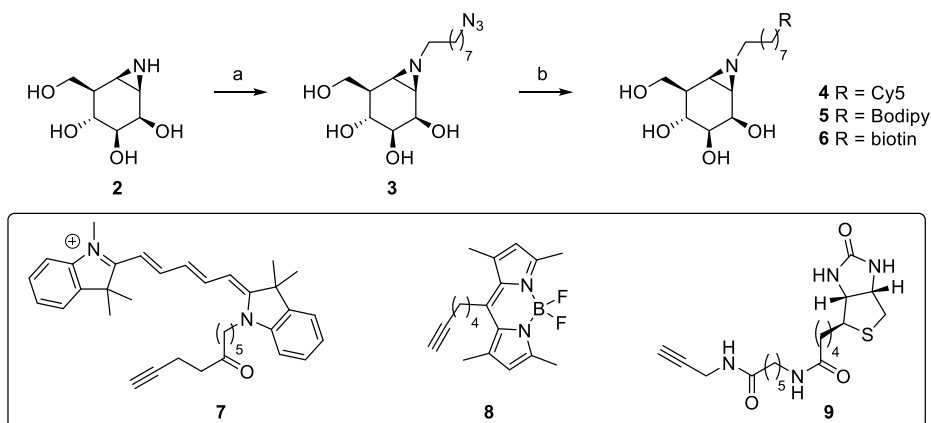
oxycarbenium ion reacts with the catalytic nucleophile (Glu-519⁹) to form a covalent enzyme- β -mannopyranosyl adduct. Upon deprotonation of a water molecule and substitution of the anomeric carboxylate, the mannose is released from the enzyme.

Based on this catalytic mechanism, cyclophellitol based β -mannosidase inhibitors can be designed. Cyclophellitol, isolated from mushroom *Phellinus* sp., proved to be a potent¹⁰, selective¹¹ covalent inhibitor of almond β -glucosidase^{12,13}, and in later studies to be an inhibitor of retaining β -glucosidases in general (Figure 1C).¹⁴ Cyclophellitol aziridine derivatives inhibit retaining β -glucosidases¹⁵ as well and can be modified to become viable β -glucosidase activity-based probes (ABPs) by functionalizing the aziridine moiety (Figure 1D).¹⁶ Various applications including selective fluorescent labeling and pull-down experiments have been performed to detect and monitor the specific enzymes of interest *in vitro*, *in situ* and *in vivo*.^{17,18} Based on these promising results configurational cyclophellitol aziridine analogues have been developed for ABP studies on several retaining glycosidase families.¹⁹⁻²¹ Interestingly, the configuration of the cyclophellitol warhead determines which glycosidase is targeted. Through obtaining β -mannopyranose-configured cyclophellitol based ABPs, labeling and detecting of β -mannosidases would come within reach and would allow to investigate and monitor β -mannosidase activity *in vitro*, *in situ* and *in vivo*. In this chapter a straightforward synthesis of β -mannopyranose-configured cyclophellitol aziridine ABPs (Figure 1E) and the biological validation of these probes in various biological tissues is presented.

5.2 Results and discussion

β -Mannopyranose-configured cyclophellitol aziridine **2** was obtained according to the efficient 13 steps synthesis described previously.²⁴ *N*-alkylation with 1-azido-8-iodooctane (see Chapter 3), resulted in chemical ligation handle **3** (Scheme 5.1). Azide-alkyne Huisgen cycloaddition with fluorophore **7** (see Chapter 2), **8**²⁵, or biotin **9**²⁶, resulted in the corresponding ABPs **4** – **6**.

The configuration of cyclophellitol based inhibitors confers glycosidase selectivity in most examples studied.^{17,19-21} In order to determine the selectivity of the obtained compounds, biotin probe **6** was subjected to a mouse kidney homogenate. The pull-down experiment was performed with streptavidin coated paramagnetic beads. The obtained proteins were subjected to trypsin digestion and the peptide fragments were analysed by LC-MS/MS and compared with the UniProt database by employing the Mascot search engine.²⁰ In addition, control samples were generated by performing this

Scheme 5.1 Synthesis of β -mannopyranose-configured cyclophellitol aziridine inhibitors and ABPs.

Reagents and conditions: a) 1-azido-8-iodooctane (see Chapter 3), K_2CO_3 , DMF, 80 °C, overnight, 64%; b) **7** (see Chapter 2), **8**²⁵ or **9**²⁶, $Cu.SO_4 \cdot 5H_2O$, sodium ascorbate, **4** 17%, **5** 36% and **6** 32%.

protocol in lysate which was pre-incubated with ABP **4** prior to subjecting ABP **6** to the lysate or performing this protocol with DMSO only. After comparing with these control samples, 45 unique proteins were identified (See experimental Table S1). Of these proteins, MANBA, the exo- β -mannosidase present in mammals was the single glycosidase identified.

After having identified MANBA as the specific target of biotin probe **6** in mouse kidney homogenate, the potency of all compounds (**1** – **6**) was determined as inhibitors of the retaining β -mannosidase obtained from snail (*Helix pomatia*), which is commercially available. The inhibition potential was assessed at pH 4.2, according to literature procedure.²⁷ The inhibitor was pre-incubated for 30 min with varying concentrations after which the residual enzymatic activity was measured using the fluorogenic substrate 4-methylumbelliferyl β -D-mannoside (4- μ β -man). However, up to 50 μ M no inhibition was detected. The activity of glycosidases is pH dependent and therefore the potency of Cy5 tagged probe **4** was investigated at various pH by treatment of the enzyme at various pH followed by SDS-PAGE and fluorescence scanning of the gels (Figure 5.2A). The efficiency of MANBA labeling appeared maximal at pH 5.5 whereas it is negligible at pH 4.2. As a control, the activity of the enzyme at various pH was determined with the 4- μ β -man substrate and compared with the quantification of the fluorescence band in Figure 5.2A (Figure 5.2B). Although the enzyme activity pattern does not correspond with the inhibition potency pattern of compound **4** under strong acidic conditions, at pH 5.5 the enzyme is active and compound **4** is a potent inhibitor. Therefore, the apparent IC_{50} values of compounds **1** – **6** were reassessed at

pH 5.5 (Figure 5.2C). In this setting, *N*-alkylation of the cyclophellitol aziridine turned out to be low micromolair active probes (**3** – **6**). Finally, the labeling of MANBA with compound **4** at the optimal pH with various probe concentrations is depicted in Figure 5.2D.

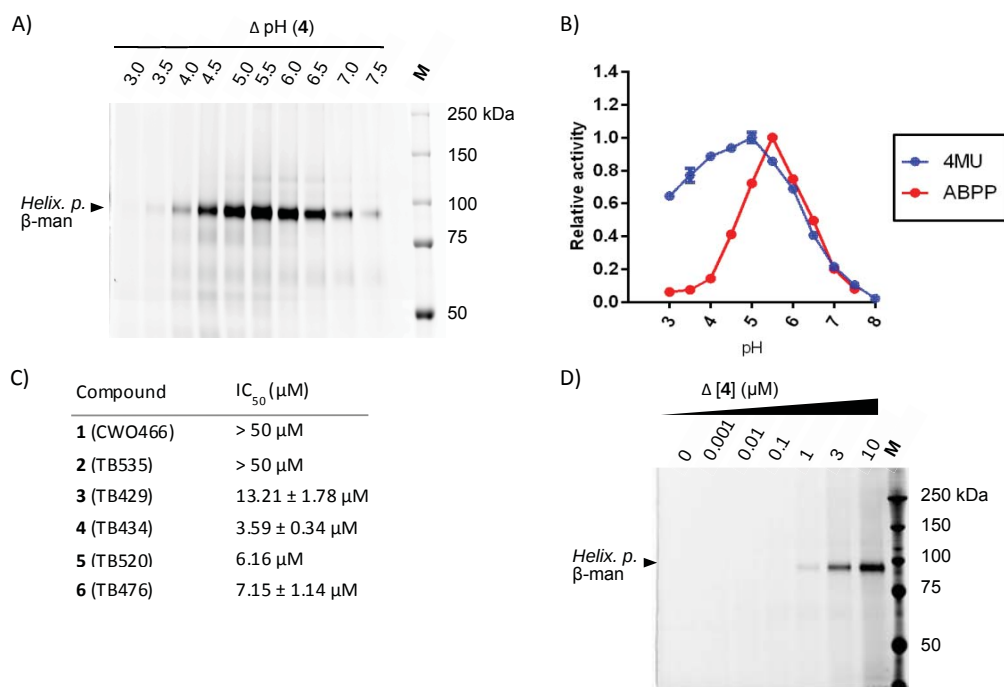


Figure 5.2 A) ABPP of β -mannosidases from *Helix p.* with 10 μ M compound **4** at various pH. After incubation for 1 h, the samples were resolved by SDS-PAGE and scanned for Cy5 fluorescence. B) Quantification of the fluorescent read-out (A) compared with the hydrolysis of the fluorogenic substrate 4-methylumbelliferyl β -D-mannoside. C) Apparent IC_{50} values towards β -mannosidase from snail (*Helix p.*) of compounds **1** – **6** at pH 5.5. D) Labeling of β -mannosidases in *Helix p.* at various concentrations of compound **4** after incubation of 1 h at 37 °C and pH 5.5.

The next pursued aim was activity-based protein profiling in lysates of kidney from mice with ABP **4**. To this end, probe concentration dependent labeling was examined and from 1 μ M of probe, labeling of MANBA (100.8 kDa) was observed (Figure 5.3A). Next, the pH dependent activity was examined, Figure 5.3B. Indeed, a similar pH tendency as towards β -mannosidases from *Helix p.* was obtained. Finally, the mechanism-based nature of the observed labeling was confirmed by an incubation time dependent experiment (Figure 5.3C). Labeling of MANBA is more intense after extended incubation time. This result indicates that ABP **4** binds in a covalent fashion to MANBA.

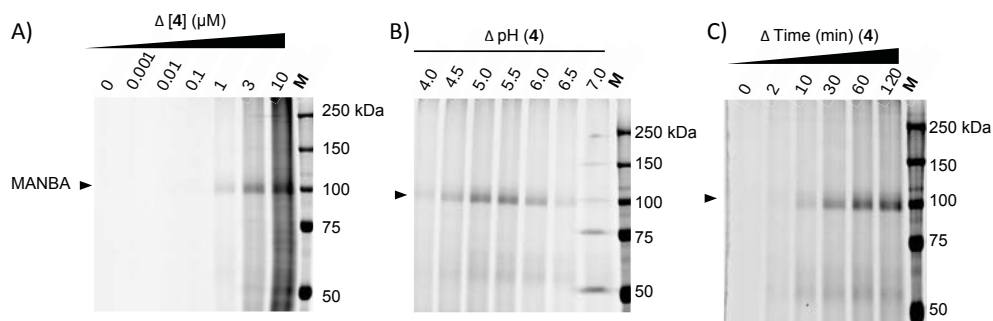


Figure 5.3 Activity-based protein profiling with ABP **4** in mouse kidney homogenates. A) Concentration dependent labeling up to 10 μM probe for 1 h at pH 5.5. B) pH dependent labeling from pH 4.0 – 7.0 with 1 μM probe for 1 h. C) Incubation time dependent labeling at pH 5.5 with 1 μM probe. Gels are 7.5% SDS-PAGE and scanned for Cy5 fluorescence.

5.3 Conclusion

In this chapter various β -mannopyranose-configured cyclophellitol aziridine based probes were synthesized in order to specifically target β -mannosidases. Biotin probe **6** successfully identified MANBA, a retaining exo- β -mannosidase, as the specific target. The pH optimum activity and labeling efficiency with ABP **4** was determined to be at pH 5.5 for β -mannosidase from *Helix p.* At this condition, ABPs **3** – **6** proved to be low micromolar inhibitors. Finally, *in vitro* activity-based protein profiling was performed with ABP **4** in homogenized mouse kidney resulting in effective labeling of MANBA in pH-dependent and incubation time dependent fashion. These ABPs can be employed for further ABPP and used as tools to examine β -mannosidosis.

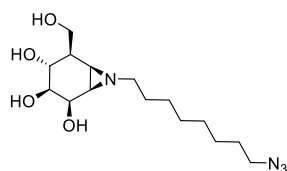
Experimental

General: Chemicals were purchased from Acros, Sigma Aldrich, Biosolve, VWR, Fluka, Merck and Fisher Scientific and used as received unless stated otherwise. *N,N*-dimethylformamide (DMF) and toluene were stored over flame-dried 4 Å molecular sieves 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. 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 $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}\cdot 4\text{H}_2\text{O}$ (25 g/L) and $(\text{NH}_4)_4\text{Ce}(\text{SO}_4)_4\cdot 2\text{H}_2\text{O}$ (10 g/L) in 10% sulfuric acid or a solution of KMnO_4 (20 g/L) and K_2CO_3 (10 g/L) in water, followed by charring at $\sim 150^\circ\text{C}$. Column chromatography was performed using Screening Device b.v. Silica Gel (particle size of 40 – 63 μm , pore diameter of 60 Å) in the indicated solvents. For reversed-phase HPLC purifications an Agilent Technologies 1200 series instrument equipped with a semi-preparative column (Gemini C18, 250 x 10

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 Bruker AV-400 (400 and 101 MHz respectively), a Bruker DMX-600 (600 and 151 MHz respectively) or a Bruker AV-850 (850 and 214 MHz respectively) spectrometer in the given solvent. Chemical shifts are given in ppm (δ) relative to the residual solvent peak of methanol-*d*. 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).

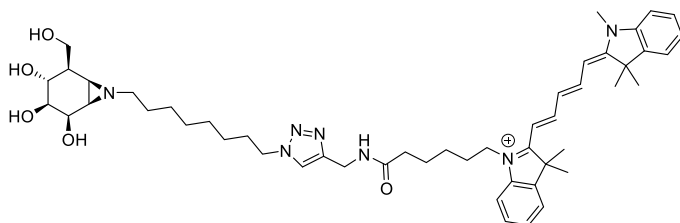
General click procedure

To a solution of aziridine-azide (1 equiv.) and desired alkyne (1 equiv.) in DMF (2.0 mL) were added copper(II)-sulfate pentahydrate (0.66 equiv., 1 M in H₂O) and sodium ascorbate (0.76 equiv., 1 M in H₂O). After stirring overnight at room temperature, the reaction mixture was concentrated *in vacuo*, purified with HPLC (linear gradient, A: 50 mM NH₄HCO₃ in H₂O, B: MeCN, in 12 min) and lyophilized.



(1R,2R,3S,4R,5R,6R)-7-(8-azido-octyl)-5-(hydroxymethyl)-7-azabicyclo[4.1.0]heptane-2,3,4-triol (3, TB429)

Cyclophellitol aziridine **2**²⁴ (0.4 mmol), 1-azido-8-iodooctane (see Chapter 3, 0.6 mmol, 168 mg) and K₂CO₃ (237 mg, 1.72 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 (6% MeOH in DCM $\rightarrow$ 8% MeOH in DCM) gave **3** (32.1 mg, 98 μ mol, 64%) as a clear oil. ¹H NMR (400 MHz, MeOD) δ 4.14 (t, $J = 3.8$ Hz, 1H), 3.82 (dd, $J = 10.2, 6.0$ Hz, 1H), 3.70 (dd, $J = 8.3, 11.7$ Hz, 1H), 3.51 (dd, $J = 6.7, 4.7$ Hz, 1H), 3.45 (dd, $J = 6.6, 4.1$ Hz, 1H), 3.28 (t, $J = 6.8$ Hz, 2H), 2.42 – 2.33 (m, 1H), 2.23 – 2.13 (m, 1H), 2.10 – 2.05 (m, 2H), 2.05 – 1.96 (m, 1H), 1.58 (t, $J = 6.9$ Hz, 4H), 1.44 – 1.34 (m, 8H). ¹³C NMR (101 MHz, MeOD) δ 103.8, 98.3, 93.8, 92.6, 89.6, 80.6, 73.0, 72.3, 71.3, 58.6, 58.4, 58.1, 56.5, 55.9. HRMS: calculated for [C₁₅H₂₉N₄O₄]⁺ 329.21833, found 329.21823.

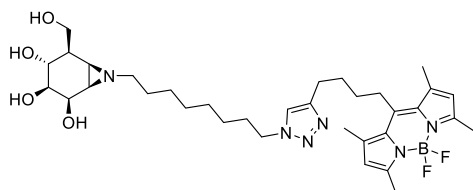


ABP 4 (TB434)

Aziridine-azide **3** (13.1 mg, 39.8 μ mol) and Cy5-alkyne **7** (see Chapter 2, 20 mg, 36 μ mol) were treated according to General Click Procedure. Purification by HPLC (40% $\rightarrow$

60%, A in B) followed by lyophilizing gave ABP **4** (5.3 mg, 6.0 μ mol, 17%) as a blue powder. ¹H NMR

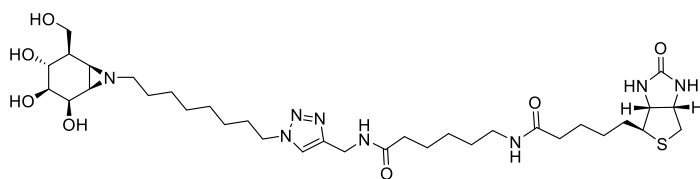
(850 MHz, MeOD) δ 8.25 (dt, J = 3.6, 2.0 Hz, 2H), 7.85 (s, 1H), 7.49 (d, J = 7.4 Hz, 2H), 7.44 – 7.39 (m, 2H), 7.32 – 7.24 (m, 4H), 6.62 (t, J = 12.4 Hz, 1H), 6.29 (d, 2.2 Hz, 1H), 6.27 (d, 2.2 Hz, 1H), 4.41 (s, 2H), 4.37 (t, J = 7.1 Hz, 2H), 4.14 (t, J = 4.1 Hz, 1H), 4.10 (d, J = 7.5 Hz, 1H), 4.09 (d, J = 7.9 Hz, 1H), 3.81 (dd, J = 10.2, 6.0 Hz, 1H), 3.68 (dd, J = 8.0, 9.1 Hz, 1H), 3.63 (s, 3H), 3.50 (dd, J = 6.5, 4.7 Hz, 1H), 3.45 (dd, J = 6.6, 4.2 Hz, 1H), 2.37 – 2.32 (m, 1H), 2.25 (t, J = 7.3 Hz, 2H), 2.18 – 2.13 (m, 1H), 2.08 – 2.02 (m, 2H), 2.01 – 1.98 (m, 1H), 1.87 – 1.85 (m, 2H), 1.82 – 1.79 (m, 2H), 1.79 – 1.70 (m, 14H), 1.58 – 1.52 (m, 2H), 1.49 – 1.42 (m, 2H), 1.35 – 1.28 (m, 8H). ^{13}C NMR (214 MHz, MeOD) δ 175.7, 175.4, 174.6, 155.6, 155.5, 144.2, 143.5, 142.6, 142.5, 129.8, 129.7, 126.6, 126.3, 126.2, 123.4, 123.2, 112.0, 111.9, 104.4, 104.2, 75.6, 70.1, 65.7, 64.4, 61.4, 51.3, 44.9, 44.8, 44.2, 43.2, 36.5, 35.6, 31.5, 31.3, 30.3, 30.3, 29.9, 28.2, 28.1, 27.9, 27.8, 27.4, 27.3, 26.4. LC/MS analysis: R_t 6.18 min (linear gradient 10 $\rightarrow$ 90% B in 12.5 min), m/z 848.27 $[\text{M}]^+$. HRMS: calculated for $[\text{C}_{50}\text{H}_{70}\text{N}_7\text{O}_5]^+$ 848.54329, found 848.54278.



ABP 5 (TB520)

Aziridine-azide **3** (7.1 mg, 22 μmol) and Bodipy **8**²⁵ (7.1 mg, 22 μmol) were treated according to General Click Procedure. Purification by HPLC (42% $\rightarrow$ 48%, A in B) followed by lyophilizing gave ABP **5** (5.13 mg, 7.82 μmol , 36%) as an orange

powder. ^1H NMR (600 MHz, MeOD) δ 7.74 (s, 1H), 6.11 (s, 2H), 4.35 (d, J = 7.2 Hz, 1H), 4.34 (d, J = 6.6 Hz, 1H), 4.13 (t, J = 4.1 Hz, 1H), 3.81 (dd, J = 10.2, 6.0 Hz, 1H), 3.68 (dd, J = 10.2, 8.4 Hz, 1H), 3.50 (dd, J = 6.7, 4.7 Hz, 1H), 3.47 – 3.42 (m, 1H), 3.35 (s, 3H), 3.32 – 3.28 (m, 2H), 3.04 – 2.97 (m, 2H), 2.78 (d, J = 7.2 Hz, 1H), 2.77 (d, J = 7.2 Hz, 1H), 2.44 (s, 6H), 2.38 (s, 6H), 2.36 – 2.30 (m, 1H), 2.14 (dt, J = 11.7, 7.1 Hz, 1H), 2.07 – 1.97 (m, 3H), 1.93 – 1.83 (m, 4H), 1.68 – 1.61 (m, 2H), 1.57 – 1.50 (m, 2H), 1.36 – 1.24 (m, 8H). ^{13}C NMR (151 MHz, MeOD) δ 154.9, 148.5, 147.9, 142.2, 132.6, 123.4, 122.6, 75.6, 70.1, 65.7, 64.4, 61.4, 51.9, 49.9, 44.9, 44.1, 43.2, 32.2, 31.2, 30.8, 30.3, 30.3, 29.9, 29.1, 28.2, 27.3, 25.9, 16.5, 14.4. LC/MS analysis: R_t 6.15 min (linear gradient 10 $\rightarrow$ 90% B in 12.5 min), m/z 657.07 $[\text{M} + \text{H}]^+$. HRMS: calculated for $[\text{C}_{34}\text{H}_{52}\text{BF}_2\text{N}_6\text{O}_4]^+$ 657.41117, found 657.41122.



ABP 6 (TB476)

Aziridine-azide **3** (9.1 mg, 28 μmol) and biotin **9**²⁶ (11.5 mg, 28 μmol) were treated according to General Click Procedure. Purification by

HPLC (18% $\rightarrow$ 24%, A in B) followed by lyophilizing gave ABP **6** (6.5 mg, 8.9 μmol , 32%) as a white powder. ^1H NMR (500 MHz, MeOD) δ 7.84 (s, 1H), 4.49 (dd, J = 7.8, 4.9 Hz, 1H), 4.42 (s, 1H), 4.38 (d, J = 7.6 Hz, 1H), 4.36 (d, J = 7.6 Hz, 1H), 4.30 (dd, J = 7.9, 4.4 Hz, 1H), 4.14 (t, J = 3.9 Hz, 1H), 3.81 (dd, J = 10.2, 5.9 Hz, 1H), 3.69 (dd, J = 10.1, 8.8 Hz, 1H), 3.54 – 3.43 (m, 2H), 3.24 – 3.18 (m, 1H), 3.17 – 1.12 (m, 2H), 2.92 (dd, J = 12.7, 5.0 Hz, 1H), 2.70 (d, J = 12.7 Hz, 1H), 2.39 – 2.32 (m, 1H), 2.26 – 2.13 (m, 5H), 2.10 – 2.03 (m, 2H), 2.03 – 1.96 (m, 1H), 1.90 (s, 5H), 1.79 – 1.70 (m, 1H), 1.65 – 1.53 (m, 6H), 1.52 – 1.48 (m, 2H), 1.43 – 1.39 (m, 2H), 1.36 – 1.25 (m, 9H). ^{13}C NMR (126 MHz, MeOD) δ

176.0, 175.9, 146.2, 124.1, 75.6, 70.1, 65.7, 64.4, 63.4, 61.6, 61.4, 57.0, 51.3, 44.9, 44.1, 43.2, 41.0, 40.2, 36.8, 36.7, 35.6, 31.2, 30.3, 30.3, 30.1, 29.9, 29.8, 29.5, 28.2, 27.5, 27.3, 26.9, 26.5. LC/MS analysis: R_t 3.98 min (linear gradient 10 $\rightarrow$ 90% B in 12.5 min), m/z 723.20 $[M + H]^+$. HRMS: calculated for $[C_{34}H_{59}N_8O_7S]^+$ 723.42219, found 723.42220.

Pull-down and LC/MS analysis with ABP 6 (TB476)

4.0 mg total protein from mouse kidney homogenates were incubated with either DMSO, 10 μ M biotin probe **6** (TB476), or 5 μ M Cy5 probe **4** (TB434) followed by 10 μ M **6**, each step was taking 1 h at 37 °C in a total volume of 500 μ L McIlvaine buffer pH 5.5 (750 mM citric acid/ Na_2HPO_4). Samples were subsequently denatured with the addition of 125 μ L 10% (w/v) SDS and boiling for 5 min at 100 °C. From here on, samples were prepared for pull-down with streptavidin beads as published earlier.²⁸ After the pull-down procedure, half of the samples were treated by the trypsin digestion buffer (100 mM Tris-HCl pH 7.8, 100 mM NaCl, 1 mM $CaCl_2$, 2% (v/v) acetonitrile (ACN) and 10 ng/ μ L trypsin) and the bead suspension was incubated in a thermoshaker at 37 °C overnight. The supernatant containing the trypsin-digested peptides was desalted using stage tips, followed by evaporation and re-dissolving in 75 μ L sample solution (H_2O /ACN/formic acid, 95/3/0.1, v/v/v). The beads containing active-site peptides were eluted with elution buffer (5% (v/v) formic acid, 25% (v/v) ACN, 70% (v/v) H_2O , 10 μ M biotin) for 30 min at 37 °C, after which the supernatant was desalted using stage tips and prepared for LC/MS analysis using the above mentioned procedures.

For the LC/MS analysis, 1 μ L of sample was injected with phase A (0.1 % (v/v) formic acid in ultrapure H_2O) on a C18 column (Acquity UPLC M-Class 300 μ m x 50 mm, packed with BEH C18 material of 1.7 μ m diameter and 300 Å pore size particles), eluted with a 50 min gradient of 10% to 60% phase B (0.1 % (v/v) formic acid in ACN), followed by 10 min equilibration to 1% phase B at a flow of 0.4 μ L/min, hyphenated with Electro-spray ionization (ESI) via Nano-spray source with ESI emitters (New Objectives) fused silica tubing 360 μ m OD x 25 μ m ID tapered to 5 ± 0.5 μ m (5 nl/cm void volume) to a Synapt G2Si mass spectrometer (Waters) operating with Masslynx for acquisition and Ent3 software for polymer envelope signal deconvolution. The following settings in positive resolution mode were used: source temperature of 80 °C, capillary voltage 4.5 kV, nano flow gas of 0.25 Bar, purge gas 250 L/h, trap gas flow 2.0 ml/min, cone gas 100 L/h, sampling cone 25V, source offset 25, trap CE 32V, scan time 3.0 sec, mass range 400-2400 m/z . Lock mass acquiring was done with a mixture of Leu Enk (556.2771) and Glu Fib (785.84265), lockspray voltage 3.5 kV, Glufib fragmentation was used as calibrant. The PLGS (Waters) program was used for data analysis, protein ID or extraction of mgf files for further Mascot (Matrix Science) search analysis. The identification results were exported as Excel file including protein accession numbers, mass of the protein, pI, PLGS score, and % coverage of the protein by amino acids identified by LC/MS.

Comparing TB434 (4) labeling with enzymatic activity at varying pH

For the enzymatic activity determination, 52 ng (0.55 pmole) β -mannosidase from *Helix pomatia* (Sigma) were equilibrated in 25 μ L McIlvaine buffer at varying pH (3.0 – 8.0) at 37°C for 15 min, then

assayed with 4-mu- β -D-man substrate prepared at the respective pHs. All measurements were performed in triplicates. For the TB434 (4) labeling, 2.6 μ g (27.5 pmole) of the same β -mannosidase were equilibrated in 10 μ L McIlvaine buffer at varying pH (3.0 - 7.5) at 37 °C for 15 min, incubated with 10 μ M TB434 (end concentration; prepared in McIlvaine buffer at pH 3.0 - 7.5) at 37 °C for 1 h, denatured and resolved by SDS-PAGE, scanned for cy5 fluorescence with a Typhoon FLA 9500 imager (GE Healthcare), and fluorescent signals were quantified with the ImageQuant TL software (GE Healthcare). Both γ values derived from activity measurement and the gel were normalized against the highest value in their respective experiment.

The concentration dependent labeling of β -mannosidase from *Helix pomatia* with TB434 (4) was performed with varying probe concentrations (0 – 10 μ M) for 1 h at 37 °C at pH 5.5.

Enzyme activity assays

The β -mannosidase activity was assayed at 37 °C by incubating 25 μ L enzyme (with or without inhibitor pre-incubation) in a black 96-well plate (Greiner, medium binding) with 100 μ L 2 mM 4-methylumbelliferyl- β -D-mannopyranoside (4-mu- β -D-man) as substrate in 150 mM McIlvaine buffer (pH 4.2 or 5.5) for 30 min or 1 h, and stopping by adding 200 μ L 1 M glycine-NaOH. Fluorescence was measured with a LS55 fluorometer (Perkin Elmer) using $\lambda_{\text{Ex}} = 366$ nm and $\lambda_{\text{Em}} = 445$ nm.

For the determination of apparent IC₅₀ values from the inhibitors, 47 ng (0.5 pmol) β -mannosidase from *Helix pomatia* were equilibrated in 12.5 μ L McIlvaine buffer (150 mM, pH 5.5) for 15 min at 37 °C, then with 12.5 μ L 2x ABP dilution (final [ABP] = 0.1 – 50 μ M; diluted in McIlvaine buffer pH 5.5) for 2 h at 37 °C, followed by an 1 h incubation with the 4-mu- β -D-man substrate at 37 °C and pH 5.5. Apparent IC₅₀ values for each inhibitor were calculated by fitting a non-linear [Inhibitor] vs response function (variable slope, four parameters, least squares fit, without constraint) to the measured values (in triplicates) at each inhibitor concentration, using Prism (GraphPad, version 7.0).

In vitro ABPP with TB434 (4) in mouse kidney homogenates

Kidneys from wild type mice were homogenized in KPi buffer (25 mM K₂HO₄/KH₂PO₄, 0.1% (v/v) Triton X-100, protease inhibitor cocktail (Roche, version 12)) with 1mm glass beads and a FastPrep-24 homogenizer (MP Biomedicals). The basic labeling condition consists of equilibrating 25 μ g total protein from the homogenate in McIlvaine buffer (150 mM, various pH) in a total volume of 10 μ L, and ABP labeling with 5 μ L of 3x concentrated ABP prepared in McIlvaine buffer. For concentration dependent labeling, mouse tissue homogenates were equilibrated in McIlvaine buffer pH 5.5 for 15 min at 37 °C, followed by incubation with various probe concentrations up to 10 μ M TB434 (end concentration, pH 5.5) at 37 °C for 1 h. For labeling at varying pH, mouse tissue homogenates were equilibrated in McIlvaine buffer pH 4.0 – 7.0 for 15 min at 37 °C, followed by incubating with 1 μ M TB434 (prepared in McIlvaine buffer pH 4.0 -7.0) for 1 h at 37 °C. For labeling at varying incubation

Table S1. Proteins identified with LC/MS analysis after pull-down with ABP 6.

N°	Accession	Entry	Description	PGLS Score	Cover (%)	N°	Accession	Entry	Description	PGLS Score	Cover (%)
1	Q9R0H5	K2C71	Keratin, type II cytoskeletal 71	1204	16	24	P35700	PRDX1	Peroxioredoxin-1	174	36
2	Q6NXH9	K2C73	Keratin, type II cytoskeletal 73	1199	18	25	P25444	RS2	40S ribosomal protein S2	172	8
3	P02535	K1C10	Keratin, type I cytoskeletal 10	414	15	26	Q922K1	K1C16	Keratin, type I cytoskeletal 16	166	15
4	Q64462	CP4B1	Cytochrome P450 4B1	410	11	27	Q9JW6	ALRF2	Allyl/REF export factor 2	155	18
5	P08249	MDHM	Malate dehydrogenase	397	20	28	Q920B0	FRM4B	FERM domain-containing protein 4B	145	7
6	Q64436	ATP4A	K-transporting ATPase alpha chain	380	16	29	Q9DCY0	KEG1	Glycine N-acyltransferase-like protein KEG1	145	12
7	P17182	ENOA	Alpha-enolase	347	36	30	Q8K2I4	MANBA	Beta-mannosidase	142	11
8	Q9QXD6	F16P1	Fructose-1,6-bisphosphatase 1	327	33	31	Q9DCL4	MET15	Probable methyltransferase-like protein 15	136	22
9	P63268	ACTH	Actin, gamma-enteric smooth muscle	316	27	32	Q9DCW4	ETFB	Electron transfer flavoprotein subunit beta	135	16
10	Q61414	K1C15	Keratin, type I cytoskeletal 15	313	20	33	Q64505	CP7A1	Cholesterol 7-alpha-monooxygenase	134	16
11	Q922U2	K2C5	Keratin, type II cytoskeletal 5	268	19	34	Q9DB77	QCR2	Cytochrome b-c1 complex subunit 2	133	21
12	Q8BGZ7	K2C75	Keratin, type II cytoskeletal 75	267	15	35	P17183	ENOG	Gamma-enolase	129	25
13	P10922	H10	Histone H1.0	254	7	36	Q9CPR4	RL17	60S ribosomal protein L17	129	29
14	P63017	HSP7C	Heat shock cognate 71 kDa protein	245	20	37	Q8R050	ERF3A	Eukaryotic peptide chain release factor	128	17
15	P17156	HSP72	Heat shock-related 70 kDa protein 2	211	24				GTP-binding subunit ERF3A		
16	Q3UV17	K220	Keratin, type II cytoskeletal 2 oral	211	19	38	P12970	RL7A	60S ribosomal protein L7a	126	14
17	Q61361	PGCB	Brevican core protein	204	15	39	Q6IMP4	PANX2	Pannexin-2	120	16
18	Q8BH95	ECHM	Enoyl-CoA hydratase	193	12	40	Q91YT0	NDUV1	NADH dehydrogenase [UQ] flavoprotein 1	118	13
19	P16125	LDHB	L-lactate dehydrogenase B chain	186	22	41	P20029	GRP78	78 kDa glucose-regulated protein	118	16
20	Q80WM3	CPLX4	Complexin-4	186	29	42	Q68FD5	CLH1	Clathrin heavy chain 1	112	16
21	P05784	K1C18	Keratin, type I cytoskeletal 18	184	27	43	P29351	PTN6	Tyr.-protein phosphatase non-receptor type 6	112	15
22	Q99JRI	SFXN1	Sideroflexin-1	180	24	44	Q3V124	EID3	EP300-interacting inhibitor of diff. 3	109	13
23	Q91Y10	ARLY	Argininosuccinate lyase	179	23						

time, mouse kidney homogenates were prepared as above, and incubated with 1 μ M TB434 (end concentration; pH 5.5) at 37 °C for 2 min to 2 h. After incubation, samples were denatured with 3.75 μ L 5X Laemmli's sample buffer at 98 °C for 5 min, resolved in 7.5% SDS-PAGE gels, and wet slab gels were scanned for Cy5 fluorescence by a Typhoon FLA 9500 imager (GE Healthcare). Coomassie stain were carried out as loading control.

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6

Synthesis of carba-cyclophellitols: a new class of cyclophellitol derivatives¹

6.1 Introduction

Cyclophellitol (**1**, Figure 6.1), isolated in 1990 from the mushroom *Phellinus* sp., is a potent mechanism-based inhibitor of retaining β -glucosidases.² Cyclophellitol is a configurational analogue of β -glucopyranoses, but its conformational behaviour is different. Whereas β -glucopyranoses preferably adopt a $^4C_1$ conformation, the epoxide annulation in **1** enforces a preferred $^4H_3$ half-chair conformation onto the cyclitol moiety. A similar half-chair conformation is thought to occur during hydrolysis of β -glucosidic linkages as effected by retaining β -glucosidases and for this reason cyclophellitol is thought to bind well within β -glucosidase active sites. After protonation of the epoxide oxygen by the acid-base catalyst residing within β -glucosidase active sites and subsequent S_N2 displacement by the active site nucleophile a covalent enzyme-inhibitor adduct emerges.^{3,4} This adduct is stable over time leading to irreversible retaining β -glucosidase inhibition.

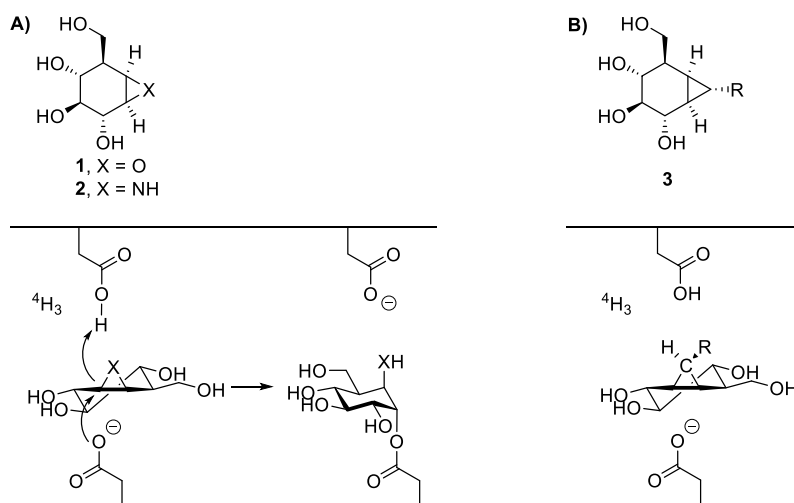
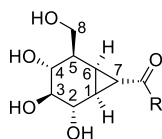


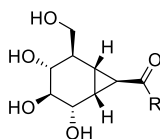
Figure 6.1 A) Cyclophellitol (1) and cyclophellitol aziridine (2) inhibit retaining β -glucosidases by initial binding in $^4H_3$ conformation, followed by S_N2 displacement of the (protonated) epoxide-oxygen or aziridine-nitrogen. B) Carba-cyclophellitols (3) are proposed to be competitive retaining β -glucosidase inhibitors by mimicry of the $^4H_3$ transition state. X = O or NH; R = alkyl or acyl.

Following the discovery of cyclophellitol, a number of synthetic analogues were reported.^{5,6} Cyclophellitol aziridine (2), the cyclophellitol analogue in which the epoxide oxygen in cyclophellitol 1 is substituted by nitrogen, proved to inhibit β -glucosidases covalently and irreversibly as well.⁷ The potency and specificity of cyclophellitol (1) and cyclophellitol aziridine (2), and their putative mode of action (entering the enzyme active site as a $^4H_3$ half-chair transition state analogue followed by S_N2 displacement of the epoxide/aziridine heteroatom) invited the question whether the corresponding carba analogue (3) (that is, substitution of the oxygen/nitrogen for a carbon atom) would yield viable retaining β -glucosidase inhibitors (Figure 6.1B). And, if so, whether such compounds would act as competitive inhibitors by transition state mimicry without – due to lack of a suitable leaving group – ensuing covalent bond formation. To test this hypothesis, a set of carba-cyclophellitols was designed (Figure 6.2). Next to carba-cyclophellitols featuring a retaining β -glucopyranose configuration, analogues having an α -glucopyranose configuration, a β -galactopyranose configuration and an α -galactopyranose configuration were included in the synthetic targets, leading to the focused carba-cyclophellitol library 4-23 (Figure 6.2). In this chapter, the synthesis of these carba-cyclophellitols is presented.

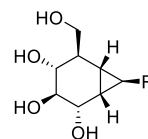
glucopyranose-configured carba-cyclophellitol


 4 (DW172), R = O(CH₂)₂

 5 (DW174), R = (CH₂)₂

 6 (DW082), R = NH(CH₂)₄N₃

 7 (DW064), R = O(CH₂)₂

 8 (DW170), R = (CH₂)₂

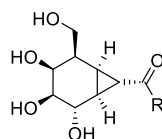
 9 (DW068), R = NH(CH₂)₄N₃


10 (TB280), R = H

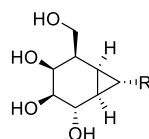
 11 (DW167), R = CH₂OH

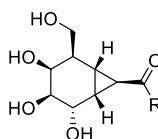
 12 (DW153), R = CH₂O(CH₂)₂

galactopyranose-configured carba-cyclophellitol

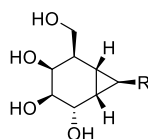

 13 (TB304), R = O(CH₂)₂

 14 (TB405), R = (CH₂)₂

 15 (TB292), R = NH(CH₂)₄N₃

 16 (TB283), R = CH₂OH

 17 (TB290), R = CH₂O(CH₂)₂

 18 (TB343), R = O(CH₂)₂

 19 (TB297), R = (CH₂)₂

 20 (TB291), R = NH(CH₂)₄N₃


21 (TB350), R = H

 22 (TB286), R = CH₂OH

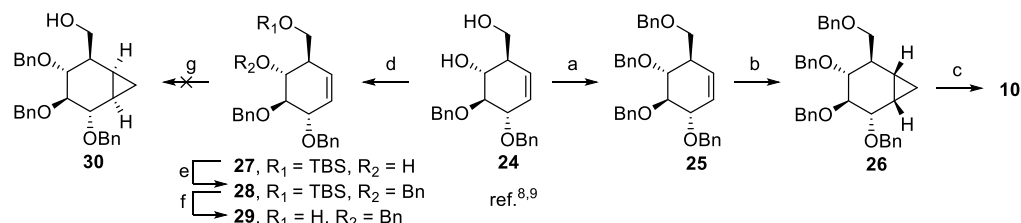
 23 (TB287), R = CH₂O(CH₂)₂

Figure 6.2 Structures of the carba-cyclophellitols (4-23) that are subject of the studies presented in this chapter.

6.2 Results and discussion

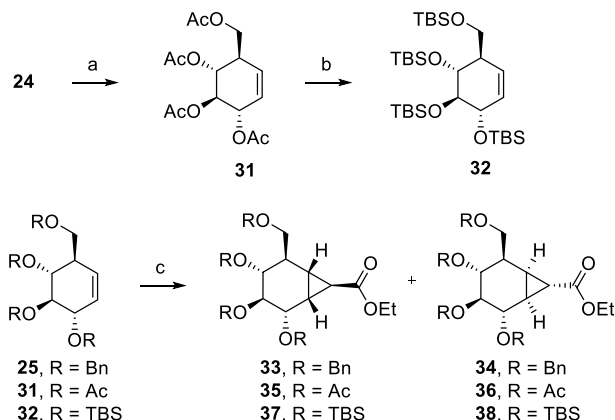
The synthesis of compound **10**, the most simple carba-cyclophellitol commenced with the preparation of key intermediate **24** following the procedure first described by the group of Madsen⁸ and optimized⁹ in more recent years (Scheme 6.1). Global benzylation of **24** was followed by a Simmons-Smith cyclopropanation⁶ to afford cyclopropane **26** in 46% yield as the single product. After palladium-catalyzed hydrogenolysis and subsequent acetylation, the acetylated intermediate was purified by silica gel column chromatography. Deacetylation under Zemplén conditions resulted in compound **10**. After several protection group manipulations, compound **29** was obtained. Attempts to synthesize cyclopropane **30**, an isomer of cyclopropane **10**, from compound **29** following literature procedures (40%)⁶, however, turned out to be abortive, in that compound **30** could not be synthesized.

As a next research objective, the discovery of a versatile intermediate as starting point to obtain various carba-cyclophellitol derivatives was undertaken. Ethyl diazoacetate (EDA)^{10,11} was employed as the cyclopropanylating agent for this purpose. When the conditions developed for cyclopropanation of glucals with Rh₂(OAc)₄ as catalyst¹²⁻¹⁴ were applied to benzylated **25**, trace amounts of the desired product were detected after TLC-MS analysis of the reaction mixture. Several other products with higher

Scheme 6.1 Synthesis of cyclopropane **10**.

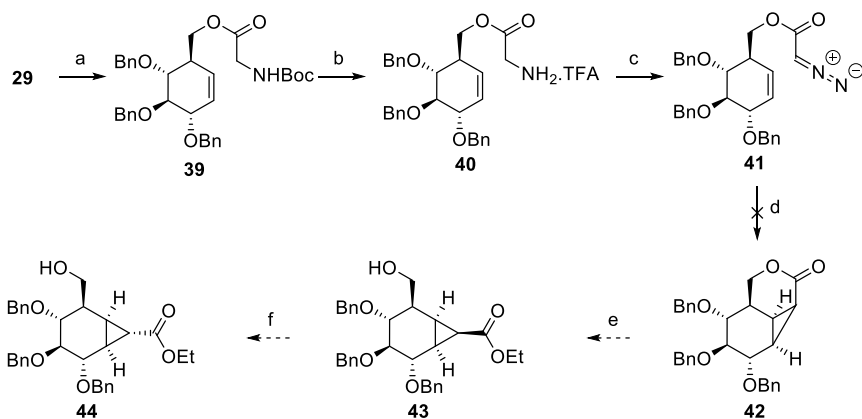
Reagents and conditions: a) BnBr, NaH, TBAI, DMF, 0 °C to rt, overnight, 94%; b) 1,2-dimethoxyethane, boron trifluoride diethyl etherate, Et₂Zn, CH₂I₂, CH₂Cl₂, rt, 3 h, 46%; c) (i) Pd/C, H₂, MeOH, rt, overnight, (ii) Ac₂O, pyridine, rt, 48 h, (iii) NaOMe, MeOH, rt, 2 h, 66% over three steps; d) TBS-Cl, imidazole, DMF, rt, 1 h; e) BnBr, NaH, TBAI, DMF, 0 °C to rt, overnight; f) TBAF, THF, rt, 2 h, 83% over 3 steps; g) 1,2-dimethoxyethane, Et₂Zn, CH₂I₂, CH₂Cl₂, MeOH, rt, 5h.

(compared to the desired product) molecular masses were detected (TLC-MS), which may be the result of Büchner-type ring expansion^{15,16}, in which the benzyl ethers in **25** may have reacted with EDA under the applied conditions.^{17,18} Furthermore, after adding the 3 eq. EDA over 6 h to the reaction mixture as is advocated in the literature, dimerization of EDA to ethyl maleate was observed to occur as a major event. The latter was circumvented by addition of less EDA (1.5 eq. EDA) over 6 h. A comparative study in which a number of transition metal catalysts were compared side-by-side was performed to further optimize the reaction conditions (Scheme 6.2) in terms of yield. Rh₂(OAc)₄, Cu(acac)₂ and Pd(OAc)₂¹⁹, which are widely applied catalysts for EDA-mediated cyclopropanation of various substrates²⁰⁻²², were employed in this study. Palladium-based catalysts are, in general, optimal for addition to electron-poor alkenes whereas rhodium- and copper-based catalysts are more effective in catalyzing cyclopropanation of electron-rich olefins. The influence of the electron density of the alkene was studied by comparing peracetylated cyclohexene **31** and persilylated cyclohexene **32** (which were prepared by means of standard protective group manipulations from **24** – see Scheme 6.2) with perbenzylated cyclohexene **25**. The combination of Cu(acac)₂ as catalyst and tetra-O-benzyl-cyclohexene **25** came out as the optimal one based on TLC-MS analysis, even though, in contrast to compounds **31** and **32**, and as shown before, compound **25** can undergo Büchner-type reactions. With the aim to minimize such side reactions, which also occurred in Cu(acac)₂/EDA-mediated cyclopropanations, EDA is best added over time to a mixture of **25** and the copper(II) catalyst in ethyl acetate. Upon TLC-MS detection of Büchner adducts, the reaction was worked up by concentration, the desired product isolated, the side products removed and the remainder of starting material reused. In this manner and over three reaction cycles, compounds **33** and **34** can be obtained as a mixture (in a ratio of 2:1) in 35% yield.

Scheme 6.2 EDA based cyclopropanation on cyclohexenes.


Reaction conditions: a) (i) Li (s), NH_3 (l), THF, -60°C , 35 min, (ii) Ac_2O , pyridine, rt, overnight, 79% over two steps; b) (i) NaOMe, MeOH, rt, overnight, (ii) TBS-Cl, imidazole, DMF, rt to reflux temperature, 7 days, 35%; c) To 0.1 mmol of substrate (**25**, **31**, or **32**) and 5 mol% of catalyst ($\text{Rh}(\text{OAc})_2$, $\text{Cu}(\text{acac})_2$ or $\text{Pd}(\text{OAc})_2$) in DCE (90 μL) at reflux temperature was added EDA (0.3 mmol) in DCE (300 μL) over 6 h.

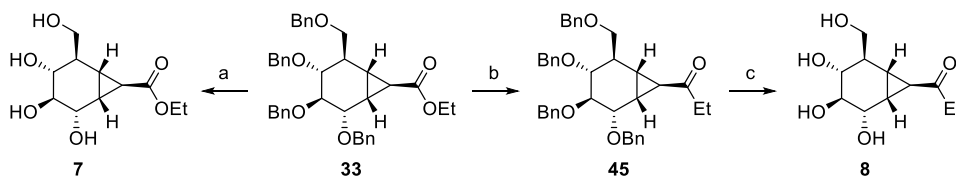
An alternative strategy is intramolecular cyclopropanation (Scheme 6.3). Alcohol **29** was coupled with *N*-Boc-glycine to obtain **39**. Treatment with TFA gave amine **40**, which was subsequently subjected to diazotization to give **41**. Based on the work of Corey^{23,24} internal cyclopropanation with $\text{Cu}(\text{N-tert-butylsalicylaldiminato})_2$ in toluene or $\text{Cu}(\text{acac})_2$ in EtOAc, was attempted. However, no product other than dimerisation could be detected (TLC) in this experiment.

Scheme 6.3 An attempt to achieve intramolecular cyclopropanation.


Reagents and conditions: a) *N*-Boc-glycine, DIC, DMAP, rt, overnight, quant.; b) TFA, CH₂Cl₂, rt, 45 min, quant.; c) NaNO₂, monosodium citrate, CH₂Br₂, H₂O, 0 °C, 1 h, 99%; d) Cu(acac)₂ or Cu(*N*-*tert*-butylsalicylaldiminato)₂, toluene, EtOAc or DCE, reflux temperature; e) EtOH, H₂SO₄; f) KHMDS, THF, -78 °C.

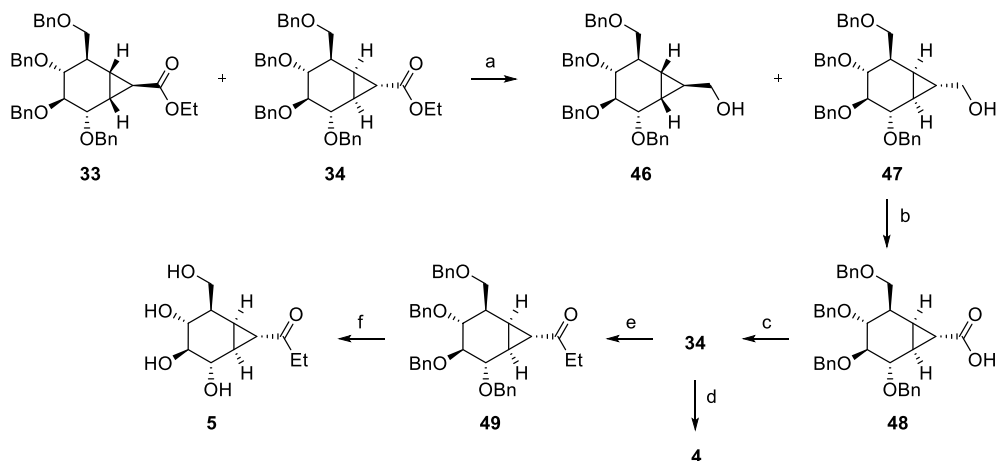
The versatility of carba-cyclophellitols **33** and **34** as starting materials for the synthesis of a number of functional analogues is demonstrated in Schemes 6.4 – 6.7. Separation of the stereoisomers **33** and **34** by silica gel column chromatography or HPLC was not successful. Crystallization of the mixture of compounds obtained after Cu(acac)₂/EDA-mediated cyclopropanation of **25** from ethanol, however, resulted in isolation of compound **33** as the sole isomer. Global debenzoylation of **33** under standard palladium-catalyzed hydrogenation conditions in EtOAc and AcOH resulted in compound **7** (Scheme 6.4). Ester **33** was converted into ketone **45** and subsequently subjected to palladium-catalyzed hydrogenolysis in MeOH to obtain compound **8**.

Scheme 6.4 Synthesis of **7** and **8**.



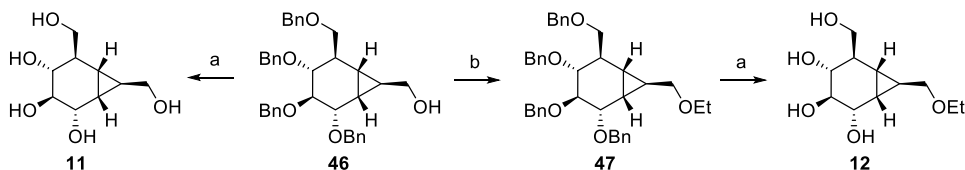
Reagents and conditions: a) Pd(OH)₂/C, H₂, EtOAc, AcOH, rt, overnight, 97%; b) *N*,*O*-dimethylhydroxylamine hydrochloride, EtMgBr, THF, -5 °C to rt, overnight, 56%; c) Pd(OH)₂/C, H₂, MeOH, rt, overnight, 96%.

After reduction of the mixture of esters **33** and **34**²⁵, alcohols **46** and **47** were obtained and successfully separated by column chromatography (Scheme 6.5). In the next steps, alcohol **47** was converted into compounds **4** and **5**. Oxidation of alcohol **47** with aqueous sodium dichromate–sulphuric acid (Jones reagent) afforded acid **48**. Esterification of acid **48** with ethanol gave ester **34**, which was subjected to palladium-catalyzed hydrogenolysis in EtOAc and AcOH to obtain compound **4** (81% yield). After conversion of ester **34** into ketone **49** and global debenzoylation under standard palladium-catalyzed hydrogenation conditions in MeOH compound **5** was synthesized.

Scheme 6.5 Synthesis of **4** and **5**.


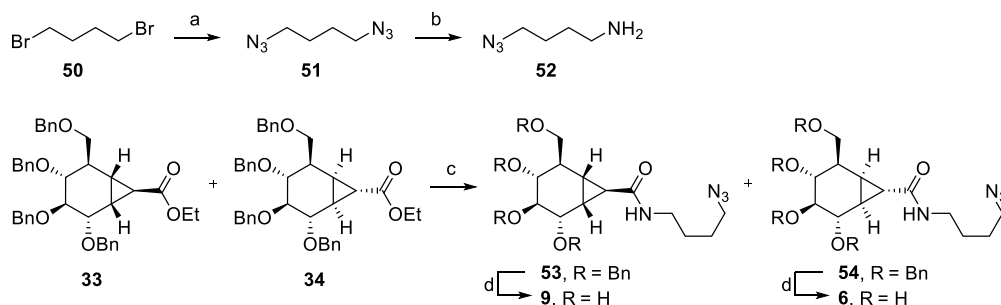
Reagents and conditions: a) DIBAL, THF, 30 min at 0 °C and then 1 h at rt, **46** (28%) and **47** (13%); b) Jones reagent, acetone, 0 °C, 3 h, 53%; c) EtOH, *N,N'*-diisopropylcarbodiimide, 4-dimethylaminopyridine, toluene, rt, 4 h, 62%; d) Pd(OH)₂/C, H₂, EtOAc, AcOH, rt, overnight, 81%; e) *N,O*-dimethylhydroxylamine hydrochloride, EtMgBr, THF, -5 °C to rt, overnight, 45%; f) Pd(OH)₂/C, H₂, MeOH, rt, overnight, 58%.

Alcohol **11** and ether **12** were synthesized from compound **46** (Scheme 6.6). Subjection of compound **46** to palladium-catalyzed hydrogenolysis in MeOH gave compound **11**. Alkylation of compound **46** with bromoethane followed by global debenzoylation afforded compound **12**.

Scheme 6.6 Synthesis of **11** and **12**.


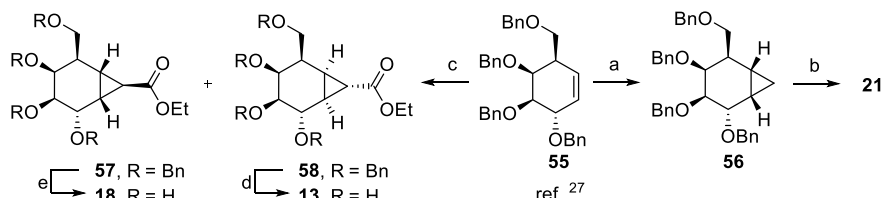
Reagents and conditions: a) Pd(OH)₂/C, H₂, MeOH, rt, overnight, **11** (90%), **12** (94%); b) EtBr, NaH, TBAI, DMF, 0 °C to rt, 4 h, 59%.

Next, the mixture of carba-cyclophellitols **33** and **34** was treated with LiOH and subsequently coupled to 1-azido-4-aminobutane linker **52**, which was synthesized following the procedure of Ma *et al* (Scheme 6.7).²⁶ Preparative HPLC allowed separation of **53** and **54**. The thus purified compounds were treated with anhydrous BCl₃ in dichloromethane to afford **6** and **9** in 99% and 88% yield.

Scheme 6.7 Synthesis of amide **6** and **9**.

Reagents and conditions: a) NaN_3 , DMF, H_2O , 80 °C, overnight, 98%; b) PPh_3 , Et_2O , EtOAc, 1 M HCl, 0 °C to rt, 20 h, 77%; c) (i) LiOH, THF, MeOH, H_2O , rt, overnight, 82%; (ii) **52**, HCTU, DIPEA, DCM, rt, overnight, 78%; d) BCl_3 , DCM, **9** (88%), **6** (99%).

The galactopyranose-configured carba-cyclophellitol derivatives were synthesized in a similar fashion. Cyclohexene **55** was synthesized in large quantities as described by Willems²⁷ based on the method of Llebaria.²⁸ Simmons-Smith cyclopropanation of cyclohexene **55** afforded cyclopropane **56**⁶ in stereoselective fashion. In the next step, compound **56** was subjected to palladium-catalyzed hydrogenolysis to obtain target compound **21** (Scheme 6.8). The optimized conditions for EDA-mediated cyclopropanation of **25** as described before were applied on **55**, yielding **57** and **58**, which could, in contrast to the glucopyranose-configured isomer, be separated by silica gel column chromatography. Palladium-catalyzed hydrogenation in MeOH of compound **58** afforded compound **13**. Compound **18** was obtained after palladium-catalyzed hydrogenation of compound **57** in EtOAc with AcOH.

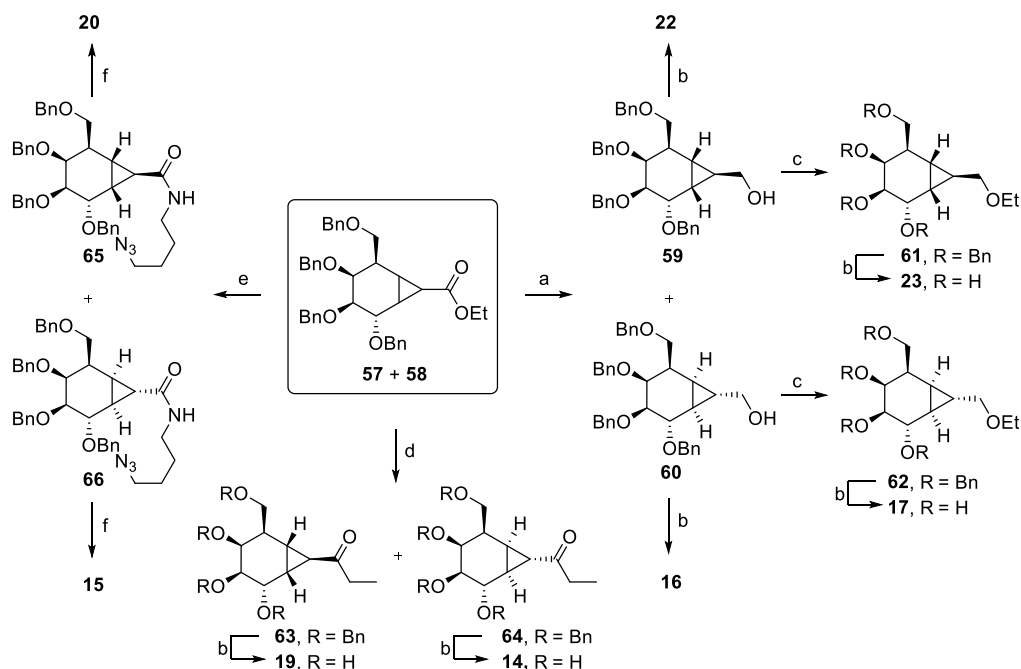
Scheme 6.8 Synthesis of cyclopropane **21** and esters **13** and **18**.

Reagents and conditions: a) 1,2-dimethoxyethane, boron trifluoride diethyl etherate, Et_2Zn , CH_2I_2 , CH_2Cl_2 , 84%; b) (i) $\text{Pd}(\text{OH})_2/\text{C}$, H_2 , MeOH, (99%); c) EDA, $\text{Cu}(\text{acac})_2$, EtOAc, **52** and **53** (3:1, 13%); d) Pd/C , MeOH, H_2 , overnight, 14%; e) $\text{Pd}(\text{OH})_2$, H_2 , EtOAc, AcOH, rt, overnight, 9%.

A mixture of **57** and **58** was used as a starting point to synthesize various other galactopyranose-configured carba-cyclophellitols (Scheme 6.9). DIBAL-mediated

reduction of such a mixture provided alcohol **59** and alcohol **60** which were separated by silica gel column chromatography. Palladium-catalyzed hydrogenolysis of compound **59** resulted in compound **22**. The free alcohol of **59** was alkylated with bromoethane to afford ether **61**, which was subsequently debenzylated to obtain compound **23**. Global debenzylation of alcohol **60** gave compound **16** in 87% yield. Treatment of alcohol **60** with ethylbromide, sodium hydride and tetrabutylammonium iodide gave compound **62**. Debenzylation using palladium-catalyzed hydrogenolysis conditions afforded compound **17**. The esters **57** and **58** were converted into the corresponding ketones **63** and **64** and subsequently separated by HPLC. Compound **63** was deprotected by palladium-catalyzed hydrogenation to obtain compound **19**. In a similar fashion compound **64** was converted into compound **14**. Finally, a mixture of **57** and **58** was treated with LiOH and coupled with **52**.²⁶ The obtained compounds **65** and **66** were separated by HPLC. The benzyl protection groups of compound **65** were removed by

Scheme 6.9 Synthesis of **14-17**, **19**, **20**, **22** and **23**.



Reagents and conditions: a) DIBAL, CH₂Cl₂, **59** (40%) and **60** (36%); b) Pd/C, H₂, MeOH, rt, overnight, **14** (88%), **16** (87%), **17** (quant.), **19** (80%), **22** (quant.), **23** (quant.); c) EtBr, NaH, TBAI, DMF, 0 °C to rt, **61** (80%) **62** (74%); d) *N,O*-dimethylhydroxylamine hydrochloride, EtMgBr, THF, -8

°C to rt, overnight, **63** (16%) and **64** (21%); e) (i) LiOH, THF, EtOH, H₂O, rt, overnight; (ii) **52**, HCTU, DIPEA, DCM, rt, overnight, **65** (24%) and **66** (18%); d) BCl₃, DCM, **15** (70%), **20** (quant.). BCl₃ to afford compound **20**. Treatment of compound **66** with BCl₃ afforded compound **15**. The configuration of the obtained carba-cyclophellitol products was determined by nuclear Overhauser effect spectroscopy (NOESY), Figure 6.3. Spatial couplings between H-1 and H-2 and between H-3 and H-7 were observed in compound **33**. The observed spatial couplings in compound **34**, a stereoisomer of **33**, are between H-2 and H-7 and between H-5 and H-6. These observations were consistent throughout the described library.

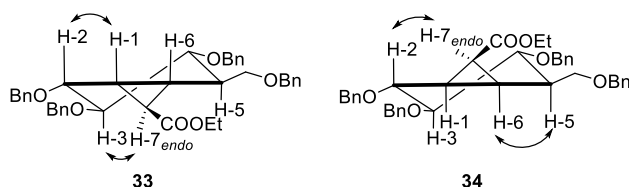


Figure 6.3 Configuration determination by spatial couplings of nuclear Overhauser effect spectroscopy (NOESY) experiments.

6.3 Conclusion

Cyclophellitols and cyclophellitol aziridines are potent, selective and covalent glycosidases inhibitors that act by mimicking the oxocarbenium ion transition state. Carba-cyclophellitol, an analogue of cyclophellitol and cyclophellitol aziridine, is expected to be a stable competitive inhibitor of glycosidases. In order to examine their inhibition properties, a set of 20 different carba-cyclophellitols is synthesized and described in this chapter. The described β -glucopyranose-configured carba-cyclophellitols are further evaluated towards *Thermotoga maritima* TmGH1 in Chapter 7 as potential retaining β -glucosidase inhibitors.

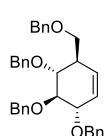
Experimental

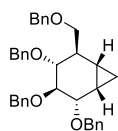
General: All chemicals were purchased from Acros, Sigma Aldrich, Biosolve, VWR, Fluka, Merck and Fisher Scientific and used as received unless stated otherwise. *N,N*-Dimethylformamide (DMF) and toluene were stored over flame-dried 4 Å molecular sieves before use. Traces of water from reagents were removed by co-evaporation with toluene in reactions that require anhydrous conditions. All reactions were performed under an argon atmosphere. TLC analysis was conducted using Merck aluminium 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 or a

solution of KMnO_4 (20 g/L) and K_2CO_3 (10 g/L) in water, followed by charring at $\sim 150^\circ\text{C}$. Column chromatography was performed using Screening Device b.v. Silica Gel (particle size of 40 – 63 μm , pore diameter of 60 Å) in the indicated solvents. For reversed-phase HPLC purifications an Agilent Technologies 1200 series instrument equipped with a semiprep column (Gemini C18, 250 x 10 mm, 5 μm particle size, Phenomenex) was used. LC/MS analysis was performed on a Surveyor HPLC system (Thermo Finnigan) equipped with a C_{18} column (Gemini, 4.6 mm x 50 mm, 5 μm particle size, Phenomenex), coupled to a LCQ Advantage Max (Thermo Finnigan) ion-trap spectrometer (ESI^+). The applied buffers were H_2O , MeCN and 1% aqueous TFA. ^1H NMR and ^{13}C NMR spectra were recorded on a Bruker AV-400 (400 and 101 MHz respectively), a Bruker DMX-600 (600 and 151 MHz respectively) or a Bruker AV-850 (850 and 214 MHz respectively) spectrometer in the given solvent. Chemical shifts are given in ppm (δ) relative to the residual solvent peak or to 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).

General procedure for global debenzylation.

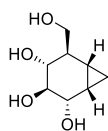
To a solution of the benzyl ether in MeOH was added a catalytic amount of 10% Pd on carbon or $\text{Pd}(\text{OH})_2$ on carbon. The reaction vessel was purged with hydrogen gas and the mixture was vigorously stirred overnight. After TLC analysis showed full conversion to a lower running spot, the palladium catalyst was filtered off over a pad of Celite followed by concentration *in vacuo*, which gave the corresponding product.


 (((1*R*,2*R*,3*S*,6*R*)-6-((benzyloxy)methyl)cyclohex-4-ene-1,2,3-triyl)tris(oxy))tris(methylene) tribenzene (**25**)
 Diol **24** (2.21 g, 6.50 mmol) was dissolved in DMF (33 mL) at 0°C . TBAI (22.0 mg, 60 μmol , 0.01 eq.), BnBr (1.86 mL, 15.6 mmol, 2.4 eq.) and NaH (60% dispersion in mineral oil, 0.629 g, 15.7 mmol, 2.4 eq.) were added. After stirring overnight, additional BnBr (0.93 mL, 7.80 mmol, 1.2 eq.) and NaH (60% dispersion in mineral oil, 0.315 g, 7.68 mmol, 1.0 eq.) were added at 0°C . After the mixture was stirred for 4 h, it was quenched with MeOH (2 mL) and concentrated *in vacuo*. The crude residue was redissolved in Et_2O (100 mL) and washed with H_2O (1 x 100 mL, 3 x 50 mL). The aqueous layers were extracted with Et_2O (50 mL) and the combined organic layers were dried over MgSO_4 , filtered and concentrated *in vacuo*. Purification by column chromatography (3% EtOAc in pentane $\rightarrow$ 6% EtOAc in pentane) gave fully benzylated **25** as a yellow oil (3.17 g, 6.08 mmol, 94%). ^1H NMR (400 MHz, CDCl_3): δ (ppm) 7.56 – 7.00 (m, 20H), 5.83 – 5.56 (m, 2H), 4.98 – 4.86 (m, 3H), 4.70 (s, 2H), 4.53 – 4.36 (m, 3H), 4.31 – 4.22 (m, 1H), 3.81 (dd, $J = 10.1, 7.8$ Hz, 1H), 3.67 (t, $J = 9.8$ Hz, 1H), 3.52 (d, $J = 4.4$ Hz, 2H), 2.64 – 2.42 (m, 1H). ^{13}C NMR (100 MHz, CDCl_3): δ (ppm) 129.3, 128.5, 128., 128.1, 128.0, 127.9, 127.8, 127.7, 127.0, 85.5, 81.0, 78.5, 75.5, 75.5, 73.2 72.2, 69.3, 44.5. HRMS: calculated for $[\text{C}_{35}\text{H}_{37}\text{O}_4]^+$ 520.14791, found 521.26883.



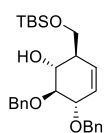
(1S,2S,3R,4R,5R,6R)-2,3,4-tris(benzyloxy)-5-((benzyloxy)methyl)bicyclo[4.1.0]heptane (26)

To a solution of 1,2-dimethoxyethane (72 μ L) in DCM (0.35 mL) was added boron trifluoride ethyl etherate (43 μ L) and diethyl zinc (1 M in hexane, 0.7 mL, 0.7 mmol) at room temperature. After stirring for 5 min, diiodomethane (112 μ L, 1.4 mmol) was added and the reaction mixture was stirred an additional 5 min. Compound **24** (36.3 mg, 70 μ mol) was dissolved in DCM (0.85 mL) and added dropwise to the reaction mixture. After stirring for 3 h, the reaction mixture was quenched with a saturated aqueous NH_4Cl solution and diluted with EtOAc. The aqueous layer was extracted with EtOAc (3x) and the combined organic layers were dried over MgSO_4 , filtered and concentrated *in vacuo*. Purification by column chromatography (pentane $\rightarrow$ 8% EtOAc in pentane) gave cyclopropane **26** (17.3 mg, 32 μ mol, 46%) as a yellow oil. ^1H NMR (400 MHz, CDCl_3): δ (ppm) 7.40 (d, J = 7.0 Hz, 2H), 7.37 – 7.27 (m, 16H), 7.18 – 7.12 (m, 2H), 4.89 – 4.75 (m, 4H), 4.69 (d, J = 12 Hz, 1H), 4.55 – 4.36 (m, 3H), 4.14 – 4.04 (m, 1H), 3.59 (d, J = 4.2 Hz, 2H), 3.46 – 3.24 (m, 2H), 1.93 – 1.85 (m, 1H), 1.44 – 1.34 (m, 1H), 1.17 – 1.07 (m, 1H), 0.82 – 0.74 (m, 1H), 0.40 – 0.35 (m, 1H). ^{13}C NMR (101 MHz, CDCl_3): δ (ppm) 128.5, 128.5, 128.2, 128.1, 128.0, 127.8, 127.7, 127.6, 84.4, 80.5, 79.5, 75.6, 75.3, 73.3, 71.4, 44.1, 16.2, 14.2, 10.4.



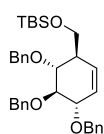
(1S,2S,3R,4R,5R,6R)-5-(hydroxymethyl)bicyclo[4.1.0]heptane-2,3,4-triol (10)

Compound **26** (760 mg, 1.4 mmol) was dissolved in MeOH (20 mL). The reaction mixture was purged with argon gas and 10% palladium on carbon (373 mg) was added. After the reaction vessel was purged with hydrogen gas and vigorously stirring overnight, the palladium catalyst was filtered off over a pad of Celite followed by concentration *in vacuo*. Purification by column chromatography (EtOAc $\rightarrow$ 30% MeOH in EtOAc) gave a crude product which was dissolved in pyridine (4.2 mL) and acetic anhydride (0.67 mL, 7.1 mmol) was added. After stirring for 2 days at room temperature, the reaction mixture was diluted with EtOAc (30 mL) and washed with H_2O (3x). The combined water layers were extracted with EtOAc (2x) and the combined organic layers were dried over MgSO_4 , filtered, concentrated *in vacuo*. Purification by column chromatography (pentane $\rightarrow$ 20% EtOAc in pentane) gave the corresponding acetylated product **10** (0.36 g, 1.0 mmol, 71%) as a clear oil. ^1H NMR (400 MHz, CDCl_3): δ (ppm) 5.39 (dd, J = 8.7, 6.2 Hz, 1H), 5.00 – 4.84 (m, 2H), 4.18 – 4.05 (m, 2H), 2.19 – 2.13 (m, 1H), 2.09 (s, 3H), 2.06 (s, 3H), 2.00 (s, 6H), 1.68 – 1.56 (m, 2H), 1.10 – 1.02 (m, 1H), 0.93 – 0.85 (m, 1H), 0.53 (q, J = 5.7 Hz, 1H). ^{13}C NMR (101 MHz, CDCl_3): δ (ppm) 171.2, 170.8, 170.2, 169.9, 72.9, 71.6, 70.2, 64.6, 41.0, 21.2, 21.0, 20.8, 20.8, 15.9, 13.6, 10.7. HRMS: calculated for $[\text{C}_{16}\text{H}_{22}\text{O}_8\text{Na}]^+$ 365.12069, found 365.12048. The acetylated product (25 mg, 73 μ mol) was dissolved in MeOH (10 mL) and a catalytic amount of NaOMe was added. After TLC analysis showed full conversion to a lower running spot, the reaction mixture was neutralized with Amberlite-H $^+$ IR-120, filtered and concentrated *in vacuo* to obtain compound **10** (12 mg, 68 μ mol, 93%). ^1H NMR (400 MHz, D_2O): δ (ppm) 3.99 (dd, J = 8.8, 5.9 Hz, 1H), 3.83 (dd, J = 10.9, 3.5 Hz, 1H), 3.70 (dd, J = 10.9, 6.3 Hz, 1H), 3.18 (t, J = 10.2 Hz, 1H), 3.09 (dd, J = 10.2, 8.9 Hz, 1H), 1.77 – 1.66 (m, 1H), 1.39 – 1.31 (m, 1H), 1.05 – 0.93 (m, 1H), 0.79 – 0.72 (m, 1H), 0.29 (q, J = 5.4 Hz, 1H). ^{13}C NMR (101 MHz, D_2O): δ (ppm) 74.5, 72.1, 71.0, 63.1, 45.1, 17.6, 12.6, 9.2. HRMS: calculated for $[\text{C}_8\text{H}_{14}\text{O}_4\text{Na}]^+$ 197.07843, found 197.07845.



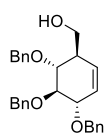
(1R,2R,5S,6S)-5,6-bis(benzyloxy)-2-(((tert-butyldimethylsilyl)oxy)methyl)cyclohex-3-en-1-ol (27)

Diol **24** (0.558 g, 1.64 mmol) was dissolved in DMF (8.2 mL), after which TBS-Cl (0.271 g, 1.80 mmol, 1.1 eq.) and imidazole (0.279 g, 4.10 mmol, 2.5 eq.) were added. The reaction mixture was stirred at room temperature for 1 h and was then partitioned between Et₂O (40 mL) and H₂O (40 mL). The organic layer was separated, washed with H₂O (2x), dried over MgSO₄, filtered and concentrated *in vacuo* to give crude title compound **27**, which was continued without further purification. HRMS: calculated for [C₂₇H₃₉O₄Si]⁺ 455.26121, found 455.26129.



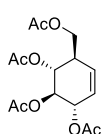
tert-butyldimethyl(((1R,4S,5R,6R)-4,5,6-tris(benzyloxy)cyclohex-2-en-1-yl)methoxy)silane (28)

Crude compound **27** was dissolved in DMF (8.0 mL) at 0 °C, after which TBAI (catalytic amount), BnBr (0.23 mL, 1.97 mmol, 1.2 eq.) and NaH (60% dispersion in mineral oil, 79.2 mg, 1.98 mmol, 1.21 eq.) were added. After stirring at room temperature overnight, the reaction mixture was concentrated *in vacuo* and partitioned between Et₂O (25 mL) and H₂O (25 mL). The organic layer was washed with H₂O (3x) and the resulting aqueous layers were extracted with Et₂O. The combined organic layers were dried over MgSO₄, filtered and concentrated *in vacuo* to give crude title compound **28** as a thick yellow oil, which was continued without further purification. HRMS: calculated for [C₃₄H₄₄O₄SiNa]⁺ 567.29011, found 567.28989.



((1R,4S,5R,6R)-4,5,6-tris(benzyloxy)cyclohex-2-en-1-yl)methanol (29)

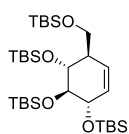
Crude compound **28** (0.893 g, 1.64 mmol) was dissolved in THF (8.2 mL), after which TBAF (1 M in THF, 9.8 mL, 9.8 mmol, 6.0 eq.) was added. After the mixture was stirred at room temperature for 2 h, it was quenched with 4 drops of H₂O and concentrated *in vacuo*. Purification by column chromatography (30% EtOAc in pentane → 50% EtOAc in pentane) gave title compound **29** as a yellow oil (0.585 g, 1.36 mmol, 83% over 3 steps). ¹H NMR (400 MHz, CDCl₃): δ (ppm) 7.45 – 7.07 (m, 15H), 5.72 (dt, *J* = 10.0, 3.0 Hz, 1H), 5.53 (dt, *J* = 10.2, 2.6 Hz, 1H), 5.03 – 4.84 (m, 3H), 4.74 – 4.57 (m, 3H), 4.28 – 4.17 (m, 1H), 3.83 (dd, *J* = 10.6, 7.4 Hz, 1H), 3.68 – 3.50 (m, 3H), 2.46 (ddd, *J* = 14.3, 7.4, 3.7 Hz, 1H). ¹³C NMR (100 MHz, CDCl₃): δ (ppm) 138.8, 138.5, 138.4, 128.6, 128.5, 128.5, 128.4, 128.3, 128.0, 128.0, 127.9, 127.7, 127.7, 85.2, 80.8, 78.7, 75.3, 75.2, 72.1, 63.1, 45.8. HRMS: calculated for [C₂₈H₃₁O₄]⁺ 431.22169, found 431.22174.



(1R,2R,3S,6R)-6-(acetoxymethyl)cyclohex-4-ene-1,2,3-triyl triacetate (31)

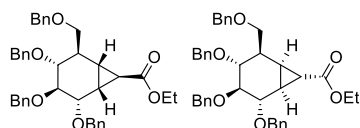
NH₃ (20 mL) was condensed at -60 °C. Lithium (250 mg) was added and the reaction mixture was stirred until the lithium was completely dissolved. To this solution was added a solution of compound **24** (340 mg, 1.00 mmol) in THF (22.5 mL). The reaction mixture was stirred for 30 min at -60 °C and subsequently quenched with MeOH (10 mL). The solution was allowed to come to room temperature and stirred until all NH₃ had evolved. The resulting crude was then dissolved in pyridine (6.0 mL) and acetic anhydride (5.0 mL) was added. After stirring overnight, additional acetic anhydride (9.0 mL) was added. The reaction mixture was partitioned between EtOAc (25 mL) and H₂O (10 mL). The organic layer was washed with H₂O (3x), dried over MgSO₄ and concentrated *in vacuo*. The residue was taken up in pyridine (3.0 mL) and

Ac₂O (2.0 mL). After stirring overnight at room temperature, the reaction mixture was partitioned between EtOAc (25 mL) and H₂O (10 mL). The organic layer was washed with H₂O (3x), dried over MgSO₄, filtered and concentrated *in vacuo*. Purification by column chromatography (10% EtOAc in pentane → 40% EtOAc in pentane) and coevaporation with toluene (to remove any residual pyridine) gave title compound **31** as a yellow oil (0.258 g, 0.786 mmol, 79% over 2 steps). ¹H NMR (400 MHz, CDCl₃): δ (ppm) 5.72 – 5.68 (m, 1H), 5.67 – 5.61 (m, 1H), 5.58 – 5.54 (m, 1H), 5.32 (dd, *J* = 10.6, 7.9 Hz, 1H), 5.28 – 5.18 (m, 1H), 4.15 (dd, *J* = 11.3, 4.1 Hz, 1H), 4.02 (dd, *J* = 11.3, 5.1 Hz, 1H), 2.83 – 2.76 (m, 1H), 2.03 (s, 12H). ¹³C NMR (100 MHz, CDCl₃): δ (ppm) 170.9, 170.5, 170.3, 170.1, 128.5, 126.5, 72.8, 72.2, 69.2, 63.1, 41.4, 21.0, 20.8, 20.8, 20.8. HRMS: calculated for [C₁₅H₂₁O₈]⁺ 329.12309, found 329.12336.



(((1*R*,2*R*,3*S*,6*R*)-6-(((*tert*-butyldimethylsilyl)oxy)methyl)cyclohex-4-ene-1,2,3-triyl)tris(oxy))tris(*tert*-butyldimethylsilane) (32)

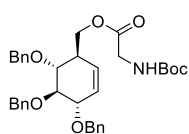
Compound **31** (69.5 mg, 0.434 mmol) was dissolved in MeOH (4.0 mL) and NaOMe (catalytic amount) was added. After stirring overnight, the reaction mixture was concentrated *in vacuo* and dissolved in DMF (3.1 mL). After addition of imidazole (0.708 g, 10.4 mmol, 24 eq.) and a solution of TBS-Cl (0.864 g, 5.73 mmol, 13.2 eq.) in DMF (2.0 mL) the mixture was stirred at room temperature for 5 days and subsequently refluxed over 2 nights. The reaction mixture was partitioned between Et₂O (10 mL) and H₂O (10 mL). The organic layer was separated, washed with H₂O (2x), dried over MgSO₄, filtered and concentrated *in vacuo*. Purification by column chromatography (10% toluene in pentane) gave title compound **32** as a slightly yellow oil (94.0 mg, 0.152 mmol, 35%). ¹H NMR (400 MHz, CDCl₃): δ (ppm) 5.74 (d, *J* = 3.6 Hz, 1H), 5.73 (d, *J* = 3.6 Hz, 1H), 3.93 (d, *J* = 2.5 Hz, 1H), 3.90 (d, *J* = 2.0 Hz), 3.83 (d, *J* = 3.2 Hz, 1H), 3.61 (dd, *J* = 9.6, 8.0 Hz, 1H), 3.52 (dd, *J* = 9.2, 7.2, 1H), 2.35 – 2.30 (m, 1H), 0.87 (s, 18H), 0.86 – 0.83 (m, 18H), 0.10 – 0.05 (m, 18H), 0.01 (s, 3H), 0.00 (s, 3H). ¹³C NMR (100 MHz, CDCl₃): δ (ppm) 127.3, 127.2, 76.0, 70.5, 69.2, 65.4, 46.4, 26.3, 26.3, 26.2, 26.1, 18.6, 18.5, 18.2, -3.9, -4.1, -4.2, -4.5, -4.6, -5.0, -5.1. HRMS: calculated for [C₃₁H₆₉O₈Si₄]⁺ 617.42674, found 617.42689.



ethyl (1*R*,2*S*,3*R*,4*R*,5*R*,6*R*,7*R*)-2,3,4-tris(benzyloxy)-5-((benzyloxy)methyl) bicyclo[4.1.0]heptane-7-carboxylate (33) and ethyl (1*S*,2*S*,3*R*,4*R*,5*R*,6*S*,7*S*)-2,3,4-tris(benzyloxy)-5-((benzyloxy)methyl)bicyclo[4.1.0]heptane-7-carboxylate (34)

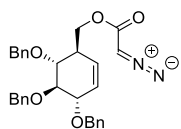
EtOAc was dried over flame dried 4 Å molsieves overnight before use. To a solution of cyclic alkene **25** (1.57 g, 3.01 mmol) in EtOAc (2.7 mL) in a 2-necked pear flask, was added Cu(acac)₂ (79.0 mg, 0.301 mmol, 0.1 eq.). The reaction mixture was stirred at 90 °C and a solution of ethyl diazoacetate (13 wt% DCM, 4.52 mmol, 0.55 mL, 1.5 eq.) in EtOAc (9.0 mL) was added by syringe pump over 6 h. TLC-MS analysis indicated the presence of starting material, so an equal batch of ethyl diazoacetate diluted with EtOAc was added. After addition of a total of 6 eq. of ethyl diazoacetate, the formation of a product with *m/z* 715 [M + Na]⁺ was detected by TLC-MS analysis. The reaction was concentrated *in vacuo* and purification by column chromatography (3% EtOAc in pentane → 7% EtOAc in pentane) to give the desired product as a crude mixture of 2 isomers. In addition, recovered starting material **25**

(0.433 g, 0.832 mmol, 28%) was obtained and was subjected to the same conditions as stated above. After addition of 4.5 eq. of ethyl diazoacetate, significant byproduct formation was observed by TLC-MS analysis. After this cycle was repeated a second time a total crude mixture of α -ester **33** and β -ester **34** (0.642 g, 1.06 mmol, 35%, 2:1, as a mixture of α/β) was obtained as a light yellow oil. Crystallization of the combined crude isomeric product mixture from ethanol gave **33** as a white solid (0.274 g, 0.452 mmol, 15%) and a mixture of **33** and **34** as a light yellow oil (0.368 g, 0.606 mmol, 20%). **33** ^1H NMR (400 MHz, CDCl_3): δ (ppm) 7.30 (m, 16H), 7.14 (m, 2H), 4.89 – 4.69 (m, 4H), 4.64 (d, J = 11.8 Hz, 1H), 4.53 – 4.34 (m, 3H), 4.22 – 4.03 (m, 3H), 3.66 – 3.52 (m, 2H), 3.40 (t, J = 10.2 Hz, 1H), 3.25 (dd, J = 10.1, 8.3 Hz, 1H), 2.05 – 1.97 (m, 1H), 1.94 – 1.88 (m, 1H), 1.76 (ddd, J = 9.5, 4.7, 2.3 Hz, 1H), 1.67 (t, J = 4.7 Hz, 1H), 1.27 (t, J = 7.1 Hz, 3H). ^{13}C NMR (100 MHz, CDCl_3): δ (ppm) 173.4, 139.0, 138.6, 136.6, 138.3, 128.5, 128.5, 128.5, 128.4, 128.2, 128.0, 128.0, 127.8, 127.7, 127.6, 127.5, 84.1, 79.2, 78.5, 75.7, 75.4, 73.3, 71.6, 70.2, 60.8, 43.1, 26.9, 25.0, 25.0, 14.4. HRMS: calculated for $[\text{C}_{39}\text{H}_{43}\text{O}_7]^+$ 607.30542, found 607.30589.



((1R,4S,5R,6R)-4,5,6-tris(benzyloxy)cyclohex-2-en-1-yl)methyl (tert-butoxycarbonyl) glycinate (39**)**

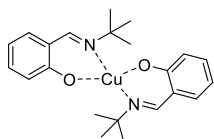
Compound **29** (51.9 mg, 0.120 mmol), N-Boc-glycine (31.5 mg, 0.18 mmol, 1.5 eq.) and DMAP (catalytic amount) were dissolved in toluene (0.6 mL) and DIC (38 μL , 2 eq.) was subsequently added dropwise. After stirring overnight at room temperature, the reaction mixture was filtered over Celite, concentrated *in vacuo* and purified by column chromatography (8% EtOAc in pentane $\rightarrow$ 25% EtOAc in pentane) to give compound **39** as a yellow oil (69.4 mg, 0.120 mmol, quant.). ^1H NMR (400 MHz, CDCl_3): δ (ppm) 7.42 – 7.21 (m, 15H), 5.72 (dt, J = 10.2, 2.4 Hz, 1H), 5.58 – 5.46 (m, 1H), 5.04 – 4.49 (m, 6H), 4.28 (dd, J = 10.8, 3.2 Hz, 1H), 4.23 (ddd, J = 7.7, 3.3, 1.9 Hz, 1H), 4.14 (dd, J = 10.9, 5.0 Hz, 1H), 3.80 (td, J = 13.3, 11.6, 7.5 Hz, 3H), 3.53 (t, J = 9.8 Hz, 1H), 2.71 – 2.52 (m, 1H), 1.45 (d, J = 2.5 Hz, 9H). ^{13}C NMR (75 MHz, CDCl_3): δ (ppm) 170.5, 138.9, 138.0, 128.6, 128.6, 128.5, 128.2, 128.0, 128.0, 127.9, 127.8, 127.5, 104.8, 101.9, 85.4, 80.8, 72.3, 64.5, 43.3, 42.4, 28.5. HRMS: calculated for $[\text{C}_{35}\text{H}_{42}\text{NO}_7]^+$ 588.29558, found 588.29600.



((1R,4S,5R,6R)-4,5,6-tris(benzyloxy)cyclohex-2-en-1-yl)methyl 2-diazoacetate (41**)**

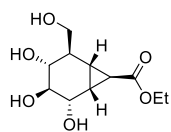
To a solution of compound **39** (69.4 mg, 0.120 mmol) in DCM (0.3 mL) was added TFA (0.3 mL). After stirring for 45 min at room temperature, the reaction mixture was concentrated *in vacuo* to give compound **40** as a light yellow solid which was used without further purification (72.2 mg, 0.120 mmol, quant.). HRMS (as the free amine): calculated for $[\text{C}_{30}\text{H}_{34}\text{NO}_5]^+$ 488.24315, found 488.24285. Compound **40** (60.0 mg, 0.0990 mmol) was suspended in H_2O (0.4 mL), after which monosodium citrate (31.7 mg, 0.149 mmol, 1.5 eq.) and CH_2Br_2 (0.5 mL) were added. The reaction was cooled to 0 $^\circ\text{C}$ and NaNO_2 (8.19 mg, 0.119 mmol, 1.2 eq.) was added. After stirring at 0 $^\circ\text{C}$ for 1 h, the reaction mixture was warmed up to room temperature and the organic layer was removed by syringe. Additional CH_2Br_2 was added (0.5 mL) and after stirring for 10 min the organic layer was removed again by syringe. The combined organic layers were combined and concentrated *in vacuo* to give compound **41** as a bright yellow oil (49 mg, 98.0 μmol , 99%). ^1H NMR (400 MHz, CDCl_3): δ (ppm) 7.45 – 7.16 (m, 15H), 5.73 (d, J = 10.2 Hz, 1H), 5.55 (d, J = 10.2 Hz,

1H), 4.96 (d, $J = 10.8$ Hz, 1H), 4.93 – 4.88 (m, 2H), 4.69 – 4.62 (m, 3H), 4.56 (d, $J = 9.2$ Hz, 1H), 4.33 (dd, $J = 10.8, 3.0$ Hz, 1H), 4.23 – 4.18 (m, 2H), 3.82 (t, $J = 8.4$ Hz, 1H), 3.54 (t, $J = 9.8$ Hz, 1H), 2.61 (bs, 1H). ^{13}C NMR (100 MHz, CDCl_3): δ (ppm) 138.9, 138.5, 138.4, 128.6, 128.6, 128.4, 128.1, 128.0, 127.9, 127.8, 127.7, 85.3, 80.9, 77.9, 75.5, 74.4, 72.3, 63.9, 46.4, 43.6.



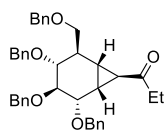
Bis(*N*-tert-butylsalicylaldiminato)copper(II)

$\text{Cu}(\text{OAc})_2$ (0.399 g, 2.00 mmol) was dissolved in H_2O (5 mL) and a solution of salicylaldehyde (435 μL , 4.00 mmol, 2 eq.) in EtOH (2 mL) was added. After stirring for 1 h at 55 $^\circ\text{C}$, the precipitate was filtered off and subsequently suspended in EtOH (2 mL). After addition of tert-butylamine (525 μL , 5.00 mmol, 2.25 eq.), the reaction mixture was refluxed for 1.5 h and concentrated *in vacuo* to give the title compound as black crystals (0.680 g, 1.64 mmol, 82%). m.p.: 185 $^\circ\text{C}$ (literature values 185–186 $^\circ\text{C}$).⁷⁴



ethyl (1*R*,2*S*,3*R*,4*R*,5*R*,6*R*,7*R*)-2,3,4-trihydroxy-5-(hydroxymethyl)bicyclo[4.1.0]heptane-7-carboxylate (7)

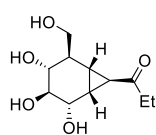
Benzylated **33** (72.9 mg, 0.120 mmol) was dissolved in EtOAc (1.25 mL) and AcOH (0.25 mL) was added. After addition of $\text{Pd}(\text{OH})_2/\text{C}$ (catalytic amount) the reaction mixture was purged with H_2 . After stirring overnight at room temperature, the palladium catalyst was filtered off over a pad of Celite. Concentration *in vacuo* of the filtrate gave compound **7** as a brown thick oil (29.1 mg, 0.118 mmol, 97%). ^1H NMR (400 MHz, CD_3OD): δ (ppm) 4.12 (d, $J = 7.2$ Hz, 1H), 4.09 (d, $J = 7.2$ Hz, 1H), 3.90 (dd, $J = 8.7, 5.6$ Hz, 1H), 3.83 (dd, $J = 10.6, 3.9$ Hz, 1H), 3.65 (dd, $J = 10.6, 6.6$ Hz, 1H), 3.12 (t, $J = 10.1$ Hz, 1H), 3.04 – 2.94 (m, 1H), 1.94 – 1.85 (m, 1H), 1.78 – 1.70 (m, 1H), 1.66 (t, $J = 4.6$ Hz, 1H), 1.61 (ddd, $J = 9.1, 4.4, 1.6$ Hz, 1H), 1.24 (t, $J = 7.1$ Hz, 3H). ^{13}C NMR (100 MHz, MeOD): δ (ppm) 175.2, 76.2, 72.4, 72.0, 64.4, 61.8, 46.3, 30.4, 25.7, 25.0, 14.5. HRMS: calculated for $[\text{C}_{11}\text{H}_{18}\text{O}_6\text{Na}]^+$ 269.09956, found 269.09967.



1-((1*R*,2*S*,3*R*,4*R*,5*R*,6*R*,7*R*)-2,3,4-tris(benzyloxy)-5-((benzyloxy)methyl)bicyclo[4.1.0]heptan-7-yl)propan-1-one (45)

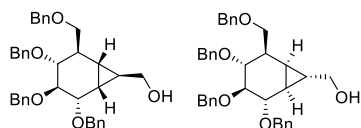
Ester **33** (60.8 mg, 0.100 mmol) was added to $\text{Me}(\text{MeO})\text{NH}\cdot\text{HCl}$ (12.2 mg, 0.125 mmol, 1.25 eq.) in THF (0.5 mL). After addition of EtMgBr (0.5 M in THF, 0.840 mmol, 8.4 eq.) over 2 h at $-5 - 0$ $^\circ\text{C}$, the reaction mixture was stirred overnight, quenched with aqueous HCl (3 M, 3 mL) and extracted with EtOAc (10 mL). The organic layer was dried, concentrated *in vacuo* and redissolved in THF (0.8 mL). After addition of EtMgBr (1 M in THF, 0.300 mmol, 3 eq.) over 2 min at -20 $^\circ\text{C}$, the reaction mixture was allowed to come to room temperature and was stirred for 75 min before quenching with aqueous HCl (3 M, 3 mL). The reaction mixture was extracted with EtOAc (10 mL) after which the organic layer was dried and concentrated *in vacuo*. Purification by column chromatography (6% EtOAc in pentane $\rightarrow$ 8% EtOAc in pentane) gave compound **45** as a white solid (32.8 mg, 55.6 μmol , 56%). ^1H NMR (400 MHz, CDCl_3): δ (ppm) 7.39 – 7.18 (m, 18H), 7.15 – 7.12 (m, 2H), 4.92 – 4.76 (m, 3H), 4.74 – 4.57 (m, 2H), 4.50 – 4.38 (m, 3H), 4.06 (dd, $J = 7.9, 5.8$ Hz, 1H), 3.61 – 3.50 (m, 2H), 3.39 (t, $J = 10.0$ Hz, 1H), 3.34 – 3.24 (m, 1H), 2.58 (dd, $J = 14.4, 7.6$ Hz, 2H), 2.09 – 2.00 (m, 1H), 1.98 (t, $J = 4.5$ Hz, 1H), 1.95 – 1.89 (m, 1H), 1.86 – 1.78 (m, 1H), 1.08 (t, $J = 7.3$ Hz, 3H). ^{13}C NMR (100 MHz, CDCl_3): δ (ppm) 209.6, 139.1, 138.7, 138.6, 138.4,

128.6, 128.5, 128.5, 128.2, 128.0, 127.9, 127.8, 127.7, 84.3, 79.4, 78.7, 75.7, 75.5, 73.5, 71.6, 70.4, 43.5, 37.3, 32.6, 29.6, 27.4, 8.2. HRMS: calculated for $[C_{39}H_{42}O_5Na]^+$ 613.29245, found 613.29242.



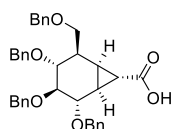
1-((1R,2S,3R,4R,5R,6R,7R)-2,3,4-trihydroxy-5-(hydroxymethyl)bicyclo[4.1.0]heptan-7-yl)propan-1-one (8)

Compound **45** (32.8 mg, 55.6 μ mol) was treated according to General procedure for global debenzoylation with $Pd(OH)_2/C$ to obtain title compound **8** as a clear oil (12.3 mg, 53.4 μ mol, 96%). ^1H NMR (400 MHz, D_2O): δ (ppm) 4.04 (dd, J = 8.6, 5.5 Hz, 1H), 3.84 (dd, J = 11.0, 3.6 Hz, 1H), 3.72 (dd, J = 11.0, 6.2 Hz, 1H), 3.37 – 3.09 (m, 2H), 2.72 (dd, J = 7.2, 14.8 Hz, 2H), 2.25 (t, J = 4.5 Hz, 1H), 2.11 – 1.98 (m, 1H), 1.90 – 1.83 (m, 1H), 1.68 – 1.61 (m, 1H), 1.04 (t, J = 7.3 Hz, 3H). ^{13}C NMR (100 MHz, D_2O): δ (ppm) 216.1, 74.4, 70.7, 70.4, 62.5, 44.3, 36.6, 32.1, 31.9, 26.8, 7.4. HRMS: calculated for $[C_{11}H_{19}O_5]^+$ 231.12270, found 231.12270.



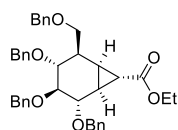
((1R,2S,3R,4R,5R,6R,7R)-2,3,4-tris(benzyloxy)-5-(benzyloxy)methyl)bicyclo[4.1.0]heptan-7-yl)methanol (46) and ((1S,2S,3R,4R,5R,6S,7S)-2,3,4-tris(benzyloxy)-5-(benzyloxy)methyl)bicyclo[4.1.0]heptan-7-yl)methanol (47)

A crude mixture of **33** and **34** (0.142 g, 0.234 mmol) was dissolved in THF (1 mL) at 0 °C, after which DIBAL (1 M in hexanes, 2.1 mL, 2.1 mmol, 9.0 eq.) was added dropwise. After the mixture was stirred for 30 min at 0 °C followed by 1 h at room temperature, the reaction was quenched with EtOAc. The mixture was concentrated *in vacuo* and the residue was partitioned between EtOAc (20 mL) and 1 M aqueous HCl (20 mL). The aqueous layer was extracted with EtOAc (20 mL) and the combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by column chromatography (20% EtOAc in pentane $\rightarrow$ 25% EtOAc in pentane) gave title compounds **46** (36.6 mg, 64.8 μ mol, 28%) and **47** (17.1 mg, 30.2 μ mol, 13%) as white solids. **46** ^1H NMR (400 MHz, $CDCl_3$): δ (ppm) 7.46 – 7.20 (m, 18H), 7.19 – 7.11 (m, 2H), 4.96 – 4.64 (m, 5H), 4.55 – 4.29 (m, 3H), 4.06 (dd, J = 7.9, 6.2 Hz, 1H), 3.59 (d, J = 4.1 Hz, 1H), 3.51 (dd, J = 11.2, 6.3 Hz, 1H), 3.40 – 3.18 (m, 3H), 1.93 – 1.89 (m, 1H), 1.30 – 1.20 (m, 1H), 1.12 – 1.03 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 139.2, 139.1, 138.8, 138.5, 128.6, 128.5, 128.5, 128.2, 128.1, 128.1, 128.0, 127.9, 127.8, 127.8, 127.7, 84.8, 80.1, 79.3, 75.7, 75.4, 73.4, 71.7, 70.9, 66.5, 43.6, 26.4, 22.0, 19.8. HRMS: calculated for $[C_{37}H_{41}O_6]^+$ 565.29485, found 565.29462. **47** ^1H NMR (400 MHz, $CDCl_3$): δ (ppm) 7.63 – 6.95 (m, 20H), 4.95 – 4.60 (m, 5H), 4.53 – 4.31 (m, 3H), 3.75 – 3.62 (m, 3H), 3.58 – 3.42 (m, 2H), 3.07 – 2.99 (m, 2H), 2.41 – 2.30 (m, 1H, H-5), 1.14 (dd, J = 8.2, 4.7 Hz, 1H), 1.02 (dd, J = 8.9, 4.8 Hz, 1H), 0.97 – 0.78 (m, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 139.0, 138.6, 138.5, 138.2, 128.7, 128.6, 128.6, 128.5, 128.3, 128.2, 128.1, 128.1, 128.0, 127.9, 127.9, 127.7, 127.7, 86.4, 82.3, 75.5, 75.3, 73.5, 72.5, 71.4, 66.7, 40.2, 22.9, 21.2, 20.6. HRMS: calculated for $[C_{37}H_{41}O_6]^+$ 565.29485, found 565.29526.



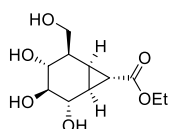
(1S,2S,3R,4R,5R,6S,7S)-2,3,4-tris(benzyloxy)-5-((benzyloxy)methyl)bicyclo[4.1.0]heptane-7-carboxylic acid (48)

Chromic acid stock solution (1.0 M) was prepared (*Caution*: Chromic acid is corrosive, toxic and carcinogenic). Concentrated H_2SO_4 (2.25 mL, 40.5 mmol) is taken up in H_2O (12.5 mL). To this solution was added CrO_3 (2.50 g, 25.0 mmol) and the resulting bright red solution was stirred until all solids were completely dissolved. The solution was then diluted with H_2O to a total volume of 25 mL. Compound **47** (261 mg, 0.462 mmol) was dissolved in acetone (9.2 mL) and cooled to 0 °C, after which the chromic acid stock solution (0.92 mL, 0.920 mmol, 2 eq.) was added. After stirring for 3 h, the reaction mixture was diluted with EtOAc (150 mL) and washed with aqueous HCl (3 M, 2 x 150 mL) and brine (150 mL). The organic layer was dried over MgSO_4 , filtered and concentrated *in vacuo*. Purification by column chromatography (15% EtOAc in pentane $\rightarrow$ 35% EtOAc in pentane) gave carboxylic acid **48** as a white solid (141 mg, 0.244 mmol, 53%). ^1H NMR (400 MHz, CDCl_3): δ (ppm) 7.44 – 7.10 (m, 20H), 4.90 – 4.71 (m, 4H), 4.69 – 4.57 (m, 1H), 4.54 – 4.34 (m, 3H), 3.75 (d, J = 8.1 Hz, 1H), 3.65 (dd, J = 8.9, 2.7 Hz, 1H), 3.60 – 3.51 (m, 2H), 3.11 (t, J = 10.2 Hz, 1H), 2.45 – 2.33 (m, 1H), 2.03 – 1.98 (m, 1H), 1.80 (dd, J = 9.5, 4.5 Hz, 1H), 1.60 (t, J = 4.6 Hz, 1H). ^{13}C NMR (100 MHz, CDCl_3): δ (ppm) 179.4, 138.9, 138.6, 138.5, 138.1, 128.7, 128.6, 128.6, 128.5, 128.5, 128.2, 128.2, 128.1, 128.0, 127.9, 127.7, 85.8, 81.3, 76.2, 75.5, 75.4, 73.3, 72.5, 70.2, 40.6, 27.3, 26.0, 22.0. HRMS: calculated for $[\text{C}_{37}\text{H}_{39}\text{O}_6]^+$ 579.27412, found 579.27438.



ethyl (1S,2S,3R,4R,5R,6S,7S)-2,3,4-tris(benzyloxy)-5-((benzyloxy)methyl)bicyclo[4.1.0]heptane-7-carboxylate (34)

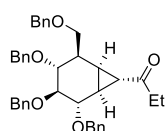
To a solution of carboxylic acid **48** (141 mg, 0.244 mmol) in toluene (1.2 mL) was added ethanol (66 μL , 0.488 mmol, 2 eq.) and DMAP (catalytic amount). After DIC (75 μL , 0.484 mmol, 2.0 eq.) was added dropwise, the reaction mixture was stirred for 4 h at room temperature. The reaction mixture was filtered over Celite, concentrated *in vacuo* and purification by column chromatography (7% EtOAc in pentane $\rightarrow$ 10% EtOAc in pentane) gave title compound **34** as a white solid (91.4 mg, 0.151 mmol, 62%). ^1H NMR (400 MHz, CDCl_3): δ (ppm) 7.44 – 7.14 (m, 20H), 4.89 – 4.72 (m, 4H), 4.64 (d, J = 11.6 Hz, 1H), 4.52 – 4.36 (m, 3H), 4.15 (d, J = 7.2 Hz, 1H), 4.12 (d, J = 7.2), 3.75 (d, J = 7.8 Hz, 1H), 3.65 (dd, J = 8.9, 2.7 Hz, 1H), 3.61 – 3.48 (m, 2H), 3.14 (t, J = 10.2 Hz, 1H), 2.45 – 2.29 (m, 1H), 1.96 – 1.84 (m, 1H), 1.74 (dd, J = 9.3, 4.5 Hz, 1H), 1.61 (t, J = 4.7 Hz, 1H), 1.26 (t, J = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, CDCl_3): δ (ppm) 173.5, 138.9, 138.7, 138.6, 138.2, 128.6, 128.5, 128.5, 128.4, 128.2, 128.1, 128.0, 128.0, 127.9, 127.8, 127.8, 127.7, 127.6, 127.5, 85.9, 81.5, 76.3, 75.4, 75.4, 73.3, 72.4, 70.4, 60.8, 40.6, 26.4, 24.9, 22.2, 14.4. HRMS: calculated for $[\text{C}_{39}\text{H}_{43}\text{O}_7]^+$ 607.30542, found 607.30589.



ethyl (1S,2S,3R,4R,5R,6S,7S)-2,3,4-trihydroxy-5-(hydroxymethyl)bicyclo[4.1.0]heptane-7-carboxylate (4)

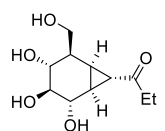
Benzylated **34** (20.9 mg, 34.4 μmol) was dissolved in mixture of EtOAc (0.4 mL) and AcOH (0.1 mL). After addition of $\text{Pd}(\text{OH})_2/\text{C}$ (catalytic amount), the reaction mixture was brought under H_2 atmosphere. After stirring overnight, the palladium catalyst was filtered off over a pad of Celite. The filtrate was concentrated *in vacuo* and purification by column chromatography (10% MeOH in EtOAc) gave compound **4** as a clear oil (6.90 mg, 28.0 μmol , 81%).

^1H NMR (400 MHz, D_2O): δ (ppm) 4.22 – 4.09 (m, 2H), 3.94 (dd, J = 11.0, 3.9 Hz, 1H), 3.69 (d, J = 8.6 Hz, 1H), 3.61 (dd, J = 11.0, 8.2 Hz, 1H), 3.30 (dd, J = 10.2, 8.7 Hz, 1H), 3.01 (t, J = 10.3 Hz, 1H), 2.20 – 2.16 (m, 1H), 2.00 – 1.89 (m, 1H), 1.68 – 1.61 (m, 2H), 1.26 (t, J = 7.2 Hz, 3H). ^{13}C NMR (100 MHz, D_2O): δ (ppm) 175.8, 77.3, 72.3, 68.1, 62.7, 62.1, 41.8, 27.4, 25.2, 21.7, 13.3. HRMS: calculated for $[\text{C}_{11}\text{H}_{19}\text{O}_7]^+$ 247.11761, found 247.11774.



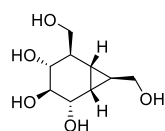
1-((1S,2S,3R,4R,5R,6S,7S)-2,3,4-tris(benzyloxy)-5-((benzyloxy)methyl)bicyclo[4.1.0]heptan-7-yl)propan-1-one (49)

Ethyl ester **34** (60.8 mg, 0.100 mmol) was added to $\text{Me}(\text{MeO})\text{NH}\cdot\text{HCl}$ (12.2 mg, 0.125 mmol, 1.25 eq.) in THF (0.5 mL). Subsequently, EtMgBr (0.5 M in THF, 0.840 mmol, 8.4 eq.) was added over 2 h at -5 to 0°C . After stirring overnight, the reaction mixture was quenched with aqueous HCl (3 M, 3 mL). The reaction mixture was extracted with EtOAc (10 mL), after which the organic layer was dried and concentrated *in vacuo*. Purification by column chromatography (15% EtOAc in pentane) gave compound **49** as a white solid (29.4 mg, 47.9 μmol , 48%). ^1H NMR (400 MHz, CDCl_3): δ (ppm) 7.42 – 7.14 (m, 20H), 4.95 – 4.69 (m, 4H), 4.60 (d, J = 11.5 Hz, 1H), 4.52 – 4.30 (m, 3H), 3.72 (d, J = 7.8 Hz, 1H), 3.69 – 3.46 (m, 3H), 3.26 (t, J = 10.2 Hz, 1H), 2.38 (dd, J = 8.7, 7.4 Hz, 2H), 2.36 – 2.29 (m, 1H), 1.89 (t, J = 4.7 Hz, 1H), 1.84 – 1.82 (m, 1H), 1.82 – 1.79 (m, 1H), 1.00 (t, J = 7.3 Hz, 3H). ^{13}C NMR (101 MHz, CDCl_3): δ (ppm) 209.7, 139.0, 138.7, 138.6, 138.2, 128.6, 128.6, 128.5, 128.5, 128.2, 128.2, 128.1, 127.9, 127.9, 127.8, 127.8, 127.7, 127.6, 86.1, 81.6, 76.3, 75.4, 73.4, 72.2, 70.3, 40.7, 37.0, 29.8, 29.3, 26.4, 8.0. HRMS: calculated for $[\text{C}_{39}\text{H}_{42}\text{O}_5\text{Na}]^+$ 613.29245, found 613.29257.



1-((1S,2S,3R,4R,5R,6S,7S)-2,3,4-trihydroxy-5-(hydroxymethyl)bicyclo[4.1.0]heptan-7-yl)propan-1-one (5)

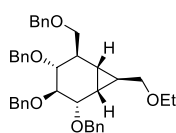
Compound **49** (29.4 mg, 49.8 μmol) was treated according to General procedure for global debenzoylation with $\text{Pd}(\text{OH})_2/\text{C}$ to obtain title compound **5** as a clear oil (6.70 mg, 29.1 μmol , 58%). ^1H NMR (400 MHz, D_2O): δ (ppm) 3.92 (dd, J = 11.2, 4.0 Hz, 1H), 3.89 (dd, J = 0.8 Hz, 8.6 Hz, 1H), 3.60 (dd, J = 10.8, 8.2 Hz, 1H), 3.28 (dd, J = 10.1, 8.6 Hz, 1H), 3.05 (t, J = 10.0 Hz, 1H), 2.69 (dd, J = 14.6, 7.4 Hz, 2H), 2.21 – 2.15 (m, 1H), 2.13 (t, J = 4.2 Hz, 1H), 1.93 (ddd, J = 9.7, 5.0, 5.0, 1H), 1.67 (dd, J = 9.1, 4.3 Hz, 1H), 1.03 (t, J = 7.2 Hz, 3H). ^{13}C NMR (100 MHz, D_2O): δ (ppm) 216.2, 77.3, 72.4, 68.2, 62.7, 42.1, 36.6, 30.1, 29.2, 28.4, 7.4. HRMS: calculated for $[\text{C}_{11}\text{H}_{19}\text{O}_5]^+$ 231.12270, found 231.12276.



(1R,2S,3R,4R,5R,6R,7R)-5,7-bis(hydroxymethyl)bicyclo[4.1.0]heptane-2,3,4-triol (11)

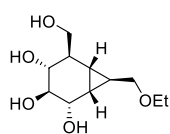
Compound **46** (25.6 mg, 45.1 μmol) was treated according to General procedure for global debenzoylation with $\text{Pd}(\text{OH})_2/\text{C}$ to obtain title compound **11** as a clear oil (8.30 mg, 40.6 μmol , 90%). ^1H NMR (400 MHz, D_2O): δ (ppm) 4.03 (dd, J = 8.7, 6.0 Hz, 1H), 3.90 (dd, J = 10.9, 3.5 Hz, 1H), 3.78 (dd, J = 10.9, 6.1 Hz, 1H), 3.62 (dd, J = 11.6, 6.1 Hz, 1H), 3.33 (dd, J = 11.5, 7.7 Hz, 1H), 3.25 (t, J = 10.1 Hz, 1H), 3.21 – 3.11 (m, 1H), 1.86 – 1.77 (m, 1H), 1.35 (dt, J = 9.0, 5.5 Hz, 1H), 1.13 (dt, J = 11.3, 5.3 Hz, 1H), 1.05 – 0.97 (m, 1H). ^{13}C NMR (100 MHz, D_2O):

δ (ppm) 70.5, 67.0, 66.5, 60.8, 58.6, 40.2, 20.2, 18.8, 14.1. HRMS: calculated for $[\text{C}_9\text{H}_{17}\text{O}_6]^+$ 205.10705, found 205.10701.



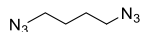
(1R,2S,3R,4R,5R,6R,7R)-2,3,4-tris(benzyloxy)-5-((benzyloxy)methyl)-7-(ethoxymethyl)bicyclo[4.1.0]heptane (46)

Compound **46** (18.0 mg, 32.0 μmol), TBAI (catalytic amount) and NaH (60%, 2.55 mg, 2.0 eq.) were dissolved in DMF (0.3 mL) at 0 °C. After stirring for 5 min EtBr (21 μL , 0.287 mmol, 9.0 eq.) was added and the reaction mixture was allowed to stir at room temperature for 4 h. The reaction mixture was partitioned between H_2O (10 mL) and EtOAc (10 mL) and the organic layer was washed with H_2O (2x) and all the aqueous layers were extracted with EtOAc (1x). The combined organic layers were dried over MgSO_4 , filtered and concentrated *in vacuo*. Purification by column chromatography (10% EtOAc in pentane $\rightarrow$ 20% EtOAc in pentane) gave title compound **47** as a clear oil (11.1 mg, 18.7 μmol , 59%). ^1H NMR (400 MHz, CDCl_3): δ (ppm) 7.48 – 7.11 (m, 20H), 4.91 – 4.72 (m, 4H), 4.66 (d, $J = 11.7$ Hz, 1H), 4.55 – 4.36 (m, 3H), 4.07 (dd, $J = 8.0, 6.2$ Hz, 1H), 3.66 – 3.60 (m, 2H), 3.56 – 3.42 (m, 2H), 3.42 – 3.32 (m, 2H), 3.32 – 3.22 (m, 2H), 1.89 (dd, $J = 6.6, 3.5$ Hz, 1H), 1.34 – 1.28 (m, 1H), 1.17 (t, $J = 7.0$ Hz, 3H), 1.12 – 1.04 (m, 2H). ^{13}C NMR (101 MHz, CDCl_3): δ (ppm) 139.3, 139.2, 138.9, 138.6, 128.6, 128.5, 128.5, 128.2, 128.2, 128.1, 128.0, 127.9, 127.8, 127.7, 127.6, 127.1, 84.9, 80.2, 79.3, 75.7, 75.3, 74.1, 73.3, 71.1, 70.9, 65.8, 43.6, 23.5, 21.6, 20.4, 15.5. HRMS: calculated for $[\text{C}_{39}\text{H}_{45}\text{O}_5]^+$ 593.32615, found 593.32647.



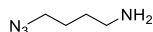
(1R,2S,3R,4R,5R,6R,7R)-7-(ethoxymethyl)-5-(hydroxymethyl)bicyclo[4.1.0]heptane-2,3,4-triol (12)

Compound **47** (11.1 mg, 18.7 μmol) was treated according to General procedure for global debenzoylation with $\text{Pd}(\text{OH})_2/\text{C}$ to obtain title compound **12** as a clear oil (4.1 mg, 17.7 μmol , 94%). ^1H NMR (400 MHz, D_2O): δ (ppm) 4.00 (dd, $J = 8.8, 6.0$ Hz, 1H), 3.87 (dd, $J = 10.9, 3.5$ Hz, 1H), 3.75 (dd, $J = 10.9, 6.0$ Hz, 1H), 3.65 – 3.55 (m, 3H), 3.24 – 3.16 (m, 2H), 3.16 – 3.10 (m, 1H), 1.83 – 1.72 (m, 1H), 1.36 – 1.30 (m, 1H), 1.20 (t, $J = 7.1$ Hz, 3H), 1.13 – 1.05 (m, 1H), 1.08 – 0.95 (m, 1H). ^{13}C NMR (100 MHz, D_2O): δ (ppm) 79.3, 78.2, 75.8, 75.3, 70.5, 67.3, 49.0, 27.6, 26.6, 23.4, 18.5. HRMS: calculated for $[\text{C}_{11}\text{H}_{21}\text{O}_6]^+$ 233.13835, found 233.13843.



1,4-diazidobutane (51)

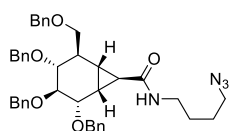
To a solution of 1,4-dibromobutane (2.38 mL, 20.0 mmol) in DMF (20 mL) was added a solution of NaN_3 (2.73 g, 42.0 mmol, 2.1 eq.) in H_2O (10 mL). After stirring overnight at 80 °C, the reaction mixture was cooled down to room temperature and diluted with brine (40 mL) and hexanes (40 mL). The organic layer was separated and the aqueous layer was extracted with hexane (2 x 40 mL). The combined organic layers were dried over MgSO_4 , filtered and concentrated *in vacuo* to give title compound **51** as a clear oil (2.73 g, 19.6 mmol, 98%). Analytical data are in agreement with literature precedence.²⁶



4-azidobutan-1-amine (52)

1,4-Diazidobutane (2.72 g, 19.4 mmol) **51** was dissolved in mixture of Et_2O (12 mL), EtOAc (12 mL) and aqueous HCl (1 M, 40 mL). Triphenylphosphine (5.09 g, 19.4 mmol, 1.00 eq.) was

added portion wise over 1 h at 0 °C. The aqueous layer was separated and then washed with Et₂O (2 x 40 mL). The pH of the aqueous layer was adjusted to pH 13 with aqueous NaOH (2 M). After extraction with DCM (3 x 60 mL), the combined organic layers were dried over MgSO₄, filtered and concentrated *in vacuo* to give title compound as a clear oil **52** (1.70 g, 14.9 mmol, 77%). Analytical data are in agreement with literature precedence.²⁶

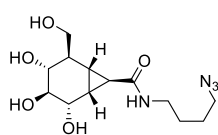


(1R,2S,3R,4R,5R,6R,7R)-N-(4-azidobutyl)-2,3,4-tris(benzyloxy)-5-((benzyloxy)methyl)bicyclo[4.1.0]heptane-7-carboxamide (53) and (1S,2S,3R,4R,5R,6S,7S)-N-(4-azidobutyl)-2,3,4-tris(benzyloxy)-5-((benzyloxy)methyl)bicyclo[4.1.0]heptane-7-carboxamide (54)

N-(4-azidobutyl)-2,3,4-tris(benzyloxy)-5-((benzyloxy)methyl)bicyclo[4.1.0]heptane-7-carboxamide (54)

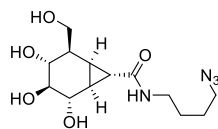
To a mixture of **33** and **34** (0.142 g, 0.234 mmol) in THF (8 mL), was added MeOH (2 mL), H₂O (1 mL) and LiOH (22.4 mg, 0.94 mmol, 4 eq.). After stirring overnight at room temperature, 1 M aqueous HCl solution was used to acidify the reaction mixture to pH 2. The reaction mixture was diluted with EtOAc (20 mL) and washed with brine (10 mL). The aqueous layer was extracted with EtOAc (10 mL) and the combined organic layers were dried over MgSO₄, filtered and concentrated *in vacuo*. Purification by column chromatography (30% EtOAc in pentane) gave the carboxylic acid derivatives as a mixture of alpha- and beta-isomers (119 mg, 0.206 mmol, 82%) as a clear oil. The carboxylic acid derivatives (0.346 g, 0.600 mmol) were dissolved in DCM (6.0 mL) and 4-azidobutan-1-amine **52** (82.2 mg, 0.720 mmol, 1.2 eq.) was added. After addition of DIPEA (364 µL, 2.10 mmol, 3.5 eq.) and HCTU (298 mg, 0.720 mmol, 1.2 eq.), the reaction mixture was stirred overnight at room temperature. The reaction mixture was concentrated *in vacuo*, dissolved in EtOAc (40 mL) and subsequently washed with aqueous HCl (1 M, 2 x 40 mL), saturated aqueous NaHCO₃ (40 mL) and brine (2 x 40 mL), dried over MgSO₄, filtered and concentrated *in vacuo*. Purification by column chromatography (30% EtOAc in pentane) gave the desired product as alpha- and beta-isomers (0.341 g, 0.505 mmol, 78%) as a white solid, which were separated by HPLC purification (C18, linear gradient: 50-90% B in A, solutions used A: H₂O, B: acetonitrile, 0.5% TFA, 15 min). **53** ¹H NMR (400 MHz, CDCl₃): δ (ppm) 7.38 – 7.10 (m, 20H), 5.66 (t, *J* = 5.8 Hz, 1H), 4.90 – 4.69 (m, 4H), 4.61 (d, *J* = 11.6 Hz, 1H), 4.53 – 4.32 (m, 3H), 4.10 (dd, *J* = 8.2, 5.9 Hz, 1H), 3.59 (qd, *J* = 8.8, 3.8 Hz, 2H), 3.42 (t, *J* = 10.1 Hz, 1H), 3.30 (t, *J* = 6.1 Hz, 4H), 3.26 – 3.20 (m, 1H), 2.04 (dt, *J* = 10.0, 5.2 Hz, 1H), 1.94 – 1.85 (m, 1H), 1.82 (ddd, *J* = 9.2, 4.4, 2.2 Hz, 1H), 1.65 – 1.57 (m, 4H), 1.35 (t, *J* = 4.5 Hz, 1H). ¹³C NMR (100 MHz, CDCl₃): δ (ppm) 172.1, 139.1, 138.6, 128.6, 128.6, 128.6, 128.5, 128.3, 128.1, 128.0, 127.9, 127.9, 127.7, 84.4, 79.4, 78.8, 75.7, 75.5, 73.5, 71.6, 70.5, 51.3, 43.4, 39.4, 27.3, 27.1, 26.5, 25.7, 23.8. HRMS: calculated for [C₄₁H₄₇N₄O₅]⁺ 675.35410, found 675.35411. **54** ¹H NMR (400 MHz, CDCl₃): δ (ppm) 7.45 – 7.12 (m, 20H), 5.22 (t, *J* = 5.8 Hz, 1H), 4.87 (d, *J* = 12.3 Hz, 2H), 4.83 – 4.74 (m, 2H), 4.60 (d, *J* = 11.5 Hz, 1H), 4.50 (d, *J* = 11.0 Hz, 1H), 4.37 (d, *J* = 2.4 Hz, 2H), 3.83 (dd, *J* = 9.1, 3.6 Hz, 1H), 3.75 (d, *J* = 7.6 Hz, 1H), 3.66 – 3.52 (m, 2H), 3.34 (t, *J* = 10.2 Hz, 1H), 3.23 (t, *J* = 6.7 Hz, 2H), 3.19 – 3.10 (m, 1H), 3.10 – 3.00 (m, 1H), 2.29 – 2.24 (m, 1H), 1.79 (dd, *J* = 9.1, 4.8 Hz, 1H), 1.70 – 1.63 (m, 1H), 1.55 – 1.44 (m, 2H), 1.43 – 1.30 (m, 3H). ¹³C NMR (100 MHz, CDCl₃): δ (ppm) 172.1, 139.0, 139.0, 138.7, 138.3, 128.7, 128.6, 128.5, 128.3, 128.1, 128.1, 127.9, 127.9, 127.8, 127.7, 127.4, 86.3,

81.6, 75.9, 75.4, 75.4, 73.2, 71.9, 70.3, 51.2, 40.5, 39.2, 27.1, 26.3 25.2, 24.5, 21.5. HRMS: calculated for $[C_{41}H_{47}N_4O_5]^+$ 675.35410, found 675.35436.



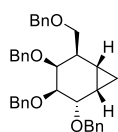
(1R,2S,3R,4R,5R,6R,7R)-N-(4-azidobutyl)-2,3,4-trihydroxy-5-(hydroxymethyl)bicyclo[4.1.0]heptane-7-carboxamide (9)

To a cooled (-78 °C) solution of benzylated **53** (12.7 mg, 18.8 μ mol) in DCM (0.2 mL) was added slowly BCl_3 (1 M in DCM, 0.400 mL, 0.40 mmol, 20 eq.). After stirring for 5 h at -78 °C, the reaction was quenched with MeOH (5 mL) and allowed to warm to room temperature overnight. Concentration *in vacuo*, co-evaporation with toluene (3x) and purification by column chromatography (20% MeOH in DCM) gave compound **9** as a white solid (5.20 mg, 16.5 μ mol, 88%). ^1H NMR (400 MHz, MeOD): δ (ppm) 3.81 (dd, J = 8.7, 5.7 Hz, 1H), 3.72 (dd, J = 10.6, 3.9 Hz, 1H), 3.55 (dd, J = 10.5, 6.5 Hz, 1H), 3.34 – 3.30 (m, 2H), 3.09 (t, J = 6.6 Hz, 2H), 3.03 (t, J = 10.1 Hz, 1H), 2.97 – 2.85 (m, 1H), 1.85 – 1.80 (m, 1H), 1.74 – 1.70 (m, 1H), 1.63 – 1.54 (m, 5H), 1.53 – 1.49 (m, 1H). ^{13}C NMR (100 MHz, MeOD): δ (ppm) 174.8, 76.5, 72.6, 72.2, 64.7, 52.2, 46.4, 40.1, 28.7, 27.9, 27.3, 27.2, 23.2. HRMS: calculated for $[C_{13}H_{22}N_4O_5]^+$ 315.16630, found 315.16635.



(1S,2S,3R,4R,5R,6S,7S)-N-(4-azidobutyl)-2,3,4-trihydroxy-5-(hydroxymethyl)bicyclo[4.1.0]heptane-7-carboxamide (6)

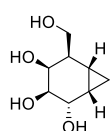
To a cooled (-78 °C) solution of benzylated **54** (12.7 mg, 18.8 μ mol) in DCM (0.2 mL) was added slowly BCl_3 (1 M in DCM, 0.400 mL, 0.40 mmol, 20 eq.). After stirring for 3 h at -78 °C, additional BCl_3 (1 M in DCM, 0.400 mL, 0.376 mmol, 20 eq.) was added. After stirring overnight at -20 °C, the reaction was quenched with MeOH (5 mL) and allowed to warm to room temperature. Concentration *in vacuo*, co-evaporation with MeOH (3 x) and purification by column chromatography (20% MeOH in DCM) gave compound **6** as a slightly yellow solid (5.9 mg, 18.6 μ mol, 99%). ^1H NMR (400 MHz, MeOD): δ (ppm) 3.80 (d, J = 6.3 Hz, 1H), 3.53 – 3.44 (m, 2H), 3.17 – 3.01 (m, 3H), 2.83 (t, J = 9.7 Hz, 1H), 2.07 – 1.90 (m, 1H), 1.76 – 1.61 (m, 1H), 1.54 – 1.44 (m, 4H), 1.44 – 1.38 (m, 1H), 1.34 – 1.28 (m, 1H). ^{13}C NMR (100 MHz, MeOD): δ (ppm) 79.6, 74.5, 70.7, 65.1, 52.3, 43.8, 40.2, 27.9, 27.9, 27.5, 25.0, 24.5. HRMS: calculated for $[C_{13}H_{22}N_4O_5]^+$ 315.16630, found 315.16635.



(1S,2S,3R,4S,5R,6R)-2,3,4-tris(benzyloxy)-5-((benzyloxy)methyl)bicyclo[4.1.0]heptane (56)

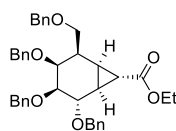
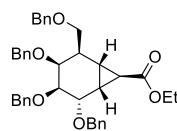
To a solution of DCM (0.5 mL) and 1,2-dimethoxyethane (100 μ L) was added consecutively boron trifluoride ethyl etherate (62 μ L) and diethylzinc (1 M in hexane, 1.0 mL, 1.0 mmol) at room temperature. After stirring for 5 min, diiodomethane (161 μ L, 2.0 mmol) was added and the reaction mixture was stirred for additional 5 min. Compound **55** (52.0 mg, 0.100 mmol) was dissolved in DCM (1.0 mL) and added dropwise to the reaction mixture. After stirring overnight, the reaction mixture was quenched with a saturated aqueous NH_4Cl solution and diluted with EtOAc. The aqueous layer was extracted with EtOAc (3x) and the combined organic layers were washed with brine, dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by column chromatography (pentane $\rightarrow$ 8% EtOAc in pentane) gave benzylated cyclopropane **56** (45.1 mg, 84.3

μmol , 84%) as a clear oil. ^1H NMR (400 MHz, CDCl_3): δ (ppm) 7.47 – 7.21 (m, 20H), 4.86 (dd, $J = 27.0, 11.7$ Hz, 2H), 4.76 – 4.62 (m, 3H), 4.58 (d, $J = 11.7$ Hz, 1H), 4.47 (d, $J = 4.4$ Hz, 2H), 4.39 (dd, $J = 8.4, 6.6$ Hz, 1H), 3.90 (s, 1H), 3.66 – 3.52 (m, 2H), 3.19 (dd, $J = 8.4, 1.1$ Hz, 1H), 1.90 – 1.85 (m, 1H), 1.55 – 1.40 (m, 1H), 0.84 – 0.69 (m, 2H), 0.30 (q, $J = 5.2$ Hz, 1H). ^{13}C NMR (101 MHz, CDCl_3): δ (ppm) 139.4, 139.4, 139.2, 138.4, 128.5, 128.4, 128.4, 128.3, 128.1, 128.0, 127.9, 127.8, 127.5, 127.5, 127.4, 83.3, 76.8, 76.3, 74.0, 73.3, 72.8, 71.3, 42.4, 16.3, 14.0, 11.5. HRMS: calculated for $[\text{C}_{36}\text{H}_{39}\text{O}_4]^+$ 557.26623, found 557.26551.



(1S,2S,3R,4S,5R,6R)-5-(hydroxymethyl)bicyclo[4.1.0]heptane-2,3,4-triol (21)

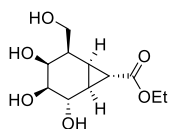
Compound **56** (40.0 mg, 74.8 μmol) was treated according to General procedure for global debenzoylation with $\text{Pd}(\text{OH})_2/\text{C}$ to obtain title compound **21** as a clear oil (13.0 mg, 74.7 μmol , 99%). ^1H NMR (400 MHz, D_2O): δ (ppm) 4.20 (dd, $J = 9.0, 6.6$ Hz, 1H), 3.80 (s, 1H), 3.76 – 3.62 (m, 2H), 3.17 (dd, $J = 9.1, 1.7$ Hz, 1H), 1.83 – 1.78 (m, 1H), 1.42 – 1.32 (m, 1H), 0.82 – 0.75 (m, 2H), 0.25 – 0.18 (m, 1H). ^{13}C NMR (101 MHz, D_2O): δ (ppm) 73.6, 71.8, 69.4, 62.9, 43.3, 17.6, 12.5, 10.4. HRMS: calculated for $[\text{C}_8\text{H}_{14}\text{O}_4\text{Na}]^+$ 197.07843, found 197.07839.



(1R,2S,3R,4S,5R,6R,7R)-Ethyl 2,3,4-tris(benzyloxy)-5-((benzyloxy)methyl)bicyclo[4.1.0]heptane-7-carboxylate and (57) (1R,2S,3R,4S,5R,6S,7S)-Ethyl 2,3,4-tris(benzyloxy)-5-((benzyloxy)methyl)bicyclo[4.1.0]heptane-7-carboxylate

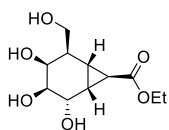
(58)

A 2-necked pear flask was charged with cyclic alkene **55** (0.52 g, 1.0 mmol), $\text{Cu}(\text{acac})_2$ (26 mg, 0.1 mol, 0.1 eq.) and EtOAc (2.2 mL, dried over activated 4 Å molsieves overnight). After stirring under reflux at 90 °C a solution of ethyl diazoacetate (13 wt% DCM, 1.5 mmol, 0.18 mL, 1.5 eq.) in EtOAc (3.1 mL) was added by syringe pump over 6 h. The reaction was concentrated *in vacuo* and purification by column chromatography (50 cv of 5% EtOAc in pentane) gave the desired products **57** (65 mg, 0.107 μmol , 10%), **58** (23 mg, 0.038 μmol , 3.5%) and crude recovered starting material **55** (~80%). **57** ^1H NMR (400 MHz, CDCl_3): δ (ppm) 7.34 – 7.23 (m, 20H), 4.88 (d, $J = 11.6$ Hz, 1H), 4.81 – 4.60 (m, 4H), 4.56 (d, $J = 11.6$ Hz, 1H), 4.50 – 4.40 (m, 2H), 4.37 (dd, $J = 8.2, 6.4$ Hz, 1H), 4.14 – 4.08 (m, 2H), 3.91 (s, 1H), 3.64 (t, $J = 9.0$ Hz, 1H), 3.56 (dd, $J = 8.6, 6.3$ Hz, 1H), 3.15 (d, $J = 8.2$ Hz, 1H), 2.13 – 2.04 (m, 1H), 1.99 – 1.93 (m, 1H), 1.62 – 1.58 (m, 1H), 1.40 – 1.34 (m, 1H), 1.28 – 1.25 (m, 3H). ^{13}C NMR (101 MHz, CDCl_3): δ (ppm) 173.5, 139.2, 139.0, 139.0, 138.2, 128.6, 128.4, 128.3, 128.0, 128.0, 127.9, 127.6, 127.5, 82.9, 76.2, 75.7, 74.1, 73.4, 73.0, 71.6, 70.5, 60.7, 41.4, 27.1, 26.0, 24.5, 14.4. HRMS: calculated for $[\text{C}_{39}\text{H}_{43}\text{O}_6]^+$ 607.30542, found 607.30560. **58** ^1H NMR (400 MHz, CDCl_3): δ (ppm) 7.40 – 7.24 (m, 20H), 4.87 (d, $J = 11.6$ Hz, 1H), 4.78 – 4.65 (m, 4H), 4.53 – 4.40 (m, 3H), 4.14 – 4.08 (m, 2H), 4.08 – 4.02 (m, 1H), 4.00 (d, $J = 8.8$ Hz, 1H), 3.66 – 3.58 (m, 1H), 3.57 – 3.51 (m, 1H), 3.47 (dd, $J = 8.8, 1.7$ Hz, 1H), 2.47 – 2.38 (m, 1H), 2.09 (t, $J = 4.6$ Hz, 1H), 1.72 (dd, $J = 9.4, 4.4$ Hz, 1H), 1.59 – 1.52 (m, 1H), 1.22 (s, 3H). ^{13}C NMR (101 MHz, CDCl_3): δ (ppm) 174.0, 139.2, 138.9, 138.6, 138.3, 128.6, 128.5, 128.4, 128.3, 128.0, 127.8, 127.8, 127.7, 127.6, 127.6, 127.6, 127.4, 84.3, 77.7, 74.9, 74.8, 73.4, 72.9, 72.7, 70.4, 60.7, 38.4, 26.1, 23.1, 22.6, 14.4. HRMS: calculated for $[\text{C}_{39}\text{H}_{43}\text{O}_6]^+$ 607.30542, found 607.30550.



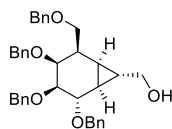
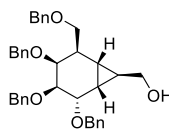
(1S,2S,3R,4S,5R,6S,7S)-Ethyl 2,3,4-trihydroxy-5-(hydroxymethyl)bicyclo[4.1.0]heptane-7-carboxylate (13)

Ester **58** (20 mg, 33 μ mol) was treated according to the General procedure for global debenzoylation with 10% Pd on carbon. Purification by column chromatography (acetone $\rightarrow$ 1% MeOH in acetone) gave β -debenzylated ester **13** (1.1 mg, 4.5 μ mol, 14%) as a clear oil. ^1H NMR (400 MHz, D_2O): δ (ppm) 4.17 – 4.07 (m, 2H), 4.01 – 3.97 (m, 1H), 3.88 (d, J = 9.1 Hz, 1H), 3.78 – 3.65 (m, 2H), 3.43 (dd, J = 9.0, 2.3 Hz, 1H), 2.38 – 2.30 (m, 1H), 2.02 (t, J = 4.7 Hz, 1H), 1.74 – 1.67 (m, 1H), 1.66 – 1.60 (m, 1H), 1.24 (t, J = 7.2 Hz, 3H). ^{13}C NMR (101 MHz, D_2O): δ (ppm) 176.5, 75.5, 69.0, 68.7, 62.0, 61.9, 38.6, 27.7, 23.3, 22.7, 13.3. HRMS: calculated for $[\text{C}_{11}\text{H}_{19}\text{O}_6]^+$ 247.11761, found 247.11773.



(1R,2S,3R,4S,5R,6R,7R)-Ethyl 2,3,4-trihydroxy-5-(hydroxymethyl)bicyclo[4.1.0]heptane-7-carboxylate (18)

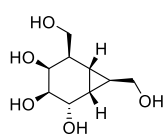
Benzylated ester **57** (115 mg, 190 μ mol) was dissolved in EtOAc (2 mL) and AcOH (0.4 mL) was added. The reaction mixture was purged with argon gas and Pd(OH) $_2$ /C (20 mg) was added. After the reaction vessel was purged with hydrogen gas and vigorously stirring overnight, the palladium catalyst was filtered off over a pad of Celite followed by concentration *in vacuo*. Purification by column chromatography (acetone $\rightarrow$ 2% MeOH in acetone) gave compound **18** (4.2 mg, 17.0 μ mol, 9%) as a clear oil. ^1H NMR (400 MHz, D_2O): δ (ppm) 4.25 (dd, J = 9.1, 6.3 Hz, 1H), 4.17 – 4.10 (m, 2H), 3.86 (s, 1H), 3.78 – 3.66 (m, 2H), 3.22 (dd, J = 9.1, 1.8 Hz, 1H), 2.09 – 2.02 (m, 1H), 2.01 – 1.93 (m, 1H), 1.70 (t, J = 4.6 Hz, 1H), 1.47 – 1.40 (m, 1H), 1.24 (t, J = 7.2 Hz, 3H). ^{13}C NMR (101 MHz, D_2O): δ (ppm) 175.7, 73.3, 71.1, 67.7, 62.2, 62.0, 42.2, 28.8, 25.5, 23.7, 13.4. HRMS: calculated for $[\text{C}_{11}\text{H}_{19}\text{O}_6]^+$ 247.11761, found 247.11729



((1R,2S,3R,4S,5R,6R,7R)-2,3,4-tris(benzyloxy)-5-((benzyloxy)methyl)bicyclo[4.1.0]heptan-7-yl)methanol (59) and ((1S,2S,3R,4S,5R,6S,7S)-2,3,4-tris(benzyloxy)-5-((benzyloxy)methyl)bicyclo[4.1.0]heptan-7-yl)methanol (60)

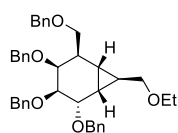
A mixture of **57** and **58** (0.254 g, 0.420 mmol) was dissolved in DCM (2.1 mL) at 0 $^\circ\text{C}$, after which DIBAL (1 M in hexanes, 1.05 mmol, 2.5 eq.) was added dropwise. After the mixture was stirred for 1 h at 0 $^\circ\text{C}$ the reaction was quenched with EtOAc, diluted with DCM and washed with 1 M aqueous HCl. The layers were separated and the aqueous layer was extracted three times with DCM. The combined organic layers were washed with saturated aqueous NaHCO_3 and brine, dried over MgSO_4 , filtered and concentrated *in vacuo*. Purification by column chromatography (pentane $\rightarrow$ 30% EtOAc in pentane) yielded compound **59** 94.5 mg, 0.167 mmol, 40%) and compound **60** (85.7 mg, 0.152 mmol, 36%) as yellow oils. **59** ^1H NMR (400 MHz, CDCl_3): δ (ppm) 7.42 – 7.21 (m, 20H), 4.88 (d, J = 11.6 Hz, 1H), 4.78 (d, J = 11.6 Hz, 1H), 4.73 – 4.64 (m, 3H), 4.57 (d, 11.6 Hz, 1H), 4.52 – 4.40 (m, 2H), 4.35 (t, J = 8.0 Hz, 1H), 3.89 (s, 1H), 3.65 – 3.51 (m, 2H), 3.44 (dd, J = 11.2, 6.7 Hz, 1H), 3.35 (dd, J = 11.2, 6.8 Hz, 1H), 3.17 (d, J = 8.3 Hz, 1H), 1.93 – 1.79 (m, 1H), 1.36 – 1.30 (m, 1H), 1.07 – 0.99 (m, 1H), 0.72 – 0.63 (m, 1H). ^{13}C NMR (101 MHz, CDCl_3): δ (ppm) 139.3, 139.3, 139.0, 138.3, 128.5, 128.5, 128.4,

128.3, 128.1, 128.0, 127.9, 127.8, 127.6, 127.5, 127.5, 83.6, 76.9, 76.1, 74.0, 73.3, 72.9, 71.7, 71.1, 66.6, 41.8, 27.4, 22.1, 19.5. HRMS: calculated for $[C_{37}H_{41}O_5]^+$ 565.29485, found 565.29480. **60** ^1H NMR (400 MHz, $CDCl_3$): δ (ppm) 7.41 – 7.20 (m, 20H), 4.85 (d, J = 11.6 Hz, 1H), 4.78 – 4.67 (m, 4H), 4.53 – 4.41 (m, 3H), 4.01 – 3.92 (m, 2H), 3.64 – 3.52 (m, 2H), 3.50 – 3.41 (m, 2H), 3.16 (dd, J = 11.1, 7.7 Hz, 1H), 2.47 – 2.38 (m, 1H), 1.51 – 1.48 (m, 1H), 1.01 – 0.95 (m, 1H), 0.86 – 0.79 (m, 1H). ^{13}C NMR (101 MHz, $CDCl_3$): δ (ppm) 139.3, 139.1, 139.0, 138.4, 128.6, 128.5, 128.4, 128.3, 128.0, 127.9, 127.8, 127.7, 127.6, 127.6, 127.5, 127.4, 84.4, 78.2, 75.4, 74.7, 73.5, 72.8, 72.8, 71.0, 67.0, 37.9, 23.2, 21.5, 18.3. HRMS: calculated for $[C_{37}H_{41}O_5]^+$ 565.29485, found 565.29472.



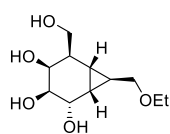
(1R,2S,3R,4S,5R,6R,7R)-5,7-bis(hydroxymethyl)bicyclo[4.1.0]heptane-2,3,4-triol (22)

Alcohol **59** (30.0 mg, 53 μ mol) was treated according to General procedure for global debenzoylation with 10% Pd on carbon to obtain the title compound **22** (10.9 mg, 53 μ mol, quant.) as a clear oil. ^1H NMR (400 MHz, D_2O): δ (ppm) 4.20 (dd, J = 9.1, 6.6 Hz, 1H), 3.85 – 3.82 (m, 1H), 3.78 – 3.66 (m, 2H), 3.58 (dd, J = 11.6, 6.1 Hz, 1H), 3.27 (dd, J = 11.6, 7.8 Hz, 1H), 3.20 (dd, J = 9.1, 1.8 Hz, 1H), 1.90 – 1.82 (m, 1H), 1.36 – 1.29 (m, 1H), 1.05 – 0.97 (m, 1H), 0.77 – 0.70 (m, 1H). ^{13}C NMR (101 MHz, D_2O): δ (ppm) 74.0, 71.6, 68.6, 65.3, 62.7, 42.7, 25.8, 23.2, 18.3. HRMS: calculated for $[C_9H_{16}O_5Na]^+$ 227.08899, found 227.08899



(1R,2S,3R,4S,5R,6R,7R)-2,3,4-tris(benzyloxy)-5-((benzyloxy)methyl)-7-(ethoxymethyl)bicyclo[4.1.0]heptane (61)

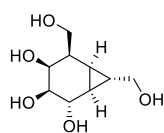
To a solution of alcohol **59** (33.9 mg, 60.0 μ mol) and TBAI (10 mg) in DMF (0.8 mL) was added NaH (60% dispersion in mineral oil, 19.2 mg, 0.480 mmol, 8 eq.) at 0 $^{\circ}C$. After stirring the reaction mixture for 5 min, EtBr (20 μ L, 0.36 mmol, 6 eq.) was added and the reaction mixture was stirred overnight at room temperature. The reaction mixture was quenched with H_2O (3 mL) and diluted with EtOAc (20 mL). The organic layer was separated and washed with saturated aqueous $NaHCO_3$, H_2O (3x) and brine. The combined aqueous layers were extracted with EtOAc and the combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by column chromatography (pentane $\rightarrow$ 20% EtOAc in pentane) gave ether **61** as a yellow oil (28.5 mg, 48.1 μ mol, 80%). ^1H NMR (400 MHz, $CDCl_3$): δ (ppm) 7.43 – 7.21 (m, 20H), 4.88 (d, J = 11.6 Hz, 1H), 4.83 (d, J = 11.6 Hz, 1H), 4.77 – 4.61 (m, 3H), 4.58 (d, J = 11.7 Hz, 1H), 4.52 – 4.40 (m, 2H), 4.39 – 4.33 (m, 1H), 3.90 (s, 1H), 3.68 – 3.53 (m, 2H), 3.52 – 3.37 (m, 2H), 3.35 – 3.22 (m, 2H), 3.18 (d, J = 8.3 Hz, 1H), 1.97 – 1.87 (m, 1H), 1.41 – 1.35 (m, 1H), 1.15 (t, J = 7.0 Hz, 3H), 1.04 (p, J = 6.2 Hz, 1H), 0.70 – 0.64 (m, 1H). ^{13}C NMR (101 MHz, $CDCl_3$): δ (ppm) 139.5, 139.4, 139.2, 138.4, 128.5, 128.4, 128.3, 128.3, 128.1, 127.9, 127.8, 127.5, 127.5, 127.4, 83.6, 76.9, 76.2, 74.2, 74.0, 73.3, 72.9, 71.1, 71.0, 65.8, 41.8, 24.5, 21.6, 20.0, 15.4. HRMS: calculated for $[C_{39}H_{45}O_5]^+$ 593.32615, found 593.32617



(1R,2S,3R,4S,5R,6R,7R)-7-(ethoxymethyl)-5-(hydroxymethyl)bicyclo[4.1.0]heptane-2,3,4-triol (23)

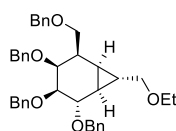
Ether **61** (20 mg, 34 μ mol) was treated according to General procedure for global debenzoylation with 10% Pd on carbon to obtain the title compound **23** (8.0 mg, 35

μmol , quant.) as a clear oil. ^1H NMR (400 MHz, D_2O): δ (ppm) 4.22 – 4.18 (dd, $J = 6.4, 9.2$ Hz, 1H), 3.83 (s, 1H), 3.75 – 3.67 (m, 2H), 3.63 – 3.51 (m, 3H), 3.23 – 3.13 (m, 2H), 1.91 – 1.83 (m, 1H), 1.38 – 1.28 (m, 1H), 1.17 (t, $J = 7.1$ Hz, 3H), 1.05 – 0.96 (m, 1H), 0.80 – 0.73 (m, 1H). ^{13}C NMR (101 MHz, D_2O): δ (ppm) 73.9, 73.8, 71.5, 68.6, 66.1, 62.6, 42.6, 23.3, 23.2, 18.7, 14.1. HRMS: calculated for $[\text{C}_{11}\text{H}_{20}\text{O}_5\text{Na}]^+$ 255.12029, found 255.12034



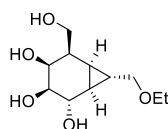
(1S,2S,3R,4S,5R,6S,7S)-5,7-bis(hydroxymethyl)bicyclo[4.1.0]heptane-2,3,4-triol (16)

Alcohol **60** (35 mg, 62 μmol) was treated according to General procedure for global debenzoylation with 10% Pd on carbon to obtain the title compound **16** (11.2 mg, 54 μmol , 87%) as a clear oil. ^1H NMR (400 MHz, D_2O): δ (ppm) 4.01 – 3.95 (m, 1H), 3.83 (d, $J = 8$ Hz 1H), 3.78 – 3.64 (m, 2H), 3.41 – 3.31 (m, 3H), 2.29 – 2.20 (m, 1H), 1.31 – 1.21 (m, 1H), 1.02 – 0.92 (m, 1H), 0.92 – 0.84 (m, 1H). ^{13}C NMR (101 MHz, D_2O): δ (ppm) 76.0, 69.6, 69.5, 65.6, 62.4, 38.8, 22.6, 21.9, 17.8. HRMS: calculated for $[\text{C}_9\text{H}_{17}\text{O}_5]^+$ 205.10705, found 205.10753



(1S,2S,3R,4S,5R,6S,7S)-2,3,4-tris(benzyloxy)-5-((benzyloxy)methyl)-7-(ethoxymethyl)bicyclo[4.1.0]heptane (62)

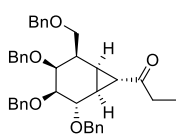
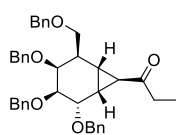
To a solution of alcohol **60** (38.0 mg, 67.3 μmol) and a catalytic amount of TBAI in DMF (0.8 mL) was added NaH (60% dispersion in mineral oil, 21.4 mg, 0.536 mmol 8 eq.) at 0 $^\circ\text{C}$. After stirring the reaction mixture for 5 min, EtBr (50 μL , 0.66 mmol, 10 eq.) was added and the reaction mixture was stirred overnight. The reaction mixture was quenched with H_2O (3 mL) and diluted with EtOAc (20 mL). The organic layer was separated and washed with saturated aqueous NaHCO_3 , H_2O (3x) and brine. The combined aqueous layers were extracted with EtOAc and the combined organic layers were dried over MgSO_4 , filtered and concentrated *in vacuo*. Purification by column chromatography (pentane $\rightarrow$ 20% EtOAc in pentane) gave ether **62** as a yellow oil (29.6 mg, 50.0 μmol , 74%). ^1H NMR (400 MHz, CDCl_3): δ (ppm) 7.40 – 7.22 (m, 20H), 4.87 (d, $J = 11.6$ Hz, 1H), 4.80 – 4.67 (m, 4H), 4.49 – 4.44 (m, 3H), 4.03 (dd, $J = 3.8, 1.7$ Hz, 1H), 3.99 (d, $J = 8.7$ Hz, 1H), 3.68 – 3.61 (m, 1H), 3.59 – 3.50 (m, 1H), 3.47 – 3.40 (m, 3H), 3.20 (d, $J = 6.8$ Hz, 2H), 2.40 – 2.37 (m, 1H), 1.51 – 1.43 (m, 1H), 1.17 (t, $J = 7.0$ Hz, 3H), 0.99 (dd, $J = 8.9, 4.4$ Hz, 1H), 0.85 – 0.77 (m, 1H). ^{13}C NMR (101 MHz, CDCl_3): δ (ppm) 139.5, 139.2, 139.0, 138.5, 128.5, 128.4, 128.4, 128.3, 128.3, 128.2, 128.1, 127.9, 127.9, 127.7, 127.6, 127.5, 127.5, 127.5, 127.4, 127.3, 84.8, 78.3, 75.4, 74.8, 74.4, 73.5, 72.7, 72.4, 71.0, 65.6, 38.6, 21.3, 20.5, 18.4, 15.4. HRMS: calculated for $[\text{C}_{39}\text{H}_{45}\text{O}_5]^+$ 593.32615, found 593.32603.



(1S,2S,3R,4S,5R,6S,7S)-7-(ethoxymethyl)-5-(hydroxymethyl)bicyclo[4.1.0]heptane-2,3,4-triol (17)

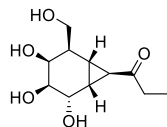
Ether **62** (23.0 mg, 39 μmol) was treated according to General procedure for global debenzoylation with 10% Pd on carbon to obtain the title compound **17** (9.0 mg, 39 μmol , quant.) as a clear oil. ^1H NMR (400 MHz, D_2O): δ (ppm) 4.02 – 3.95 (m, 1H), 3.84 (d, $J = 8.9$ Hz, 1H), 3.75 – 3.67 (m, 2H), 3.62 – 3.52 (m, 2H), 3.42 – 3.33 (m, 2H), 3.29 – 3.22 (m, 1H), 2.31 – 2.21 (m, 1H), 1.32 – 1.26 (m, 1H), 1.18 (t, $J = 7.1$ Hz, 3H), 1.02 – 0.96 (m, 1H), 0.93 –

0.86 (m, 1H). ^{13}C NMR (101 MHz, D_2O): δ (ppm) 75.9, 74.2, 69.5, 69.4, 65.9, 62.3, 38.9, 22.3, 20.1, 17.9, 14.1. HRMS: calculated for $[\text{C}_{11}\text{H}_{21}\text{O}_5]^+$ 233.13835, found 233.13951



(1R,2S,3R,4S,5R,6R,7S)-1-(2,3,4-tris(benzyloxy)-5-((benzyloxy)methyl)bicyclo[4.1.0]heptan-7-yl)propan-1-one (63) and (1S,2S,3R,4S,5R,6S,7S)-1-(2,3,4-tris(benzyloxy)-5-((benzyloxy)methyl)bicyclo[4.1.0]heptan-7-yl)propan-1-one (64)

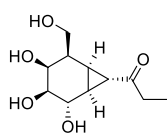
To a mixture of **57** and **58** (126 mg, 0.207 mmol) in THF (2.7 mL) was added $\text{Me}(\text{MeO})\text{NH}\cdot\text{HCl}$ (26.3 mg, 0.270 mmol, 1.3 eq.) at -8°C . Subsequently, EtMgBr (1 M in THF, 1.7 mL, 1.7 mmol, 8.4 eq.) was added over 2 h. After the mixture had stirred overnight, it was quenched with NaOH (0.5 M). The reaction mixture was extracted with EtOAc (10 mL), after which the organic layer was washed with aqueous HCl (3 M). The organic layer was dried over MgSO_4 , filtered, concentrated *in vacuo* and co-evaporated with toluene (3x). The residue was dissolved in THF (1 mL) and EtMgBr (1 M in THF, 0.60 mmol, 3 eq.) was added over 2 min at -20°C . After the reaction was allowed to come to room temperature and stirred for 3 h, the reaction was quenched with a saturated aqueous NH_4Cl solution. After extraction with EtOAc (10 mL), the organic layer was dried over MgSO_4 , filtered and concentrated *in vacuo*. Purification by column chromatography (8% EtOAc in pentane $\rightarrow$ 10% EtOAc in pentane) followed by purification by HPLC (C18, linear gradient: 50-90% B in A, solutions used A: H_2O , B: acetonitrile, 0.1% TFA, 15 min) gave compound **63** (19.4 mg, 32.8 μmol , 16%) and compound **64** (25.2 mg, 42.6 μmol , 21%). **63** ^1H NMR (400 MHz, CDCl_3): δ (ppm) 7.39 – 7.22 (m, 20 H), 4.88 (d, $J = 11.6$ Hz, 1H), 4.76 – 4.52 (m, 5H), 4.44 (d, $J = 3.1$ Hz, 2H), 4.35 (dd, $J = 8.3$, 6.3 Hz, 1H), 3.92 (s, 1H), 3.63 (t, $J = 9.0$ Hz, 1H), 3.57 – 3.50 (m, 1H), 3.19 (d, $J = 7.6$ Hz, 1H), 2.56 (d, $J = 8.0$ Hz, 1H), 2.52 (d, $J = 8.0$ Hz, 1H), 2.14 – 2.07 (m, 1H), 1.98 – 1.87 (m, 2H), 1.48 – 1.39 (m, 1H), 1.06 (t, $J = 7.3$ Hz, 3H). ^{13}C NMR (101 MHz, CDCl_3): δ (ppm) 209.8, 139.2, 139.0, 138.9, 138.2, 128.6, 128.5, 128.4, 128.3, 128.0, 127.9, 127.9, 127.9, 127.7, 127.5, 127.5, 83.2, 76.5, 75.6, 74.1, 73.5, 72.9, 71.6, 70.6, 41.7, 37.1, 33.7, 29.9, 26.6, 8.1. HRMS: calculated for $[\text{C}_{39}\text{H}_{43}\text{O}_5]^+$ 591.31050, found 591.31043. **64** ^1H NMR (400 MHz, CDCl_3): δ (ppm) 7.39 – 7.22 (m, 20H), 4.90 (d, $J = 11.6$ Hz, 1H), 4.80 – 4.60 (m, 4H), 4.53 – 4.38 (m, 3H), 4.07 – 4.01 (m, 1H), 3.96 (d, $J = 8.7$ Hz, 1H), 3.58 (t, $J = 8.8$ Hz, 1H), 3.54 – 3.44 (m, 2H), 2.50 – 2.39 (m, 3H), 1.78 (dd, $J = 9.2$, 4.4 Hz, 1H), 1.60 – 1.5 (m, 1H), 1.03 (t, $J = 7.3$ Hz, 3H). ^{13}C NMR (101 MHz, CDCl_3): δ (ppm) 210.4, 139.4, 138.9, 138.5, 138.3, 128.6, 128.5, 128.4, 128.4, 128.0, 127.9, 127.8, 127.7, 127.6, 127.5, 127.4, 84.2, 77.9, 75.1, 74.9, 73.5, 72.9, 72.5, 70.4, 38.5, 37.1, 30.0, 28.1, 25.9, 8.2. HRMS: calculated for $[\text{C}_{39}\text{H}_{43}\text{O}_5]^+$ 591.31050, found 591.31025.



(1R,2S,3R,4S,5R,6R,7R)-1-(2,3,4-trihydroxy-5-(hydroxymethyl)bicyclo[4.1.0]heptan-7-yl)propan-1-one (19)

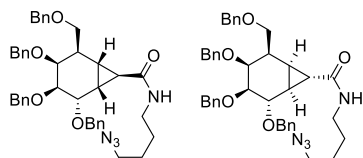
Ethyl ketone **63** (14.8 mg, 25 μmol) was treated according to General procedure for global debenzoylation with 10% Pd on carbon to obtain the title compound **19** (4.6 mg, 20 μmol , 80%) as a white solid. ^1H NMR (400 MHz, D_2O): δ (ppm) 4.26 (dd, $J = 9.0$, 6.1 Hz, 1H), 3.89 (s, 1H), 3.81 – 3.65 (m, 2H), 3.29 (dd, $J = 9.1$, 1.6 Hz, 1H), 2.74 (d, $J = 8.0$ Hz, 1H), 2.70 (d, $J = 8.0$ Hz, 1H), 2.18 (t, $J = 4.6$ Hz, 1H), 2.11 – 2.04 (m, 1H), 2.03 – 1.96 (m, 1H), 1.50

– 1.42 (m, 1H), 1.04 (t, $J = 7.3$ Hz, 3H). ^{13}C NMR (101 MHz, D_2O): δ (ppm) 216.2, 73.4, 71.1, 68.0, 62.3, 42.4, 36.5, 33.2, 31.8, 26.5, 7.4. HRMS: calculated for $[\text{C}_{11}\text{H}_{19}\text{O}_5]^+$ 231.12270, found 231.12260.



(1S,2S,3R,4S,5R,6S,7S)-1-(2,3,4-trihydroxy-5-(hydroxymethyl)bicyclo[4.1.0]heptan-7-yl)propan-1-one (14)

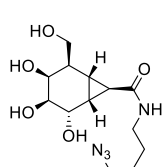
Ethyl ketone **64** (12.0 mg, 20 μmol) was treated according to General procedure for global debenzoylation with 10% Pd on carbon to obtain the title compound **14** (4.0 mg, 18 μmol , 88%) as a white solid. ^1H NMR (400 MHz, D_2O): δ (ppm) 3.93 (t, $J = 2.8$ Hz, 1H), 3.81 (d, $J = 9.2$ Hz, 1H), 3.72 – 3.55 (m, 2H), 3.36 (dd, $J = 9.0, 2.3$ Hz, 1H), 2.64 – 2.54 (m, 2H), 2.37 (t, $J = 4.6$ Hz, 1H), 2.30 – 2.21 (m, 1H), 1.66 – 1.56 (m, 2H), 0.96 (t, $J = 7.4$ Hz, 3H). ^{13}C NMR (101 MHz, D_2O): δ (ppm) 217.1, 75.4, 69.0, 68.8, 62.0, 38.8, 36.5, 30.4, 26.6, 7.6. HRMS: calculated for $[\text{C}_{11}\text{H}_{19}\text{O}_5]^+$ 231.12270, found 231.12283.



(1R,2S,3R,4S,5R,6R,7R)-N-(4-azidobutyl)-2,3,4-tris(benzyloxy)-5-((benzyloxy)methyl)bicyclo[4.1.0]heptane-7-carboxamide (65)
(1S,2S,3R,4S,5R,6S,7S)-N-(4-azidobutyl)-2,3,4-tris(benzyloxy)-5-((benzyloxy)methyl)bicyclo[4.1.0]heptane-7-carboxamide (66)

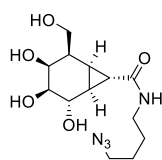
To a mixture of **57** and **58** (0.260 g, 0.428 mmol) in THF (16 mL), EtOH (4 mL), H_2O (3 mL) and MeOH (0.5 mL), was added LiOH (150 mg, 6.3 mmol, 14.7 eq.). After the mixture was stirred overnight at room temperature, 1 M aqueous HCl solution was used to acidify to pH 1, and the reaction mixture was partitioned between EtOAc (125 mL) and brine (30 mL). The aqueous layer was extracted with EtOAc (50 mL) and the combined organic layers were dried over MgSO_4 , filtered and concentrated *in vacuo*, resulting in a yellow oil. The crude acid was used without further purification. HRMS: calculated for $[\text{C}_{37}\text{H}_{39}\text{O}_6]^+$ 579.27412, found 579.27384. The crude acid was dissolved in DCM (7.6 mL) to this were added 4-azidobutan-1-amine **52** (52 mg, 0.456 mmol, 1.1 eq.), *N,N*-diisopropylethylamine (230 μL , 1.33 mmol, 3.1 eq.) and HCTU (188 mg, 0.456 mmol, 1.1 eq.). After stirring overnight at room temperature, the reaction mixture was concentrated *in vacuo*, redissolved in EtOAc (40 mL), washed with 1 M aqueous HCl and saturated aqueous NaHCO_3 , dried over MgSO_4 , filtered and concentrated *in vacuo*. Purification by column chromatography (pentane $\rightarrow$ 30% EtOAc in pentane) followed by purification by HPLC (C18, linear gradient: 70-90% B in A, , solutions used A: H_2O , B: acetonitrile, 0.1% TFA, 15 min) afforded compound **65** (68.9 mg, 0.102 mmol, 24%) and compound **66** (51.0 mg, 75.6 μmol , 18%) as white solids. **65** ^1H NMR (400 MHz, CDCl_3): δ (ppm) 7.39 – 7.21 (m, 20H), 5.70 – 5.63 (m, 1H), 4.88 (d, $J = 11.6$ Hz, 1H), 4.75 – 4.55 (m, 5H), 4.44 (d, $J = 2.7$ Hz, 2H), 4.39 (dd, $J = 8.3, 6.5$ Hz, 1H), 3.93 (s, 1H), 3.66 (t, $J = 8$ Hz, 1H), 3.58 – 3.54 (m, 1H), 3.31 – 3.22 (m, 4H), 3.13 (d, $J = 8.4$ Hz, 1H), 2.15 – 2.05 (m, 1H), 1.93 – 1.85 (m, 1H), 1.64 – 1.48 (m, 4H), 1.45 – 1.36 (m, 1H), 1.31 – 1.23 (m, 1H). ^{13}C NMR (101 MHz, CDCl_3): δ (ppm) 172.1, 139.2, 138.9, 138.8, 138.2, 128.5, 128.4, 128.4, 128.3, 128.1, 127.9, 127.9, 127.7, 127.5, 127.5, 83.1, 76.4, 75.5, 74.1, 73.5, 72.7, 71.5, 70.6, 51.1, 41.6, 39.2, 28.1, 27.2, 26.3, 26.0, 23.0. HRMS: calculated for $[\text{C}_{41}\text{H}_{47}\text{N}_4\text{O}_5]^+$ 675.35410, found 675.35401. **66** ^1H NMR (400 MHz, CDCl_3): δ (ppm) 7.40 – 7.24 (m, 20H), 5.56 (t, $J = 5.6$ Hz, 1H), 4.86 (d, $J = 11.2$ Hz, 1H), 4.81 – 4.61 (m, 4H), 4.54 – 4.42 (m, 3H), 4.06 – 4.05 (m, 1H), 3.96 (d, $J = 14.8$ Hz, 1H), 3.60 (t, $J = 9.0$ Hz, 1H), 3.56 – 3.52 (m, 1H), 3.49 (dd, $J = 8.7, 1.6$ Hz, 1H),

3.31 – 3.16 (m, 4H), 2.51 – 2.42 (m, 1H), 1.77 – 1.72 (m, 2H), 1.60 – 1.51 (m, 5H). ^{13}C NMR (101 MHz, CDCl_3): δ (ppm) 172.8, 139.4, 138.9, 138.6, 138.3, 128.5, 128.4, 128.4, 128.4, 128.0, 128.0, 127.9, 127.9, 127.8, 127.8, 127.6, 127.5, 84.3, 77.7, 75.2, 75.1, 73.4, 72.9, 72.2, 70.3, 51.1, 39.3, 38.1, 27.1, 26.3, 24.5, 24.4, 21.7. HRMS: calculated for $[\text{C}_{41}\text{H}_{47}\text{N}_4\text{O}_5]^+$ 675.35410, found 675.35385.



(1R,2S,3R,4S,5R,6R,7R)-N-(4-azidobutyl)-2,3,4-trihydroxy-5-(hydroxymethyl)bicyclo[4.1.0]heptane-7-carboxamide (20)

To a solution of benzylated **65** (62.5 mg, 93 μmol) in DCM (0.46 mL) was added slowly BCl_3 (1 M in DCM, 1.9 mL, 1.9 mmol, 20 eq.) at -78°C . After stirring for 4 h at -78°C , the reaction mixture was quenched with MeOH (3 mL), concentrated *in vacuo* and co-evaporated with toluene (3x). Purification by column chromatography (EtOAc $\rightarrow$ 20% MeOH in EtOAc) gave title compound **20** (29.0 mg, 93 μmol , quant.) as a white solid. ^1H NMR (400 MHz, D_2O): δ (ppm) 4.25 (dd, $J = 9.1, 6.3$ Hz, 1H), 3.85 (s, 1H), 3.77 – 3.65 (m, 2H), 3.33 (t, $J = 6.2$ Hz, 2H), 3.28 – 3.14 (m, 3H), 1.97 – 1.87 (m, 2H), 1.66 – 1.52 (m, 5H), 1.31 – 1.25 (m, 1H). ^{13}C NMR (101 MHz, D_2O): δ (ppm) 174.6, 73.5, 71.1, 67.8, 62.3, 50.8, 42.2, 39.1, 27.0, 27.0, 25.8, 25.4, 21.8. HRMS: calculated for $[\text{C}_{13}\text{H}_{23}\text{N}_4\text{O}_5]^+$ 315.16630, found 315.16638.



(1S,2S,3R,4S,5R,6S,7S)-N-(4-azidobutyl)-2,3,4-trihydroxy-5-(hydroxymethyl)bicyclo[4.1.0]heptane-7-carboxamide (15)

To a solution of benzylated **66** (45.0 mg, 66 μmol) in DCM (0.50 mL) was added slowly BCl_3 (1 M in DCM, 1.5 mL, 1.5 mmol, 23 eq.). After stirring for 4 h at -78°C , the reaction mixture was quenched with MeOH (3 mL), concentrated *in vacuo* and co-evaporated with toluene (3x). Purification by column chromatography (EtOAc $\rightarrow$ 20% MeOH in EtOAc) gave title compound **15** (14.6 mg, 46.4 μmol , 70%) as a white solid. ^1H NMR (400 MHz, D_2O): δ (ppm) 3.98 (t, $J = 4.0$ Hz, 1H), 3.84 (d, $J = 9.0$ Hz, 1H), 3.74 – 3.62 (m, 2H), 3.41 (dd, $J = 9.0, 2.3$ Hz, 1H), 3.31 (t, $J = 6.4$ Hz, 2H), 3.18 (t, $J = 6.3$ Hz, 2H), 2.34 – 2.25 (m, 1H), 1.91 (t, $J = 4.7$ Hz, 1H), 1.64 – 1.43 (m, 6H). ^{13}C NMR (101 MHz, D_2O): δ (ppm) 175.3, 75.5, 69.0, 68.9, 61.9, 50.7, 38.9, 38.5, 25.8, 25.7, 25.3, 24.0, 21.4. HRMS: calculated for $[\text{C}_{13}\text{H}_{23}\text{N}_4\text{O}_5]^+$ 315.16630, found 315.16615.

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7

Biological evaluation of carba-cyclophellitols¹

7.1 Introduction

The diverse conformational pathways of glycosidases^{2,3} (for example Figure 7.1A) coupled to their phenomenal transition-state stabilization⁴ offers a powerful route to selective enzyme inhibition. One of the main goals of the field, very rarely achieved, is to design and apply conformationally-restricted inhibitors in order to provide both potency and specificity; conformationally-biased inhibitors that target specific classes of glycoside hydrolase (GH) would be of considerable use as cellular and mechanistic probes and potential as starting points for therapeutic compounds. Cyclophellitol (**1**) satisfy the criterion as a potential conformational analogue of the oxocarbenium ion transition-state during β -glucosidase-mediated hydrolysis of a β -glucosidic linkage. Herein, the enzyme catalytic nucleophile attacks at “C1” of cyclophellitol forming a

covalent enzyme-substrate adduct (Figure 7.1B). In a similar fashion cyclophellitol aziridine (**2**) is a potent covalent inhibitor. Carba-cyclophellitols (compounds in which the epoxide- or aziridine moiety in **1** or **2**, respectively, is substituted with a cyclopropane moiety), are cyclophellitol analogues that, while harboring the conformational properties, would be inert towards nucleophiles. Indeed, mimicking the transition-state conformation of the oxocarbenium ion of β -glucosidases by carba-cyclophellitol would result in a new class of potential competitive β -glucosidase inhibitors (Figure 7.1C).

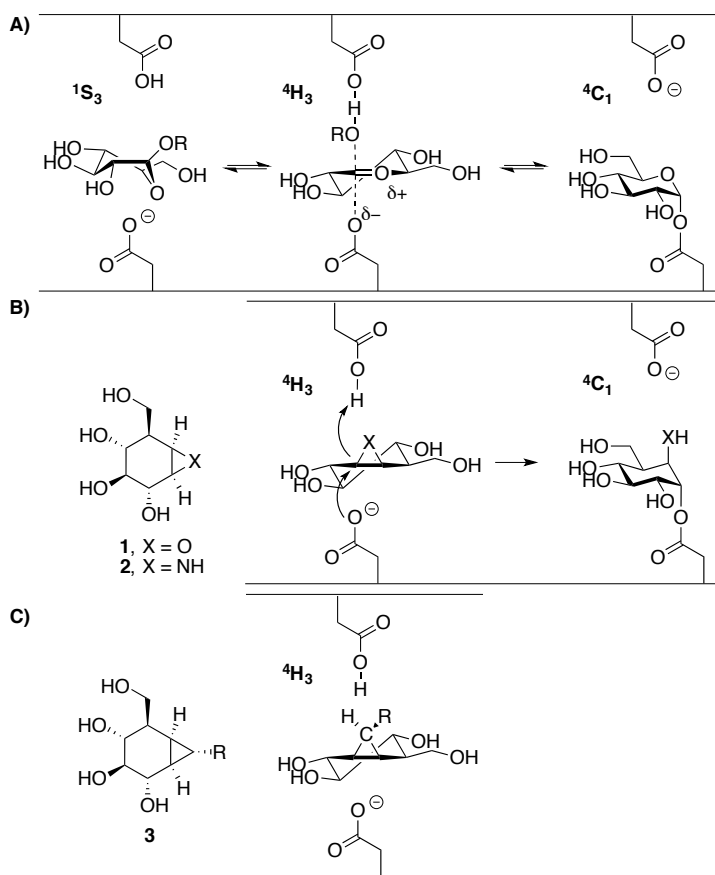


Figure 7.1 A) Mechanistic itinerary of retaining β -glucosidases. B) Structure of cyclophellitol (**1**) and cyclophellitol aziridine (**2**) adopting a $4H_3$ conformation and its proposed mechanism of binding. C) Structure of carba-cyclophellitol (**3**) adopting a $4H_3$ conformation, positioned in the active site. X = O, NH; R = acyl.

Bicyclic cyclopropyl glucosidase inhibitors, with the bridge between the “C6” and “O5” atoms were first proposed by Tanaka and co-workers⁵ (**4**) and later developed in *galacto*

configuration by Bennet and co-workers (5) and found to be good α -glucosidase and galactosidase inhibitors, respectively (Figure 7.2).⁶ More recently, the activated form of compound 5 (6) have been used as a covalent inhibitor.⁷ In these cases the conformational restriction limits the accessible conformations to “off-pathway” $^3H_2$ and $^2H_3$ half chairs⁷ (or perhaps their related 1,4 boats) recently elegantly revealed by X-ray crystallography.⁸ Further, Stick and Stubbs⁹ synthesized a bicyclic cyclopropyl inhibitor (7) with the bridge between the “anomeric” C1 carbon position and the “C2” atom with a millimolar K_i value.

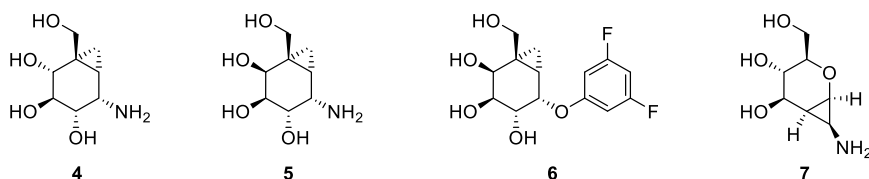


Figure 7.2 Potent bicyclic carbacycle based glucosidase- (4⁵ and 7⁹) and galactosidase inhibitors (5⁶ and 6⁷).

The carba-cyclophellitol derivatives (8 – 10, Figure 7.3) could be by virtue of the advantage of their conformational restriction between the “O5” and “anomeric” C1 carbon positions a potent inhibitor in which the conformational restraint is a glycosidase reaction coordinate relevant $^4H_3$. In this chapter, carba-cyclophellitols (8 – 10) were examined towards *Thermotoga maritima* TmGH1, a classical model β -glucosidase, human lysosomal retaining β -glucosidase, GBA1 (deficiency of which is causative of the human lysosomal storage disorder, Gaucher disease) and human lysosomal α -glucosidase, GAA (deficient in the human glycogen storage disease, Pompe

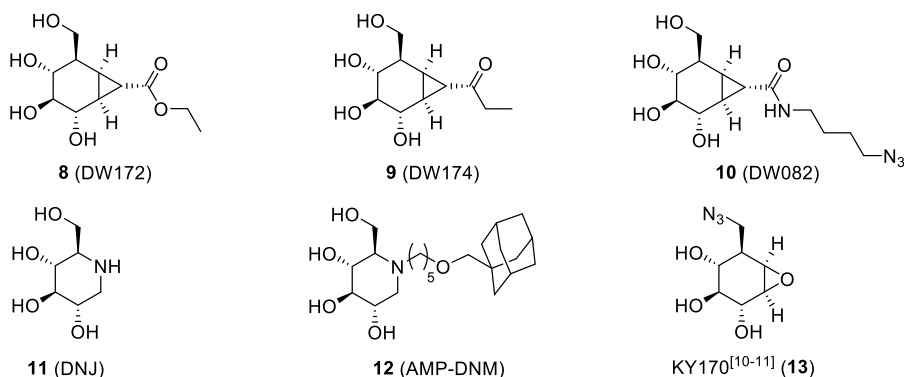


Figure 7.3 Structures of carba-cyclophellitol 8 – 10, DNJ (11), AMP-DNM (12, MZ-21) and KY170^{10,11} (13) used in this study.

disease). After analyzing carba-cyclophellitols by ^1H NMR analysis, quantum mechanical analysis and DFT calculations, carba-cyclophellitol **10** was soaked in *TmGH1* to elucidate the conformation binding mode in the active site.

7.2 Results and discussion

The synthesis of carba-cyclophellitols, including β -glucopyranose configured **8** – **10**, is described in Chapter 6. After having carba-cyclopropane **8-10** in hand, their inhibition potency was studied in comparison with deoxynojirimycin (DNJ), a known competitive *TmGH1* inhibitor and AMP-DNM (MZ-21), a known human retaining β -glucosidase inhibitor.¹³ Initial binding constant (K_i) values were determined on *TmGH1* by monitoring the UV-absorbance of *p*-nitrophenolate from *p*-nitrophenyl β -D-glucopyranoside using the Lineweaver–Burk method. Carba-cyclophellitol **8** and **9** showed micromolar inhibition, consistent with our design strategy and similar to that displayed by the charged species DNJ, whereas **10** proved to be a strong reversible binding *TmGH1* inhibitor with a K_i value of 8.2 nM, much more potent than DNJ¹⁴ and AMP-DNM (low micromolar) (Table 7.1 and Figure 7.4). We then explored the activity of compound **10** in human lysosomal retaining β -glucosidase, GBA1 which resulted into an apparent IC_{50} of around 100 μM . No apparent inhibition of the human lysosomal α -glucosidase, GAA was observed at final concentrations of **10** up to 150 μM . Thus, although less potent for GBA1 than for the bacterial enzyme tested, compound **10** appears to have selectivity for the human lysosomal β -glucosidase over the human lysosomal α -glucosidase, which is opposite of the selectivity observed for DNJ (Table 7.1).

Table 7.1 Apparent IC_{50} values and inhibitory constants (K_i) for *in vitro* inhibition of α - and β -glucosidase activity by compounds **8-10**, DNJ (**11**) and AMP-DNM (**12**).

Compound	$K_i^{[a]}$	apparent IC_{50}	
	<i>TmGH1</i> ^[b]	GBA1 ^[c]	GAA ^[c]
8 (DW172)	22.3 μM	>150 μM	> 150 μM
9 (DW174)	88.9 μM	>150 μM	> 150 μM
10 (DW082)	8.20 nM	99 $\pm$ 1.9 μM	> 150 μM
11 (DNJ)	2.50 $\mu\text{M}^{[d]}$	109 $\pm$ 1.0 $\mu\text{M}^{[e]}$	1.5 $\mu\text{M}^{[g]}$
12 (AMP-DNM)	4.97 μM	156 $\pm$ 16 nM ^[f]	0.4 $\mu\text{M}^{[g]}$

^a K_m *TmGH1* = 0.24 mM. ^bThe assay was performed with *p*-NPG as substrate. ^cThe assay was performed with 2,4-DNPG as substrate. ^eValues in agreement with literature. ^d K_i DNJ = 3.8 μM in

TmGH1,¹⁴ $^{4}IC_{50}$ DNJ = 250 μ M in GBA1,¹⁵ $^{4}IC_{50}$ AMP-DNM = 100-200 nM in GBA1.^{15,16} ^gValues from reference.¹⁷

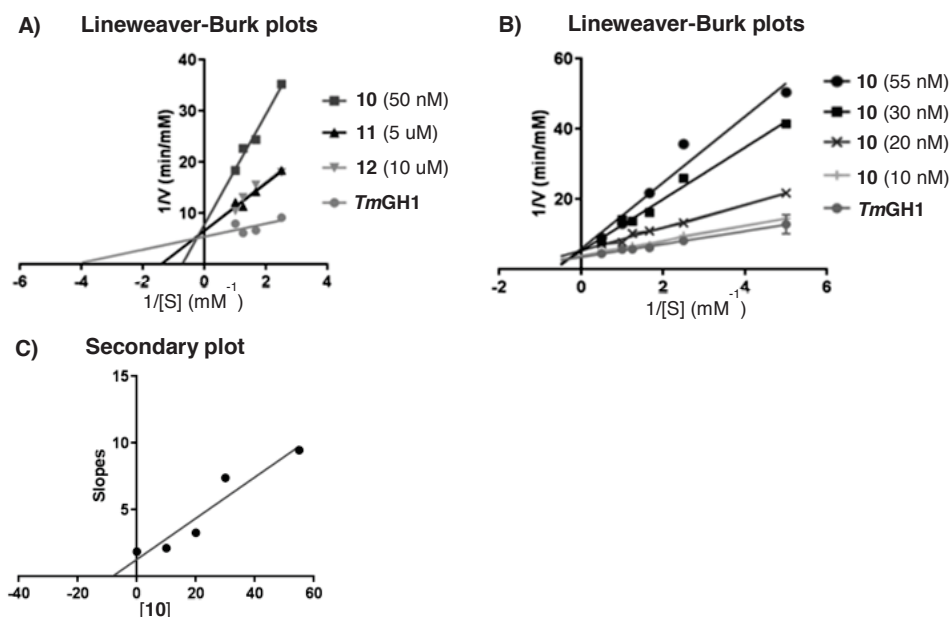


Figure 7.4 A) Lineweaver-Burk plots of β -glucosidase *TmGH1* in the presence of inhibitors **10** (50 nM), **11** (5 μ M) and **12** (10 μ M). B) Lineweaver-Burk plots of β -glucosidase *TmGH1* in the presence of inhibitor **10** at different concentrations (10, 20, 30 and 55 nM). C) Secondary plot for inhibitor **10**.

Inspired by the low μ M to nM inhibition of *TmGH1* by the carba-cyclopropanes, we sought to determine whether the cyclopropyl moiety indeed biased the conformation to $^4H_3$. The conformational free energy landscape (FEL) for generic cyclopropyl (**3**, R = H) was calculated by *ab initio* metadynamics, and the Cremer-Pople puckering coordinates θ and ϕ were used as collective variables, yielding a Mercator representation for the FEL (as used previously for diverse glycosidase inhibitors¹⁴⁻¹⁶), Figure 7.5A. Compound **3** clearly favors the $^4H_3$ conformation *in vacuo*, with the flipped $^3H_4$ form in another local energy minimum. Subsequent to FEL calculation, the experimental J values are compared of several (cyclohexane) ring protons of compound **9** with their calculated counterparts, in which calculations were performed on compound **9** in the $^4H_3$ conformation (Table 7.1). Both sets of values are in good agreement, which underscores the notion that compound **9**, and by extension also the other compounds subject of this chapter (whose proton NMR of **10** give broadened signals due to the amide present) do indeed adopt the $^4H_3$ conformation in solution (Table 7.2, Figure 7.6).

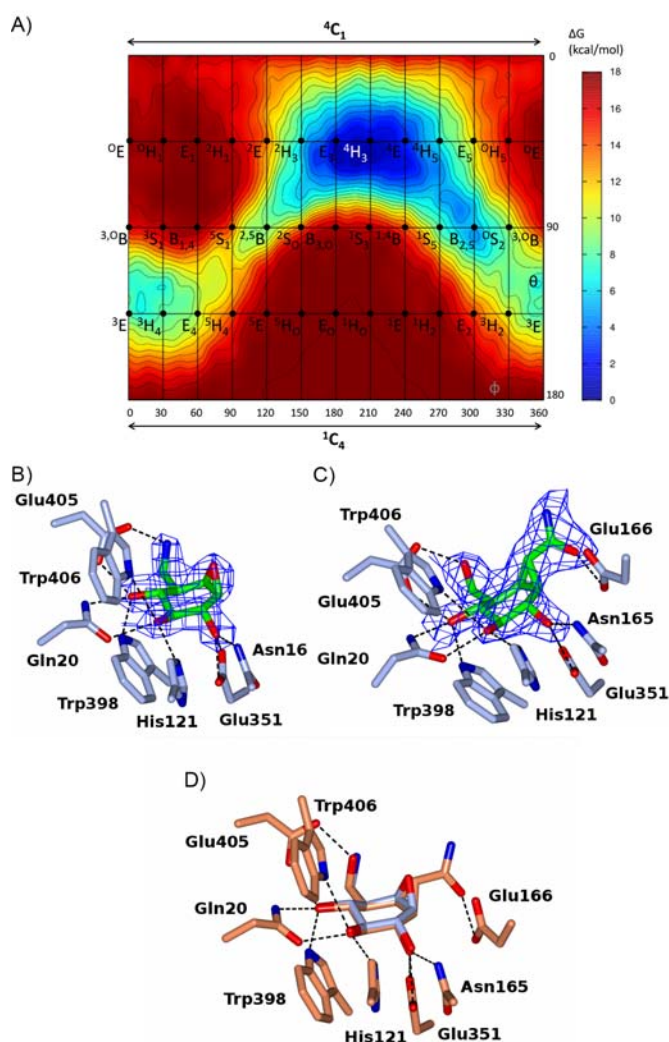
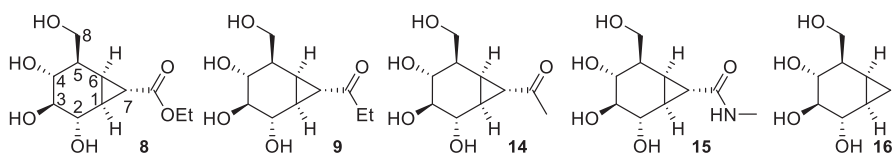


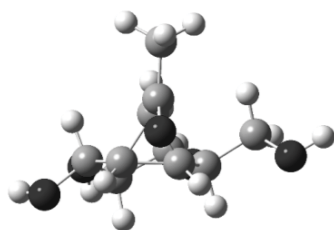
Figure 7.5 A) A mercurator representation for the computed free energy landscape (FEL) of cyclopropyl (3, R = H) (θ and ϕ are given in degrees). B) Crystal structure of *TmGH1* in complex with unreacted KY170 (13). C) Crystal structure of *TmGH1* in complex with carba-cyclophellitol 10, showing the carba-cyclophellitol-CO-NH group. Electron density maps for both B and C are maximum likelihood / σ_A weighted $2F_{\text{obs}} - F_{\text{calc}}$ syntheses contoured at 1.4σ . D) Overlay of (B) in ice blue and (C) in coral.

Structural dissection of the inhibitory action of 10, and the conceptual link through to cyclophellitol 1, was achieved first by rapid soaking (as opposed to pre-incubation as used previously to trap the covalent adduct¹⁷) of crystals of *TmGH1* with cyclophellitol derivative KY170^{10,11} (13). Serendipitously, this indeed afforded the unreacted

Table 7.2 The experimental (8 and 9) and calculated coupling constants of carba-cyclophellitols (14 - 16) in Hz.

					
Coupling	<i>J</i> exp. 8	<i>J</i> exp. 9	<i>J</i> calc. 14	<i>J</i> calc. 15	<i>J</i> calc. 16
H1-H6	n.d.	9.1	9.0	8.7	7.9
H1-H2	n.d.	0.8	0.5	0.6	0.5
H2-H3	8.6	8.6	6.9	6.7	6.6
H3-H4	10.3	10.1	8.0	8.1	8.1
H4-H5	10.3	9.7	8.8	8.4	8.4
H5-H6	n.d.	5.0	5.0	5.1	5.2
H5-H8a	8.2	8.2	9.0	8.0	8.1
H5-H8b	3.9	4.0	3.3	2.3	2.4
H1-H7a	n.d.	4.3	3.6	3.8	4.5
H6-H7a	n.d.	4.6	4.0	4.1	5.0
H1-H7b					8.6
H6-H7b					8.2

Coupling constants were determined by ^1H NMR experiments (exp.) or obtained from DFT calculations (calc.). n.d.: values could not be observed because of peak overlap or small couplings.

**Figure 7.6** Optimized conformation of carba-cyclophellitol 14.

cyclophellitol KY170 in $^4\text{H}_3$ conformation, with the nucleophile poised to attack, Figure 7.5B, confirming our hypothesis that (unreacted) cyclophellitols adopt a transition state-like $^4\text{H}_3$ conformation. In order to dissect similar mimicry by carba-cyclopropane **10**, and confirm the FEL calculated by *ab initio* metadynamics, *TmGH1* crystals were soaked with carba-cyclophellitol **10** and the subsequently obtained structure was analyzed and solved with X-ray crystallography. The obtained electron density pattern clearly demonstrates the presence of carba-cyclophellitol **10** in the active site in $^4\text{H}_3$

conformation (Figure 7.5C; the butyl azide moiety is highly mobile and differently disordered in the structure and not shown for clarity).

Overlay of cyclophellitol derivative KY170 with carba-cyclophellitol **10** (Figure 7.5D) shows almost perfect coincidence of atomic positions, showing that, as suggested by the FEL, **10** is a permanent mimic of cyclophellitol posted in the active site prior to nucleophilic attack.

The improved binding of **10**, relative to **8** and **9** presumably stems from desolvation caused by the alkyl-azido “tail” sitting in the aglycone site. One of the design advantages of the carba-cyclopropanes is that any pendent R groups are disposed pseudo-axial to the sugar ring, consistent with the distortions seen during catalysis which presumably adds to their augmentation of binding. The 3-D structure with **10** confirms this and shows a lateral, anti-trajectory interaction of the catalytic amino acid Glu166 with the pseudo-axially disposed amide of **10**. There are 4 molecules of *TmGH1* in the crystallographically-observed asymmetric unit. While they all show the R group axial, they all show different degrees of disorder of this alkyl region itself. In one molecule, there is essentially no electron density for the tail, while in two molecules the chain passes through the aglycon region (that is flanked by Val169, Trp168 and Trp324) making nonspecific interactions with this region. In the fourth molecule of the AU, the alkyl azido chain appears to follow two separate routes along each hydrophobic flank of the substrate binding cleft.

7.3 Conclusion

Carba-cyclophellitols are potent β -glucosidase inhibitors (K_i of 8.2 nM for the most potent derivative with an *N*-(4-azidobutyl)carboxamide moiety (**10**) towards *TmGH1* β -glucosidase). NMR analysis, *ab initio* metadynamics simulation of the conformational free energy landscape and DFT calculations indicated that carba-cyclophellitols favors the relevant $^4H_3$ conformation of the glycosidase reaction coordinate. 3-D structural analysis and comparison with unreacted epoxides show that compound **10** indeed bind in $^4H_3$ conformation suggesting that conformational strain induced through a cyclopropyl unit may add to the armory of tight binding inhibitor designs. Given the large number of glycosidase inhibitors in medical use, including those being developed as pharmacological chaperones and as diagnostic tools, the harnessing of appropriate conformation restraint, coupled to correct stereochemistry, should add greatly to the enzymological, cellular, and, ultimately, therapeutic toolbox.

Experimental

Determination of the conformation of carba-cyclophellitol

The calculated ^1H NMR coupling constants were obtained by first finding the lowest energy conformation of carba-cyclophellitol **14**, **15** or **16**, for which a library of gas phase conformations was generated using conformer distribution option included in Spartan 04¹⁸ program, employing Semiempirical/RM1. All conformers were further optimized by Gaussian 03¹⁹ at DFT/B3LYP 6-311++G(d,p), their zero-point energies were calculated and the energies corrected for solvent by another optimization step employing a Polarizable Continuum Model set for water. The energies of these conformers, corrected for their zero-point energies, were compared and of the lowest energy conformer (Figure 7.6) an NMR calculation was performed using Gauge-Independent Atomic Orbital (GIAO) method with added spin-spin coupling calculation.

TmGH1 preparation

TmGH1 construct pET-28a-TmGH1-His₆ was cloned, expressed and purified as described by Zechel *et al.*¹³

Enzyme activity assays and IC₅₀ measurements

Enzyme preparations of recombinant human GBA1 (Cerezyme) and recombinant human GAA (Myozyme) used for IC₅₀ measurements were obtained from Genzyme, USA.

The activity of β -D-glucosidase GBA1 was assayed at 37 °C by monitoring the UV-absorbance of 2,4-dinitrophenolate released from 2,4-dinitrophenyl β -D-glucopyranoside. To determine the apparent IC₅₀ values, 2.6 μM of enzyme in 150 mM McIlvaine buffer pH 5.2 with 0.2% Taurocholate (w/v), 0.1% Triton X-100 (v/v) and 0.1% Bovine Serum albumin (BSA) (w/v) was pre-incubated with a range of inhibitor dilutions (with 1% DMSO) for 15 min at 37 °C. The reaction was started by adding 5 μL of this enzyme-inhibitor mixture to 95 μL of 2,4-dinitrophenyl β -D-glucopyranoside substrate (200 μM) in 150 mM McIlvaine buffer (pH 5.2) 0.2% Taurocholate (w/v), 0.1% Triton X-100 (v/v), 0.1% Bovine Serum albumin (BSA) (w/v).

The activity of β -D-glucosidase GAA was assayed at 37 °C by monitoring the UV-absorbance of 2,4-dinitrophenolate released from 2,4-dinitrophenyl α -D-glucopyranoside. To determine the apparent IC₅₀ values, 8.9 μM of enzyme in 150 mM McIlvaine buffer pH 4.8 with 0.1% BSA (w/v) was pre-incubated with a range of inhibitor dilutions (with 1% DMSO) for 15 min at 37 °C. The reaction was started by adding 5 μL of this enzyme-inhibitor mixture to 95 μL of 2,4-dinitrophenyl α -D-glucopyranoside substrate (200 μM) in 150 mM McIlvaine buffer (pH 4.8) with 0.1% BSA (w/v).

The release of 2,4-dinitrophenolate was monitored via absorbance at 400 nm every 2s for 60 s to determine the hydrolysis rate. All IC₅₀ values were determined in triplicate. The data were normalized to the untreated control condition and finally the residual activity was calculated through equation 1, plotted against the $\log[I]$ and curve-fitted with one-phase exponential decay function (GraphPad Prism 5.0).

Equation 1: Residual activity % = $(V/V_0) \times 100$

Kinetic studies

The kinetics studies of reversible inhibitors in *TmGH1* were performed by monitoring the UV-absorbance of *p*-nitrophenolate released from *p*-nitrophenyl- β -D-glucopyranoside. *TmGH1* at 3.7 μ M in 50 mM phosphate buffer (pH 6.8) was pre-incubated with a range of inhibitor dilutions for 15 min at 25 °C. The reaction was then started by adding 5 μ L of this enzyme-inhibitor mixture to 95 μ L of *p*-nitrophenyl β -D-glucopyranoside substrate (from 0.2 to 1 mM) in 50 mM phosphate buffer (pH 6.8). Following the onset inhibition (one minute incubation), the release of *p*-nitrophenolate was monitored via absorbance at 400 nm every 2s for 60 s to determine the hydrolysis rate. The K_i values of reversible inhibition were determined by taking the slopes from each Lineweaver-Burk plots and plotting them vs the inhibitor concentration (Figure 7.4). After fitting the data into a straight line by linear regression, the [I]-intercept gave the desired $-K_i$ value. The K_m of *TmGH1* was determined from the value of the horizontal axis intercept ($-1/K_m$).

TmGH1 crystallization, data collection and structure refinement methods

Crystals were grown using the Sitting Drop vapour diffusion method, with *TmGH1* at 12.5 mg/ml, mixed in a volume ratio 0.6:0.1:0.4 protein: seed: well solution in the drop, over a well comprised of 0.2 M K/Na tartrate, 0.1 M bis-tris propane pH 7.4, 20% polyethylene glycol (PEG) 3350. The seed stock was prepared using crystals grown over 0.2 M Na malonate, 0.1 M bis-tris propane pH 6.6, 26% PEG 3350, which were vortexed with approximately 50 μ L well solution, in an Eppendorf tube containing a polystyrene bead, for 1.5 min. A crystal was soaked with a speck of solid powder of KY170 (**13**) for an hour, and then harvested into liquid nitrogen with a nylon CryoLoopTM via a cryoprotectant solution containing 0.2 M K/Na tartrate, 0.1 M bis-tris propane pH 7.4, 20% PEG 3350, 25% (v/v) ethylene glycol. X-ray diffraction data were collected at the European Synchrotron Radiation Facility beamline id 23-2, and the space group was $P2_12_12_1$, with approximate unit cell dimensions $a = 94.8$ Å, $b = 94.9$ Å, $c = 112.9$ Å, $\alpha = \beta = \gamma = 90^\circ$. The data were processed using *iMOSFLM*²⁰, and scaled using *AIMLESS*²¹ to a resolution of 2.1 Å.

Further crystals were grown using a similar method, with *TmGH1* in a ratio of 0.5:0.1:0.6 protein: seed: well solution, over a well containing 11% PEG 4000, 0.1 M imidazole pH 7.0, 0.1 M Ca acetate, 0.1 M trimethylamine N-oxide (TMAO), and with a seed bead stock prepared as above using crystals grown over 15% PEG 4000, 0.1 M imidazole pH 7.0, 0.22 M Ca acetate. The crystals were soaked with 2.5 mM **10** in 11% PEG 4000, 0.1 M imidazole pH 7.0, 0.1 M Ca acetate, 0.1 M TMAO, for 4 hours, and harvested into liquid nitrogen with a nylon CryoLoopTM via a cryoprotectant solution containing 11% PEG 4000, 0.1 M imidazole pH 7.0, 0.2 M calcium acetate, 25% ethylene glycol. X-ray data were collected at the Diamond Light Source beamline IO2, and processed with *DIALS*²², and scaled with *AIMLESS* to a resolution of 2.0 Å. The space group was $P1$ and the approximate unit cell dimensions, $a = 71.6$ Å, $b = 76.7$ Å, $c = 95.3$ Å, $\alpha = 72.3^\circ$, $\beta = 86.4^\circ$, $\gamma = 85.5^\circ$.

Both structures were solved using programs from the *CCP4* suite²³. The native *TmGH1* structure PDB entry 1OD0¹³ was used as the model for molecular replacement using *PHASER*²⁴. The structures were refined with cycles of maximum-likelihood refinement using *REFMAC*²⁵, interspersed with manual rebuilding with *Coot*²⁶.

Table 7.2 Data collection and refinement statistics.

	10	13
Data collection		
Space group	<i>P</i> 1	<i>P</i> 2 ₁ 2 ₁ 2 ₁
Cell dimensions		
<i>a</i> , <i>b</i> , <i>c</i> (Å)	71.6, 76.7, 95.3	94.8, 94.8, 112.9
α , β , γ (°)	72.3, 86.4, 85.6	90.0, 90.0, 90.0
Resolution (Å)	72.9-2.10 (2.14-2.10)	56.5-2.10 (2.15-2.10)
<i>R</i> _{sym} or <i>R</i> _{merge}	9.7 (51.7)	10.5 (96.6)
<i>I</i> / σ <i>I</i>	5.2 (1.4)	12.8 (2.3)
Completeness (%)	97.3 (95.4)	100.0 (100.0)
Redundancy	2.7 (2.5)	7.4 (7.5)
Refinement		
No. reflections	109393	60171
<i>R</i> _{work} / <i>R</i> _{free}	0.24/0.31	0.18/0.24
No. atoms		
Protein	14155	7175
Ligand/ion	222	53
Water	403	263
<i>B</i> -factors		
Protein	31.8	37.0
Ligand/ion	34.0	38.7
Water	29.8	36.15
R.m.s deviations		
Bond lengths (Å)	0.018	0.016
Bond angles (°)	1.879	1.729
Ramachandran plot residues		
In most favorable regions (%)	96.2	97.7
In allowed regions (%)	3.2	1.7
PDB code	5N6S	5N6T

Metadynamics simulations

In order to obtain the free energy landscape of carba-cyclophellitol (**3**, R = H), quantum mechanical calculations were performed using Density Functional Theory-based molecular dynamics (MD), according to the Car-Parrinello method.²⁷ All systems were enclosed in an isolated cubic box of 12.0

$\text{\AA} \times 12.0 \text{ \AA} \times 12.0 \text{ \AA}$, using a fictitious electron mass of 700 au and a time step of 0.12 fs. The Kohn-Sham orbitals were expanded in a plane wave (PW) basis set with a kinetic energy cutoff of 70 Ry. Ab initio pseudopotentials generated within the Troullier-Martins scheme were employed.²⁸ The Perdew, Burke and Ernzerhoff generalized gradient-corrected approximation (PBE)²⁹ was selected in view of its good performance in previous work on isolated sugars,¹⁴ glycosidases³⁰ and glycosyltransferases.³¹ The metadynamics algorithm^{32,33} was used to explore the conformational free energy landscape of the system, taking as collective variables θ and ϕ of the puckering coordinates of Cremer and Pople.³⁴ Initially, the height of the Gaussian terms was set at $0.6 \text{ kcal}\cdot\text{mol}^{-1}$ and a new Gaussian-like potential was added every 250 MD steps. Once the whole free energy space was explored, the height of the Gaussian terms was reduced to the half ($0.3 \text{ kcal}\cdot\text{mol}^{-1}$) and a new Gaussian-like potential was added every 500 MD steps. The width of the Gaussian terms was set to $0.10 \text{ \AA}$. The simulation was stopped according to the invariance of the energy differences between wells, which was further confirmed by a time-independent free energy estimator (Figure 7.7).³⁵ The phase space was fully explored in less than 60 ps and the simulation was further extended up to 180 ps. The errors in the energy minima, taken as standard deviation, are below $1 \text{ kcal}\cdot\text{mol}^{-1}$.

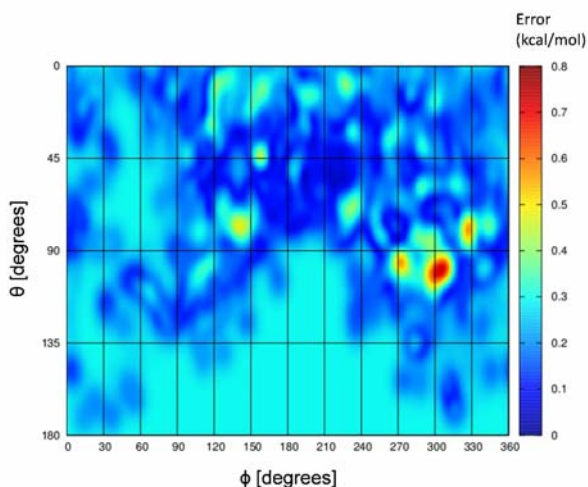


Figure 7.7 Error surface for carba-cyclophellitol. Plot calculated according to the time-independent free energy estimator described by Tiwary and Parrinello.³⁵

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8

Synthesis of potential glycosyltransferase inhibitors based on carba-cyclophellitol¹

8.1 Introduction

The enzymatic formation of glycosidic linkages is achieved by glycosyltransferases (GTs). At the end of their lifetime, glycosidic linkages within oligosaccharides and glycoconjugates (combined termed glycans) are hydrolysed by glycosidases (GHs), and thus GTs and GHs combined control glycan levels and diversity within cells and organisms. Just as is the case with GH inhibitors, GT inhibitors are widely sought after with the aim to exert control over glycan metabolism. Indeed, numerous potent and selective GH inhibitors have been discovered in the past decades, and some are in clinical use.^{2,3} However, and despite the therapeutic potential of GT inhibitors, potent GT inhibitors are scarce.^{4,5}

Retaining galactosyltransferases (GalT) catalyse the transfer of galactose from UDP- α -D-galactose (donor) to an acceptor molecule such as proteins, (oligo)saccharides and lipids (Figure 8.1). A double displacement mechanism is suggested for galactosyltransferases of the GT6 family, including α 3GalT (E.C. 2.4.1.87), α -1,3-*N*-acetylgalactosaminyltransferase (GTA, E.C. 2.4.1.40) and α -1,3-galactosyltransferase (GTB, E.C. 2.4.1.37).⁶ In this (putative) mechanism (Figure 8.1A), a covalent enzyme-donor intermediate is formed via an oxocarbenium ion-like transition state. An incoming nucleophile (the acceptor-OH to be glycosylated) would then release the enzyme-bound galactoside to yield the galactosylated product with concomitant reforming of the enzyme active site.

An alternative mechanism (Figure 8.1B), is suggested for lipopolysaccharyl- α -1,4-galactosyltransferase C (LgtC, GT8 family).⁷ In this S_Ni -like mechanism, a front-side

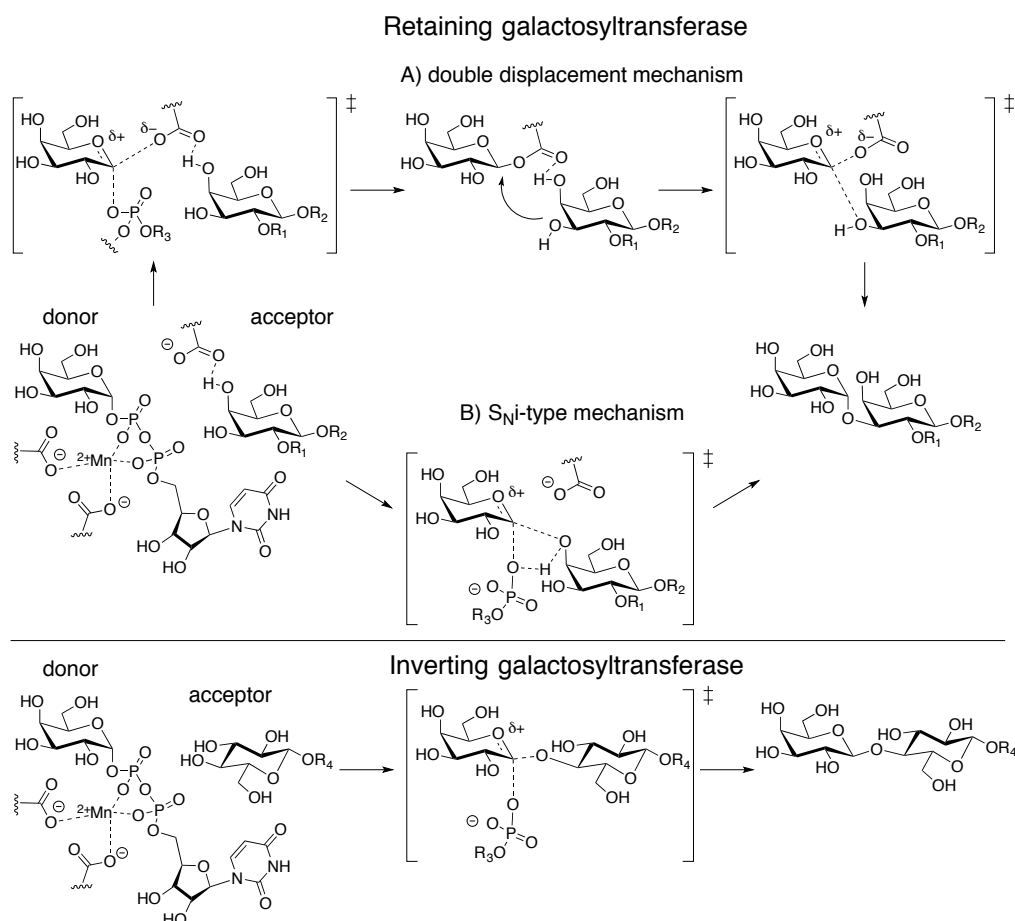


Figure 8.1 Putative mechanisms of retaining and inverting galactosyltransferases.

attack of the acceptor on the anomeric position of the donor takes place, while the leaving group departs simultaneously from the same face resulting in the formation of the product with retention of configuration. This mechanism, implies an oxocarbenium ion-like transition state or a potentially short-lived oxocarbenium ion intermediate.

Finally, inverting galactosyltransferases, such as β -1,4-galactosyltransferase T1 (β 4Gal-T1), employ a S_N2 nucleophilic attack of the acceptor at the anomeric center (Figure 8.1). Indeed, the oxocarbenium-like transition state is formed as well in this mechanism, followed by expulsion of the UDP-moiety, resulting in product formation with inverted configuration at the anomeric center.⁸

The promising results of carba-cyclophellitol derivatives as described in Chapter 6 and 7 wherein the oxocarbenium ion-like transition state was mimicked by carba-cyclophellitols, which employs a $^4H_3$ conformation, served as a source of inspiration for the here-described research. Known α -galactosidase inhibitors, such as 1-deoxy-galactosnojirimycin (DGJ, with an IC_{50} value of 4.7 nM for lysosomal α -galactosidase A⁹), do not inhibit galactosyltransferases, such as α 3GalT.¹⁰ On the other hand, uridine diphosphate (UDP, **1**) is a relatively potent α 3GalT inhibitor with an IC_{50} of 13 μ M (Figure 8.2A).¹⁰ Based on these considerations, compound **3** (AB209) was designed as a potential inhibitor of galactosyltransferases (Figure 8.2B). Furthermore, compound **4** (AB209) was synthesized, based on **2** which is a moderately potent α 3GalT inhibitor (IC_{50} value of 320 μ M).¹¹ In compound **2**, an *O*-sulfamoyl amide bond was introduced to mimic the pyrophosphate moiety of the natural donor, UDP-galactose.

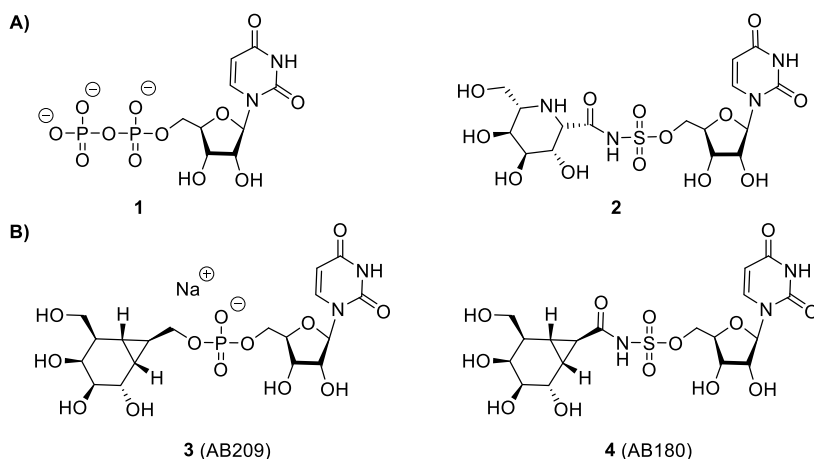


Figure 8.2 A) Galactosyltransferase inhibitors described in the literature.^{10,11} B) Potential galactosyltransferase inhibitors that are the subject of the research described in this chapter.

In the second part of this chapter the synthesis of glucose-configured isomer **5** is described (Figure 8.3). Glucopyranose-configured carba-cyclophellitol derivatives are described in Chapter 6 and were used as a starting point to obtain this potential inhibitor towards glucosyltransferases including glucosylceramide synthase.

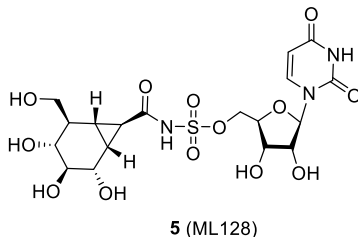
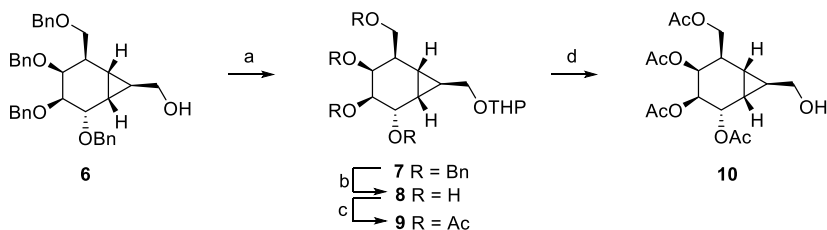


Figure 8.3 Structure of compound **5**.

8.2 Results and discussion

The synthesis of compound **3** was inspired by the phosphoramidite-based sugar nucleotide synthesis as described by Gold and co-workers.¹² α -Galactopyranose-configured carba-cyclophellitol **6** was synthesized as described in Chapter 6. Once a phosphodiester bond is formed however, all protective groups in the penultimate intermediate are ideally removed in a single (basic) step. Protection group manipulations were therefore executed to transform **6** into a suitable substrate (**10**) in which all alcohols barring the one intended for phosphorylation are masked as the acetate (Scheme 8.1). Briefly, the primary alcohol of compound **6** was reacted with 3,4-dihydro-2H-pyran (DHP) and catalytic *para*-toluenesulfonic acid followed by removal of the benzyl ethers in the resulting THP ether **7** under Birch reduction conditions to give compound **8**. Global acetylation and acid catalyzed THP deprotection afforded key intermediate **10**.

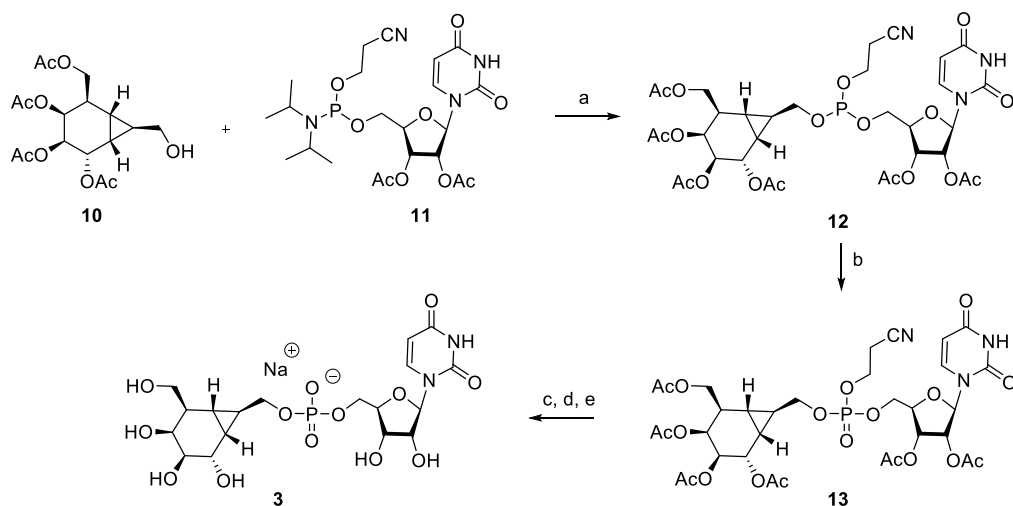
Scheme 8.1 Synthesis of alcohol **10**.



Reagents and conditions: a) 3,4-dihydro-2H-pyran, *p*TsOH, CH₂Cl₂, rt, 30 min, 79%; b) Li, NH₃ (l), -60 °C, 1 h; c) Ac₂O, 4-dimethylaminopyridine, pyridine, rt, overnight, 88% over 2 steps; d) *p*TsOH, MeOH, rt, 1 h, 85%.

The obtained alcohol **10** was then coupled with uridine phosphoramidite **11**¹³ (Scheme 8.2). After 30 min, the phosphite moiety was oxidized with *tert*-butylhydroperoxide giving phosphate diester **13**. In the following three-step-procedure all protecting groups were removed. Treatment with DBU resulted in the removal of the 2-cyanoethyl group and partial deacetylation was observed by LC/MS analysis. Next, aqueous ammonia was added to the reaction mixture. However, after stirring overnight the deacetylation process was not completed as determined by LC/MS analysis. Therefore, the reaction mixture was concentrated *in vacuo* and stirred overnight in Et₃N/H₂O/MeOH, providing the target compound. The crude product mixture was purified by size-exclusion chromatography followed by HPLC purification. Finally, treatment with Dionex sodium exchange resin provided compound **3** as the sodium salt in 22% yield.

Scheme 8.2 Synthesis of compound **3**.

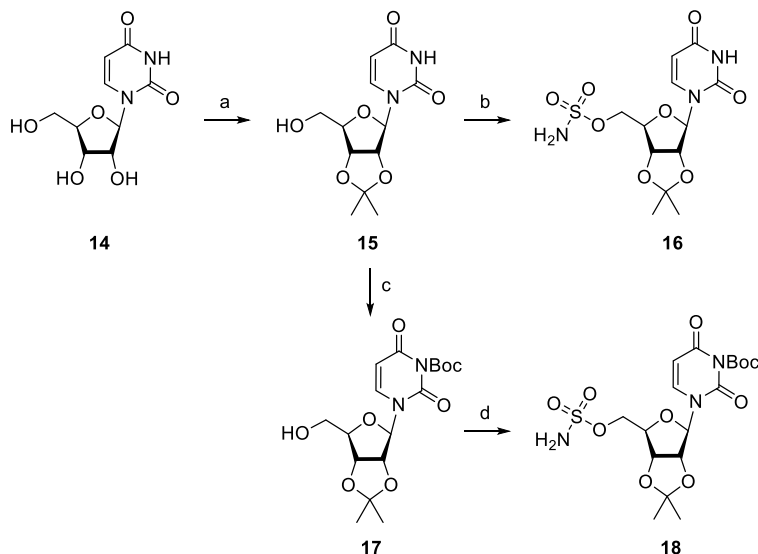


Reagents and conditions: a) DCI, acetonitrile, rt, 30 min; b) ^tBuOOH, rt, 30 min; c) DBU, rt, 1h; d) aqueous NH₃, rt, 24 h; e) i) Et₃N/H₂O/MeOH (1:1:3), rt, overnight; ii) size-exclusion, HPLC purification, Dionex sodium exchange, 22% over 6 steps.

In order to prepare compound **4**, the UDP mimic had to be synthesized first (Scheme 8.3). The synthesis of *O*-sulfamoylamide **16** was accomplished according to the literature procedure.¹⁴ Iodine-catalyzed acetalization of uridine **14** and acetone gave 2',3'-isopropylidene-uridine **15**. Installment of the sulfamoyl functionality to give **16** was accomplished by first generation of sulfamoyl chloride from chlorosulfamoyl isocyanate and formic acid followed by addition to the reaction mixture of compound

15. Purification of *O*-sulfamoylamide **16** however turned out to be troublesome. An alternative strategy comprises the protection of the uracil-nitrogen as the Boc derivative (**15** to **17**). *O*-sulfamoylation and ensuing purification now proceeded uneventfully yielding compound **18**, which was used in the following steps instead of **16**.

Scheme 8.3 Synthesis of *O*-sulfamoyluridines.

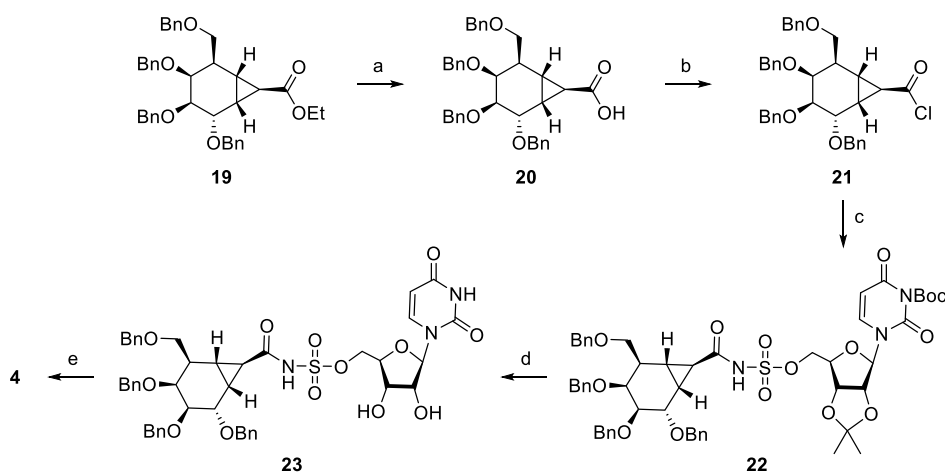


Reagents and conditions: a) I_2 , acetone, rt, overnight, 83%; b) formic acid, chlorosulfamoyl isocyanate, NaH, THF, overnight, 0 °C to rt, 79%; c) i) TBDMS-Cl, *N,N*-dimethylpyridin-4-amine, pyridine, overnight, 88%; ii) Et_3N , *N,N*-dimethylpyridin-4-amine, di-*tert*-butyl dicarbonate, CH_2Cl_2 , rt, overnight, quantitative; iii) tetra-*n*-butylammoniumfluoride (TBAF), THF, 0 °C to rt, 40 min, 81%; d) formic acid, chlorosulfamoyl isocyanate, pyridine, 0 °C to reflux, overnight, 30%;

α -Galactopyranose-configured carba-cyclophellitol **19** was synthesized in sufficient quantities following the procedure described in Chapter 6. Treatment with lithium hydroxide in a mixture of THF, MeOH and H_2O resulted in acid **20** (Scheme 8.4). Acylation of **20** using oxalyl chloride in toluene gave acid chloride **21**. After having acid chloride **21** and *O*-sulfamoylamide **18** available, the key step in the sequence was investigated. Coupling of acid chloride **21** with *O*-sulfamoylamide **18** resulted in compound **22**, which was purified by size-exclusion chromatography followed by silica gel column chromatography. In contrast to cyclophellitol and cyclophellitol aziridine¹⁵⁻¹⁷, carba-cyclophellitol derivatives are stable towards acidic conditions. Therefore, the isopropylidene and *tert*-butyloxycarbonyl protecting groups of **22** could be successfully removed by treatment with TFA and H_2O in CH_2Cl_2 . The remaining benzyl groups

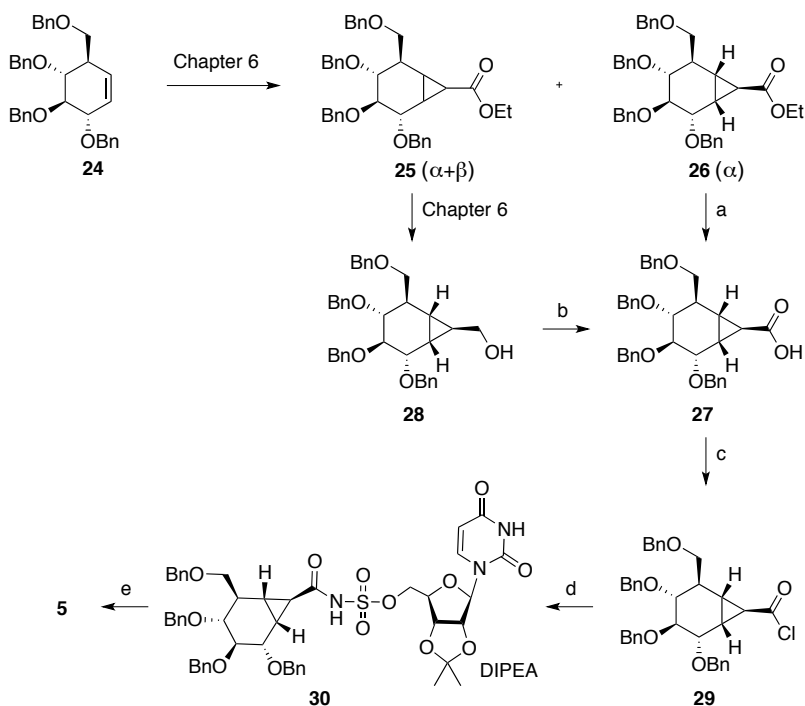
were removed under the agency of BCl_3 as the Lewis acid. Purification by HPLC provided compound **4** in 69% yield.

Scheme 8.4 Synthesis of compound **4**.



Reagents and conditions: a) LiOH , $\text{THF}/\text{MeOH}/\text{H}_2\text{O}$ (8:2:1), rt, overnight, 86%; b) oxalyl chloride, toluene, 40°C , overnight; c) **18**, N,N -diisopropylethylamine, CH_2Cl_2 , rt, 3 h, 60% over 2 steps; d) $\text{TFA}/\text{H}_2\text{O}$ (9:1), CH_2Cl_2 , 0°C to rt, 4.5 h, 81%; e) BCl_3 , CH_2Cl_2 , -78°C to 0°C , 4.5 h, 69%.

The synthesis of α -glucopyranose-configured carba-cyclophellitol **5** (Scheme 8.5), was based on the strategy as described above towards α -galactopyranose-configured analogue **4**. The synthesis of compound **5** requires access to sufficient quantities of acid chloride **29**. Cyclopropanation of cyclohexene **24** yielded ethyl ester **25** as an α/β -mixture (see also Chapter 6). Crystallization in ethanol resulted in obtaining a pure fraction of α -ethyl ester **25**. Hydrolysis by LiOH resulted in carboxylic acid **27**. In order to obtain larger quantities of carboxylic acid **26**, ethyl ester **25** as an α/β -mixture was first reduced with di-isobutyl aluminum hydride after which alcohol **27** was isolated by column chromatography (Chapter 6). Alcohol **27** was oxidized to carboxylic acid **26** using Jones oxidation conditions. Having large quantities of carboxylic acid **26** available, the synthesis was continued as described for compound **4**. Briefly, acylation of acid **26** using oxalyl chloride afforded compound **28**, which was coupled with previously synthesized uridine sulfamoyl amide **16** to obtain compound **29** as the DIPEA salt. Finally, global deprotection by treatment with BCl_3 , followed by quenching with MeOH gave compound **5**, which was purified by precipitation in chloroform, followed by HPLC purification.

Scheme 8.5 Synthesis of compound 5.

Reagents and conditions: a) LiOH, THF/MeOH/H₂O (6.5:1.5:1), rt, 5 h, 91%; b) Jones reagent, acetone, 0 °C, 70 min, 75%; c) oxalyl chloride, toluene, 40 °C, 4 h; d) **16**, *N,N*-diisopropylethylamine, CH₂Cl₂, rt, overnight, 70% over 2 steps; e) i) BCl₃, CH₂Cl₂, 0 °C, 3 h; ii) MeOH, 30 min, 54% over 2 steps.

The potency of the synthesised UDP-glucose and UDP-galactose mimetics to inhibit GH activities was established next (Table 8.1). Compound **4** turned out to be a potent human lysosomal α-galactosidase (α-GalA) inhibitor, with considerable less potency towards human lysosomal β-galactosidase (GALC). As can be seen from Table 8.1, no inhibition was observed for any of the α-glucosidases (GAA, GANAB) or β-glucosidases (GBA1, GBA2) tested for compound **5** (ML128). Furthermore, **5** failed to inhibit glucosylceramide synthase (GCS), a glucosyltransferase. Inhibition assays to assess the potential of the compounds to target other transferases, including the α-galactosyl transferases for which the galactose-configured compounds were designed, could not be performed in the timeframe of the PhD research described here, though compounds have been shared with interested groups who may shed light on their GT inhibitory properties, or lack thereof, in the (near) future.

Table 8.1 Apparent IC₅₀ values of compounds **3** – **5** towards glycosidases and glucosyltransferase (μM).

Compound	<u>α-galactosidase</u>	<u>β-galactosidase</u>	<u>GlucT</u>	<u>α-glucosidase</u>		<u>β-glucosidase</u>	
	α-GalA	GALC	GCS	GAA	GANAB	GBA1	GBA2
3 (AB209)	>500	>500	--	--	--	--	--
4 (AB180)	18.84 ± 1.33	180.27 ± 4.52	--	--	--	--	--
5 (ML128)	--	--	>50	>100	>100	>100	>100

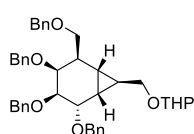
8.3 Conclusion

In this chapter the versatility of carba-cyclophellitol as analogues of the oxocarbenium ion-like transition state is demonstrated by synthesizing potential inhibitors (**3** and **4**) towards galactosyltransferases. In the (near) future, their GT specific inhibition potency will be examined. Furthermore, compound **4** turned out to be a potent carba-cyclophellitol-based α-galactosidase inhibitor. In a similar fashion compound **5** (ML128) was successfully synthesized. However, inhibition towards GCS or various glucosidases (GAA, GANAB, GBA1 or GBA2) was not observed.

Experimental

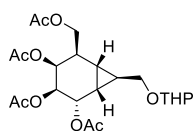
General methods: All chemicals were purchased from Acros, Sigma Aldrich, Biosolve, VWR, Fluka, Merck and Fisher Scientific and used as received unless stated otherwise. Dichloromethane (DCM), tetrahydrofuran (THF) and toluene were stored over flame-dried 4 Å molecular sieves before use. Pyridine and 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 aluminium 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 μm, pore diameter of 60 Å) in the indicated solvents. For reversed-phase HPLC purifications a HPLC system equipped with a C18 semiprep column (Gemini C18, 250x10 mm, 5 μm 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 μm 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. ³¹P NMR, ¹H NMR and ¹³C NMR spectra were recorded on a Brüker AV-400 (161.7, 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 $m/z = 150\text{--}2000$) 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).



2-(((1R,2S,3R,4S,5R,6R,7R)-2,3,4-tris(benzyloxy)-5-((benzyloxy)methyl)bicyclo[4.1.0]heptan-7-yl)methoxy)tetrahydro-2H-pyran (7)

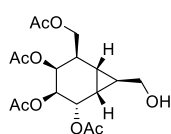
Alcohol **6** (see Chapter 6, 708 mg, 1.25 mmol) was dissolved in DCM (15 mL) and 3,4-dihydropyran (170 μ L, 1.9 mmol, 1.5 eq.) and a catalytic amount of *p*TsOH were added. After stirring for 30 min at room temperature, the reaction mixture was quenched with saturated aqueous NaHCO_3 , extracted with DCM, dried with Na_2SO_4 , filtered and concentrated *in vacuo*. Purification by column chromatography (5% EtOAc in pentane $\rightarrow$ 20% EtOAc in pentane) gave a diastereomeric mixture of compound **7** (0.64 g, 0.99 mmol, 79%) as a clear oil. ^1H NMR (400 MHz, CDCl_3): δ (ppm) 7.51 – 7.19 (m, 20H, $\text{CH}_{\text{arom}}\text{-Bn}$), 5.01 – 4.81 (m, 2H, $\text{CH}_2\text{-Bn}$), 4.79 – 4.54 (m, 5H, $\text{CH}_2\text{-Bn}$, OCHO-THP), 4.38 (t, $J = 7.4$ Hz, 1H, H-2), 3.96 – 3.87 (m, 1H, H-4), 3.84 – 3.78 (m, 1H, CH_2O), 3.70 – 3.53 (m, 2H, H-8), 3.50 – 3.35 (m, 2H, CH_2O , $\text{OCH}_2\text{C-THP}$), 3.28 (dd, $J = 10.6$, 6.6 Hz, 1H, $\text{OCH}_2\text{C-THP}$), 3.20 (d, $J = 8.2$ Hz, 1H, H-3), 1.94 (m, 1H, H-5), 1.85 – 1.40 (m, 7H, H-1, 3 x $\text{CCH}_2\text{C-THP}$), 1.08 (m, 1H, H-7), 0.78 – 0.63 (m, 1H, H-6). ^{13}C NMR (101 MHz, CDCl_3): δ (ppm) 139.3, 139.3, 139.3, 139.3, 139.1, 139.1, 138.3, 138.3 ($\text{C}_q\text{-Bn}$), 128.4, 128.3, 128.2, 127.9, 127.8, 127.8, 127.7, 127.6, 127.4, 127.4, 127.3, 127.2 ($\text{CH}_{\text{arom}}\text{-Bn}$), 98.2, 97.3 (OCHO-THP), 83.5 (C-3), 76.7, 76.7 (C-2), 76.0, 76.0 (C-4), 73.9, 73.9, 73.2, 73.1, 72.8, 72.7, 71.0, 70.9 $\text{CH}_2\text{-Bn}$), 70.8, 70.8 (C-8), 70.5 ($\text{OCH}_2\text{C-THP}$), 62.0, 61.9 (CH_2O), 41.6 (C-5), 30.7, 30.6, 25.5 ($\text{CCH}_2\text{C-THP}$), 24.3, 24.1 (C-7), 21.8, 21.5 (C-1), 19.8 (C-6), 19.4, 19.3 ($\text{CCH}_2\text{C-THP}$), 19.2 (C-6). HRMS: calculated for $[\text{C}_{42}\text{H}_{48}\text{O}_6\text{Na}]^+$ 671.33431, found 671.33379.



(1R,2S,3R,4S,5R,6R,7R)-5-(acetoxymethyl)-7-(((tetrahydro-2H-pyran-2-yl)oxy)methyl)bicyclo[4.1.0]heptane-2,3,4-triyl triacetate (9)

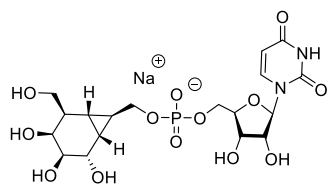
NH_3 (50 mL) was condensed in a three necked flask at -60 °C after which solid lithium (0.25 g) was added resulting in a blue solution. After addition of a solution of **7** (0.34 g, 0.52 mmol) in THF (4 mL), the reaction mixture was stirred for 1 h before cautiously quenching with MilliQ (5 mL). The reaction mixture was gradually allowed to warm to room temperature and concentrated *in vacuo* to afford debenzylated product **8**. The crude product **44** was redissolved in pyridine (15 mL) and acetic anhydride (10 mL) and a catalytic amount of DMAP were added. After stirring overnight at room temperature, the reaction was quenched with H_2O . The solution was extracted with EtOAc, washed with H_2O (3x), dried with MgSO_4 , filtered and concentrated *in vacuo*. Purification by column chromatography (10% EtOAc in pentane $\rightarrow$ 50% EtOAc in pentane) gave a diastereomeric mixture of peracetylated **9** (210 mg, 0.46 mmol, 88% over two steps) as a pale oil. ^1H NMR (400 MHz, CDCl_3): δ (ppm) 5.41 (dd, $J = 9.2$, 6.8 Hz, 1H, H-2), 5.32 (s, 1H, H-4), 4.65 – 4.57 (m, 2H, H-3, OCHO-THP), 4.14 – 4.05 (m, 2H, H-8), 3.85 – 3.75 (m, 1H,

OCH₂C-THP), 3.56 (dd, *J* = 10.7, 6.6 Hz, 0.5H, H-9), 3.50 – 3.47 (m, 1H, OCH₂C-THP), 3.40 (dd, *J* = 6.3, 2.7 Hz, 1H, H-9), 3.26 (dd, *J* = 10.7, 6.6 Hz, 0.5H, H-9), 2.35 – 2.31 (m, 1H, H-5), 2.09 (d, *J* = 15.6 Hz, 6H, CH₃-Ac), 2.02 (d, *J* = 7.6 Hz, 6H, CH₃-Ac), 1.80 – 1.54 (m, 7H, H-1, 3 x CCH₂C-THP), 1.19 – 1.15 (m, 1H, H-7), 0.79 (dd, *J* = 13.3, 5.5 Hz, 1H, H-6). ¹³C NMR (101 MHz, CDCl₃): δ (ppm) 170.7, 170.7, 170.7, 170.4, 170.2 (C=O-Ac), 98.3, 97.4 (OCHO-THP), 72.2 (C-3), 69.7 (C-2), 69.6, 69.1 (C-9), 69.1 (C-4), 62.9, 62.9 (C-8), 62.0, 61.5 (OCH₂C-THP), 38.2, 38.1 (C-5), 30.5, 30.4, 25.4, 25.3 (CCH₂C-THP), 24.4, 24.2 (C-7), 21.1, 21.0 (C-1), 20.8, 20.7, 20.7, 20.7 (CH₃-Ac), 19.3, 19.0 (CCH₂C-THP), 18.2, 17.9 (C-6). HRMS: calculated for [C₂₂H₃₂O₁₀Na]⁺ 479.18877, found 479.18854.



(1R,2S,3R,4S,5R,6R,7R)-5-(acetoxymethyl)-7-(hydroxymethyl)bicyclo[4.1.0]heptane-2,3,4-triyl triacetate (10)

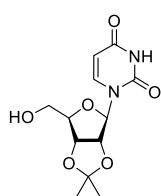
A solution of compound **9** (150 mg, 0.33 mmol) in MeOH (10 mL) was acidified to pH 2 with *p*-TsOH. After stirring for 1 h at room temperature, the reaction mixture was quenched with saturated aqueous NaHCO₃ and extracted with DCM (3x). The combined organic layers were washed with brine, dried with MgSO₄, filtered and concentrated *in vacuo*. Purification by column chromatography (50% DCM in pentane → 10% MeOH in DCM) gave alcohol **10** (104 mg, 0.28 mmol, 85%) as a white solid. ¹H NMR (400 MHz, CDCl₃): δ (ppm) 5.39 (dd, *J* = 9.2, 6.8 Hz, 1H, H-2), 5.31 (s, 1H, H-4), 4.64 (dd, *J* = 9.3, 1.6 Hz, 1H, H-3), 4.11 (d, *J* = 8.1 Hz, 2H, H-8), 3.49 (dd, *J* = 11.4, 6.4 Hz, 1H, CH₂), 3.42 (dd, *J* = 11.4, 6.4 Hz, 1H, CH₂), 2.36 – 2.25 (m, 1H, H-5), 2.09 (d, *J* = 15.6 Hz, 6H, CH₃-Ac), 2.02 (d, *J* = 7.6 Hz, 6H, CH₃-Ac), 1.67 (dt, *J* = 8.8, 6.5 Hz, 1H, H-1), 1.16 (t, *J* = 5.5 Hz, 1H, H-7), 0.78 (ddd, *J* = 8.2, 4.7, 2.6 Hz, 1H, H-6). ¹³C NMR (101 MHz, CDCl₃): δ (ppm) 171.2, 170.9, 170.6, 170.3 (C=O-Ac), 72.3 (C-3), 69.9 (C-2), 69.1 (C-4), 65.1 (CH₂O), 62.9 (C-8), 38.2 (C-5), 27.0 (C-7), 21.2 (C-1), 20.8, 20.8, 20.8, 20.7 (CH₃-Ac), 17.9 (C-6). HRMS: calculated for [C₁₇H₂₄O₉Na]⁺ 395.13125, found 395.13103.



((2R,5R)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl (((1R,2S,3R,4S,5R,6R,7R)-2,3,4-trihydroxy-5-(hydroxymethyl)bicyclo[4.1.0]heptan-7-yl)methyl) phosphate sodium salt (3)

To a solution of phosphoramidite **11**¹³ (52 mg, 0.09 mmol) in ACN (2 mL) was added a solution of alcohol **10** (43 mg, 0.11 mmol, 1.2 eq.) in ACN (3 mL). After stirring for 30 min at room temperature ^tBuOOH (5.5 M in decane, 70 μL, 0.40 mmol, 4.4 eq.) was added. After stirring for additional 30 min, DBU (75 μL, 0.50 mmol, 5.6 eq.) was added and the reaction mixture was stirred for 1 h before addition of aqueous NH₃ (30-35%, 0.50 mL). After stirring overnight additional aqueous NH₃ (30-35%, 0.50 mL) was added and stirred for 6 h. The reaction mixture was concentrated *in vacuo* and redissolved in MeOH (3 mL), H₂O (1 mL) and Et₃N (1 mL) in order to complete the deacetylation. After stirring overnight, the mixture was concentrated *in vacuo* and purified by size-exclusion chromatography (DCM/MeOH, 1:1) followed by HPLC purification (C18 column, linear gradient: A → 10% A in B, A = 100 mM NH₄OAc in H₂O, B = ACN, 15 min). Next, the purified product was dissolved in water and flushed through a tube of Dionex sodium exchange resin. Lyophilization afforded compound **3** (10 mg, 0.02 mmol, 22%) as a white solid. ³¹P

NMR (161.7 MHz, D₂O): δ (ppm) 0.64. ¹H NMR (400 MHz, D₂O): δ (ppm) 7.91 (d, J = 8.1 Hz, 1H, H-6), 5.97 – 5.92 (m, 1H, H-1' and H-5), 4.38 – 4.31 (m, 2H, H-2' and H-3'), 4.25 (s, 1H, H-4'), 4.22 – 4.06 (m, 3H, H-5', H-2''), 3.83 (s, 1H, H-4''), 3.80 – 3.66 (m, 4H, H-8'', H-9''), 3.20 (d, J = 9.1 Hz, 1H, H-3''), 1.90 – 1.85 (m, 1H, H-5''), 1.43 – 1.39 (m, 1H, H-1''), 1.16 – 1.12 (m, 1H, H-7''), 0.81 – 0.77 (m, 1H, H-6''). ¹³C NMR (101 MHz, D₂O): δ (ppm) 166.6 (C-4), 152.1 (C-2), 141.5 (C-6), 102.5 (C-5), 88.7 (C-1'), 83.1 (C-4'), 74.0 (C-3''), 73.7 (C-2''), 71.5 (C-4''), 69.6 (C-3'), 69.4, 69.3 (C-9''), 68.5 (C-2''), 64.4, 64.3 (C-5''), 62.5 (C-8''), 42.5 (C-5''), 24.2, 24.1 (C-7''), 23.5 (C-1''), 18.2 (C-6''). HRMS: calculated for [C₁₈H₂₈N₂O₁₃P]⁺ 511.13235, found 511.13220.



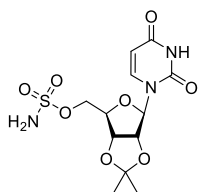
2',3'-O-Isopropylideneuridine (15)

To a solution of uridine **14** (4.88 g, 20.0 mmol) in acetone (200 mL) was added iodine (0.50 g, 2.0 mmol, 0.1 eq.). After stirring overnight at room temperature, the reaction mixture was quenched with saturated aqueous Na₂S₂O₃ (30 mL) and concentrated *in vacuo*. The crude product was redissolved in DCM, dried with MgSO₄, filtered and concentrated *in vacuo*. Purification by column chromatography (5% MeOH in DCM → 10% MeOH in DCM) gave compound **15** (4.72 g, 16.6 mmol, 83%) as a white solid. ¹H NMR (400 MHz, MeOD): δ (ppm) 7.83 (d, J = 8.0 Hz, 1H, H-6), 5.87 (d, J = 2.8 Hz, 1H, H-1'), 5.68 (d, J = 8.0 Hz, 1H, H-5), 4.91 – 4.82 (m, 2H, H-2' and H-3'), 4.21 (dd, J = 8.0, 3.6 Hz, 1H, H-4'), 3.78 (dd, J = 11.9, 3.6 Hz, 1H, H-5_a'), 3.71 (dd, J = 11.9, 4.5 Hz, 1H, H-5_b'), 1.54 (s, 3H, CH₃), 1.35 (s, 3H, CH₃). ¹³C NMR (101 MHz, MeOD): δ (ppm) 166.2 (C-4), 152.1 (C-2), 143.9 (C-6), 115.1 (C_q), 102.6 (C-5), 94.1 (C-1'), 88.4 (C-4'), 85.8 (C-2'), 82.2 (C-3'), 63.1 (C-5'), 27.5, 25.5 (CH₃). HRMS: calculated for [C₁₂H₁₇N₂O₆]⁺ 285.10811, found 285.10818.



Sulfamoyl chloride

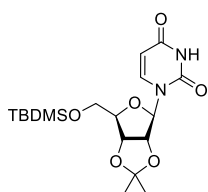
Formic acid (1 eq.) was added to chlorosulfamoyl isocyanate (1 eq.) and stirred at 0 °C. When the evolution of gas stopped, the reaction mixture was concentrated under reduced pressure at room temperature to afford sulfamoyl chloride as a clear oil, which was directly used without further purification.



2',3'-O-Isopropylidene-5'-O-sulfamoyluridine (16)

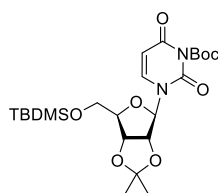
2',3'-O-isopropylideneuridine **15** (1.00 g, 3.51 mmol) was dissolved in THF (20 mL) and cooled to 0 °C, before sodium hydride (60% dispersion in oil, 0.700 g, 17.6 mmol, 5 eq.) was added. After stirring for 15 minutes, sulfamoyl chloride (7.0 mmol, 2 eq.) in THF (4 mL) was added. After stirring for 4 hours at 0 °C, the reaction mixture was allowed to come to room temperature and stirred for an additional 12 h. The reaction mixture was quenched with MeOH (5 mL) and saturated aqueous NH₄Cl (5 mL), filtrated and concentrated under reduced pressure. Purification by silica column chromatography (30% acetone in chloroform → 40% acetone in chloroform) resulted in O-sulfamoylamide **16** (1.0 g, 2.8 mmol, 79%) as a white foam. ¹H NMR (400 MHz, MeOD): δ (ppm) 7.64 (d, J = 8.1 Hz, 1H, H-2''), 5.82 (s, 1H, H-1'), 5.72 (d, J = 8.0 Hz, 1H, H-1''), 5.03 (d, J = 8.6 Hz, 1H, H-2'), 4.88 (dd, J = 6.3, 3.6 Hz, 1H, H-3'), 4.41 – 4.36 (m, 1H, H-4'), 4.36 – 4.27 (m, 2H, H-5'), 1.54 (s, 3H, CH₃), 1.35 (s, 3H, CH₃). ¹³C NMR (101 MHz, MeOD): δ (ppm) 166.2 (C=O), 152.2 (C=O), 144.3

(C-1''), 115.4 (C_q), 102.8 (C-2''), 95.1 (C-1'), 86.1 (C-4'), 85.4 (C-3'), 82.4 (C-2'), 70.0 (C-5'), 27.6, 25.5 (CH₃). HRMS: calculated for [C₁₂H₁₈N₃O₈S]⁺ 364.08091, found 364.08074.



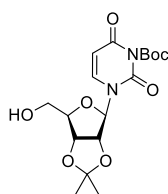
2',3'-O-Isopropylidene-5'-O-tert-butyldimethylsilyluridine (30)

To a solution of alcohol **15** (2.0 g, 7.0 mmol) in pyridine (50 mL) was added TBDMS-Cl (1.26 g, 8.4 mmol, 1.2 eq.) and a catalytic amount of 4-dimethylaminopyridine. After stirring overnight at room temperature, additional TBDMS-Cl (0.63 g, 3.5 mmol, 0.5 eq.) was added followed by stirring for 1.5 h before the reaction mixture was quenched with MeOH (5 mL) and concentrated *in vacuo*. The crude product was redissolved in EtOAc and washed with saturated aqueous NaHCO₃ and brine. The organic layer was dried with MgSO₄, filtered, and concentrated *in vacuo*. Purification by column chromatography (20% EtOAc in pentane → 50% EtOAc in pentane) gave title compound **30** (2.46 g, 6.2 mmol, 88%) as a white foam. ¹H NMR (400 MHz, CDCl₃): δ (ppm) 9.48 (s, 1H, NH) 7.71 (d, *J* = 8.1 Hz, 1H, H-6), 6.00 (d, *J* = 2.7 Hz, 1H, H-1'), 5.71 (d, *J* = 8.1 Hz, 1H, H-5), 4.78 - 4.71 (m, 2H, H-2' and H-3'), 4.35 - 4.30 (m, 1H, H-4'), 3.94 (dd, *J* = 11.5, 2.7 Hz, 1H, H-5_a'), 3.82 (dd, *J* = 11.5, 3.0 Hz, 1H, H-5_b'), 1.60 (s, 3H, CH₃), 1.37 (s, 3H, CH₃), 0.92 (s, 9H, CH₃), 0.10 (d, *J* = 2.6 Hz, 6H, CH₃). ¹³C NMR (101 MHz, CDCl₃): δ (ppm) 163.6 (C-4), 150.3 (C-2), 140.7 (C-6), 114.2 (C_q), 102.3 (C-5), 92.0 (C-1'), 86.8 (C-4'), 85.5 (C-2'), 80.4 (C-3'), 63.5 (C-5'), 27.4 (CH₃), 25.9 (C_q), 25.5 (CH₃), 18.4 (C_q), -5.3, -5.4 (CH₃). HRMS: calculated for [C₁₈H₃₁N₂O₆Si]⁺ 399.19459, found 399.19400.



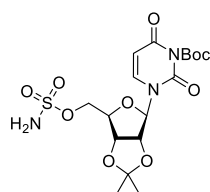
2',3'-O-Isopropylidene-5'-O-tert-butyldimethylsilyl-3-N-tert-butyloxycarbonyluridine (31)

Silyl-protected alcohol **30** (2.04 g, 5.1 mmol) was taken up in DCM (30 mL). Et₃N (2.2 mL, 15.3 mmol, 3 eq.), 4-dimethylaminopyridine (300 mg, 2.5 mmol, 0.5 eq.) and di-*tert*-butyl dicarbonate (2.22 g, 10.2 mmol, 2 eq.) were added to the solution. After stirring overnight at room temperature, the reaction mixture was concentrated *in vacuo*, and purified by column chromatography (5% EtOAc in pentane → 10% EtOAc in pentane) to yield title compound **31** (3.14 g, 5.1 mmol, quantitative) as a yellow oil. ¹H NMR (400 MHz, CDCl₃): δ (ppm) 7.71 (d, *J* = 8.2 Hz, 1H, H-6), 5.93 (d, *J* = 2.1 Hz, 1H, H-1'), 5.70 (d, *J* = 8.2 Hz, 1H, H-5), 4.75 (m, 2H, H-2' and H-3'), 4.37 - 4.34 (m, 1H, H-4'), 3.92 (dd, *J* = 11.6, 2.3 Hz, 1H, H-5_a'), 3.79 (dd, *J* = 11.6, 3.0 Hz, 1H, H-5_b'), 1.60 (s, 9H, CH₃), 1.58 (s, 3H, CH₃), 1.36 (s, 3H, CH₃), 0.90 (s, 9H, CH₃), 0.09 (d, *J* = 3.2 Hz, 6H, CH₃). ¹³C NMR (101 MHz, CDCl₃): δ (ppm) 160.5 (C-4), 148.4 (C-2), 147.6 (C=O-Boc) 139.8 (C-6), 114.0 (C_q), 101.7 (C-5), 92.8 (C-1'), 87.1 (C-4'), 86.8 (C_q), 85.6 (C-2'), 80.4 (C-3'), 63.4 (C-5'), 27.5 (CH₃), 27.3 (CH₃), 25.9 (C_q), 25.4 (CH₃), 18.4 (C_q), -5.4, -5.5 (CH₃).



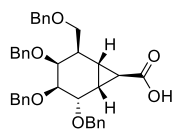
2',3'-O-Isopropylidene-3-N-tert-butyloxycarbonyluridine (17)

To a solution of Boc-protected uridine **31** (2.29 g, 4.6 mmol) in THF (50 mL) was added TBAF (6.9 mL, 6.9 mmol, 1.5 eq., 1 M in THF) at 0 °C. After stirring for 10 min, the reaction mixture was allowed to warm to room temperature and stirred for additional 30 min before the reaction mixture was partitioned between H₂O (50 mL) and EtOAc (150 mL). The aqueous layer was separated and extracted with EtOAc (3x). The combined organic layers were washed with brine, dried with MgSO₄, filtered and concentrated *in vacuo*. Purification by column chromatography (20% EtOAc in pentane → 50% EtOAc in pentane) afforded compound **17** (1.43 g, 3.7 mmol, 81%) as a white foam. ¹H NMR (400 MHz, CDCl₃): δ (ppm) 7.72 (d, *J* = 8.2 Hz, 1H, H-6), 5.82 (d, *J* = 1.8 Hz, 1H, H-1'), 5.73 (d, *J* = 8.2 Hz, 1H, H-5), 4.87 (m, 2H, H-2' and H-3'), 4.34 – 4.31 (m, 1H, H-4'), 3.88 (dd, *J* = 11.9, 2.4 Hz, 1H, H-5_a'), 3.77 (dd, *J* = 11.9, 2.4 Hz, 1H, H-5_b'), 1.59 (s, 9H, CH₃), 1.56 (s, 3H, CH₃), 1.35 (s, 3H, CH₃). ¹³C NMR (101 MHz, CDCl₃): δ (ppm) 160.6 (C-4), 148.4 (C-2), 147.5 (C=O-Boc) 141.3 (C-6), 113.9 (C_q), 101.4 (C-5), 93.8 (C-1'), 87.0 (C-4'), 86.8 (C_q), 84.5 (C-2'), 80.4 (C-3'), 62.2 (C-5'), 27.3, 27.0, 25.1 (CH₃). HRMS: calculated for [C₁₇H₂₅N₂O₈]⁺ 385.16054, found 385.16056.



2',3'-O-Isopropylidene-5'-O-sulfamoyl-3-N-tert-butyloxycarbonyluridine (18)

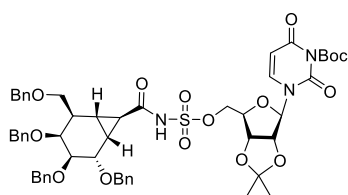
To a solution of alcohol **17** (552 mg, 1.4 mmol) in DCM (15 mL) was added pyridine (0.3 mL) and sulfamoyl chloride (345 mg, 3.0 mmol, 2.1 eq.). After the reaction mixture was refluxed overnight, the reaction mixture was concentrated *in vacuo* and purified by column chromatography (20% EtOAc in pentane → 50% EtOAc in pentane) to yield *O*-sulfamoylamide **18** (194 mg, 0.42 mmol, 30%) as a white solid. ¹H NMR (400 MHz, CDCl₃): δ (ppm) 7.40 (d, *J* = 8.2 Hz, 1H, H-6), 5.80 (d, *J* = 8.2 Hz, 1H, H-5), 5.75 (d, *J* = 1.8 Hz, 1H, H-1'), 5.00 (dd, 1H, H-2'), 4.87 (dd, 1H, H-3'), 4.43 – 4.40 (m, 1H, H-4'), 4.38 – 4.35 (m, 1H, H-5'), 1.59 (s, 9H, CH₃), 1.56 (s, 3H, CH₃), 1.35 (s, 3H, CH₃). ¹³C NMR (101 MHz, CDCl₃): δ (ppm) 160.7 (C-4), 148.5 (C-2), 147.8 (C=O-Boc) 141.7 (C-6), 114.8 (C_q), 102.3 (C-5), 94.8 (C-1'), 87.6 (C-4'), 84.7 (C_q), 84.3 (C-2'), 80.6 (C-3'), 69.5 (C-5'), 27.5, 27.1, 25.3 (CH₃). HRMS: calculated for [C₁₂H₁₈N₃O₈S]⁺ 364.08091, found 364.08088.



(1R,2S,3R,4S,5R,6R,7R)-2,3,4-tris(benzyloxy)-5-((benzyloxy)methyl)bicyclo[4.1.0]heptane-7-carboxylic acid (20)

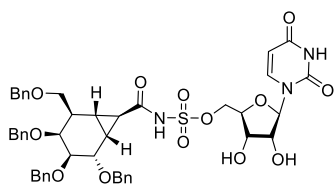
Ester **19** (see Chapter 6, 0.30 g, 0.49 mmol) was dissolved in THF/MeOH/H₂O (8:2:1, 25 mL) and LiOH (95 mg, 4 mmol, 8.0 eq.) was added. After stirring overnight at room temperature, the reaction mixture was acidified to pH 2 with aqueous HCl (1 M). The reaction mixture was diluted with H₂O and extracted with EtOAc. The organic layer was dried with MgSO₄, filtered and concentrated *in vacuo*. Purification by column chromatography (20% EtOAc in pentane → 30% EtOAc in pentane) afforded carboxylic acid **20** (0.244 g, 0.42 mmol, 86%) as a clear oil. ¹H NMR (400 MHz, CDCl₃): δ (ppm) 7.41 – 7.20 (m, 20H, H_{arom}), 4.87 (d, *J* = 11.6 Hz, 1H, CH₂Bn), 4.77 – 4.61 (m, 4H, CH₂Bn), 4.55 (d, *J* = 11.6 Hz, 1H, CH₂Bn), 4.45 (s, 2H, CH₂Bn), 4.41 – 4.29 (m, 1H, H-2), 3.89 (s, 1H, H-4), 3.63 (t, *J* = 8.9, 1H, H-8), 3.55 (t, *J* = 7.5 Hz, 1H, H-8), 3.15 (d, *J* = 8.2 Hz, 1H, H-3), 2.14 – 2.07 (m, H-1), 1.99 – 1.93 (m, 1H, H-5), 1.61 (t, *J* = 4.3 Hz, 1H, H-7), 1.40 –

1.36 (m, 1H, H-6). ^{13}C NMR (100 MHz, CDCl_3): δ (ppm) 179.4 (C=O), 139.1, 138.8, 138.1 (C_q), 128.6, 128.4, 128.3, 128.0, 127.9, 127.9, 127.6, 127.5, 127.5 (CH_{arom}), 82.7 (C-3), 76.0 (C-2), 75.6 (C-4), 74.1, 73.4, 72.9, 71.8 (CH_2Bn), 70.4 (C-8), 41.2 (C-5), 28.0 (C-1), 25.8 (C-7), 25.5 (C-6). HRMS: calculated for $[\text{C}_{37}\text{H}_{38}\text{O}_6\text{Na}]^+$ 593.30066, found 596.29994.



tert-butyl 3-(((3aR,4R,6R,6aR)-2,2-dimethyl-6-(((1R,2S,3R,4S,5R,6R,7R)-2,3,4-tris(benzyloxy)-5-((benzyloxy)methyl)bicyclo[4.1.0]heptane-7-carbonyl)sulfamoyl)oxy)methyl)tetrahydrofuro[3,4-d][1,3]dioxol-4-yl)-2,6-dioxo-3,6-dihydropyrimidine-1(2H)-carboxylate (22)

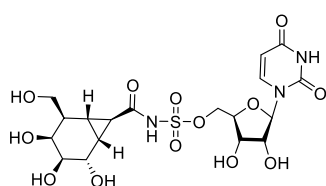
To a solution of carboxylic acid **20** (98 mg, 0.17 mmol) in toluene (5 mL) was added oxalyl chloride (0.15 mL, 1.7 mmol, 10 eq.). After stirring overnight at 40 °C, the reaction mixture was concentrated *in vacuo* followed by coevaporation with toluene (3x). The residu was redissolved in DCM (5 mL) and a solution of *O*-sulfamoylamide **18** (100 mg, 0.22 mmol, 1.3 eq.) in DCM (2 mL) and DIPEA (60 μL , 0.34 mmol, 2 eq.) were added. After stirring for 3 h at room temperature, the reaction mixture was washed with an aqueous HCl solution (1 M), saturated aqueous NaHCO_3 and brine. The organic layer was dried with Na_2SO_4 , filtered and concentrated *in vacuo*. Purification by size exclusion chromatography (DCM/MeOH, 1:1) followed by silica column chromatography (20% EtOAc in pentane $\rightarrow$ 50% EtOAc in pentane) yielded compound **22** (100 mg, 98 μmol , 60%) as a white solid. ^1H NMR (400 MHz, CDCl_3): δ (ppm) 7.36 – 7.24 (m, 21H, H-6 and H_{arom}), 5.79 (d, J = 1.8 Hz, 1H, H-1'), 5.76 (d, J = 8.2 Hz, 1H, H-5), 4.88 – 4.43 (m, 12H, CH_2Bn , H-2', H-3' and H-5'), 4.39 – 4.33 (m, 2H, H-2'' and H-4'), 3.93 (s, 1H, H-4''), 3.64 – 3.54 (m, 2H, H-8''), 3.13 (d, J = 8.5 Hz, 1H, H-3''), 2.13 – 2.09 (m, 1H, H-1''), 2.02 – 1.97 (m, 1H, H-5''), 1.66 – 1.61 (m, 1H, H-7''), 1.58 (s, 9H, CH_3), 1.55 (s, 3H, CH_3), 1.51 – 1.45 (m, 1H, H-6''), 1.33 (s, 3H, CH_3). ^{13}C NMR (101 MHz, CDCl_3): δ (ppm) 170.9 (C-9''), 160.5 (C-4), 148.6 (C-2), 147.7 (C=O-Boc), 140.8 (C-6), 138.9, 138.5, 138.1, 138.0 (C_q), 128.6, 128.5, 128.4, 128.3, 128.2, 128.0, 127.9, 127.6, 127.6, 127.5 (CH_{arom}), 114.9 (C_q), 102.6 (C-5), 93.4 (C-1'), 87.3 (C_q), 84.3 (C-2'), 83.9 (C-4'), 82.6 (C-3''), 80.3 (C-3'), 76.2 (C-2''), 75.1 (C-4''), 74.2 (CH_2Bn), 73.4 (CH_2Bn), 72.6 (C-5'), 72.5, 71.5 (CH_2Bn), 70.4 (C-8''), 45.9 (C_q), 41.0 (C-5''), 28.6 (C-1''), 27.5 (C_q), 27.4 (C-7''), 27.2, 25.3 (CH_3), 24.6 (C-6''). HRMS: calculated for $[\text{C}_{49}\text{H}_{54}\text{N}_3\text{O}_{13}\text{S}]^+$ 924.33719, found 924.33760.



((2R,5R)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl ((1R,2S,3R,4S,5R,6R,7R)-2,3,4-tris(benzyloxy)-5-((benzyloxy)methyl)bicyclo[4.1.0]heptane-7-carbonyl)sulfamate (23)

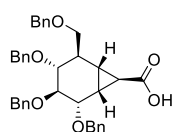
To a cooled (0 °C) solution of compound **22** (100 mg, 98 μmol) in DCM (2 mL) was dropwise added a solution of TFA/ H_2O (9:1, 500 μL). After stirring for 4.5 h the reaction mixture was concentrated *in vacuo* followed by coevaporation with toluene (3x). Purification by column chromatography (50% EtOAc in pentane $\rightarrow$ EtOAc) afforded title compound **23** (70 mg, 79 μmol , 81%) as a white solid. ^1H NMR (400 MHz, MeOD): δ (ppm) 7.69 (d, J = 8.1 Hz, 1H, H-6), 7.39 –

7.17 (m, 20H, H_{arom}), 5.89 (d, $J = 3.9$ Hz, 1H, H-1'), 5.72 (d, $J = 8.1$ Hz, 1H, H-5), 4.78 – 4.37 (m, 10H, CH₂Bn and H-5'), 4.32 (dd, $J = 8.3, 6.3$ Hz, 1H, H-2''), 4.21 – 4.10 (m, 3H, H-2', H-3' and H-4'), 3.90 (s, 1H, H-4''), 3.63 – 3.51 (m, 2H, H-8''), 3.19 (d, $J = 9.0$ Hz, 1H, H-3''), 2.17 – 2.09 (m, 1H, H-1''), 2.01 – 1.97 (m, 1H, H-5''), 1.65 (t, $J = 4.5$ Hz, 1H, H-7''), 1.50 – 1.42 (m, 1H, H-6''). ¹³C NMR (101 MHz, MeOD): δ (ppm) 173.5 (C-9''), 166.0 (C-4), 152.3 (C-2), 142.3 (C-6), 140.2, 140.0, 139.9, 139.5 (C_q), 129.5, 129.4, 129.4, 129.3, 129.1, 129.1, 129.1, 128.8, 128.7, 128.6, 128.5 (CH_{arom}), 103.1 (C-5), 90.5 (C-1'), 83.6 (C-3''), 82.7 (C-4'), 76.9 (C-2''), 76.7 (C-4''), 75.2 (C-2'), 75.0, 74.2, 73.4, 72.7 (CH₂Bn), 71.9 (C-5'), 71.8 (C-8''), 71.0 (C-3'), 42.2 (C-5''), 29.3 (C-1''), 28.4 (C-7''), 26.7 (C-6''). HRMS: calculated for [C₄₆H₅₀N₃O₁₃S]⁺ 884.30589, found 884.30684.



**((2R,5R)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl
((1R,2S,3R,4S,5R,6R,7R)-2,3,4-trihydroxy-5-(hydroxymethyl)bicyclo[4.1.0]heptane-7-carbonyl)sulfamate
(4)**

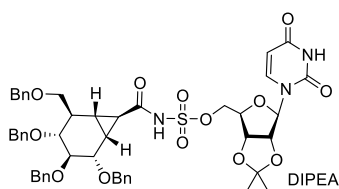
To a cooled (0 °C) solution of compound **23** (32 mg, 36 μ mol) in DCM (2 mL) was dropwise added BCl₃ (1 M in DCM, 300 μ L, 0.30 mmol, 8 eq.). After stirring for 30 min, the reaction mixture was allowed to warm to 0 °C. After stirring for additional 4 h, the reaction mixture was cooled to -78 °C and quenched with MeOH. The reaction mixture was concentrated *in vacuo*, redissolved in CHCl₃ and stirred vigorously for 30 min before the suspension was filtered. The residue was taken up in methanol and concentrated *in vacuo*. Purification by HPLC (C18, linear gradient: 5 $\rightarrow$ 10% B in A, solutions used A: H₂O, B: acetonitrile, 0.5% TFA, 15 min) afforded title compound **4** (13 mg, 25 μ mol, 69%) as a white solid. ¹H NMR (400 MHz, D₂O): δ (ppm) 7.66 (d, $J = 8.1$ Hz, 1H, H-6), 5.93 – 5.81 (m, 2H, H-1' and H-5), 4.59 (dd, $J = 11.4, 2.3$ Hz, 1H, H-5'), 4.54 (dd, $J = 11.4, 3.4$ Hz, 1H, H-5'), 4.31 – 4.27 (m, 2H, H-2' and H-4'), 4.25 – 4.21 (m, 2H, H-2' and H-3'), 3.83 (s, 1H, H-4''), 3.74 – 3.51 (m, 2H, H-8''), 3.20 (d, $J = 9.1$ Hz, 1H, H-3''), 2.08 (ddd, $J = 9.4, 6.1, 4.9$ Hz, 1H, H-1''), 1.96 – 1.90 (m, 1H, H-5''), 1.66 (t, $J = 4.5$ Hz, 1H, H-7''), 1.45 – 1.40 (m, 1H, H-6''). ¹³C NMR (101 MHz, D₂O): δ (ppm) 173.9 (C-9''), 166.1 (C-4), 151.6 (C-2), 141.6 (C-6), 102.4 (C-5), 89.3 (C-1'), 80.9 (C-4'), 73.2 (C-3''), 73.1 (C-2'), 70.9 (C-4''), 70.7 (5'), 69.3 (C-3'), 67.4 (C-2''), 62.1 (C-8''), 42.1 (C-5''), 29.8 (C-1''), 27.1 (C-7''), 24.9 (C-6''). HRMS: calculated for [C₁₈H₂₆N₃O₁₃S]⁺ 524.11808, found 524.11793.



(1R,2S,3R,4R,5R,6R,7R)-2,3,4-tris(benzyloxy)-5-((benzyloxy)methyl)bicyclo[4.1.0]heptane-7-carboxylic acid (26)

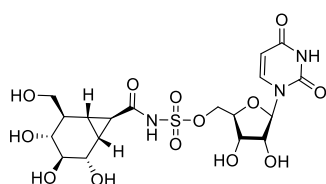
Method A: To a solution of ester **25a** (see Chapter 6, 0.202 g, 0.33 mmol) in THF/MeOH/H₂O (6.5:1.5:1, 18 mL) was added LiOH (32 mg, 1.3 mmol, 4 eq.). After stirring for 5 h at room temperature, the reaction mixture was quenched with HCl (1 M) until pH 2. The reaction mixture was extracted with EtOAc (2x) and the combined organic layers were dried over MgSO₄, filtered and concentrated *in vacuo*. Purification by column chromatography (10% EtOAc in pentane $\rightarrow$ 25% EtOAc in pentane) gave carboxylic acid **26** (0.173 g, 0.30 mmol, 91%) as a white oil.

Method B: Chromic acid stock solution (1.0 M) (*Caution:* Chromic acid is corrosive, toxic and carcinogenic): Concentrated H₂SO₄ (2.25 mL, 40.5 mmol) is taken up in H₂O (12.5 mL). To this solution was added CrO₃ (2.50 g, 25.0 mmol) and the resulting bright red solution was stirred until all solids were completely dissolved. The solution was then diluted with H₂O to a total volume of 25 mL. Alcohol **27** (see Chapter 6, 0.116 g, 0.20 mmol) was dissolved in acetone (40 mL) and cooled to 0 °C, after which chromic acid stock solution (1M, 0.4 mL, 0.40 mmol, 2 eq.) was added. After stirring for 70 min, the reaction mixture was diluted with EtOAc (150 mL) and washed with HCl (3 M, 2 x 100 mL) and brine (100 mL). The organic layer was dried over MgSO₄, filtered and concentrated *in vacuo*. Purification by column chromatography (10% EtOAc in pentane → 25% EtOAc in pentane) resulted in carboxylic acid **26** (87 mg, 0.15 mmol, 75%) as a white solid. ¹H NMR (400 MHz, CDCl₃): δ (ppm) 7.39 – 7.22 (m, 20H, H_{arom}), 4.89 – 4.62 (m, 5H, CH₂Bn), 4.43 (m, 3H, CH₂Bn), 4.09 – 4.05 (m, 1H, H-2), 3.61 (dd, *J* = 8.9, 4.5 Hz, 1H, H-8), 3.55 (dd, *J* = 8.9, 2.8 Hz, 1H, H-8), 3.40 (t, *J* = 10.2 Hz, 1H, H-4), 3.29 – 3.22 (m, 1H, H-3), 2.06 – 2.05 (m, 1H, H-1), 1.99 – 1.90 (m, 1H, H-5), 1.84 – 1.80 (m, 1H, H-6), 1.69 (t, *J* = 4.5 Hz, 1H, H-7). ¹³C NMR (101 MHz, CDCl₃): δ (ppm) 178.7 (C=O), 138.9, 138.5, 138.2, 138.2 (C_q), 128.5, 128.5, 128.5, 128.5, 128.2, 128.0, 128.0, 128.0, 127.9, 127.8, 127.7 (CH_{arom}), 84.0 (C-3), 79.0 (C-2), 78.4 (C-4), 75.7, 75.4, 73.3, 71.7 (CH₂Bn), 70.2 (C-8), 43.0 (C-5), 28.3 (C-1), 26.2 (C-9), 24.7 (C-6). HRMS: calculated for [C₃₇H₃₈O₆Na]⁺ 601.25606, found 601.25564.



DIPEA salt of ((3aR,4R,6R,6aR)-6-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-2,2-dimethyltetrahydrofuro[3,4-d][1,3]dioxol-4-yl)methyl ((1R,2S,3R,4R,5R,6R,7R)-2,3,4-tris(benzyloxy)-5-((benzyloxy)methyl)bicyclo[4.1.0]heptane-7-carbonyl)sulfamate (29**)**

Carboxylic acid **26** (74 mg, 0.12 mmol) was dissolved in toluene (6.0 mL) and oxalyl chloride (1 M in DCM, 0.24 mL, 0.24 mmol, 2.0 eq.) was added. The reaction mixture was stirred at 40 °C for 4 hours and concentrated *in vacuo*. The obtained acyl chloride was redissolved in DCM (7.0 mL) and *O*-sulfamoylamine **16** (0.100 g, 0.27 mmol, 2.3 eq.) and DIPEA (40 μL, 0.24 mmol, 2 eq.) were added. After stirring overnight at room temperature, the reaction mixture was concentrated under reduced pressure and purified by size-exclusion column chromatography (DCM/MeOH, 1:1), resulting in product **29** (89 mg, 84 μmol, 70%) as a white wax. ¹H NMR (400 MHz, MeOD): δ (ppm) 7.75 (dd, *J* = 13.8, 7.7 Hz, 1H, H-6), 7.41 – 7.18 (m, 20H, H_{arom}), 5.97 – 5.85 (m, 1H, H-1'), 5.71 (d, *J* = 8.0 Hz, 1H, H-5), 4.89 – 4.86 (m, 1H, H-2'), 4.84 – 4.70 (m, 4H, H-3', CH₂Bn), 4.59 (d, *J* = 11.5 Hz, 1H, H-4'), 4.50 – 4.33 (m, 5H, CH₂Bn), 4.31 – 4.23 (m, 2H, H-5'), 4.06 (dd, *J* = 7.9, 5.9 Hz, 1H, H-2''), 3.70 – 3.66 (m, 3H, Me-DIPEA), 3.58 – 3.57 (m, 2H, H-8''), 3.35 – 3.27 (m, 2H, H-4'' and H-3''), 3.21 – 3.15 (m, 2H, CH₂-DIPEA), 2.09 – 2.02 (m, 1H, H-1''), 1.95 (dd, *J* = 12.9, 8.3 Hz, 1H, H-5''), 1.74 (dd, *J* = 8.6, 5.3 Hz, 2H, H-7'' and H-6''), 1.56 – 1.47 (m, 3H, CH₃), 1.39 – 1.30 (m, 15H, CH₃ and isopropyl DIPEA). ¹³C NMR (101 MHz, MeOD): δ (ppm) 166.1 (C=O), 162.7 (C=O), 152.1 (C=O), 143.6 (C-2''), 140.3, 140.0, 139.9, 139.6 (C_q), 129.4, 129.3, 129.3, 129.2, 129.2, 129.1, 129.0, 129.0, 128.7, 128.5, 128.5 (CH_{arom}), 115.2 (C_q), 103.2 (C-1'), 93.7 (C-1''), 85.5 (C-2'), 85.4 (C-4'), 85.1 (C-5), 82.3 (C-3), 80.4 (C-2), 79.9 (C-4), 76.3, 76.0, 74.1, 71.9 (CH₂Bn), 71.4 (C-8), 70.1 (C-5'), 55.8 (Me-DIPEA), 44.4 (C-5), 43.8 (CH₂-DIPEA), 29.6 (C-7), 27.6 (C-1), 26.2 (CH₃), 26.2 (C-6), 25.6 (CH₃).



**((2R,5R)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl
((1R,2S,3R,4R,5R,6R,7R)-2,3,4-trihydroxy-5-(hydroxymethyl)bicyclo[4.1.0]heptane-7-carbonyl)sulfamate
(5)**

Compound **29** (86 mg, 81 μ mol) was dissolved in DCM (10 mL) and cooled to 0 °C, before BCl_3 (1 M in DCM, 1.11 mL, 1.11 mmol, 13.7 eq.) was added. After stirring for 3 h, the reaction mixture was quenched with MeOH. The reaction mixture was concentrated *in vacuo*, redissolved in CHCl_3 and stirred vigorously for 30 min before the suspension was filtered. The residue was taken up in methanol and concentrated *in vacuo*. Purification by HPLC (C18, linear gradient: 5 $\rightarrow$ 10% B in A, solutions used A: H_2O , B: acetonitrile, 0.5% TFA, 15 min) resulted into product **5** (23 mg, 43.5 μ mol, 54%) as white solid. ^1H NMR (400 MHz, MeOD): δ (ppm) 7.73 (t, J = 10.0 Hz, 1H, H-6), 5.90 (d, J = 4.4 Hz, 1H, H-1'), 5.73 (dd, J = 19.7, 8.3 Hz, 1H, H-5), 4.55 – 4.47 (m, 2H, H-5'), 4.20 (d, J = 4.1 Hz, 1H, H-4'), 4.18 – 4.11 (m, 2H, H-2', H-3'), 3.98 – 3.90 (m, 1H, H-2''), 3.84 (dd, J = 10.5, 3.6 Hz, 1H, H-8''), 3.70 – 3.59 (m, 1H, H-8''), 3.14 (t, J = 10.1 Hz, 1H, H-4''), 3.04 – 2.95 (m, 1H, H-3''), 2.03 – 1.97 (m, 1H, H-1''), 1.80 – 1.65 (m, 3H, H-5'', H-6'', H-7''). ^{13}C NMR (101 MHz, MeOD): δ (ppm) 172.8 (C=O), 152.6 (C=O), 142.4 (C-2''), 103.1 (C-1''), 90.7 (C-1'), 82.8 (C-4'), 76.2 (C-3), 75.0 (C-2'), 72.3 (C-2'), 72.1 (C-4), 71.7 (C-2), 71.1 (C-3'), 64.2 (C-8), 46.2 (C-5), 31.3 (C-1), 27.0 (C-6), 26.2 (C-7). HRMS: calculated for $[\text{C}_{18}\text{H}_{26}\text{N}_3\text{O}_{13}\text{S}]^+$ 524.11808, found 524.11807.

Materials of biological assays

Recombinant human GBA1 (Cerezyme), recombinant human GAA (Myozyme) and recombinant human α -GalA (Fabrazyme) were obtained from Genzyme, USA. Bacterial GAA homologue *CJAgd31B* was kindly provided by professor Gideon Davies (University of York, UK). Cellular homogenates of a stable human embryonic kidney 293 (HEK293) over-expressing GBA2 cell line¹⁸ were pre-incubated for 30 min with an inhibitor of GBA1 (1 mM conduritol B epoxide (CBE)). Galactocerebrosidase (GALC) was obtained by expressing recombinant murine GALC in HEK293 cells after which the secreted recombinant protein in the culture medium was directly used.¹⁹ Fibroblasts of Pompe patients diagnosed on the basis of reduced GAA activity (obtained with consent from donors) were used for GANAB determinations.

Enzyme activity assays, determination of the apparent IC_{50} values

All activity assays were performed at 37 °C. To determine the apparent IC_{50} values, enzyme preparations were pre-incubated with a range of inhibitor concentrations for 30 min, where after the residual activity was measured by addition of the appropriated 4-methylumbelliferyl-based substrate mix. GBA1 residual activity was measured with 3.7 mM 4-methylumbelliferone(4- μ)- β -D-glucopyranoside in 150 mM McIlvaine buffer, pH 5.2, supplemented with 0.2% Taurocholate (w/v), 0.1% Triton X-100 (v/v), 0.1% Bovine Serum albumin (BSA) (w/v) for 30 min. GBA2 substrate mix consisted of 3.7 mM 4- μ - β -D-glucopyranoside in 150 mM McIlvaine, pH 5.8, 0.1% BSA (w/v) and was incubated for 1 h. α -GalA activity was determined with 2 mM 4- μ - α -D-galactopyranoside dissolved in 150 mM McIlvaine, pH 4.5, 0.1 % BSA (w/v) for 30 min. GAA substrate mix consisted of 3 mM 4- μ - α -D-glucopyranoside pH 5.0, 0.1% BSA (w/v) for 1 h, GALC was assayed with 1.25 mM

4-mu- β -D-galactopyranoside in 150 mM McIlvaine pH 4.3 containing 0.2 M NaCl and 0.1% BSA (w/v) for 1h and GANAB with 3 mM 4-mu- α -D-glucopyranoside pH 7.0, 0.1% BSA (w/v) for 2 h. The bacterial enzyme activity of *CJAgd31B* was determined with 3 mM 4-mu- α -D-glucopyranoside in 50 mM citrate buffer at the optimal pH 6.5, 0.1% BSA (w/v) for 1 h. After stopping the substrate reaction with an excess of 1 M NaOH-glycine (pH 10.3), liberated 4-mu fluorescence was measured with a fluorimeter LS55 (Perkin Elmer) using λ_{Ex} 366 nm and λ_{Em} 445 nm. All IC₅₀ values were determined in triplicate with Graphpad Prism 5 software.

IC₅₀ values for GCS were determined *in situ* with NBD-ceramide as substrate as previously described.²⁰ Briefly, RAW 264.7 cells were grown to confluence in 6-well plates and pre-incubated for 1 h with an inhibitor of GBA1 activity (300 μ M CBE), followed by 1 h incubation at 37 °C with 1 nmol C6-NBD-ceramide (6-[*N*-methyl-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) aminododecanoyl] sphingosine) and in the presence of a range of inhibitor concentrations. The cells were washed 3x with PBS and harvest by scraping. After lipid extraction²¹, the C6-NBD lipids were separated and detected by HPLC (λ_{Ex} 470 nm and λ_{Em} 530 nm). IC₅₀ values were determined in duplicate from the titration curves of observed formed C6-NBD-glucosylceramide.

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9

Summary and future prospects

Malfunctioning of glycosidases causes various complex pathologies in man. Activity-Based Protein Profiling (ABPP) is a powerful technique to selectively analyze functional proteins in their physiological surroundings. Potent and selective activity-based glycosidase probes (ABPs) could help to understand the pathological processes connected with these enzymes. The first part of this Thesis describes the design, synthesis and application of a set of ABPs for retaining α -galactosidases, β -galactosidases, α -mannosidases and β -mannosidases. The second part of this Thesis focuses on the design of functionalized bicycle [4.1.0] heptanes as potential competitive inhibitors of glycosidases (α -galactosidases, β -galactosidases, α -glucosidases and β -glucosidases) and glycosyltransferases (both galactosyltransferases and glucosyltransferases).

Chapter 1 provides a brief overview of the basics of the ABPP technology. As well, information is provided on the reaction itineraries of retaining glycosidases, which are at the basis of the design of the covalent and competitive glycosidase inhibitors presented in this Thesis. Finally, the potencies of transition-state based inhibitors are described including therapeutic examples. **Chapter 2** describes the development and validation of α -galactosidase selective probes that may be used to study the lysosomal storage disorders (LSDs), Fabry disease and Schindler disease. Both *N*-acylated- and *N*-alkylated versions of α -galactose-configured cyclophellitol aziridines were examined on their capacity to inhibit and label, in an ABPP setting, retaining α -galactosidases. The *N*-acylated version is more prone to hydrolysis during purification and lyophilisation than its *N*-alkylated counterpart. However, this probe also proved the most effective in labeling retaining α -galactosidases.

The synthesis and validation of a new β -galactosidase selective probe is presented in **Chapter 3**. Once the synthesis of the one-step ABP using the conventional method turned out to be abortive, a two-step bioorthogonal labeling approach was attempted. In this way, the human retaining β -galactosidase, GALT (lysosomal galactocerebrosidase) could be detected in kidney lysate by in-gel labeling. In a final attempt to obtain a one-step ABP, inverse-electron-demand Diels-Alder (IEDDA) ligation was successfully applied to obtain a potent one-step β -galactosidase probe for labeling of GLB1 and GALT in mouse kidney homogenates. This probe may in turn be used as tool to study GM1 gangliosidosis, Morquio B syndrome and Krabbe disease which are caused by mutations in GH35 and GH59 galactosidases, GBL1 and GALT, respectively.

Looking forward to the sections on competitive inhibitors, Birch reduction of compound **1**¹, which featured as an intermediate in the synthesis of galactose-configured cyclophellitol, may yield cyclohexene **2** (Figure 9.1). Compound **2** may adopt the ⁴H₃ conformation and thereby mimic the oxocarbenium ion transition-state of α -galactosidases (**3**) and β -galactosidases (**4**). Therefore, compound **2** may act as a potential reversible galactosidase inhibitor.

The library of glycosidase ABPs is extended in **Chapter 4** with the synthesis and validation of ABPs for retaining α -mannosidases. The reaction itinerary of α -mannosidases is different from the previously described glycosidases, and a boat (B_{2,5}) transition-state conformation is expected to emerge during substrate processing. A covalent enzyme-inhibitor complex was obtained after soaking of α -mannosidase configured cyclophellitol aziridine in GH38 Golgi α -mannosidase II from drosophila.

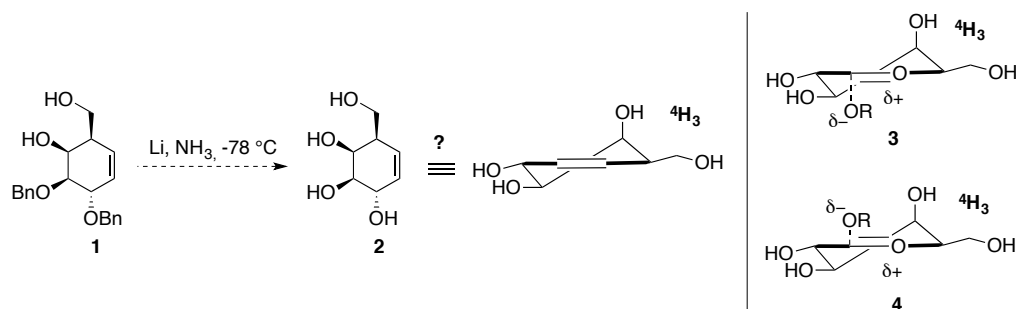


Figure 9.1 Cyclohexene **2** may adopt the $^4H_3$ conformation and therefore conformationally mimic the oxocarbenium ion transition-state of α -galactosidases (**3**) and β -galactosidases (**4**).

Herein, the cyclohexane has adopted the skew boat ($^1S_5$) conformation as expected from the reaction itinerary. The corresponding Cy5-functionalized ABP proved to label GH38 jack bean α -mannosidase in an activity-dependent fashion. Follow-up ABPP experiments *in vitro* and *in vivo* will reveal whether the α -mannosidases probes will be of use in diagnosing α -mannosidase connected diseases such as α -mannosidosis or larynx cancer, and the identification of therapeutics aimed at correcting these diseases.² The first part of this Thesis ends with the description, in **Chapter 5**, of a set of GH2 exo- β -mannosidase selective ABPs. The β -mannopyranose-configured cyclophellitol aziridine based probes were validated in ABPP experiments with exo- β -mannosidases from *Helix p.* Furthermore, ABPP experiments in lysates of mice kidney resulted in labeling of MANBA, a GH2 β -mannosidase that is deficient in β -mannosidosis.

Based on the results for mannosidases presented in this Thesis, it would be of interest to develop cyclophellitol-based ABPs (**5** – **7**, Figure 9.2) for other retaining exoglycosidases for which a boat transition-state conformation is expected as part of the reaction itinerary.³ In case of GH39 α -idurodinase, for example, such a $^2,5B$ transition-state is expected to occur during substrate processing.⁴ α -Idurodinase selective ABPs (**5**) for ABPP would be of interest to investigate mucopolysaccharidosis

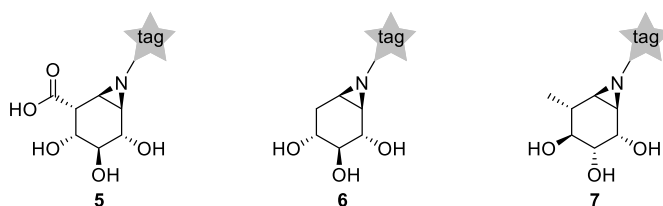
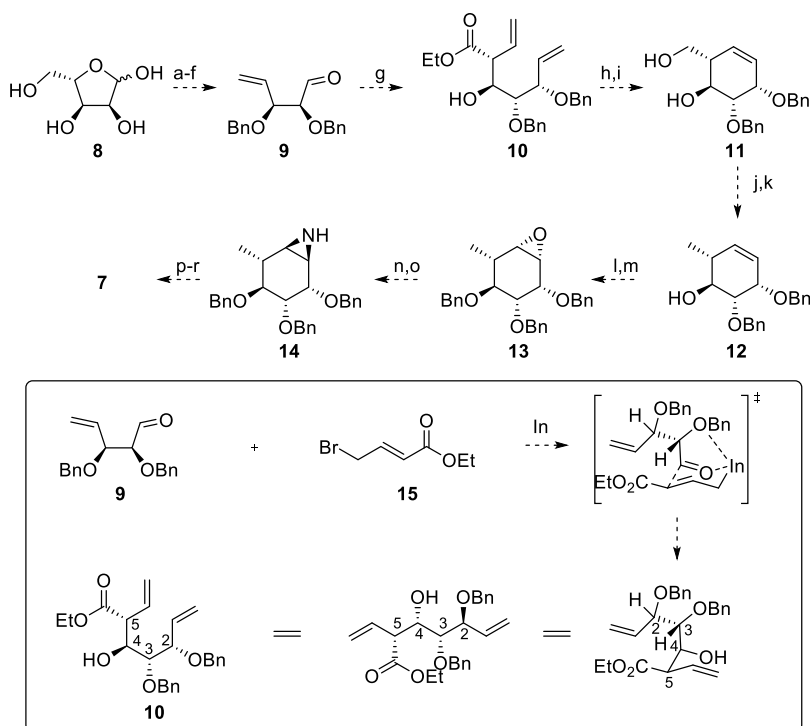


Figure 9.2 Putative ABPs to target GH39 α -idurodinase (**5**), GH120 β -xylosidase (**6**) and GH78 α -rhamnosidase (**7**).

I, caused by α -idurodinase deficiency.⁵ Another target could be GH120 β -xylosidase, found in *Bifidobacterium adolescentis*. Monitoring this enzyme with ABPs (**6**) could be of interest as this bacteria is present in the human gut.⁶ Finally, GH78 α -rhamnosidase is of industrial interest as this enzyme is used in the debittering of citrus juices.⁷⁻⁹ With ABPP using ABP **7** the enzyme activity could be monitored under various conditions.

A putative synthesis of the α -L-rhamnopyranose-configured cyclophellitol aziridine based ABP **7** is depicted in Scheme 9.1. The synthetic strategy is based on the synthesis of cyclophellitol as described by Madsen and co-workers¹⁰, which has recently been

Scheme 9.1 Synthesis of α -L-rhamnopyranose-configured cyclophellitol aziridine based ABP **7**.

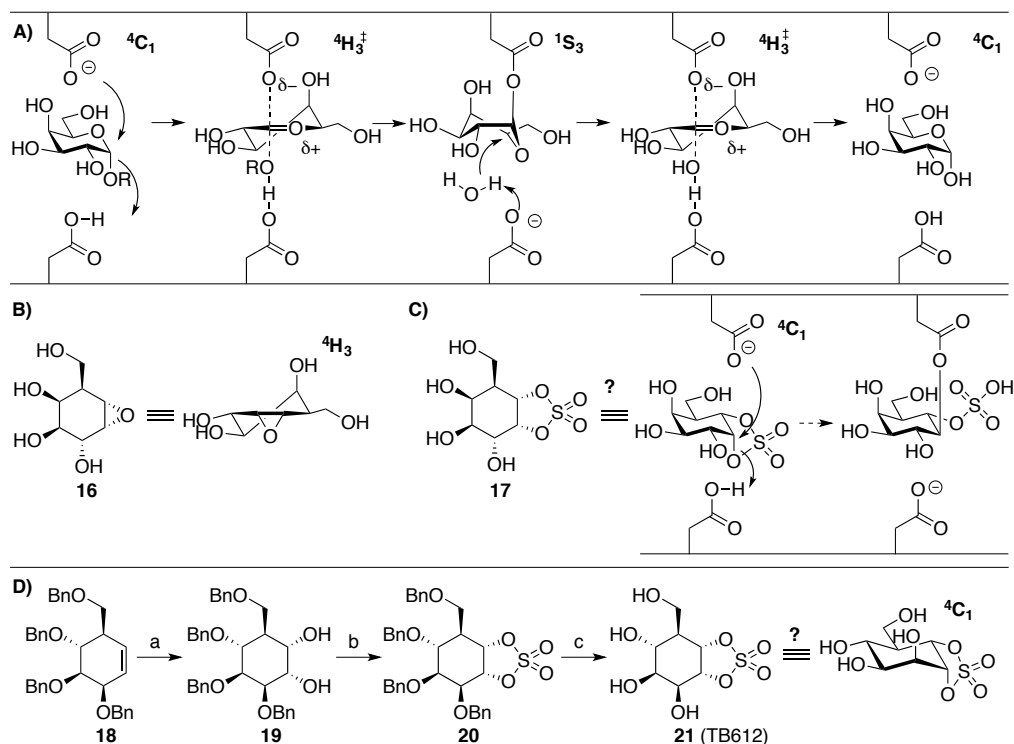


Reagents and conditions: a) acetyl chloride, MeOH, 0 °C to rt; b) trityl chloride, pyridine, rt; c) BnBr, NaH, DMF, 0 °C to rt; d) *p*TsOH, DCM/MeOH (1:1), rt; e) *i*. PPh₃, imidazole, THF, reflux; *ii*. I₂, THF, reflux; f) Zn, THF/H₂O (9:1), 50 °C, sonicate; g) ethyl 4-bromocrotonate, In (powder), La(OTf)₃; h) Grubbs II, DCM, reflux; i) *i*. DIBAL, THF, 0 °C to rt; *ii*. NaBH₄, EtOAc/H₂O (2:1); j) *p*TsCl, Et₃N, CH₂Cl₂; k) LiAlH₄, THF, 0 °C to rt; l) BnBr, NaH, DMF, 0 °C to rt; m) *m*CPBA, DCE, reflux; n) NaN₃, LiClO₄, MeCN, 80 °C; o) PPh₃ polymer-bound on styrene-divinylbenzene copolymer, CH₃CN, reflux, overnight; p) Li, THF, NH₃ (l), - 60 °C; q) 1-azido-8-iodooctane, K₂CO₃, DMF, 80 °C, r) alkyne tags (see Chapter 4), Cu.SO₄.5H₂O, sodium ascorbate, DMF, rt.

optimized.¹¹ Commencing with L-ribose¹² (**8**) as starting material, aldehyde **9** may be obtained in 6 steps. Subsequently the stereoselective indium-catalyzed Barbier reaction with ethyl-4-bromocrotonate may be performed to obtain compound **10**. The proposed stereoselective outcome of this reaction can be explained by the formed 6-membered transition state (Scheme 9.1). Grubbs II catalyzed ring closure metathesis of **10** followed by reduction of the ethyl ester may give compound **11**. Tosylation of the primary alcohol followed by treatment with lithium aluminium hydride¹³ may afford L-rhamnopyranose-configured cyclohexene **12**. After benzylation of **12**, epoxidation with *m*CPBA may give the desired epoxide **13** and the diastereomeric isomer which may be separated by silica column chromatography. Treatment of epoxide **13** with sodium azide and lithium perchlorate¹⁴, followed by a Staudinger reaction using triphenylphosphine on beads may yield aziridine **14**. After the Birch reduction, *N*-alkylation with 1-azido-8-iodooctane and azide-alkyne Huisgen cycloaddition with a specific alkyne-tag (see Chapter 4), ABP **7** may be obtained.

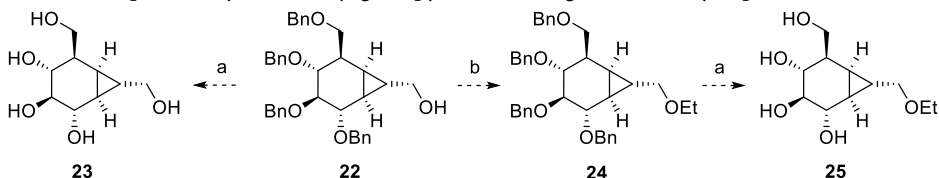
The galactopyranose-configured cyclophellitol (**16**) is an oxocarbenium ion transition-state mimic (Scheme 9.2A and B). The introduction of a sulfate group, as in compound **17**, may result in a ⁴C₁ conformational substrate mimic, which is susceptible to form a covalent enzyme-substrate adduct (Scheme 9.2C). The synthesis of this new class of cyclophellitols was explored starting from *manno*-configured cyclohexene **18** (Scheme 9.2D). Ruthenium(III)-catalyzed *syn*-dihydroxylation¹⁵ of **18** proceeded in a stereoselective fashion and resulted into compound **19**. After treatment with thionyl chloride and subsequent ruthenium catalyzed oxidation, cyclic sulfate **20** was obtained. Palladium-catalyzed hydrogenation gave target compound **21** (TB612). The conformation of TB612 may be confirmed by DFT calculations. No inhibition of TB612 in jack bean α -mannosidase was measured up to 50 μ M. This result was expected, as the ⁴C₁ conformation is not part of the reaction itinerary of α -mannosidase. The herein described synthetic strategy could be extended to α -galactopyranose-configured cyclophellitols.

In **Chapter 6** the synthesis is described of a focused library of carba-cyclophellitol based compounds with α -glucopyranose, β -glucopyranose, α -galactopyranose and β -galactopyranose configuration. These are potential reversible inhibitors for glycosidases by mimicking the transition-state conformation of the oxocarbenium ion during the reaction itinerary. Alcohol **23** and ether **25** may be added to the described library (Scheme 9.3). Debenzylation of compound **22** using palladium-catalyzed

Scheme 9.2 Synthesis of cyclophellitol cyclosulfate **21**.

A) Proposed mechanisms of GH27 α -galactosidases. **B)** Galactopyranose-configured cyclophellitol (**16**) adopts a $^4H_3$ conformation and mimics the oxocarbenium ion transition-state of GH27 α -galactosidases. **C)** Compound **17** may adopt a $^4C_1$ conformation and mimic the substrate conformation. Reagents and conditions: a) NaIO_4 , $\text{RuCl}_3 \cdot 3\text{H}_2\text{O}$, H_2O , EtOAc , MeCN , 0°C , 1 h, 68%; b) i) SOCl_2 , Et_3N , CH_2Cl_2 , ii) NaIO_4 , $\text{RuCl}_3 \cdot 3\text{H}_2\text{O}$, H_2O , CCl_4 , MeCN , 0°C , 1.5 h, 41% over 2 steps; c) 10% Pd/C , H_2 , MeOH , 50%.

hydrogenolysis conditions may give target compound **23**. Alkylation (use of EtBr and a base) of alcohol **22**, followed by palladium-catalyzed hydrogenolysis may afford compound **25**.

Scheme 9.3 The putative synthesis of β -glucopyranose-configured carba-cyclophellitol **23** and **25**.

Reagents and conditions: a) $\text{Pd}(\text{OH})_2/\text{C}$, H_2 , MeOH , rt; b) EtBr , NaH , TBAI , DMF , 0°C to rt.

The β -glucopyranose-configured carba-cyclophellitols are biologically evaluated in **Chapter 7** on their inhibitory potency of *Thermotoga maritima* TmGH1 β -glucosidase, GBA1 and GAA. Carba-cyclophellitols turned out to be selective β -glucosidase inhibitors. Furthermore, carba-cyclophellititol (**26**) with an *N*-(4-azidobutyl)carboxamide moiety is the most potent TmGH1 inhibitor (K_i of 8.2 nM, Figure 9.3). Next, the conformation of the carba-cyclophellititol derivatives were analysed by ^1H NMR analysis, quantum mechanical analysis and DFT calculations, which all supported the $^4\text{H}_3$ conformation as the preferential conformation adopted by β -glucopyranose-configured carba-cyclophellitols. After soaking of carba-cyclophellititol DW082 in TmGH1, the obtained structure was analysed by X-ray crystallography, revealing that DW082 also shows up in $^4\text{H}_3$ conformation when bound to the active site of this enzyme. As various glycosidase inhibitors are in clinical use or under examination as inhibitors, probes or pharmacological chaperones, the selectivity and tight binding properties of cyclopropyl sugars may be an attractive class for further investigation. Intestinal α -glucosidases, for example, is a target for treatment of diabetes mellitus type 2. Reversible inhibitors have been approved and are applied in the clinic.¹⁶ The α -glucopyranose-configured carba-cyclophellitols, as described in **Chapter 6**, are designed as reversible α -glucosidase inhibitors and were examined as such. However, no inhibition up to 100 μM was observed using the 4-methylumbelliferyl- α -D-glucopyranoside as substrate in α -glucosidases (GAA or GANAB). Similarly the α -galactopyranose-configured carba-cyclophellitols were examined as potential α -galactosidase inhibitors and no inhibition up to 500 μM was observed using the 4-methylumbelliferyl- α -D-galactopyranoside as substrate and α -GalA as the glycosidase. The described β -galactopyranose-configured carba-cyclophellitols, however, do inhibit β -galactosidases in the micromolar range (Figure 9.3 and Table 9.1).

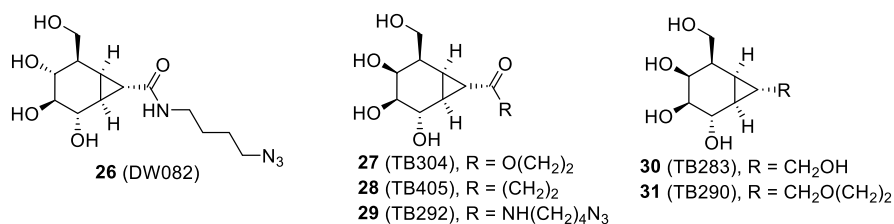
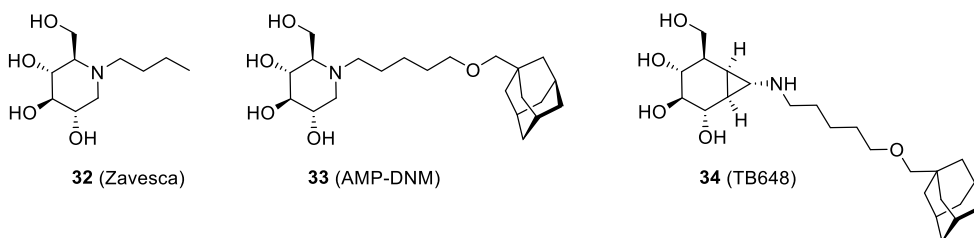


Figure 9.3 The structures of DW082 (**26**) and β -galactopyranose configured carba-cyclophellitols (**27** – **31**).

Table 9.1 The apparent IC_{50} values of compound **27** - **31** towards GALC and α -GalA in μ M with 4-methylumbelliferyl- α -D-galactopyranoside as substrate.

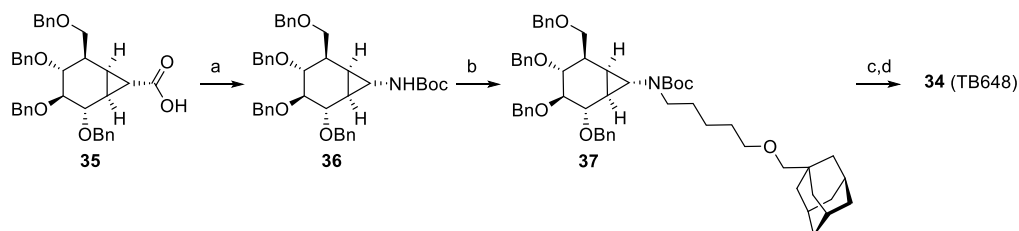
Compound	β -galactosidase (GALC)	α -galactosidase (α -GalA)
27 (TB304)	>500	>500
28 (TB405)	205.40 ± 10.95	>500
29 (TB292)	39.91 ± 2.86	>500
30 (TB283)	76.67 ± 0.89	>500
31 (TB290)	349.53 ± 25.25	>500

N-butyl-1-deoxynojirimycin (**32**, Zavesca, Figure 9.4) is an approved drug for the treatment of Gaucher disease in a substrate reduction therapy.^{17,18} This iminosugar is a potent ($IC_{50} = 50 \mu M$ ¹⁹) reversible inhibitor of glucosylceramide synthase (GCS), which significantly relieved the symptoms of Gaucher patients. Later, the more hydrophobic analogue, AMP-DNM (**33**) was reported to be a 100-fold more potent GCS inhibitor ($IC_{50} = 0.2 \mu M$ ¹⁹). Therefore, adamantane functionalized carba-cyclophellitol **34** (TB648) was designed as a potential GCS inhibitor.

**Figure 9.4** The structures of potent (**32** and **33**) and potential (**34**) GCS inhibitors.

The synthesis of TB648 is described in Scheme 9.4. Treatment of compound **35** (**Chapter 6**) with diphenyl phosphoryl azide in the presence of triethylamine and *tert*-butanol resulted into compound **36**. Alkylation with 5-(adamant-1-yl-methoxy)-1-bromopentane²⁰, followed by global debenzoylation and treatment with TFA gave target compound TB648 (**34**). The inhibition studies of TB648 towards GCS could not be performed in the timeframe of the here presented PhD research and will be performed in the near future.

Zavesca and AMP-DNM are potential β -glucosidase inhibitors.^{20,21} The apparent IC_{50} values were reassessed together with TB648 and extended to lysosomal α -glucosidase (GAA, Table 9.2). Zavesca and AMP-DNM inhibit GBA1 and GAA and more potently

Scheme 9.4 The synthesis of TB648 (**34**).

Reagents and conditions: a) t -BuOH, Et_3N , diphenyl phosphoryl azide, 90°C , overnight, 51%; b) 5-(adamant-1-yl-methoxy)-1-bromopentane²⁰, NaH, DMF, rt, 3 h, 81%; c) 10% Pd/C, H_2 , AcOH, MeOH, rt, 5 h; d) TFA, CH_2Cl_2 , rt, 45 min, 80% over 2 steps.

GBA2. TB648 turned out to be selective for GBA1 with potency in the nanomolar range (Table 9.2). Selective inhibitors of lysosomal glucocerebrosidase (GBA1) are pursued as they may be exploited in pharmacological chaperone therapy for Gaucher disease. Herein, the folding of the enzyme in the endoplasmic reticulum may be stabilized to restore the GBA1 activity.²¹ TB648 may therefore be exploited as a selective modulator of GBA1 in a clinical setting.

Table 9.2 The apparent IC_{50} values of Zavesca (**32**), AMP-DNM (**33**) and TB648 (**34**) towards GBA1, GBA2 and GAA in μM .

Compound	Human β -glucosidases		Human α -glucosidase
	GBA1	GBA2	GAA
Zavesca (32)	604.40 ± 12.59	0.14 ± 0.01	39.37 ± 2.45
AMP-DNM (33)	0.178 ± 0.003	0.0007 ± 0.0003	2.96 ± 0.07
TB648 (34)	0.0111 ± 0.0008	47.91 ± 2.72	>1000

The $\text{B}_{2,5}$ transition-state is expected for mannosidases, unlike β -glucosidases or α -galactosidases. This may explain the lower potency of mannopyranose-configured cyclophellitols towards mannosidases (**Chapter 4** and **Chapter 5**) and therefore $\text{B}_{2,5}$ conformed compounds are interesting targets. The conformation of the bicyclic aziridine **38** was determined by J -coupling constant analysis and turned out to be predominantly $\text{B}_{1,4}$ (Figure 9.5).²² This conformation resembles the conformation of oxocarbenium ion transition-state (**39**) present in the itinerary of α -mannosidases. This led to the design of bicyclic cyclopropyl **40** which may adopt a boat conformation as well. By altering of the hydroxyl conformations, a close mimic of **39** could be obtained and as such be a potential reversible α -mannosidases inhibitor.

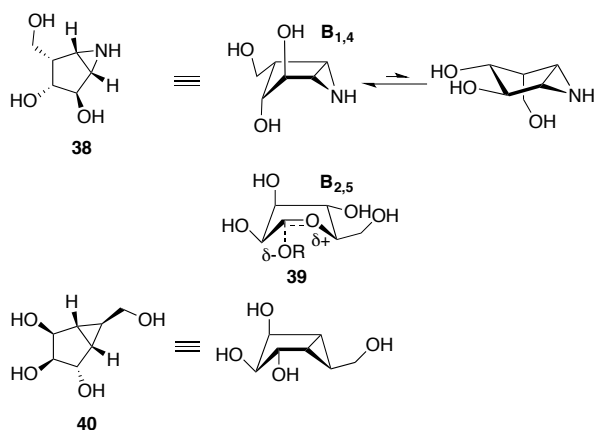
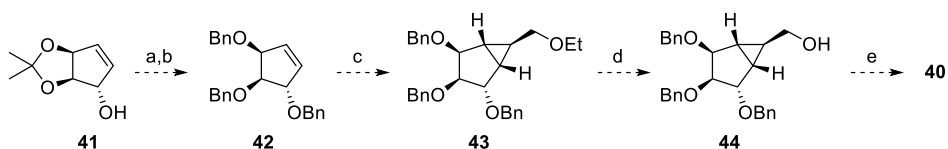


Figure 9.5 Bicyclic aziridine **38** adopts predominantly the $B_{1,4}$ conformation²² and therefore bicyclic cyclopropyl **40** is proposed to be a conformational mimic of the oxocarbenium ion transition-state of α -mannosidases.

The putative synthesis of bicyclic cyclopropyl **40** is depicted in Scheme 9.5. Cyclopentene **41**²³ could be treated with HCl in a mixture of THF and H₂O and subsequently benzylated to obtain compound **42**. Cyclopropanation with EDA as described in **Chapter 6** may give compound **43**. Reduction of the ester followed by palladium-catalyzed hydrogenation may give target compound **40**.

Scheme 9.5 The putative synthesis of bicyclic cyclopropyl **40**.



Reagents and conditions: a) HCl, THF, MeOH; b) BnBr, NaH, DMF; c) EDA, Cu(acac)₂, EtOAc; d) DIBAL, THF; e) Pd(OH)₂/C, H₂, MeOH.

The substrate specificity of β -mannosidase over α -mannosidases is determined in part by the conformation of the aglycon. Bicyclic cyclopropyl **46** with the 2-hydroxyl group in axial position may result in a selective β -mannosidases inhibitor (Figure 9.6). The synthesis of this conformational analogue can be commenced with cyclopentene **47**.²⁴ Global benzylation of compound **47**, followed by cyclopropanation with EDA may give bicyclic cyclopropyl **49**. After DIBAL-mediated reduction of **49** and palladium-catalyzed hydrogenolysis target compound **46** could be obtained.

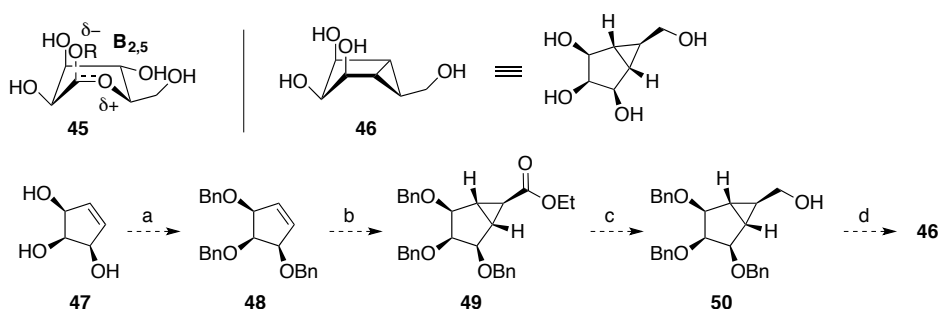


Figure 9.6 Bicyclic cyclopropyl **46** is proposed to be a conformational mimic of the oxocarbenium ion transition-state of β -mannosidases (**45**). Reagents and conditions: a) BnBr, NaH, DMF; b) EDA, Cu(acac)₂, EtOAc; c) DIBAL, THF; d) Pd(OH)₂/C, H₂, MeOH.

The glycan metabolism is controlled by glycosidases together with glycosyltransferases (GTs). Potent GTs inhibitors, however, are scarce and often sought after as potential GT regulators. Based on the results of carba-cyclophellitols as analogues of the oxocarbenium ion-like transition-state, the synthesis of two potential carba-cyclophellitol based galactosyltransferase (GalT) inhibitors (**51** and **52**, Figure 9.7) is described in **Chapter 8**. A *O*-sulfamoyl- (**51**) or a phosphate moiety (**52**) was introduced to mimic the substrate and enhance the potency towards GalTs.

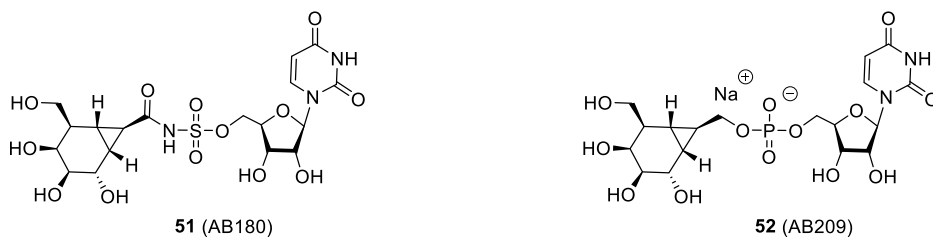
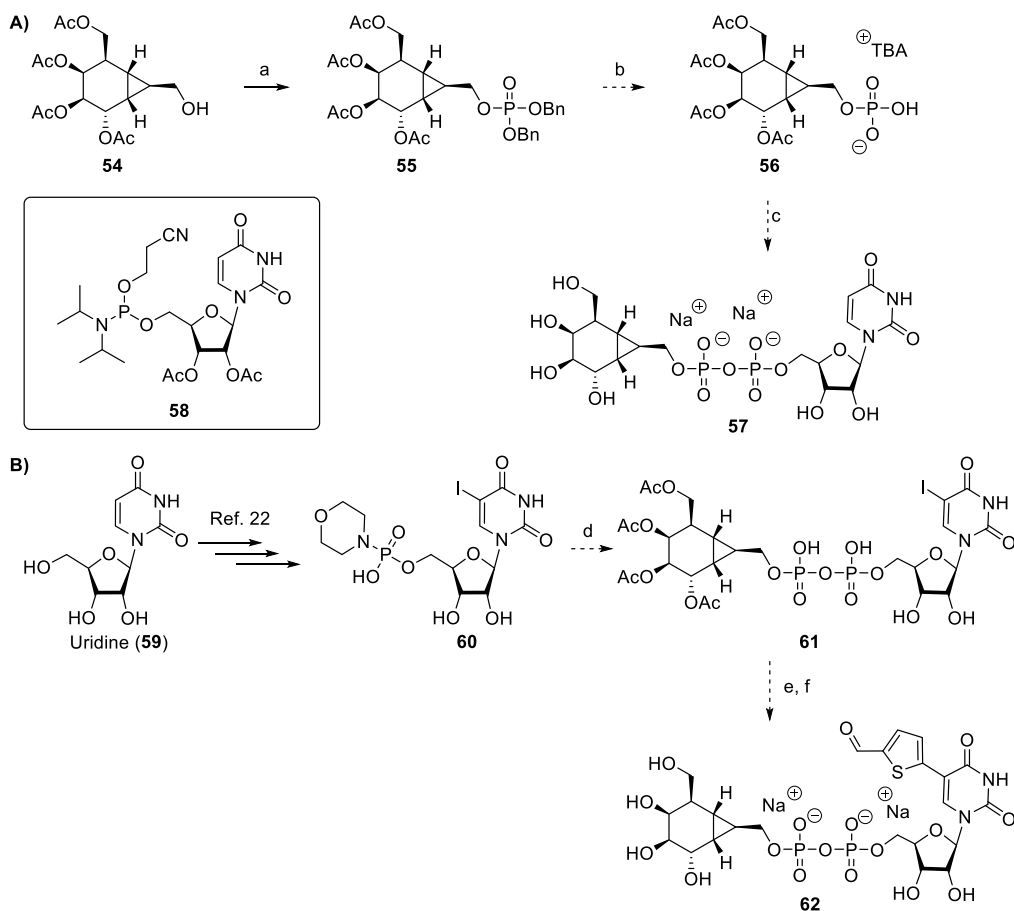


Figure 9.7 Potential carba-cyclophellitol based galactosyltransferase (GalT) inhibitors.

Carba-cyclophellitol UDP **57** may be an attractive target that could be obtained following the same synthetic strategy (Scheme 9.6A). After obtaining monophosphate **55**, the benzyl ethers may be removed with palladium-catalyzed hydrogenation. Coupling of phosphate **56** with phosphoramidite **58** using the previously described methodology could result into carba-cyclophellitol UDP **57**. As described by Wagner and co-workers, introduction of a formylthienyl moiety at position 5 of the uracil base of Gal-UDP resulted in an up to 10-fold lower K_i compared to the K_m of the natural donor towards various galactosyltransferases.²⁵ Therefore, Gal-UPD derivative **62** may be a more potent inhibitor towards galactosyltransferases (Scheme 9.6B). The proposed

synthetic route towards compound **62** starts with 5-iodouridine-5'-phosphoromorpholidate **60** which can be obtained from uridine (**59**) in 48% over three steps.²⁵ Subsequently, tetrazole catalyzed coupling with carba-cyclophellitol **56**, Suzuki coupling with (5-formyl-2-thienyl)boronic acid, deacetylation and treatment with Dionex sodium exchange resin may result in 5-(5-formylthien-2-yl) UDP carba-cyclophellitol **62** as a sodium salt.

Scheme 9.6 The putative synthesis compound **57** and compound **62**.



Reagents and conditions: a) tetrabenzyl pyrophosphate, LDA, THF, - 78 °C to rt, 2 h, 17%; b) i) H₂, Pd/C 10%, MeOH, rt; ii) tertbutylammonium hydroxide (TBA-OH) 40% aqueous solution, lyophilisation; c) i) **58**²⁶, DCI, MeCN, rt; ii) 'BuOOH, rt; iii) DBU, rt; iv) Et₃N/H₂O/MeOH (1:1:3), rt; d) **56**, tetrazole, DMF, rt; e) (5-formyl-2-thienyl)boronic acid, Na₂Cl₄Pd, trisodium salt of triphenylphosphine-3,3',3''-trisulfonic acid

(TPPTS), Cs_2CO_3 , H_2O , 50°C ; f) $\text{Et}_3\text{N}/\text{H}_2\text{O}/\text{MeOH}$ (1:3:1), rt, size-exclusion, HPLC purification, Dionex sodium exchange.

To determine the potency of GalT inhibitors, the donor and acceptor substrate are required. The donor substrate of GTB, for example, is commercially available (UDP- α -D-galactose as a disodium salt). A simplified acceptor substrate, α -L-Fucp-(1 $\rightarrow$ 2)- β -D-Galp-O-(CH_2) $_8\text{N}_3$ **63** (Figure 9.8), can be synthesized (see experimental). Although this is a simplified structure of the acceptor, similar structures were tolerated by GTB.^{27,28}

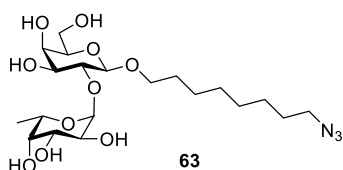


Figure 9.8 Structure of acceptor substrate **63**.

After having the donor and acceptor analogue **63** in hand a spectroscopic assay with a cascade of reactions, such as the commercial available Malachite green assay, can be performed to determine the inhibition potency of a potential inhibitor by a fluorescent output (Figure 9.9). In this assay, phosphatases will give inorganic phosphate (P_i) from the GTB produced UDP. After formation of P_i , a solution of ammonium molybdate in diluted sulfuric acid will be added followed by the addition of malachite green oxalate. Finally, the absorbance at 620 nm of the obtained malachite phosphomolybdate complex is proportional with the conversion of GTB.

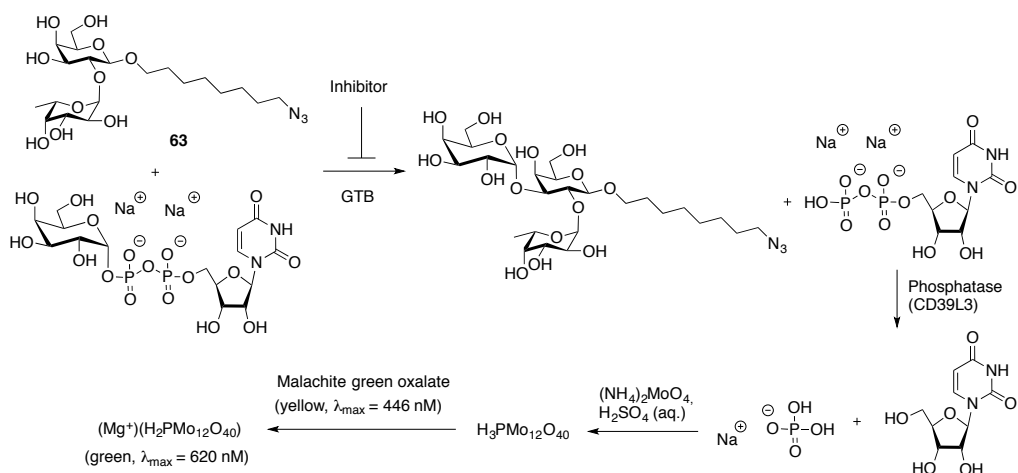


Figure 9.9 GTB based biochemical activity assay.

Additionally compound **51** (AB180) and **52** (AB209) were examined towards galactosidases. AB180 turned out to be the first potent carba-cyclophellitol based α -GalA inhibitor (Table 9.3).

Table 9.3 The apparent IC₅₀ values of **51** and **52** towards GALC and α -GalA in μ M.

Compound	β -galactosidase (GALC)	α -galactosidase (α -GalA)
51 (AB180)	180.27 $\pm$ 4.52	18.84 $\pm$ 1.33
52 (AB209)	>500	>500

In the second part of **Chapter 8**, compound ML128 (**64**) was synthesized and analysed towards glucosylceramide synthase (GCS) as well as towards α -glucosidases (GAA and GANAB) and β -glucosidases (GBA1 and GBA2). However, no inhibition was observed. ML131 (**65**), an analogue of **64**, was synthesized in a similar manner (Figure 9.10).

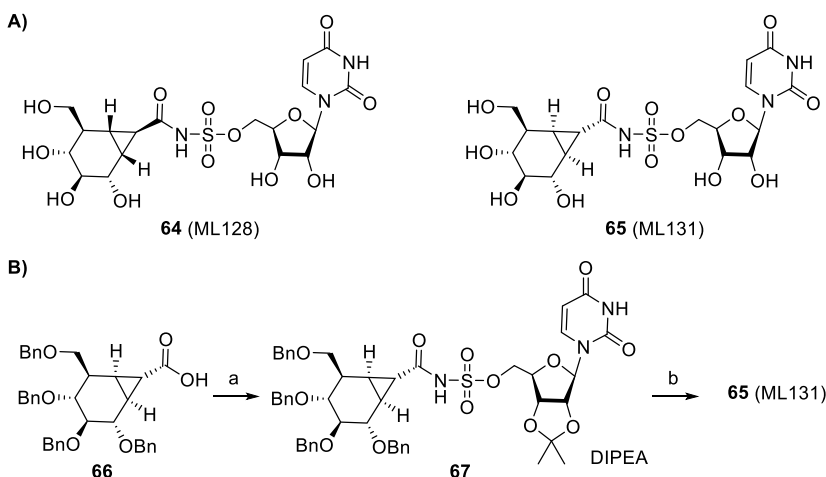


Figure 9.10 A) Structures of compound **64** (ML128) and **65** (ML131). B) Synthesis of β -glucopyranose-configured carba-cyclophellitol **65** (ML131). Reagents and conditions: a) i) oxalyl chloride, toluene, 40 °C, 5 h; ii) 2,3'-O-isopropylidene-5'-O-sulfamoyluridine, N,N-diisopropylethylamine, CH₂Cl₂, rt, overnight, size-exclusion, 86%; b) i) BCl₃, CH₂Cl₂, 0 °C, 3 h; ii) MeOH, HPLC purification, 20%.

The inhibitory potency of ML131 **65** was determined towards GBA1, GBA2, GAA and GANAB (all glycosidases) and GCS (a glycosyl transferase) in a similar fashion as for compound **64**. ML131 turned out to be micromolar active towards GBA1 and GBA2

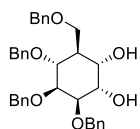
(Table 9.4), similar to the most potent carba-cyclophellitol analogue reported in **Chapter 7**, DW082.

Table 9.4 Apparent IC₅₀ values of compound **65** towards glucosyltransferase and glycosidases (μM).

Compound	β-glucosidase		α-glucosidase		GlucT
	GBA1	GBA2	GAA	GANAB	GCS
65 (ML131)	26.18 ± 0.75	138.90 ± 8.48	>100	>100	>50

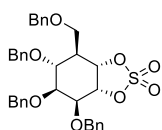
Experimental

General methods: All 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 was 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 aluminium 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. Column chromatography was performed using Screening Device b.v. Silica Gel (particle size of 40 – 63 μm, pore diameter of 60 Å) in the indicated solvents. For reversed-phase HPLC purifications an Agilent Technologies 1200 series instrument equipped with a semiprep column (Gemini C18, 250 x 10 mm, 5 μm 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 μm particle size, Phenomenex), coupled to a LCQ Advantage Max (Thermo Finnigan) ion-trap spectrometer (ESI⁺). The applied buffers were H₂O (A) and MeCN with 1% aqueous TFA (B). Optical rotations were measured on a Propol automatic polarimeter. ³¹P NMR, ¹H NMR and ¹³C NMR spectra were recorded on a Brüker AV-400 (161.7, 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).



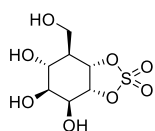
(1S,2S,3R,4S,5R,6S)-3,4,5-tris(benzyloxy)-6-((benzyloxy)methyl)cyclohexane-1,2-diol (4)

To a solution of cyclohexene **3** (1.04 g, 2.0 mmol) in EtOAc/ACN (1:1, 60 mL) was added a solution of sodium periodate (0.641 g, 3 mmol, 1.5 eq.) and catalytic amount of ruthenium(III) chloride trihydrate (36 mg, 0.14 mmol, 0.07 eq.) in H₂O (16 mL) at 0 °C. After vigorously stirring for 1 h, the reaction mixture was quenched with 10% aqueous Na₂S₂O₃ (15 mL) and diluted with saturated aqueous NaHCO₃ and EtOAc. The aqueous layer was separated and extracted with EtOAc (3 x). The combined organic layers were washed with brine, dried over MgSO₄ and concentrated *in vacuo*. Purification by column chromatography (30% EtOAc in pentane → 40% EtOAc in pentane) gave diol **4** (0.746 g, 1.34 mmol, 68%) as a clear oil. ¹H NMR (400 MHz, CDCl₃) δ 7.40 – 7.24 (m, 18H), 7.22 – 7.17 (m, 2H), 4.89 (d, *J* = 11.0 Hz, 1H), 4.78 (d, *J* = 12.2 Hz, 1H), 4.69 – 4.59 (m, 3H), 4.49 (s, 2H), 4.45 (d, *J* = 10.9 Hz, 1H), 4.06 – 3.91 (m, 5H), 3.64 (t, *J* = 9.3 Hz, 1H), 3.56 (t, *J* = 9.0 Hz, 1H), 2.60 (s, 1H), 2.23 (qd, *J* = 9.4, 3.3 Hz, 1H). ¹³C NMR (101 MHz, CDCl₃) δ 138.7, 138.5, 138.5, 137.4, 128.6, 128.4, 128.1, 128.0, 127.8, 127.7, 127.6, 81.2, 75.9, 75.7, 75.0, 73.6, 73.3, 72.6, 71.9, 71.8, 71.8, 70.0, 42.7. HRMS: calculated for [C₃₅H₃₈O₆]⁺ 555.27412, found 555.27433.

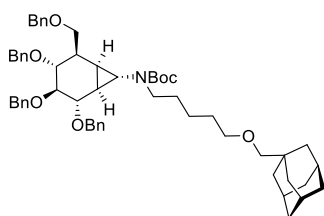


(3aR,4S,5S,6R,7R,7aS)-4,5,6-tris(benzyloxy)-7-((benzyloxy)methyl)hexahydrobenzo[d][1,3,2]dioxathiole 2,2-dioxide (5)

Diol **4** (0.36 g, 0.65 mmol) was dissolved in CH₂Cl₂ (30 mL) and Et₃N (0.33 mL, 2.4 mmol, 3.7 eq.) was added at 0 °C. Next, thionyl chloride (0.15 mL, 2.1 mmol, 3.2 eq.) was slowly added over 5 min. After stirring for 1 h, additional Et₃N (90 μL, 0.65 mmol, 1 eq.) and thionyl chloride (45 μL, 0.65 mmol, 1 eq.) was added. The reaction mixture was stirred for an additional 30 min before quenching with cold H₂O and dilution with cold Et₂O. The layers were separated and the organic layer was washed with H₂O (2 x), dried over MgSO₄, filtered and concentrated *in vacuo*. Purification by column chromatography (3.5% EtOAc in pentane) gave the sulphite diastereomeric mixture (390 mg) as an orange oil. HRMS: calculated for [C₃₅H₃₇O₇S]⁺ 601.22545, found 601.22584. To a solution of the sulphite diastereomeric mixture (192 mg, 0.32 mmol) in CCl₄/MeCN (1:1, 24 mL) was added a solution of sodium periodate (0.137 g, 0.64 mmol, 2.0 eq.) and a catalytic amount of ruthenium(III) chloride trihydrate (6 mg, 22 μmol, 0.07 eq.) in H₂O (12 mL) at 0 °C. After vigorously stirring for 1.5 h, the reaction mixture was diluted with Et₂O and H₂O. The organic layer was separated, washed with brine, dried over MgSO₄ and concentrated *in vacuo*. Purification by column chromatography (8% EtOAc in pentane) gave title compound (81 mg, 0.131 mmol, 41% over 2 steps) as a white solid. ¹H NMR (400 MHz, CDCl₃) δ 7.40 – 7.20 (m, 18H), 7.14 (dd, *J* = 7.3, 2.2 Hz, 2H), 5.29 (dd, *J* = 11.2, 6.4 Hz, 1H), 5.20 (t, *J* = 6.2 Hz, 1H), 4.73 (dd, *J* = 19.8, 11.8 Hz, 1H), 4.59 (d, *J* = 4.2 Hz, 2H), 4.59 – 4.51 (m, 1H), 4.43 (d, *J* = 11.7 Hz, 2H), 4.31 (d, *J* = 11.9 Hz, 1H), 4.22 (d, *J* = 11.5 Hz, 1H), 4.11 (d, *J* = 1.6 Hz, 1H), 3.82 – 3.77 (m, 2H), 3.62 – 3.46 (m, 2H), 2.26 – 2.16 (m, 1H). ¹³C NMR (101 MHz, CDCl₃) δ 138.0, 137.6, 137.5, 137.4, 128.7, 128.6, 128.5, 128.2, 128.1, 128.0, 127.9, 127.7, 83.6, 80.0, 76.5, 74.2, 73.2, 72.9, 72.8, 72.6, 64.8, 42.5. HRMS: calculated for [C₃₅H₃₆O₈SNa]⁺ 639.20231, found 639.20238.

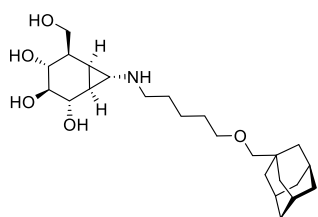
**(3aR,4S,5S,6R,7aS)-4,5,6-trihydroxy-7-****(hydroxymethyl)hexahydrobenzo[d][1,3,2]dioxathiole 2,2-dioxide (6, TB612)**

Benzylated sulphate **5** (70 mg, 0.11 mmol) was dissolved in MeOH (10 mL). The reaction mixture was purged with nitrogen gas and 10% palladium on carbon (15 mg) was added. After the reaction vessel was purged with hydrogen gas and vigorously stirring overnight, the palladium catalyst was filtered off followed by concentration *in vacuo*. Purification by column chromatography (10% MeOH in DCM → 20% MeOH in DCM) resulted compound **6** (14 mg, 55 μmol, 50%) as a clear oil. ¹H NMR (500 MHz, MeOD) δ 4.20 (d, *J* = 2.0 Hz, 1H), 4.15 (d, *J* = 5.2 Hz, 1H), 4.08 (d, *J* = 5.3 Hz, 1H), 4.04 – 3.97 (m, 1H), 3.79 – 3.73 (m, 1H), 3.59 (dd, *J* = 10.6, 6.3 Hz, 1H), 3.51 – 3.43 (m, 1H), 2.25 – 2.20 (m, 1H). ¹³C NMR (126 MHz, MeOD) δ 88.3, 83.2, 82.6, 76.2, 74.3, 64.1, 45.7.

**tert-butyl (5-(((3R,5R)-adamantan-1-****yl)methoxy)pentyl)((1S,2S,3R,4R,5R,6S,7S)-2,3,4-****tris(benzyloxy)-5-((benzyloxy)methyl)bicyclo[4.1.0]heptan-7-yl)carbamate (37)**

To a solution of carboxylic acid **35** (80 mg, 0.138 mmol) in *tert*-butanol (4 mL) was added triethylamine (21 μL, 0.152 mmol, 1.1 eq.) and diphenylphosphorylazide (33 μL, 0.152 mmol, 1.1 eq.).

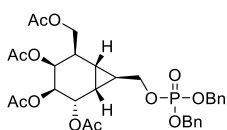
The reaction mixture was heated to 90 °C and stirred overnight. The solution was concentrated *in vacuo*, redissolved in EtOAc (15 mL) and washed with HCl (1 M) and saturated aqueous NaHCO₃. The organic layer was dried over MgSO₄ and concentrated *in vacuo*. Purification by column chromatography (10% EtOAc in pentane → 20% EtOAc in pentane) gave compound **36** (45 mg, 0.069 mmol, 51%) as a white solid. HRMS: calculated for [C₄₁H₄₈NO₆]⁺ 650.34761, found 650.34826. Compound **36** (9.1 mg, 14.1 μmol) was taken up in DMF (0.14 mL) and subsequently 5-(adamant-1-yl-methoxy)-1-bromopentane (18 mg, 56 μmol, 4 eq.) and NaH (60% dispersion in mineral oil, 4.0 mg, 0.10 mmol, 7 eq.) were added at room temperature. After stirring for 3 h, the reaction mixture was diluted with Et₂O and washed with aqueous HCl (0.1 M). The water layer was extracted with Et₂O (3 x) and the combined organic layers were dried over MgSO₄ and concentrated *in vacuo*. Purification by column chromatography (4% EtOAc in pentane → 8% EtOAc in pentane) gave title compound **37** (10.1 mg, 11.4 μmol, 81%) as a clear oil. ¹H NMR (400 MHz, CDCl₃) δ 7.40 (d, *J* = 7.1 Hz, 2H), 7.37 – 7.20 (m, 16H), 7.13 (dd, *J* = 6.7, 2.9 Hz, 2H), 4.88 (d, *J* = 11.0 Hz, 2H), 4.82 – 4.73 (m, 3H), 4.49 (d, *J* = 2.5 Hz, 2H), 4.36 (d, *J* = 10.8 Hz, 1H), 3.88 (d, *J* = 8.0 Hz, 1H), 3.78 (dd, *J* = 8.7, 2.9 Hz, 1H), 3.66 (t, *J* = 6.7 Hz, 1H), 3.54 (d, *J* = 7.8 Hz, 1H), 3.50 (d, *J* = 8.5 Hz, 1H), 3.30 (t, *J* = 6.5 Hz, 2H), 3.04 – 2.98 (m, 1H), 2.93 (d, *J* = 2.5 Hz, 2H), 2.42 – 2.35 (m, 2H), 1.94 (s, 3H), 1.75 – 1.16 (m, 22H). HRMS: calculated for [C₅₇H₇₄NO₇]⁺ 884.54598, found 884.54680.



(1S,2S,3R,4R,5R,6S,7S)-7-((5-(((3R,5R)-adamantan-1-yl)methoxy)pentyl)amino)-5-(hydroxymethyl)bicyclo[4.1.0]heptane-2,3,4-triol (34**, TB648)**

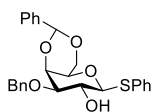
Compound **37** (9.5 mg, 10 μ mol) was dissolved in MeOH (1 mL). A few drops of acetic acid (glacial) and 10% Pd on carbon (8 mg) were added and the reaction vessel was purged with hydrogen gas.

After vigorously stirring for 5 h at room temperature, the solution was filtered and concentrated *in vacuo*. The residue was dissolved in CH₂Cl₂/trifluoroacetic acid (1.6 mL, 1:1) and stirred for 45 min at room temperature. The reaction mixture was concentrated *in vacuo* and purification by HPLC purification (C18, linear gradient: 30–36% B in A, solutions used A: H₂O, B: acetonitrile, 0.5% TFA) yielded adamantane **34** (3.4 mg, 8.1 μ mol, 80%) as a clear oil. LC/MS analysis: R_t 5.6 min (linear gradient 10% $\rightarrow$ 90% B in 12.5 min,). ¹H NMR (850 MHz, MeOD) δ 4.62 (s, 1H), 3.93 (dd, *J* = 10.5, 4.5 Hz, 1H), 3.61 (dd, *J* = 10.5, 7.9 Hz, 1H), 3.47 (dd, *J* = 8.4, 1.2 Hz, 1H), 3.39 (t, *J* = 6.4 Hz, 2H), 3.09 (dd, *J* = 10.0, 8.5 Hz, 1H), 2.97 (s, 2H), 2.80 (t, *J* = 10.2 Hz, 1H), 2.69 (t, *J* = 7.6 Hz, 2H), 2.03 – 1.98 (m, 1H), 1.94 (s, *J* = 7.9 Hz, 1H), 1.91 (d, *J* = 7.5 Hz, 1H), 1.77 (s, 1H), 1.75 (s, 1H), 1.69 (s, 1H), 1.68 (s, 1H), 1.61 – 1.57 (m, 2H), 1.56 (s, 2H), 1.56 (s, 2H), 1.55 – 1.51 (m, 2H), 1.44 – 1.38 (m, 2H), 1.37 – 1.30 (m, 2H), 1.22 (s, 2H), 1.06 (dd, *J* = 9.6, 1.0 Hz, 1H). ¹³C NMR (214 MHz, MeOD) δ 83.1, 79.4, 74.6, 72.5, 71.0, 64.9, 50.1, 49.3, 49.2, 49.1, 49.0, 48.9, 48.8, 48.7, 43.6, 40.8, 39.7, 38.3, 31.1, 30.6, 30.2, 29.8, 26.2, 25.1, 23.8. HRMS: calculated for [C₂₄H₄₂NO₅]⁺ 424.30575, found 424.30580



(1R,2S,3R,4S,5R,6R,7R)-5-(acetoxymethyl)-7-(((bis(benzyloxy)phosphoryl)oxy)methyl)bicyclo[4.1.0]heptane-2,3,4-triyl triacetate (55**)**

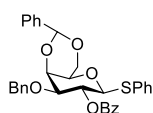
Alcohol **54** (142 mg, 0.38 mmol) was dissolved in THF (5 mL), cooled to -78 °C and lithium diisopropylamide (2 M in THF, 0.21 mL, 0.42 mmol, 1.1 eq.) was added. After stirring for 5 min, tetrabenzyl pyrophosphate (0.246 g, 0.46 mmol, 1.2 eq.) in THF (2 mL) was added. The reaction mixture was stirred for 3 h, before being quenched with saturated aqueous NaHCO₃, extracted with EtOAc and washed with brine. The aqueous layer was extracted once with EtOAc. The combined organic layers were dried over MgSO₄, filtered and concentrated *in vacuo*. Purification by column chromatography (20% EtOAc in pentane $\rightarrow$ 50% EtOAc in pentane) gave compound **55** (42 mg, 66 μ mol, 17%) as a white solid. ³¹P NMR (162 MHz, CDCl₃): δ = -0.47. ¹H NMR (400 MHz, CDCl₃): δ 7.37 (s, 10H), 5.36 (dd, *J* = 9.2, 6.8 Hz, 1H), 5.26 (s, 1H), 5.08 – 4.98 (m, 4H), 4.55 (dd, *J* = 9.3, 1.6 Hz, 1H), 4.10 – 3.94 (m, 2H), 3.85 – 3.67 (m, 2H), 2.16 – 2.13 (m, 1H), 2.10 – 2.00 (m, 12H), 1.67 – 1.63 (m, 1H), 1.11 – 1.03 (m, 1H), 0.80 – 0.71 (m, 1H). ¹³C NMR (100 MHz, CDCl₃): δ 171.0, 170.8, 170.6, 170.3, 135.9, 135.8, 128.8, 128.1, 72.1, 70.2, 70.2, 69.5, 69.4, 69.4, 69.2, 69.0, 62.8, 38.0, 24.4, 24.3, 21.3, 21.2, 20.9, 20.9, 20.8, 18.5. HRMS: Calculated for [C₃₁H₃₈O₁₂P]⁺ 633.20954, found 633.21000.



Phenyl 3-O-benzyl-4,6-O-benzylidene-1-thio-β-D-galactopyranoside (68**)**

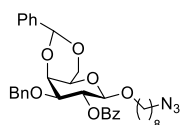
To a solution of phenyl 4,6-O-benzylidene-1-thio-β-D-galactopyranoside²⁹ (6.49 g, 18.0 mmol) in toluene (45 mL) was added dibutyltin oxide (4.52 g, 18.2 mmol).³⁰ After reflux overnight in a Dean-Stark apparatus the reaction mixture was

concentrated *in vacuo*. The resulting mixture was redissolved in DMF (45 mL) and CsF (5.46 g, 36.0 mmol) and BnBr (3.21 mL, 27.0 mmol) were added. After stirring for 3 h at room temperature the reaction mixture was quenched with MeOH, filtered over celite and concentrated *in vacuo*. Purification by silica chromatography (5% EtOAc in toluene → 6% EtOAc in toluene) resulted in compound **68** (5.18 g, 11.5 mmol, 64%) as a white solid. $[\alpha]_D^{20} = -8.0$ ($c = 0.1$, CHCl_3). ^1H NMR (400 MHz, CDCl_3): $\delta = 7.71 - 7.64$ (m, 2H), 7.47 – 7.11 (m, 13), 5.41 (s, 1H), 4.71 (d, $J = 4.0$ Hz, 2H), 4.50 (d, $J = 9.5$ Hz, 1H), 4.34 (dd, $J = 12.3, 1.3$ Hz, 1H), 4.12 (d, $J = 3.2$ Hz, 1H), 4.00 – 3.89 (m, 2H), 3.49 (dd, $J = 9.3, 3.3$ Hz, 1H), 3.41 (s, 1H), 2.47 (s, 1H). ^{13}C NMR (101 MHz, CDCl_3): $\delta = 138.0, 137.9, 133.8, 130.7, 129.1, 129.0, 128.6, 128.2, 128.2, 128.0, 126.6, 101.2, 87.1, 80.3, 73.3, 71.7, 70.1, 69.5, 67.2$. HRMS: calculated for $[\text{C}_{26}\text{H}_{27}\text{O}_5\text{S}]^+$ 451.15737; found 451.15749.



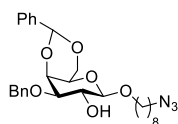
Phenyl 2-O-benzoyl-3-O-benzyl-4,6-O-benzylidene-1-thio-β-D-galactopyranoside (69)

To a solution alcohol **68** (90 mg, 0.2 mmol) in pyridine (0.5 mL) was added benzoyl chloride (26 μL , 0.22 mmol). After stirring overnight at room temperature the reaction mixture was diluted with EtOAc, washed with H_2O and brine. The organic layer was dried with MgSO_4 , filtered and concentrated *in vacuo*. Purification by silica chromatography (5% EtOAc in toluene) yielded in compound **69** (110 mg, 0.2 mmol, quant.) as a white solid. $[\alpha]_D^{20} = -6.0$ ($c = 1.0$, CHCl_3). ^1H NMR (400 MHz, CDCl_3): $\delta = 8.12$ (d, $J = 7.6$ Hz, 2H), 8.04 (d, $J = 7.9$ Hz, 2H), 7.59 – 7.09 (m, 16H), 5.57 (t, $J = 9.7$ Hz, 1H), 5.48 (s, 1H), 4.80 (d, $J = 9.8$ Hz, 1H), 4.67 – 4.51 (m, 2H), 4.37 (d, $J = 12.2$ Hz, 1H), 4.24 (d, $J = 3.1$ Hz, 1H), 4.01 (d, $J = 12.2$ Hz, 1H), 3.76 (dd, $J = 9.6, 3.2$ Hz, 1H), 3.48 (s, 1H). ^{13}C NMR (101 MHz, CDCl_3): $\delta = 172.1, 165.1, 137.7, 133.90, 133.8, 133.1, 131.7, 130.3, 130.3, 130.0, 129.1, 128.8, 128.6, 128.5, 128.4, 128.2, 128.1, 127.8, 127.8, 126.7, 101.4, 85.5, 78.2, 73.2, 71.1, 70.2, 69.4, 69.1$. HRMS: calculated for $[\text{C}_{33}\text{H}_{31}\text{O}_6\text{S}]^+$ 555.18359; found 555.18380.



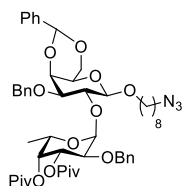
8-azido-octane 2-O-benzoyl-3-O-benzyl-4,6-O-benzylidene-β-D-galactopyranoside (70)

Compound **69** (124mg, 0.73 mmol) and 8-azido-octan-1-ol³¹ (443 mg, 0.8 mmol) were dissolved in CH_2Cl_2 (4 mL), after which activated molecular sieves (4 Å) were added. The reaction mixture was cooled to -45°C and subsequently *N*-iodosuccinimide (392 mg, 1.74 mmol) and TMSOTf (33 μL , 184 μmol) were added. After stirring for 30 min at -40°C the reaction mixture was quenched at 0°C with Et_3N (1 mL), diluted with CHCl_3 and washed with $\text{Na}_2\text{S}_2\text{O}_3$ and H_2O . The organic layer was dried with MgSO_4 , filtered and concentrated *in vacuo*. Purification by column chromatography (30% EtOAc in pentane → 50% EtOAc in pentane) gave compound **70** (380 mg, 618 μmol , 85%) as a clear yellow oil. ^1H NMR (400 MHz, CDCl_3): $\delta = 8.04$ (d, $J = 7.1$ Hz, 2H), 7.63 – 7.54 (m, 4H), 7.45 (t, $J = 7.7$ Hz, 2H), 7.40 – 7.32 (m, 3H), 7.28 – 7.14 (m, 4H), 5.63 (dd, $J = 10.0, 8.0$ Hz, 1H), 5.52 (s, 1H), 4.69 (d, $J = 12.9$ Hz, 1H), 4.60 (d, $J = 12.9$ Hz, 1H), 4.54 (d, $J = 8.0$ Hz, 1H), 4.37 – 4.29 (m, 1H), 4.24 (d, $J = 3.4$ Hz, 1H), 4.07 (dd, $J = 12.3, 1.5$ Hz, 1H), 3.94 – 3.87 (m, 1H), 3.74 (dd, $J = 10.1, 3.5$ Hz, 1H), 3.48 – 3.37 (m, 2H), 3.16 (t, $J = 7.0$ Hz, 2H), 1.47 – 1.39 (m, 3H), 1.21 – 1.00 (m, 9H). ^{13}C NMR (101 MHz, CDCl_3): $\delta = 165.2, 138.0, 137.8, 133.0, 130.4, 129.9, 129.0, 128.4, 128.4, 128.2, 127.8, 126.6, 101.4, 101.3, 77.0, 73.2, 70.9, 70.9, 69.3, 69.3, 66.7, 51.5, 29.4, 29.1, 29.0, 28.8, 26.6, 25.8$. HRMS: calculated for $[\text{C}_{35}\text{H}_{42}\text{N}_3\text{O}_7]^+$ 616.30173; found 616.30190



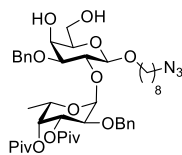
8-azido-octane 3-O-benzyl-4,6-O-benzylidene- β -D-galactopyranoside (**71**)

To a solution of **70** (0.529 g, 0.859 mmol) in MeOH (29 mL) was added NaOMe (192 mg, 3.55 mmol) and stirred for 5 h at room temperature. After neutralization with Amberlite-H⁺ IR-200 the reaction mixture was filtered and concentrated *in vacuo*. Purification by silica chromatography (50% EtOAc in pentane) resulted in compound **71** (0.369 g, 0.72 mmol, 84%) as a white solid. $[\alpha]_D^{20} = +21.6$ ($c = 1.0$, CHCl₃). ¹H NMR (400 MHz, CDCl₃): $\delta = 7.56 - 7.49$ (m, 2H), 7.48 – 7.21 (m, 8H), 5.44 (s, 1H), 4.75 (s, 2H), 4.32 – 4.24 (m, 2H), 4.11 (d, $J = 3.4$ Hz, 1H), 4.04 – 3.96 (m, 3H), 3.54 – 3.43 (m, 2H), 3.32 (s, 1H), 3.23 (t, $J = 6.9$ Hz, 2H), 2.57 (s, 1H), 1.69 – 1.52 (m, 4H), 1.38 – 1.23 (m, 8H). ¹³C NMR (101 MHz, CDCl₃): $\delta = 138.2, 137.8, 128.9, 128.5, 128.1, 127.9, 126.4, 102.9, 101.1, 79.1, 73.2, 71.5, 70.1, 69.7, 69.3, 66.6, 51.5, 29.5, 29.3, 29.1, 28.8, 26.6, 25.9$. HRMS: calculated for [C₂₈H₃₈N₃O₆]⁺ 512.27551; found 512.27551.



8-azido-octane 2-O-benzyl-3,4-O-pivaloyl- α -L-fucopyranosyl (1 $\rightarrow$ 2)-3-O-benzyl-4,6-O-benzylidene- β -D-galactopyranoside (**72**)

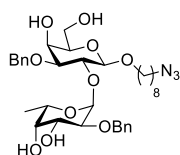
Compound **71** (0.316 g, 0.618 mmol) and 2-O-benzyl-3,4-O-pivaloyl-1-thio- α -L-fucopyranoside³² (0.477 g, 0.928 mmol) were dissolved in CH₂Cl₂ (10.3 mL), after which activated molecular sieves (3 Å) and *N*-iodosuccinimide (0.333 g, 1.48 mmol) were added. The reaction mixture was cooled to -45 °C and TMSOTf (11.2 μ L, 62 μ mol) was added. After stirring for 1 h at -40 °C the reaction mixture was warmed to -15 °C, quenched with Et₃N (3 mL) and washed with Na₂S₂O₃ and aqueous saturated NaHCO₃. The organic layer was dried with MgSO₄, filtered and concentrated *in vacuo*. Purification by column chromatography (20% EtOAc in pentane) gave disaccharide **72** as a white solid (393 mg, 429 μ mol, 69%). $[\alpha]_D^{20} = -36$ ($c = 0.4$, CHCl₃). ¹H NMR (400 MHz, CDCl₃): $\delta = 7.53$ (d, $J = 6.3$ Hz, 2H), 7.34 – 7.14 (m, 11H), 7.07 – 7.01 (m, 2H), 5.64 (d, $J = 3.4$ Hz, 1H), 5.43 (s, 2H), 5.40 – 5.34 (m, 1H), 5.24 (d, $J = 2.4$ Hz, 1H), 4.77 – 4.73 (m, 2H), 4.60 (d, $J = 12.4$ Hz, 1H), 4.52 (d, $J = 11.7$ Hz, 1H), 4.46 (d, $J = 7.8$ Hz, 1H), 4.40 (d, $J = 12.4$ Hz, 1H), 4.29 (d, $J = 12.1$ Hz, 1H), 4.22 – 4.13 (m, 2H), 4.02 (d, $J = 11.8$ Hz, 1H), 3.97 – 3.90 (m, 1H), 3.85 – 3.74 (m, 2H), 3.46 – 3.39 (m, 1H), 3.35 (s, 1H), 3.23 (t, $J = 7.0$ Hz, 2H), 1.36 – 1.30 (m, 4H), 1.36 – 1.30 (m, 8H), 1.14 (s, 9H), 1.13 (s, 9H), 1.04 (d, $J = 6.4$ Hz, 3H). ¹³C NMR (101 MHz, CDCl₃): $\delta = 177.8, 177.4, 138.2, 137.8, 137.5, 128.8, 128.5, 128.4, 128.1, 128.0, 127.8, 127.7, 127.6, 127.2, 126.2, 101.6, 100.6, 96.8, 81.3, 72.3, 72.3, 72.0, 71.5, 70.0, 71.4, 70.38, 69.7, 69.3, 66.3, 64.7, 51.4, 39.0, 38.7, 29.6, 29.4, 29.1, 28.8, 27.3, 27.2, 26.7, 26.1, 15.8$. HRMS: calculated for [C₅₁H₆₉N₃O₁₂Na]⁺ 938.47735; found 938.47678.



8-azido-octane 2-O-benzyl-3,4-O-pivaloyl- α -L-fucopyranosyl (1 $\rightarrow$ 2)-3-O-benzyl- β -D-galactopyranoside (**73**)

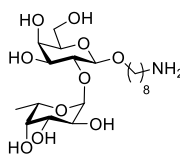
Disaccharide **72** (274 mg, 0.300 mmol) was dissolved in AcOH/H₂O (6 mL, 4:1, v/v) and heated to 50 °C. After stirring overnight the reaction mixture was concentrated and co-evaporated with toluene (7x). Purification by silica chromatography (50% EtOAc in pentane) resulted in diol **73** (176 mg, 0.213 mmol, 71%) as a clear oil. $[\alpha]_D^{20} = -60$ ($c = 1$, CHCl₃). ¹H NMR (400 MHz, CDCl₃): $\delta = 7.38 - 7.23$ (m, 5H), 7.23 – 7.13 (m, 3H), 7.09 (dd, $J = 7.2, 1.9$ Hz, 2H), 5.58 (d, $J = 3.5$ Hz, 1H), 5.39 (dd, $J = 10.8, 3.1$ Hz, 1H), 5.26 (d, $J =$

2.4 Hz, 1H), 4.72 – 4.62 (m, 2H), 4.62 – 4.48 (m, 3H), 4.46 (d, $J = 7.8$ Hz, 1H), 4.04 – 3.87 (m, 4H), 3.87 – 3.77 (m, 2H), 3.73 (dd, $J = 9.3, 3.3$ Hz, 1H), 3.52 – 3.46 (m, 2H), 3.26 (t, $J = 7.0$ Hz, 2H), 1.63 – 1.53 (m, 4H), 1.38 – 1.26 (m, 8H), 1.18 (s, 9H), 1.15 (s, 9H), 1.04 (d, $J = 6.5$ Hz, 3H). ^{13}C NMR (101 MHz, CDCl_3): $\delta = 177.4, 177.4, 137.5, 137.4, 128.7, 128.3, 128.0, 127.8, 127.7, 127.1, 101.8, 97.1, 82.8, 73.8, 72.6, 72.4, 72.3, 71.4, 71.3, 70.2, 70.1, 66.3, 64.9, 62.5, 51.5, 39.0, 38.7, 29.7, 29.4, 29.1, 28.8, 27.2, 27.2, 26.7, 26.0, 15.7$. HRMS: calculated for $[\text{C}_{44}\text{H}_{65}\text{N}_3\text{O}_{12}\text{Na}]^+$ 850.44605; found 850.44555.



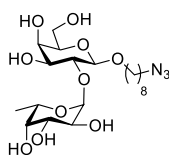
8-azido-octyl-1,2:3,6-di-O-benzyl- α -L-fucopyranosyl (1 $\rightarrow$ 2)-3-O-benzyl- β -D-galactopyranoside (74)

Diol **73** (364 mg, 0.44 mmol) was dissolved in a mixture of THF/MeOH/ H_2O (44 mL, 5:5:1, v/v/v). After addition of LiOH (197 mg, 8.23 mmol) was heated to 50 $^\circ\text{C}$ and stirred for 48 h. The reaction mixture was concentrated *in vacuo* and co-evaporated with toluene (3x). Purification by silica chromatography (100% EtOAc $\rightarrow$ 10% MeOH in EtOAc) resulted in compound **74** (267 mg, 0.40 mmol, 92%) as a clear oil. $[\alpha]_{\text{D}}^{20} = -62.4$ ($c = 0.5$, CHCl_3). ^1H NMR (400 MHz, CDCl_3): $\delta = 7.35 - 7.22$ (m, 8H), 7.14 (dd, $J = 6.9, 2.6$ Hz, 2H), 5.63 (d, $J = 3.6$ Hz, 1H), 4.75 (d, $J = 11.6$ Hz, 1H), 4.64 (d, $J = 12.0$ Hz, 1H), 4.55 – 4.48 (m, 2H), 4.45 (d, $J = 7.8$ Hz, 1H), 4.27 (d, $J = 12.0$ Hz, 1H), 4.11 – 4.07 (m, 1H), 4.06 – 3.94 (m, 3H), 3.93 – 3.88 (m, 1H), 3.87 – 3.82 (m, 1H), 3.79 – 3.75 (m, 1H), 3.73 (dd, $J = 9.3, 3.3$ Hz, 1H), 3.63 (dd, $J = 9.9, 3.6$ Hz, 1H), 3.55 – 3.50 (m, 1H), 3.49 – 3.42 (m, 1H), 3.26 (t, $J = 6.9$ Hz, 2H), 2.50 (s, 1H), 2.36 (s, 1H), 2.22 (s, 1H), 1.62 – 1.54 (m, 4H), 1.32 – 1.22 (m, 11H). ^{13}C NMR (101 MHz, CDCl_3): $\delta = 137.8, 137.4, 128.8, 128.6, 128.1, 127.9, 127.1, 102.0, 96.2, 83.2, 75.7, 73.9, 72.3, 71.9, 69.5, 71.8, 71.1, 70.0, 66.2, 65.5, 62.5, 51.5, 29.8, 29.5, 29.2, 28.9, 26.8, 26.2, 16.1$. HRMS: calculated for $[\text{C}_{34}\text{H}_{49}\text{N}_3\text{O}_{10}\text{Na}]^+$ 682.33102; found 682.33054.



8-amino-octyl-1,2:3,6-di-O-benzyl- α -L-fucopyranosyl (1 $\rightarrow$ 2)- β -D-galactopyranoside (75)

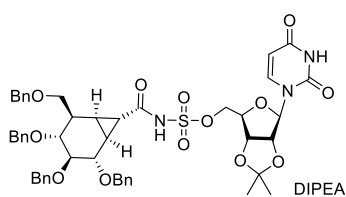
To a solution of compound **74** (59 mg, 89 μmol) in a mixture of THF/ i -BuOH/ H_2O (1:1:3, v/v/v, 2 mL) was added $\text{Pd}(\text{OH})_2/\text{C}$ (100 mg). The reaction mixture was purged with H_2 (g). After vigorous stirring overnight, the reaction mixture was filtered and concentrated *in vacuo* resulting in compound **75** (60.5 mg, 67 μmol , 75%) a clear oil. ^1H NMR (400 MHz, MeOD): $\delta = 5.22$ (s, 1H), 4.34 – 4.23 (m, 2H), 3.96 – 3.90 (m, 1H), 3.88 – 3.83 (m, 1H), 3.80 – 3.62 (m, 4H), 3.59 – 3.45 (m, 2H), 2.91 – 2.87 (m, 2H), 1.66 (m, 4H), 1.45 – 1.30 (m, 8H), 1.21 (d, $J = 6.4$ Hz, 3H). ^{13}C NMR (101 MHz, MeOD): $\delta = 103.6, 101.5, 79.0, 76.4, 75.6, 73.7, 71.7, 70.7, 70.6, 70.4, 67.8, 62.4, 49.6, 49.4, 49.2, 49.0, 48.8, 48.6, 48.4, 40.8, 30.9, 30.2, 30.1, 28.5, 27.3, 27.1, 16.8$.



8-azido-octyl-1,2:3,6-di-O-benzyl- α -L-fucopyranosyl (1 $\rightarrow$ 2)- β -D-galactopyranoside (63)

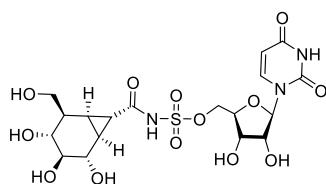
Amine **75** (28.1 mg, 61.9 μmol) was dissolved in MeOH/ H_2O (2 mL, 1:1, v/v) and K_2CO_3 (25.6 mg, 0.186 mmol) and $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (1.2 mg) were added. After addition of imidazole-1-sulfonyl azide.HCl (20 mg, 0.93 mmol) the reaction was stirred overnight. The reaction mixture was concentrated *in vacuo*, co-evaporated with toluene (3x) and redissolved in pyridine (6.2 mL). After addition of Ac_2O (1.0 mL, 10.6 mmol) the reaction mixture was stirred for 48 h. The reaction mixture was concentrated *in vacuo*

and purified by silica chromatography (30% EtOAc in pentane). ^1H NMR (400 MHz, CDCl_3): δ = 5.39 (d, J = 3.9 Hz, 1H), 5.34 – 5.26 (m, 3H), 5.06 – 4.93 (m, 2H), 4.60 (dd, J = 4.0, 13.0 Hz, 1H), 4.46 (d, J = 7.8 Hz, 1H), 4.19 (dd, J = 11.2, 6.4 Hz, 1H), 4.10 (dd, J = 11.2, 7.1 Hz, 1H), 3.99 – 3.83 (m, 3H), 3.57 – 3.48 (m, 1H), 3.27 (t, J = 7.0 Hz, 2H), 2.16 (s, 3H), 2.13 (s, 3H), 2.05 (s, 3H), 2.01 (s, 3H), 1.99 (s, 3H), 1.98 (s, 3H), 1.67 – 1.55 (m, 4H), 1.42 – 1.31 (m, 8H), 1.12 (d, J = 6.5 Hz, 3H). ^{13}C NMR (101 MHz, CDCl_3): δ = 170.8, 170.5, 101.7, 95.8, 77.5, 77.2, 76.8, 73.9, 71.78, 71.3, 70.5, 70.4, 68.4, 67.8, 67.3, 64.7, 61.3, 51.6, 29.7, 29.4, 29.2, 28.9, 26.8, 26.1, 20.8, 20.8, 15.9. Next, the residue was dissolved in MeOH (4 mL) and NaOMe (catalytic amount) was added. After stirring for 30 min, the reaction mixture was neutralized with Amberlite- H^+ IR-200, filtrated and concentrated *in vacuo* to obtain target compound **63** (8.3 mg, 17.3 μmol , 28% over three steps) as a white solid. ^1H NMR (400 MHz, MeOD): δ = 5.20 (s, 1H), 4.34 – 4.26 (m, 2H), 3.91 (m, J = 9.4, 6.7 Hz, 1H), 3.82 (d, J = 2.4 Hz, 1H), 3.78 – 3.70 (m, 4H), 3.70 – 3.63 (m, 3H), 3.57 – 3.47 (m, 2H), 3.28 (t, J = 8.0 Hz, 2H), 1.66 – 1.54 (m, 4H), 1.43 – 1.31 (m, 8H), 1.19 (d, J = 6.6 Hz, 3H). ^{13}C NMR (101 MHz, MeOD): δ = 103.6, 101.6, 79.0, 76.5, 75.8, 73.8, 71.8, 70.7, 70.6, 70.4, 67.8, 62.4, 52.4, 49.6, 49.4, 49.2, 49.0, 48.8, 48.6, 48.4, 31.0, 30.5, 30.3, 29.9, 27.8, 27.2, 16.8. HRMS: calculated for $[\text{C}_{20}\text{H}_{37}\text{N}_3\text{O}_{10}\text{Na}]^+$ 502.23712; found 502.23683.



DIPEA salt of ((3aR,4R,6R,6aR)-6-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-2,2-dimethyltetrahydrofuro[3,4-d][1,3]dioxol-4-yl)methyl ((1R,2S,3R,4R,5R,6R,7R)-2,3,4-tris(benzyloxy)-5-((benzyloxy)methyl)bicyclo[4.1.0]heptane-7-carbonyl)sulfamate (67**)**

To a solution of carboxylic acid **66**³³ (60 mg, 0.10 mmol) in toluene (6.0 mL) was added oxalyl chloride (1 M in DCM, 0.30 mL, 0.30 mmol, 3.0 eq.). After stirring for 5 at reflux conditions, the reaction mixture was concentrated *in vacuo* and redissolved in DCM (7.0 mL). 2',3'-O-Isopropylidene-5'-O-sulfamoyluridine³⁴ (0.100 g, 0.27 mmol, 2.3 eq.) and DIPEA (40 μL , 0.24 mmol, 2.4 eq.) were added. After stirring overnight at room temperature, the reaction mixture was concentrated under reduced pressure and purified by size exclusion column chromatography (DCM/MeOH, 1:1), resulting in sulfonamide **67** (90 mg, 86 μmol , 86%) as a white wax. ^1H NMR (400 MHz, MeOD): δ (ppm) 7.76 (d, J = 7.6 Hz, 1H), 7.38 – 7.17 (m, 20H), 5.94 – 5.88 (m, 1H), 5.72 (d, J = 6.4 Hz, 1H), 4.87 (d, J = 1.9 Hz, 2H), 4.84 – 4.67 (m, 4H), 4.62 – 4.54 (m, 2H), 4.52 – 4.43 (m, 2H), 4.41 – 4.35 (m, 1H), 4.30 – 4.23 (m, 2H), 3.74 (d, J = 5.8 Hz, 1H), 3.70 – 3.66 (m, 3H), 3.67 – 3.64 (m, 1H), 3.54 – 3.42 (m, 2H), 3.21 – 3.14 (m, 2H), 3.10 (t, J = 10.1 Hz, 1H), 2.39 – 2.28 (m, 1H), 1.94 (dd, J = 9.2, 4.7 Hz, 1H), 1.71 (dd, J = 9.1, 4.4 Hz, 1H), 1.61 (t, J = 4.6 Hz, 1H), 1.50 (d, J = 13.9 Hz, 3H), 1.32 (m, 15H). ^{13}C NMR (101 MHz, MeOD): δ (ppm) 179.1, 166.0, 152.0, 143.7, 143.7, 140.2, 139.9, 139.8, 139.6, 129.5, 129.5, 129.4, 129.3, 129.2, 129.1, 129.1, 128.9, 128.7, 128.7, 128.6, 128.5, 128.5, 115.2, 103.2, 93.7, 87.0, 85.4, 85.3, 82.5, 82.4, 77.7, 76.0, 75.9, 74.2, 72.6, 71.8, 70.2, 55.8, 43.8, 41.9, 27.5, 27.1, 26.5, 26.2, 25.6, 13.2.



((2*R*,5*R*)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl
 ((1*R*,2*S*,3*R*,4*R*,5*R*,6*R*,7*R*)-2,3,4-trihydroxy-5-(hydroxymethyl)bicyclo[4.1.0]heptane-7-carbonyl)sulfamate
 (65, ML131)

To a solution of sulfonamide **67** (89 mg, 84 μ mol) in DCM (10 mL) was drop-wise added BCl_3 (1 M in DCM, 1.15 mL, 1.15 mmol, 13.7 eq.) at 0 $^\circ\text{C}$. After stirring for 3 h, the reaction mixture was quenched with MeOH and stirred for 30 min. The reaction mixture was concentrated *in vacuo*, redissolved in CHCl_3 and stirred vigorously for 30 min before the suspension was filtered. The residue was taken up in methanol, concentrated *in vacuo* and purified by HPLC (C18 column, linear gradient: 5 $\rightarrow$ 10% B in A, solutions used A: H_2O , B: acetonitrile, 0.5% TFA, 15 min) to obtain compound **65** (8.6 mg, 16 μ mol, 20%) as a white solid. ^1H NMR (400 MHz, CDCl_3): δ 7.73 (d, J = 8.2 Hz, 1H), 5.90 (d, J = 4.5 Hz, 1H), 5.75 (d, J = 8.1 Hz, 1H), 4.55 – 4.48 (m, 2H), 4.20 (dd, J = 4.9, 2.5 Hz, 1H), 4.13 (dd, J = 7.0, 4.7 Hz, 2H), 3.96 – 3.92 (m, 1H), 3.57 (d, J = 8.3 Hz, 1H), 3.19 (dd, J = 10.0, 8.3 Hz, 1H), 2.89 (t, J = 10.1 Hz, 1H), 2.16 (s, 1H), 2.03 – 1.97 (m, 1H), 1.58 (s, 1H). ^{13}C NMR (101 MHz, CDCl_3): δ 172.8, 152.4, 142.4, 103.1, 90.7, 82.7, 79.3, 75.0, 74.0, 72.2, 71.0, 69.7, 64.2, 43.7, 30.2, 28.0, 24.3. HRMS: calculated for $[\text{C}_{18}\text{H}_{26}\text{N}_3\text{O}_{13}\text{S}]^+$ 524.11808, found 524.11799.

Materials of biological assays

Recombinant human GBA1 (Cerezyme), recombinant human GAA (Myozyme) and recombinant human α -Gal A (Fabrazyme) were obtained from Genzyme, USA. Cellular homogenates of a stable human embryonic kidney 293 (HEK293) over-expressing GBA2 cell line³⁵ were pre-incubated for 30 min with an inhibitor of GBA1 (1 mM conduritol B epoxide (CBE)). Galactocerebrosidase (GALC) was obtained by expressing recombinant murine GALC in HEK293 cells after which the secreted recombinant protein in the culture medium was directly used.³⁶ Fibroblasts of Pompe patients diagnosed on the basis of reduced GAA activity (obtained with consent from donors) were used for GANAB determinations.

Enzyme activity assays, determination of the apparent IC_{50} values

All activity assays were performed at 37 $^\circ\text{C}$. To determine the apparent IC_{50} values, enzyme preparations were pre-incubated with a range of inhibitor concentrations for 30 min, where after the residual activity was measured by addition of the appropriated 4-methylumbelliferyl-based substrate mix. GBA1 residual activity was measured with 3.7 mM 4-methylumbelliferone(4- μ)- β -D-glucopyranoside in 150 mM McIlvaine buffer, pH 5.2, supplemented with 0.2% Taurocholate (w/v), 0.1% Triton X-100 (v/v), 0.1% Bovine Serum albumin (BSA) (w/v) for 30 min. GBA2 substrate mix consisted of 3.7 mM 4- μ - β -D-glucopyranoside in 150 mM McIlvaine, pH 5.8, 0.1% BSA (w/v) and was incubated for 1 h. α -Gal A activity was determined with 2 mM 4- μ - α -D-galactopyranoside dissolved in 150 mM McIlvaine, pH 4.5, 0.1% BSA (w/v) for 30 min. GAA substrate mix consisted of 3 mM 4- μ - α -D-glucopyranoside pH 5.0, 0.1% BSA (w/v) for 1 h, GALC was assayed with 1.25 mM 4- μ - β -D-galactopyranoside in 150 mM McIlvaine pH 4.3 containing 0.2 M NaCl and 0.1% BSA (w/v) for 1h and GANAB with 3 mM 4- μ - α -D-glucopyranoside pH 7.0, 0.1% BSA (w/v) for 2 h. After stopping the substrate reaction with an excess of 1 M NaOH-glycine (pH 10.3), liberated 4- μ

fluorescence was measured with a fluorimeter LS55 (Perkin Elmer) using λ_{Ex} 366 nm and λ_{Em} 445 nm. All IC₅₀ values were determined in triplicate with Graphpad Prism 5 software.

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Nederlandse samenvatting

Glycosidases zijn enzymen die essentieel zijn in verschillende fundamentele biologische processen zoals de afbraak van koolhydraten (suikers), glycolipiden en glycoproteïnes. Glycosidases zijn in staat om met grote efficiëntie glycosidische bindingen te hydrolyseren, dit terwijl zulke bindingen, bestaande uit acetalen, zeer stabiel zijn als ze zich in waterig milieu bevinden, zowel in zure of basische pH, en er geen glycosidases aanwezig zijn. Genetische aberraties in glycosidases zijn de oorzaak van verschillende complexe ziektes zoals lysosomale stapelingsziektes. Met behulp van een recent ontwikkelde techniek, genaamd 'activity-based protein profiling' (ABPP), kan de enzymatische activiteit van glycosidases in kaart worden gebracht. Hiervoor zijn de zogeheten 'activity-based probes' (ABPs) ontwikkeld. Deze ABPs zijn irreversibele remmers die een biotine of fluorescente groep bevatten. Na het incuberen van een biologische monster met een gebiotinyleerde ABP kunnen de gelabelde enzymen worden geïdentificeerd met behulp van massa spectrometrie. De daadwerkelijke activiteit van deze enzymen in de fysiologische omgeving kan worden gevisualiseerd en gekwantificeerd met behulp van fluorescente ABPs.

Het onderzoek beschreven in dit proefschrift omvat de ontwikkeling en toepassingen van een set van selectieve ABPs voor verschillende 'retaining' glycosidases: α -galactosidases, β -galactosidases, α -mannosidases en β -mannosidases. In het tweede deel van dit proefschrift wordt de ontwikkeling van een nieuwe klasse competitieve remmers gepresenteerd voor glycosidases en glycosyltransferases met mogelijke klinische toepassingen. Eerder beschreven overgangstoestand-gebaseerde remmers voor glycosidases zoals voor neuraminidases (oseltamivir/Tamiflu en zanamivir/Relenza) zijn succesvol gebleken en zijn geregistreerde geneesmiddelen. Alle beschreven ABPs en competitieve remmers in dit proefschrift zijn derivaten van cyclophellitol, een natuurstof en potente β -glucosidase remmer.

In **hoofdstuk 1** worden de principes van de ABPP techniek besproken die ten grondslag liggen aan het ontwerp van de verbindingen in het eerste deel van dit proefschrift. Verder wordt er een overzicht gegeven van de voorgestelde reactie routes

van verschillende glycosidases. Deze reactie routes dienen als basis voor het ontwerp van de ABPs en de competitieve remmers. Tenslotte wordt de potentie van overgangstoestand-gebaseerde remmers beschreven met onder andere voorbeelden die een therapeutisch toepassing hebben.

Hoofdstuk 2 beschrijft de ontwikkeling van α -galactosidase selectieve ABPs die kunnen worden gebruikt om de ziekte van Fabry en de ziekte van Schindler te bestuderen. *N*-acyl- en *N*-alkyl versies van de α -galactosidase selectieve ABPs zijn hierin vergeleken. Hoewel de *N*-geacyleerde versie vatbaarder is voor hydrolyse tijdens de zuivering en lyofilisatie heeft deze ABP de voorkeur vanwege de labelings efficiëntie in de biologische omgeving.

De synthese en validatie van een β -galactosidase selectieve ABP is beschreven in **hoofdstuk 3**. Eerst is een kristalstructuur verkregen van CjGH35A galactosidase die het covalente werkingsmechanisme van de β -galactose-geconfigureerde cyclophellitol aziridines bevestigt. Vervolgens is met behulp van de Diels-Alder strategie een potente β -galactosidase ABP verkregen die GLB1 en GALC labelen in nieren van muizen. Deze ABP kan worden gebruikt als molecuulair gereedschap om GM1 gangliosidose, het syndroom van Morquio type B of de ziekte van Krabbe te bestuderen die allen worden veroorzaakt door een mutatie in ofwel GLB1 of GALC.

De bibliotheek van selectieve ABPs voor glycosidases is uitgebreid in **hoofdstuk 4** met de synthese en validatie van ABPs voor α -mannosidases om de rol van deze enzymen in α -mannosidosis of strottenhoofd kanker te onderzoeken. De reactie route van α -mannosidases, waarin een boot ($B_{2,5}$) overgangstoestand conformatie wordt verwacht, is verschillend van de eerder beschreven glycosidases. Desalniettemin is een covalente enzym-remmer complex verkregen na het doordrenken van de α -mannosidase geconfigureerde cyclophellitol aziridine in GH38 Golgi α -mannosidase II van een fruitvlieg. Zoals verwacht uit de reactie route heeft de cyclohexaan de zogenaamde 'skew-boat' conformatie aangenomen. De ABP met een Cy5 label kan goed worden gebruikt voor het zichtbaar maken van GH38 α -mannosidases.

Tenslotte vormen GH2 exo- β -mannosidase selectieve APBs het onderwerp van **hoofdstuk 5**. ABPP experimenten in lysaten van nieren afkomstig uit muizen resulteerde in het labelen van MANBA. Dit enzym is deficiënt in β -mannosidosis waardoor deze ABPs gebruikt kunnen worden als molecuulair gereedschap om de pathologie te onderzoeken.

In **hoofdstuk 6** is de synthese van carba-cyclophellitol gebaseerde verbindingen beschreven in de α -glucopyranose, β -glucopyranose, α -galactopyranose en β -galactopyranose configuratie. Deze verbindingen zijn potentiële reversibele glycosidase

remmers doordat ze de overgangstoestand conformatie van het oxocarbenium ion gedurende de reactie route nabootsen.

Hoofdstuk 7 bevat een biologische evaluatie van de β -glucopyranose-geconfigureerde carba-cyclophellitol derivaten in *Thermotoga maritima* TmGH1, GBA1 (human lysosomale β -glucosidase) en GAA (humaan lysosomale α -glucosidase). Hieruit blijkt dat deze carba-cyclophellitol derivaten potente en selectieve β -glucosidase remmers zijn. DW082 is de meest potente TmGH1 remmer met een K_i van 8.2 nM. Vervolgens is de conformatie van de carba-cyclophellitol derivaten geanalyseerd met behulp van ^1H NMR analyse, kwantum mechanische analyses en DFT berekeningen die de $^4\text{H}_3$ conformatie van carba-cyclophellitol ondersteunen. De verkregen structuur na het doordrenken van carba-cyclophellitol DW082 in TmGH1 bleek eveneens de $^4\text{H}_3$ conformatie te hebben aangenomen. Deze klasse cyclopropyl suikers zou daarom kunnen worden toegevoegd aan het arsenaal van motieven van sterk gebonden remmers.

Hoofdstuk 8 beschrijft de synthese van carba-cyclophellitol gebaseerde remmers voor galactosyltransferases welke in de (nabije) toekomst onderzocht zullen worden. AB180 bleek inmiddels de eerste potente carba-cyclophellitol gebaseerde α -galactosidase remmer te zijn. De glucosyl-analoog bleek geen potentie te hebben voor glycosidases of glucosylceramide synthase (GCS).

Tenslotte wordt in **hoofdstuk 9** een meer gedetailleerde samenvatting gegeven van het onderzoek dat beschreven is in dit proefschrift en worden er voorstellen gedaan voor vervolgonderzoek.

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Curriculum vitae

Thomas Beenakker was born in Eindhoven on the 9th of April 1989. After he obtained his VWO diploma at Adelbert College in Wassenaar in 2007, he studied Molecular Science & Technology at Leiden University and Delft University of Technology. During his bachelor studies he was selected for honour classes and he participated in the 'Crayenborgh lecture series' at the Institute for History at Leiden University. In 2010 he obtained his bachelor degree and started the master Chemistry with the specialisation Design & Synthesis at Leiden University. During his studies he did a research internship on the synthesis of teichoic acid fragments at the Bio-organic Synthesis group, under the supervision of dr. W. F. J. Hogendorf, dr. J. D. C. Codée, and prof. dr. G. A. van der Marel. Subsequently, at the Metals in Catalysis, Biomimetics & Inorganic Materials group, he worked under the supervision of dr. S. Akerboom and prof. dr. E. Bouwman. His master thesis was entitled 'Salicyl imidates as antenna for luminescent Eu(III) and Tb(III) complexes.' In July 2012 he obtained his Master of Science degree.

From September 2012 until March 2017 he was affiliated at Leiden University as a PhD candidate in the Bio-organic Synthesis group. Under the supervision of dr. J. D. C. Codée, prof. dr. G. A. van der Marel, and prof. dr. H. S. Overkleeft he conducted research presented in this thesis. He presented parts of the work as poster presentations at various national and international conferences and symposia, including: 'Design and synthesis' (2012 and 2013, Lunteren), EMBO Chemical Biology congress (2014, Heidelberg, Germany), 'CHAINS 2015' (2015, Veldhoven) and 'COST Chemical Proteomics Meeting' (2015, Oxford, UK). At the Reedijk Symposium (2013, Leiden) he won the 'best poster prize'.