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

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# 5

# Chapter 5: Design, synthesis and evaluation of potential GBA2 specific fluorogenic substrates

## 5.1 Introduction

GBA2 is the non-lysosomal β-glucosidase (EC 3.2.1.45, GH116) encoded by the *GBA2* gene located on human chromosome 9 at position p13.3.[1] It is a 927-amino-acid protein that, like GBA1, is a retaining glycosidase, processing its substrate via a classical Koshland double displacement mechanism. While both enzymes operate through a catalytic nucleophile and acid/base retaining β-glucosidase mechanism proceeding through a $^{1}S_{3}$ to a $^{4}H_{3}$ conformation to ultimately adopt a $^{4}C_{1}$ in the covalent complex, their active site compositions differ: GBA1 employs two glutamate amino acid residues as catalytic nucleophile and acid/base, whereas GBA2 employs only a glutamate as catalytic nucleophile (Glu 537) and an aspartate as acid/base residue (Asp 677).[2] It has been implied that GBA2 acts through a slightly different mechanism than GBA1. While both enzymes are beta glucosidases, GBA2 is a perpendicular protonator, whereas GBA1 acts as a lateral protonator.[3] GBA1 is localized at the luminal surface of lysosomal membranes, while GBA2 is associated with the cytoplasmic side of membranes of the endoplasmic reticulum (ER) and Golgi apparatus.[4] Both of these enzymes hydrolyse glucosylceramide (GlcCer) under normal physiological conditions.[1,5] However, unlike GBA1, GBA2 is not deficient in Gaucher disease (GD). In fact, increased levels and activity of GBA2 have been reported in GD mice and GD patients, likely as a compensatory mechanism.[5–7]

To date, no crystal structure of mammalian GBA2 exists, but bacterial homologues in *S. solfataricus* and *T. xylanolyticum* (*Tx*GH116) have provided crystal structures and insights into the catalytic mechanism of the enzyme.[3,8,9] *Tx*GH116 GBA2 shares 37% sequence homology with human GBA2.[9] Interestingly, inhibition of GBA2 has been shown to extend the life-span of Niemann pick type C (NPC) mice, a lysosomal storage disorder (LSD) characterized by cholesterol accumulation in lysosomes.[10] On the other hand, mutations in the *GBA2* gene have been associated with several disorders including hereditary spastic paraplegia (HSP), autosomal-recessive cerebellar ataxia (ARCA), and the Marinesco-Sjögren–like syndrome.[11–14] GBA2 hydrolyses GlcCer on the cytosolic side of membranes, but the physiological role of cytosolic GlcCer metabolism as well as the interplay between GBA2 and the lysosomal GBA1 remain unclear. Although no clear physiological role for GBA2 has been established yet, GBA2 inhibitors are currently being explored as potential treatments for Parkinson's disease (PD).[15]

Another key difference between GBA1 and GBA2 is their substrate preference. GBA1 has broad substrate specificity, illustrated by its ability to hydrolyse not only artificial glycosides 4-methylumbelliferyl-β-D-Glucoside (4MU-β-Glc) but also other

glycosyl derivatives such as 4-methylumbelliferyl-β-D-xyloside (4MU-β-Xyl) where the fluorogenic 4MU aglycon is often used to visualize GBA1 activity (Figure 5.1).[16–18] Furthermore, GBA1 is capable of accommodating diverse functional moieties incorporated at the *O*-6 position of functionalized glucosyl substrates. This feature has been exploited for the generation of the 6-*O*-functionalized artificial fluorogenic substrates described in chapters 2 and 3,[19] as well as fluorescent substrates[20] and activity based probes (ABPs),[21] all selective towards GBA1 over GBA2 and GBA3. Of note, the artificial glucoside 4MU-β-Xyl shows selectivity towards GBA1 over GBA2, although it is a much poorer substrate than 4MU-β-Glc, suggesting that the $CH_2OH$ group at the C-5 position plays a crucial role for GBA2 recognition.[17] Furthermore, GBA2 can hydrolyse both β-glucosidic and β-galactosidic substrates, in contrast to GBA1, which shows significantly lower hydrolysis rates for these galactosides.[5]

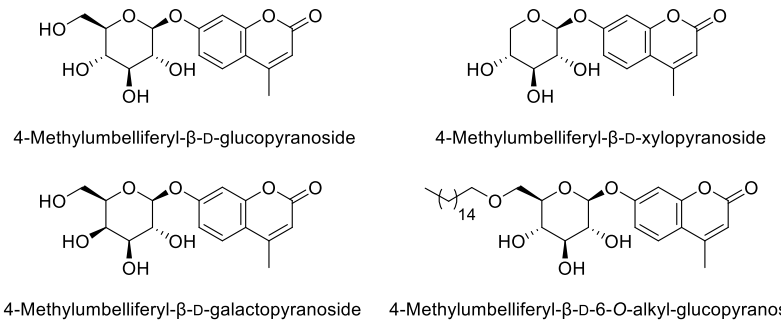
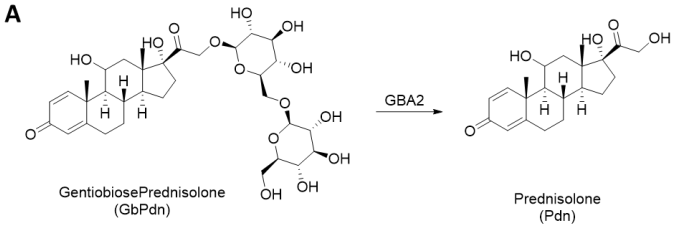



**Figure 5.1:** Chemical structures of 4-methylumbelliferyl-β-D-glucoside (4MU-β-Glc), 4-methylumbelliferyl-β-D-xyloside (4MU-β-Xyl), 4-methylumbelliferyl-β-D-galactoside (4MU-β-Gal), and 4-Methylumbelliferyl-β-D-6-*O*-alkyl-glucopyranoside (6-*O*-alkyl-4MU-β-Glc).

Although no specific fluorogenic substrate for either GBA2 or GBA3 has been identified, there have been some indications for GBA2 selective inhibition by prodrugs, ABPs and inhibitors in literature which will be discussed below. One of these examples originates from unpublished research from the Schaaf lab.[22] Glucocorticoids (GCs) are used as anti-inflammatory drugs for the treatment of inflammatory and immune conditions.[23] Their clinical use is limited however due to encountered side effects and glucocorticoid resistance.[24–28] Halima *et al.* observed that the attaching the disaccharide gentiobiose to the classical GC drug prednisolone (Pdn, Figure 5.2A) resulted in a GC prodrug that acts locally at the site of inflammation. This localized activity was considered to be exerted by GBA2, whose activity is upregulated in inflamed tissue. Given that the activation of the

Pdn-based prodrug was thought to be GBA2 dependent the synthesis and evaluation of 4MU-β-D-gentiobiose (Figure 5.3) was pursued here as a potential GBA2 selective substrate.

On the other hand, a specific ABP for GBA2 based on a β-D-arabinofuranosyl aziridine has recently been reported adopting a $^{3}E$ conformation which resembles the $^{1}S_{3}$ initial Michaelis complex conformation covalently reacting with the catalytic glutamate.[29] These aziridine-based probes, equipped with a BODIPY green or BODIPY red tag, exhibited substantial activity towards GBA2 ($IC_{50}$ values: 120–160 nM, Figure 5.2B) and good selectivity for GBA2 over GBA1 and GBA3. According to the data obtained by Su *et al*., β-D-arabinofuranose configured-glycosides might exhibit selectivity for GBA2 over GBA1 and GBA3. Furthermore, Shimokawa *et al.* recently reported the identification of an exo-β-D-arabinofuranosidase from *Microbacterium arabinogalactanolyticum* named ExoMA2, which belongs to the same GH116 enzyme class as GBA2.[30] This discovery suggests that enzymes belonging to the GH116 enzyme family may be able to process β-D-arabinofuranose-containing substrates. Based on these findings, the 4MU-β-D-arabinofuranoside substrate **3** (4MU-β-D-Araf, Figure 5.3) may hold potential as a GBA2-selective fluorogenic substrate.

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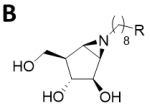
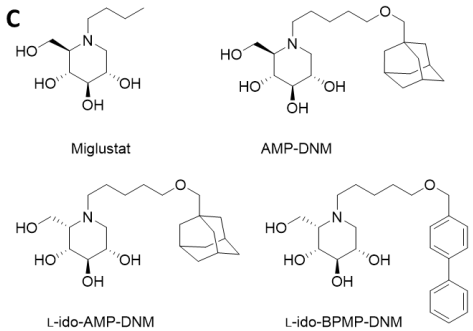



| Apparent $IC_{50}$ (nM) | GBA2 | rhGBA1 | GBA3 |
|---|---|---|---|
| R = BODIPY green | 124.4 ± 4.9 | 4292.3 ± 157.1 | >10000 |
| R = BODIPY red | 162.2 ± 34.7 | 1777.0 ± 71.0 | >10000 |

C




| $IC_{50}$ (nM) | GCS | GBA1 | GBA2 |
|---|---|---|---|
| miglustat | 50000 | 400000 | 230 |
| AMP-DNM | 200 | 100 | 2 |
| L-ido-AMP-DNM | 100 | 2000 | 1 |
| L-ido-BPMP-DNM | 8 | 20000 | 3 |

**Figure 5.2:** Chemical structures and $IC_{50}$ values for GBA2 ABPs, inhibitors and pro-drugs described in the literature. A) GBA2 selective hydrolysis and pro-drug activation of disaccharide glycosylated glucocorticoid drug prednisolone. B) β-D-Arabinofuranosyl aziridine ABPs and their apparent $IC_{50}$ values for GBA2, rhGBA1 and GBA3. C) *N*-alkyl-DNJ iminosugar derivatives (Miglustat, AMP-DNM, L-*Ido*-AMP-DNM and L-*Ido*-BPMP-DNM) and their $IC_{50}$ values for the inhibition of GCS, GBA1 and GBA2.

Overkleeft and collaborators[31,32] have reported on the synthesis and evaluation of *N*-[5-(adamantan-1-ylmethoxy)pentyl]-1-deoxynojirimycin, AMP-DNM) derivatives, as well as the DNM isomers with inversion of configuration at C-5: L-*Ido*-AMP-DNM and L-*Ido*-BPMP-DNM (Figure 5.2C). These *N*-alkyl-DNJ iminosugars are derivatives of the GCS inhibitor *N*-butyl-deoxynojirimycin (NB-DNJ, Miglustat), which inhibits GCS and is used as SRT drug in the treatment of GD.[33] Miglustat inhibits GCS at relatively high concentrations, but it is also a potent GBA2 inhibitor.[34] Its beneficial therapeutical effects are accompanied by gastrointestinal and metabolic side effects due its strongly inhibitory potential on intestinal lactase, sucrase, and isomaltase. In line with this, Wennekes *et al.* found that AMP-DNM strongly inhibits these enzymes as well, while L-ido-AMP-DNM showed good potency for the inhibition of GCS and

GBA2, with minimal to no activity against the intestinal glycosidases.[35] Since the L-ido-configured iminosugars showed GBA2 selectivity, the L-ido configured fluorogenic substrates 4MU-β-L-idose **4** (4MU-β-L-ido) and 4MU-α-L-idose **5** (4MU-α-L-ido, Figure 5.3) were also considered as interesting target compounds.

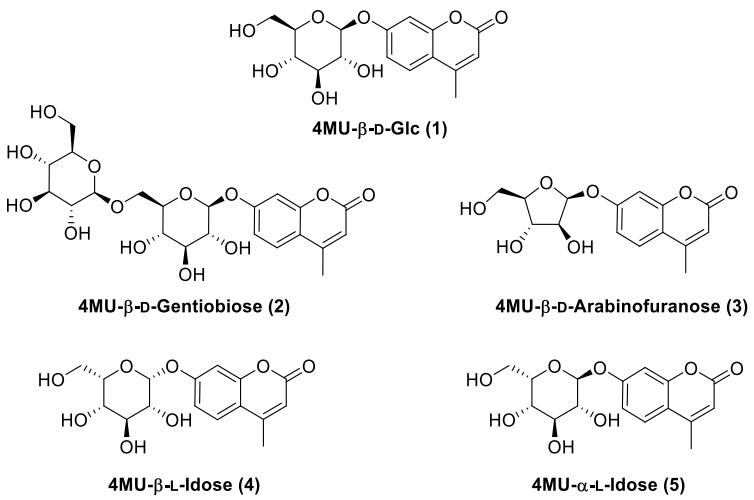



**Figure 5.3:** Chemical structures of potential GBA2 selective 4MU based glycosides: 4MU-β-D-glucose **1** (4MU-β-D-Glc), 4MU-β-D-gentiobiose **2** (4MU-β-D-Gb), 4MU-β-D-arabinofuranose **3** (4MU-β-D-Araf), 4MU-β-L-idose **4** (4MU-β-L-Ido) and 4MU-α-L-idose **5** (4MU-α-L-Ido).

Both Su *et al.* and Wennekes *et al.* observed that the introduction of a lipophilic moiety at the nitrogen of the aziridine or iminosugar was key in achieving GBA2 specificity.[29,35] Furthermore, Ghisaidoobe *et al.* also looked at the effects of introducing a biphenyl lipophilic handle and found that L-ido-BPMP-DNM presents similar inhibition activity towards GBA2 while displaying more potent GCS inhibition.[32] Based on this, another set of fluorogenic substrates was designed equipped with a 4-methylumbelliferyl (4MU) derivative functionalised at the 4-methyl position. These 4MU derivatives feature an adamantyl lipophilic handle through an amide acyl linker: Ada4MU-β-D-glucose **6** (Ada4MU-β-D-Glc), Ada4MU-β-D-arabinofuranose **7** (Ada4MU-β-D-Araf), Ada4MU-β-L-idose **8** (Ada4MU-β-L-ido) and Ada4MU-α-L-idose **9** (Ada4MU-α-L-ido).

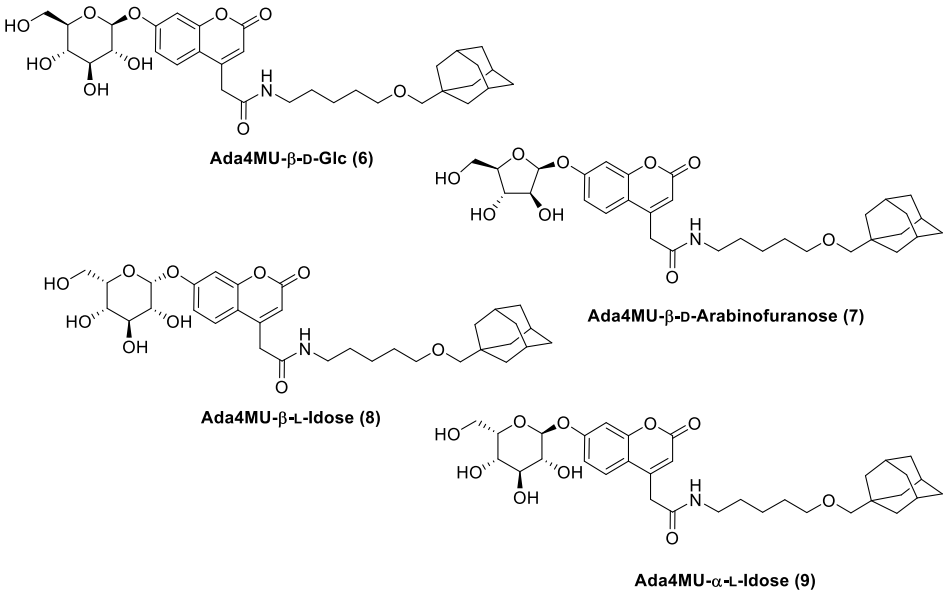


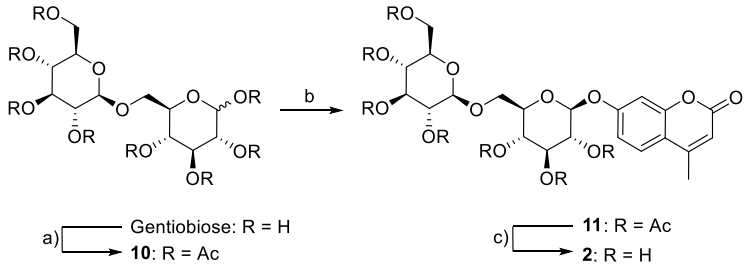

**Figure 5.4:** Chemical structures of potential GBA2 selective adamantly-4MU based glycosides: Ada4MU-β-D-glucose **6** (Ada4MU-β-D-Glc), Ada4MU-β-D-arabinofuranose **7** (Ada4MU-β-D-Araf), Ada4MU-β-L-idose **8** (Ada4MU-β-L-ido) and Ada4MU-α-L-idose **9** (Ada4MU-α-L-ido).

Given that GBA2 is a potent transglycosidase able to generate several glycosylated metabolites abnormal in several disease conditions, generating a GBA2-specific substrate to monitor the enzyme would be a valuable endeavour. Since GBA1 and GBA2 accept different substrates, likely there are also differences in transglucosylation acceptors for these enzymes. Obtaining a GBA2 specific substrate would enable future studies of GBA2-specific activity and its downstream effects. This Chapter focuses on the synthetic efforts towards the generation of fluorogenic substrates **2**-**9** and their biological evaluation against GBA1, GBA2 and GBA3.

## 5.2 Results and discussion

### 5.2.1 Synthesis of potential GBA2 specific substrates 2-7

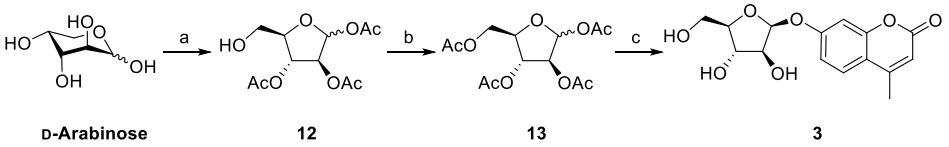
The synthesis of 4MU-β-D-gentiobiose **2** started from commercially available gentiobiose which was globally acetylated using standard acetylation conditions to yield compound **10** in quantitative yield. The fluorogenic 4MU aglycon was introduced by first brominating the anomeric position using TMSBr and bismuth tribromide in dichloromethane over 2 hours. The crude bromide was immediately coupled to 4MU using NaOH in an acetone and water mixture using TBABr as a phase transfer catalyst. After work up and purification this led to the acquisition of **11** in 38% yield over 2 steps. Finally the acetyl groups were deprotected using a catalytic amount of sodium methoxide to afford the 4MU-β-D-gentiobiose **2** in 48% yield.




**Scheme 5.1:** Synthesis towards 4MU-β-D-gentiobiose **2**. Reagents and conditions: a) $Ac_2O$, DMAP, pyridine, room temperature, 16 h, quantitative yield; b) TMSBr, $BiBr_3$, $CH_2Cl_2$, 0 °C, 2 h. ii. 4MU, NaOH, TBABr, Acetone/$H_2O$ (1:1), rt, 18 h, 38% over 2 steps. c) NaOMe, MeOH/$CH_2Cl_2$ 1:1, rt, 4 h, 48%.

The synthesis of 4MU-β-D-Araf **3** started from commercial D-arabinose. Selective silylation of the C-5 hydroxyl using *tert*-butyldiphenylsillyl chloride, followed by per-acetylation of the remaining hydroxyls using acetic anhydride in pyridine and subsequent desilylation using tetrabutylammonium fluoride, yielded intermediate **12** in 51% over 3 steps with moderate purity. The free C5-hydroxyl was then acetylated using acetic anhydride in pyridine affording per-acetylated intermediate **13** in 43% yield. The lower yield can be attributed to the impurity of the previously isolated intermediate. To generate 4MU-β-D-Araf **3**, intermediate **13** was first subjected to anomeric bromination using the addition of hydrogen bromide in acetic acid and, after a simple work-up, coupled to 4-methylumbelliferyl (4MU) using phase-transfer conditions with sodium hydroxide and tetra-butylammonium bromide in an acetone/water mixture (1:1). The peracetylated crude intermediate

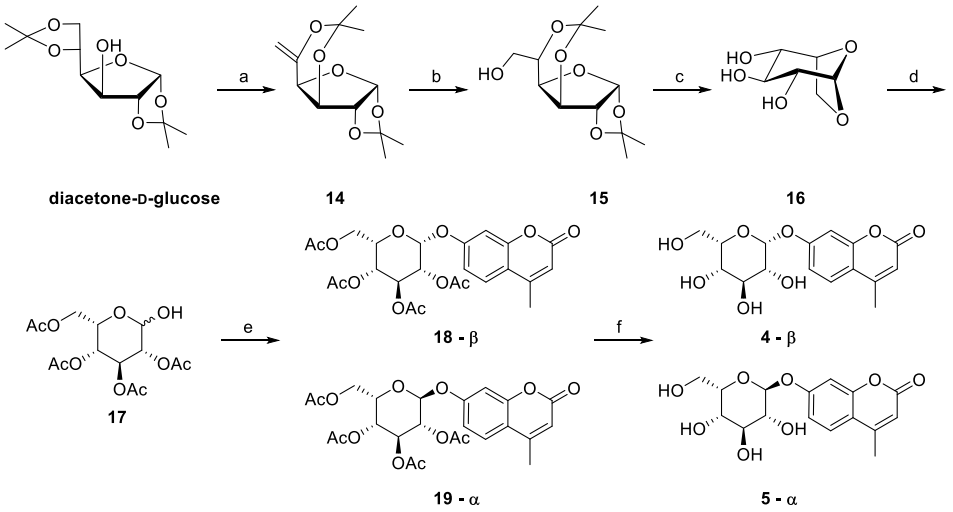
was then deprotected using sodium methoxide in a methanol/dichloromethane. Following purification by silica column chromatography and prep-HPLC, the final product, 4MU-β-D-Araf **3**, was obtained in an overall yield of 4% over three steps.




**Scheme 5.2:** Synthesis towards 4MU-β-D-arabinofuranose **3**. Reagents and conditions: a) i. TBDPSCl, pyridine, rt, 18 h. ii. $Ac_2O$, pyridine, 55 °C, 3 h. iii. TBAF (1 M in THF), THF, rt, 18 h, 51% 3 steps; b) $Ac_2O$, pyridine, rt, 6 h, 43%; c) i. HBr (30% in AcOH), $CH_2Cl_2$, 0 °C, 4 h. ii. 4MU, NaOH, TBABr, acetone/$H_2O$ (1:1), rt, 18 h. iii. NaOMe, MeOH/$CH_2Cl_2$ 1:1, rt, 4 h, 4% over 3 steps.

The synthesis of 4MU-β-L-idose **4** and 4MU-α-L-idose **5** was largely adapted from Lee *et al.*[36], who previously described the synthesis of the α- and β-isomers. Lee *et al.* based their synthetic route on a double ketal fixation strategy, targeting the 1,2- and 3,5-hydroxy groups of diacetone-α-D-glucose to form a tricyclic D-glucofuranosyl derivative **14**. Diacetone-α-D-glucose was then treated with triphenylphosphine ($PPh_3$) and *N*-bromosuccinimide (NBS) for 45 minutes in toluene after which 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) was added for an additional 2 hours which, after work up and column chromatography, yielded intermediate **14** in 45% yield. During this reaction, $PPh_3$ and NBS first generate a phosphonium salt which undergoes nucleophilic attack by the 3-hydroxy to form an alkoxyphosphonium intermediate.[37] Due to the steric constrains imposed by the fused ring system, isopropylidene rearrangement is favoured over $SN_2$ substitution by bromide. This rearrangement results in a sterically favoured $SN_2$ displacement, allowing subsequent elimination by DBU to yield the 5-exo-double bond intermediate **14**. Next, hydroboration of this intermediate using borane in THF, followed by oxidative work up with hydrogen peroxide and sodium hydroxide, led to the formation of intermediate **15** in 89% yield as a single diastereomer, as the steric environment favours borane addition from the α-face.[36] Treatment of intermediate **15** with HCl in ethanol (0.2 M) at 95 °C overnight afforded intermediate **16** in 75% yield. A subsequent one-pot acetylation and acetolysis reaction using acetic anhydride and trifluoroacetic acid, followed by an anomeric bromination, resulted in the formation of an unstable bromide intermediate. Work up with saturated aqueous sodium bicarbonate yielded the tetraacetate intermediate **17** in 48% yield. The anomeric free glycosyl intermediate was then coupled to 4MU via a

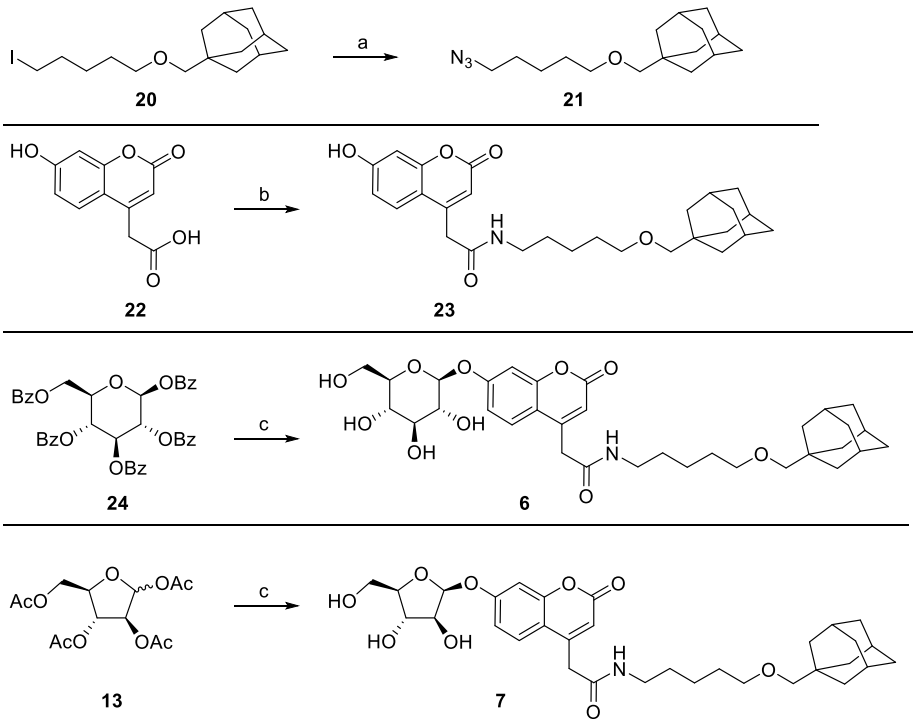
Mitsunobu-type glycosylation using diethyl azodicarboxylate (DEAD) and trimethylphosphine ($PMe_3$) in THF at room temperature for two hours to yield a mixture of α- and β-isomers (7:1) in 56% overall yield. $PMe_3$ was chosen over $PPh_3$ to facilitate purification, as trimethylphophineoxide can readily be removed by evaporation, avoiding the cumbersome purification required for triphenylphosphine oxide. The α- and β-isomers were separated by preparative HPLC, and the isolated products were deprotected using sodium methoxide in a mixture of methanol and dichloromethane to yield both the 4MU-β-L-ido **4** and 4MU-α-L-ido **5** substrates in quantitative yield.




**Scheme 5.3:** Synthesis towards 4MU-β-L-idose **4** and 4MU-α-L-idose **5**. Reagent and conditions: a) i. NBS, $PPh_3$, toluene, 90 °C, 45 min. ii. DBU, 90 °C, 2 h, 45%; b) i. $BH_3$·THF, THF, rt, 3 h. ii. $H_2O_2$ (30% in $H_2O$), NaOH (3 M), THF, 0 °C, 89%; c) HCl (0.2 M in EtOH), EtOH, 95 °C, 18 h, 75%; d) i. TFA, $Ac_2O$, rt, 18 h, ii. HBr (30% in AcOH), rt, 6 h, 48%; e) 4MU, DEAD, $PMe_3$ (1 M in THF), THF, rt, 2 h, 56% (β:α 1:7); f) NaOMe, MeOH/$CH_2Cl_2$ 1:1, rt, 4 h, quantitative yield.

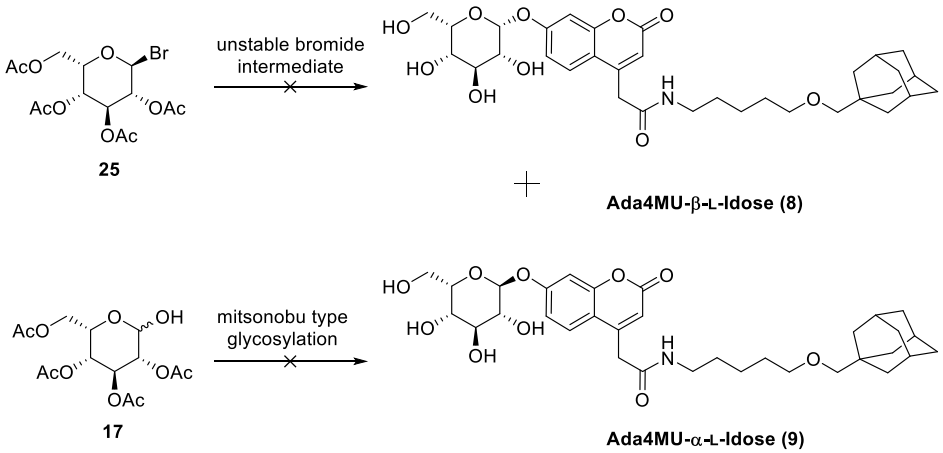
With the initial 4MU-glycosides in hand, attempts were made to synthesise the adamantly-modified 4MU (Ada4MU) glycosides. The synthesis of the Ada4MU building block **23** began with building block **20**, which was kindly provided by colleagues at the LIC and prepared through previously described synthetic routes.[38] The azido moiety was introduced by reacting intermediate **20** with sodium azide in dimethyl sulfoxide at room temperature overnight, yielding intermediate **21** in 97% yield. In parallel, the commercial 7-hydroxycoumarin-4-acetic acid building block **22** was subsequently coupled to azido intermediate **21** using hydroxybenzotriazole

(HOBt), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and tributylphosphine (P(*n*-Bu)$_3$), resulting in the formation of the adamantyl-4MU intermediate **23** in 63% yield. Perbenzoylated glucose intermediate **24** underwent anomeric bromination using hydrogen bromide, and the resulting activated glycoside was immediately coupled to **23** using sodium hydroxide in a chloroform/water mixture (1:1). The crude was deprotected using sodium methoxide in a dichloromethane/methanol (1:1) mixture to yield Ada4MU-β-D-Glc **6** in 23% yield over three steps after final silica column chromatography. A similar route was employed for the synthesis of Ada4MU-β-D-Araf **7**, starting from the earlier synthesized per-acetylated intermediate **13** (Scheme 5.2). After anomeric bromination of **13**, the crude was immediately coupled and deprotected using the same conditions described for **6**. This resulted in the formation of Ada4MU-β-D-Araf **7** in 11% yield over three steps.




**Scheme 5.4:** Synthesis towards Ada4MU **23**, Ada4MU-β-D-Glc **6** and Ada4MU-β-D-arabinofuranose **7**. Reagents and conditions: a) $NaN_3$, DMSO, rt, 20 h, 97% yield; b) **21**, HOBt, EDC, P(*n*-Bu)$_3$, Toluene/$CH_2Cl_2$ (1:1), rt, 16 h, 63%; c) i. HBr (30% in AcOH), $CH_2Cl_2$, 0 °C, 4 h. ii. **23**, NaOH, $CHCl_3/H_2O$ (1:1), rt. 18 h. iii. NaOMe, MeOH/$CH_2Cl_2$ 1:1, rt, 4 h. **6** = 23% over 3 steps; **7** = 11% over 3 steps.

The synthesis of Ada4MU-β-L-ido **8** and Ada4MU-α-L-ido **9** was also attempted; however, it proved abortive under the previous conditions. The instability of bromide intermediate **25** prevented the coupling with functionalized 4MU **23**, as was successfully performed for Ada4MU-β-D-Glc **6** and Ada4MU-β-D-Araf **7**. Additionally, the Mitsonobu-type glycosylation of intermediate **17** to **23** did not lead to any product formation (Scheme 5.3).




**Scheme 5.5**: Failed attempts at synthesizing Ada4MU-β-L-idose **8** and Ada4MU-α-L-idose **9**.

Due to the encountered difficulties in synthesizing Ada4MU-β-L-idose **8** and Ada4MU-α-L-idose **9**, the priorities were shifted to first assessing the enzymatic hydrolysis activity of GBA1, GBA2 and GBA3 towards substrates **1**-**7**. This allowed for a focus on understanding the substrate specificity and enzymatic behaviour of these enzymes, which would provide valuable insights into their potential applications and help guide future synthetic efforts.

### 5.2.2 Biochemical evaluation of 4MU based substrates 1-7

The enzymatic hydrolysis activity of all substrates was assessed using recombinant human GBA1 (rhGBA1, Cerezyme), HEK293T cell lysates overexpressing GBA2 (GBA1/2 KO), and HEK293T cell lysates overexpressing GBA3 (GBA1/2 KO) for substrate **2**, or recombinant GBA3 for substrates **3**-**7**. Enzymatic hydrolysis was determined by measuring the release of 4MU after 30 minutes of incubation, and the results were compared to the hydrolysis of the positive control 4MU-β-D-Glc **1**. As observed in Figure 5.5, no hydrolysis of 4MU-β-D-Gb **2** was detected for any of the three glucosidases, while the positive control 4MU-β-D-Glc **1** was readily hydrolysed by all three GBA enzymes. This suggests that the gentiobiose sugar is not a substrate of GBA2 when a 4MU aglycon is attached to the sugar. An important consideration here is that Halima *et al.* observed GBA2-dependent hydrolysis of GbPdn in zebrafish larvae, whereas here the hydrolysis of 4MU-β-D-Gb **2** was assayed *in vitro* against purified enzyme or HEK293T lysates overexpressing GBA enzymes. It would be interesting to investigate whether a sequential hydrolysis of the gentiobiose disaccharide in the zebrafish larvae, due to a combination of multiple glycosylhydrolases, might have resulted in what appeared to be GBA2-dependant hydrolysis of GbPdn.

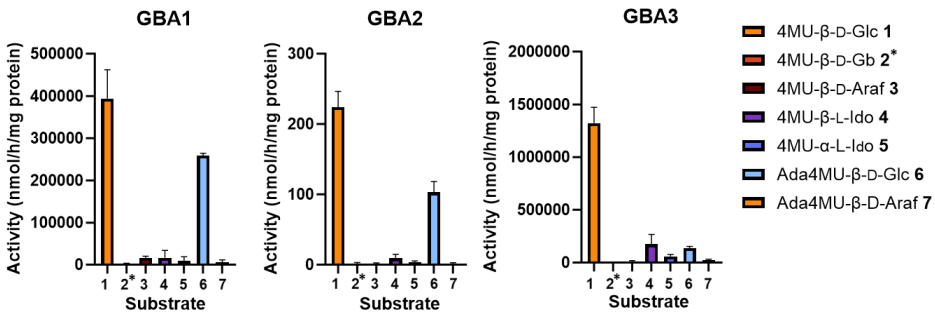



**Figure 5.5:** Hydrolysis of glucosides **1**-**7** (at 1.5 mM) by GBA1 (rhGBA1, Cerezyme®), GBA2 (GBA2 OE and GBA1/2 KO HEK293T lysates) and GBA3 (rhGBA3) over 30 minutes. *The hydrolysis of 4MU-β-D-Gb **2** was measured at 2 mM substrate concentration and GBA3 activity was measured in GBA3 OE, GBA1/2 KO HEK293T cells lysates instead of rhGBA3.

With regards to compounds **3**-**7**, unfortunately only substrate Ada4MU-β-D-Glc **6** was readily hydrolysed by rhGBA1, reaching similar levels of 4MU release as reference 4MU-β-D-Glc **1**. Similar results were observed for the hydrolysis of the

substrates by the GBA2 using GBA2 OE and GBA1/2 KO HEK293T cell lysates. For the GBA3 enzyme, only 4MU-β-D-Glc **1** showed significant 4MU release. This suggests that the Ada4MU-β-D-Glc **6** substrate, while not selective for GBA2, shows some selectivity for GBA1 and GBA2 over GBA3, and that the introduction of the lipophilic adamantane group to the fluorogenic moiety is not well tolerated by GBA3. Since GBA1 and GBA2 are membrane associated enzymes and GBA3 is a cytosolic enzyme, the introduction of this lipophilic handle to the substrate might influence its selectivity due to the inability of GBA3 to hydrolyse such lipophilic molecules.

On the other hand, 4MU-β-L-idose **4** appears to show some hydrolysis by rhGBA3 over rhGBA1 and GBA2. However, and important to note is that this substrate showed quite high background signals indicating presence of hydrolysed 4MU in the substrate stock solution. Appropriate controls and corrections were made for this when generating the data, but future experiments would benefit from the re-purification of the 4MU-β-L-idose **4** to ensure proper readout of hydrolysis by the individual enzymes.

Unfortunately, the arabinofuranose based substrates **3** and **7** showed very little to no hydrolysis by all three GBA enzymes. While the arabinofuranose-based aziridine ABPs do show GBA2 selectivity, the fluorogenic arabinofuranose-based substrates are not hydrolysed by the glucosyl hydrolases at all. GBA2 adopt the conventional Koshland two-step double-displacement conformational pathway progressing from an initial $^{1}S_{3}$ conformation to $^{4}H_{3}$ to ultimately adopt the $^{4}C_{1}$ in the covalent intermediate complex.[3] The β-D-Araf aziridines described by Su *et al.*[29] adopt an $^{3}E$ conformation[39] which resemble the initial $^{1}S_{3}$ Michaelis complex conformation when considering the aziridine fused ring. This difference in conformations between the arabinofuranose ABPs and the 4MU-based fluorogenic substrates, along with the lack of activity observed in compounds **3** and **7**, highlights the significant importance of conformational mimicking when designing substrates for these glucosyl hydrolases.

## 5.3 Conclusion

This chapter describes the design of eight potential fluorogenic substrates (**2-9**). Substrates **2**-**5** are classical 4MU-based substrates, while substrates **6**-**9** present a newly designed adamantane modified 4-methylumbellifone. The design of the gentobiose-configured 4MU substrate **2** was based on an observation made by Halima *et al.*[22] who suspected GBA2-dependent hydrolysis of the gentiobiose glycosylated classical GC drug prednisolone to occur within a zebrafish model. The L-idose configured substrates **4**,**5**,**8** and **9** were proposed based on the success of idose-configured iminosugars as dual GCS/GBA2 inhibitors.[31–35,40] On the other hand, the β-D-arabinofuranose configured substrates (**3** and **7**) were designed based on the GBA2 selective labelling observed with β-D-arabinofuranose aziridine ABPs.[29] Lastly the addition of an adamantane lipophilic handle to the fluorogenic aglycon (4MU) was considered to potentially enhance the selectivity and activity for the substrates **6**-**9**, as the addition of lipophilic extensions into the aglycon binding part has shown to increase not only selectivity but also the activity of iminosugars and ABPs.[29,40]

The synthesis of 4MU-β-D-Araf substrate **3** consisted of seven steps and was relatively straightforward. The L-idose configured fluorogenic substrates **4** and **5** were synthesized largely following the synthetic procedure described by Lee *et al*.[36] The Ada4MU fluorogenic aglycon, equipped with a lipophilic adamantane handle, was synthesized by reacting the commercial 7-hydroxycoumarin-4-acetic acid with the synthesized azido intermediate **21**. This Ada4MU aglycon was subsequently attached to the glucose and arabinofuranose by reacting the aglycon with the corresponding anomeric bromides to yield substrates **6** and **7**. Unfortunately, the designed synthesis of Ada4MU-β-L-idose **8** and Ada4MU-α-L-idose **9** could not be accomplished due to encountered difficulties with the glycosylation (Scheme 5.5).

Nevertheless, fluorogenic substrates **1**-**7** were assessed as fluorogenic substrates for GBA1, GBA2 and GBA3. This experiment revealed that the 4MU-β-D-gentiobiose substrate **2** and arabinofuranose-configured substrates **3** and **7** were not hydrolysed by any of the three glycosidases. Furthermore, while 4MU-β-L-idose **4** showed some activity and selectivity for rhGBA3. The 4MU-α-L-idose **5** substrate exhibited very little to no hydrolysis by all three enzymes. In contrast, the Ada4MU-β-D-Glc **6** showed significant hydrolysis by GBA1 and GBA2 which was comparable to released 4MU with the 4MU-β-D-Glc substrate **1**. Unlike 4MU-β-D-Glc **1**, Ada4MU-β-D-Glc **6** demonstrated very little hydrolysis by GBA3, suggesting that this substrate is not

GBA2-specific but at least GBA1/GBA2-specific over GBA3. Further research and development are necessary to generate GBA2-selective substrates.

Importantly, the research presented here indicates that while certain configurations of iminosugars and cyclophellitol aziridines exhibit GBA2 activity, one cannot directly translate this information for into substrate design, likely due to the different conformations these molecules adopt.

**5.4 Acknowledgements**

Qiang Ma is kindly acknowledged for the use of his iodo-adamantyl building block **20**. Qin Su is kindly acknowledged for the generation and use of his HEK293T cell pellets overexpressing GBA2 (GBA1/2 KO).

## 5.5 Experimental methods

### 5.5.1 Biochemical and Biological Methods

#### Materials

Cerezyme® (rhGBA1, 1.363 mg/mL) was a kind gift from Sanofi Genzyme (Amsterdam, The Netherlands). Isolated recombinant GBA3 (rhGBA3, 0.465 mg/mL) was purchased from BioTechne R&D Systems (Catalog#: 5969-GH-010). 4-Methylumbelliferyl-β-D-glucopyranoside (4MU-β-Glc) was purchased from Glycosynth (Winwick Quay, Warrington, UK). Fluorogenic substrates **2**-**7** were synthesized as described. Triton X-100 was purchased from Sigma-Aldrich. Taurocholic acid sodium salt and dimethyl sulfoxide (DMSO) were purchased from EMD Millipore Corporation (Billerica, Massachusetts, USA). Harvested cells (cell pellets) and cell lysates that were not immediately used were stored at -80 °C.

#### Cell culture

HEK293T cells overexpressing GBA2 (GBA/GBA2 KO) and GBA3 (GBA/GBA2 KO) were cultured in DMEM medium (Sigma-Aldrich) supplemented with 10% (v/v) FCS, 0.1% (w/v) penicillin/streptomycin and 1% (v/v) Glutamax at 37 °C and 5% $CO_2$. Lysates of the HEK293T cell pellets were generated by diluting the samples with 200 µL 25 mM KPI lysis buffer pH 6.5 containing 2 U/ml Benzonase® Nuclease. Afterwards the lysates were sonicated 5 times for 1 second at 20% amplitude with Vibra-CellTM VCX130 (Sonics, Newton, USA) in an ice bath. Protein concentrations were determined with a BCA protein assay (BCA kit, Pierce, Thermo Fisher), using the Emax Plus Microplate Reader (Molecular Devices, Sunnyvale, USA).

#### β-Glucosidase activity of glycosides 2-7 against GBA1, GBA2 and GBA3

The *in vitro* enzyme activity of GBA1 (rhGBA1, Cerezyme®), GBA2 (GBA2 OE and GBA1/2 KO HEK293T lysates) and GBA3 (rhGBA3) using substrates **3-7** was determined by measuring the release of the fluorescent 4-methylumbelliferyl[41] or Ada4MU **23**. The *in vitro* GBA3 activity of substrate **2** was measured in GBA3 OE and GBA1/2 KO HEK293T cell lysates instead of rhGBA3. Substrate mixes of **3**-**7** were made containing 1.5 mM of the corresponding glycoside while substrate **2** was prepared at a 2 mM concentration. 40 mM of substrates **2**-**7** were made in DMSO which were used to generate the substrate mixes (reaching 3.75% DMSO for substrate **3**-**7**, and 5% DMSO for substrate **2**) were heated at 37 °C in a water bath after which the aliquoted amounts were diluted with the appropriate enzyme buffer which were also heated at 37 °C. After dilution of the substrates with enzyme buffer

the samples were immediately vortexed for ~1 minute before being kept at 37 °C in a water bath prior to use. The Ada4MU glycoside substrate mixtures **6** and **7** were sonicated 2 seconds at 40% amplitude prior to addition to the 96 well-plate to generate a homogeneous solution. The rhGBA1 enzyme buffer consisted of 150 mM McIlvaine buffer pH 5.2 containing 0.1% (w/v) BSA, 0.2% (w/v) sodium taurocholate and 0.1% (v/v) Triton X-100. The GBA2 buffer consisted of 150 mM McIlvaine buffer pH 5.8 containing 0.1% (w/v) BSA the rhGBA3 buffer consisted of 150 mM McIlvaine buffer pH 6.0 containing 0.1% (w/v) BSA and the HEK293T cells overexpressing GBA3 were dissolved in 100 mM HEPES buffer pH 7.0. Prior to use enzymes were diluted as follows; Cerezyme® was diluted 2500x, GBA2 was diluted 2x and rhGBA3 was diluted 1600x times in 25 mM KPi pH 6.5. Per well 12.5 µL enzyme mix, 12.5 µL 150 mM McIlvaine enzyme buffer and 100 µL substrate mix were incubated for 30 minutes at 37 °C. The reaction was stopped by adding 200 µL of the stop buffer 1 M glycine-NaOH pH 10.3. As a standard and for quantification of the obtained signals 1 nmol 4MU or 1 nmol Ada4MU **23** was added. GraphPad Prism 10 was used to analyze the results. Fluorescence intensities in the fluorogenic substrate assays were measured with a fluorimeter LS55 (Perkin-Elmer, Beaconsfield, UK) at $\lambda_{ex}$ 366 nm and $\lambda_{em}$ 445 nm plus $slit_{ex}$ 10 nm and $slit_{em}$ 3.0 nm.

To ensure proper solubility of all substrates the stock solutions and enzyme buffers were heated before preparation of the sample mixtures and solutions were vortexed vigorously for around one minute after preparation. This led to the generation of clear sample solutions for substrates **3**-**5** while the Ada4MU substrates **6** and **7** remained murky white initially. All sample mixtures were kept at 37 °C prior to addition to the 96-well plate and substrate **6** and **7** were sonicated prior to use to ensure mixture homogeneity.

## 5.6 Chemical synthesis

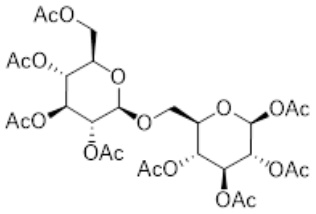
### 5.6.1 General experimental details

All reagents were of a commercial grade and were used as received unless stated otherwise. Dichloromethane ($CH_2Cl_2$), tetrahydrofuran (THF) and *N*,*N*-dimethylformamide (DMF) were stored over 4 Å molecular sieves, which were dried *in vacuo* before use. Triethylamine and di-isopropyl ethylamine (DIPEA) were dried over KOH and distilled before use. All reactions were performed under an argon atmosphere unless stated otherwise. Solvents used for flash column chromatography were of pro analysis quality. Reactions were monitored by analytical thin-layer chromatography (TLC) using Merck aluminium sheets pre-coated with silica gel 60 with detection by UV absorption (254 nm) and by spraying with a solution of $(NH_4)_6Mo_7O_{24}\cdot H_2O$ (25 g/L) and $(NH_4)_4Ce(SO_4)_4\cdot H_2O$ (10 g/L) in 10% sulfuric acid followed by charring at ~150 ˚C or by spraying with an aqueous solution of $KMnO_4$ (7%) and $K_2CO_3$ (2%) followed by charring at ~150 °C. Column chromatography was performed manually using either Baker or Screening Device silica gel 60 (0.04 - 0.063 mm) or a Biotage Isolera™ flash purification system using silica gel cartridges (Screening devices SiliaSep HP, particle size 15-40 µm, 60A) in the indicated solvents. $^1$H-NMR and $^{13}$C-NMR spectra were recorded on Bruker AV-400 (400/100 MHz) and Bruker AV-I-500 (500/125 MHz) spectrometer in the given solvent. Chemical shifts are given in ppm relative to the residual solvent peak used or tetramethylsilane (TMS) as internal standard. Coupling constants are given in Hz. All given $^{13}$C spectra are proton decoupled. The following abbreviations are used to describe peak patterns when appropriate: s (singlet), d (doublet), t (triplet), qt (quintet), m (multiplet), br (broad), Um (4-methylumbeliferone). 2D-NMR experiments (HSQC, COSY and HMBC) were carried out to assign protons and carbons of the new structures. High-resolution mass spectra (HRMS) of intermediates were recorded with a LTQ Orbitrap (Thermo Finnigan) and final compounds were recorded with an apex-QE instrument (Bruker). LC/MS analysis was performed on an LCQ Advantage Max (Thermo Finnigan) ion-trap spectrometer (ESI+) coupled to a Surveyor HPLC system (Thermo Finnigan) equipped with a C18 column (Gemini, 4.6 mm x 50 mm, 3 µm particle size, Phenomenex) equipped with buffers A: $H_2O$, B: acetonitrile (MeCN) and C: 1% aqueous TFA, or an Agilent Technologies 1260 Infinity LCMS with a 6120 Quadrupole MS system equipped with buffers A: $H_2O$, B: acetonitrile (MeCN) and C: 100 mM $NH_4OAc$. For reversed-phase HPLC-MS purifications an Agilent Technologies 1200 series prep-LCMS with a 6130

Quadrupole MS system was used equipped with buffers A: 50 mM $NH_4HCO_3$ in $H_2O$ and B: MeCN.

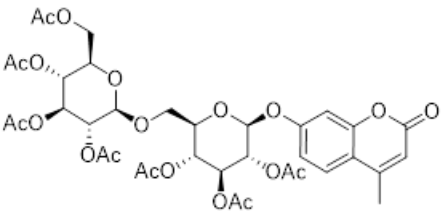
### 5.6.2 Synthesis and characterization data

**(2*S*,3*R*,4*S*,5*R*,6*R*)-6-((((2*R*,3*R*,4*S*,5*R*,6*R*)-3,4,5-Triacetoxy-6-(acetoxymethyl)tetrahydro-2*H*-pyran-2-yl)oxy)methyl)tetrahydro-2*H*-pyran-2,3,4,5-tetrayl tetraacetate (10)**



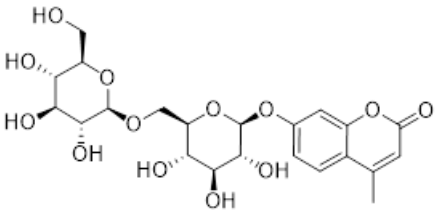

To a solution of Gentiobiose (400 mg, 1,17 mmol) in anhydrous pyridine (2 mL, 0.58 M) was added DMAP (14.3 mg, 0.12 mmol) and acetic anhydride (2 mL, 21.2 mmol) at room temperature under nitrogen atmosphere. The reaction flask was stirred overnight at room temperature. After TLC showed complete conversion of the starting material the reaction was diluted with EtOAc and washed with 1 M HCl 3 x. The combined water layers were extracted with EtOAc and the combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification of the crude material over silica column chromatography afforded the product (793 mg, 1.17 mmol, quantitative yield) as a white solid. $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 5.68 (d, $J$ = 8.3 Hz, 1H, H-1), 5.25 – 5.20 (m, 1H, H-3'), 5.20 – 5.15 (m, 1H, H-3), 5.11 – 5.03 (m, 2H, H-2 and H-4'), 4.98 (dd, $J$ = 9.5, 7.8 Hz, 2H, H-2' and H-4), 4.54 (d, $J$ = 8.0 Hz, 1H, H-1'), 4.25 (dd, $J$ = 12.3, 4.8 Hz, 1H, H-6a), 4.11 (dd, $J$ = 12.3, 2.4 Hz, 1H, H-6b), 3.93 (dd, $J$ = 11.4, 2.4 Hz, 1H, H-6'a), 3.78 (ddd, $J$ = 10.2, 5.7, 2.3 Hz, 1H, H-5'), 3.66 (ddd, $J$ = 10.0, 4.8, 2.4 Hz, 1H, H-5), 3.57 (dd, $J$ = 11.4, 5.7 Hz, 1H, H-6'b), 2.11 (s, 3H, $CH_3$-OAc), 2.09 (s, 3H, $CH_3$-OAc), 2.07 (s, 3H, $CH_3$-OAc), 2.03 (s, 3H, $CH_3$-OAc), 2.02 (s, 3H, $CH_3$-OAc), 2.01 (s, 3H, $CH_3$-OAc), 2.00 (s, 6H, 2x$CH_3$-OAc); $^{13}$C NMR (101 MHz, $CDCl_3$) $\delta$ 170.8, 170.4, 170.3, 169.7, 169.6, 169.4, 169.0 (8x$C_q$-OAc), 100.8 (C-1'), 91.7(C-1), 74.0 (C-5'), 73.0, 72.9 (C-3 and C-3'), 72.0 (C-5), 71.0, 70.4, 68.5, 68.4 (C-2, C-4, C-2' and C-4'), 67.6 (C-6'), 62.0 (C-6), 21.0, 20.9, 20.8, 20.7, 20.7 (8x$CH_3$-OAc); HRMS: calcd. for $C_{33}H_{56}O_6$ $[M+NH_4]^+$ 696,23455, found: 696,23409**.**

**(2*R*,3*R*,4*S*,5*R*,6*R*)-2-(Acetoxymethyl)-6-(((2*R*,3*R*,4*S*,5*R*,6*S*)-3,4,5-triacetoxy-6-((4-methyl-2-oxo-2*H*-chromen-7-yl)oxy)tetrahydro-2*H*-pyran-2-yl)methoxy)tetrahydro-2*H*-pyran-3,4,5-triyl triacetate (11)**



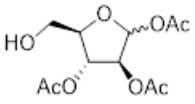
The acetylated gentiobiose **10** (375 mg, 0.55 mmol) was dissolved in dry $CH_2Cl_2$ (5.5 mL) under protected atmosphere. The solution was cooled to 0 °C followed by addition of TMS-Br (337 µL, 2.60 mmol) and tribromobismuthane (12.4 mg, 28 µmol). The reaction mixture was left stirring for 2 hours allowing to reach room temperature. The reaction mixture was diluted with $CH_2Cl_2$ and washed with sat. aq. $NaHCO_3$ and brine. The water layers were extracted with $CH_2Cl_2$ and the combined organic layers were dried ($MgSO_4$), filtered and concentrated under reduced pressure. The crude material was immediately used in the next step without further purification. The crude bromide was dissolved in a 1:1 mixture of acetone and water (12 mL) after which 4MU (228 mg, 1.29 mmol), NaOH (49 mg, 1.23 mmol) and TBABr (208 mg, 0.65 mmol) were added and the solution was stirred vigorously at room temperature in the dark overnight. After reaching completion the reaction was diluted with $CH_2Cl_2$ and washed with 1 M NaOH (2x) and brine. The water layers were extracted with $CH_2Cl_2$ and the combined organic layers were dried ($MgSO_4$), filtered and concentrated *in vacuo*. The crude was purified by silica column chromatography to yield the product **11** (165 mg, 0.21 mmol, 38% yield 2 steps) as a white solid. $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 7.58 (d, $J$ = 8.6 Hz, 1H, CH-Um), 6.94 – 6.89 (m, 2H, 2xCH-Um), 6.16 (d, $J$ = 1.3 Hz, 1H, CH-Um), 5.28 (t, $J$ = 9.3 Hz, 1H, H-3'), 5.25 – 5.18 (m, 1H, H-3), 5.12 (d, $J$ = 7.6 Hz, 1H, H-1), 5.06 (dd, $J$ = 9.4, 8.1 Hz, 2H, H-2 and H-4'), 5.02 – 4.93 (m, 2H, H-2' and H-4), 4.55 (d, $J$ = 7.9 Hz, 1H, H-1'), 4.22 (dd, $J$ = 12.4, 5.0 Hz, 1H, H-6a), 4.08 (dd, $J$ = 12.3, 2.4 Hz, 1H, H-6b), 3.94 – 3.83 (m, 2H, H-5' and H-6'a), 3.68 (dd, $J$ = 11.5, 7.3 Hz, 1H, H-6'b), 3.61 (ddd, $J$ = 9.3, 4.9, 2.3 Hz, 1H, H-5), 2.40 (d, $J$ = 1.3 Hz, 3H, $CH_3$-Um), 2.04 (s, 3H, $CH_3$-OAc), 2.04 (s, 3H, $CH_3$-OAc), 2.02 (s, 3H, $CH_3$-OAc), 2.00 (s, 3H, $CH_3$-OAc), 1.97 (s, 3H, $CH_3$-OAc), 1.96 (s, 3H, $CH_3$-OAc), 1.88 (s, 3H, $CH_3$-OAc); $^{13}$C NMR (101 MHz, $CDCl_3$) $\delta$ 170.6, 170.3, 170.2, 169.6, 169.44, 169.36, 169.3 (7x$C_q$-OAc), 160.8, 159.4, 154.8, 152.3 (4x$C_q$-Um), 126.3 (CH-Um), 115.7($C_q$-Um), 113.3, 112.9, 104.5 (3xCH-Um), 100.6 (C-1'), 98.4 (C-1), 74.0 (C-5'), 72.7, 72.5 (C-3 and C-5'), 72.1 (C-5), 71.0, 70.9, 68.6, 68.3 (C-2, C-4, C-2' and C-4'), 67.8 (C-6'), 61.9 (C-6), 20.8, 20.7, 20.6 (7x$CH_3$-OAc), 18.8 ($CH_3$-Um); HRMS not detected.

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



The protected sugar **11** (160 mg, 202 µmol) was dissolved in MeOH/$CH_2Cl_2$ (4 mL, 1:1) followed by the addition of sodium methoxide (30% in MeOH, 1 drop). The mixture was stirred for 4 h at room temperature. After reaching completion the reaction was quenched with amberlite, which was subsequently filtered off and washed with MeOH. The solution was concentrated *in vacuo* yielding the crude product which was recrystallised from hot ethanol to afford the product **2** (49 mg, 98 µmol, 48%) as a white solid. $^1$H NMR (500 MHz, DMSO) $\delta$ 7.71 (d, *J* = 8.8 Hz, 1H, CH-Um), 7.12 (dd, *J* = 8.8, 2.4 Hz, 1H, CH-Um), 7.06 (d, *J* = 2.5 Hz, 1H, CH-Um), 6.25 (d, *J* = 1.4 Hz, 1H, CH-Um), 5.45 (d, *J* = 4.8 Hz, 1H, OH), 5.21 (d, *J* = 4.0 Hz, 1H, OH), 5.17 (d, *J* = 5.3 Hz, 1H, OH), 5.04 (d, *J* = 7.2 Hz, 1H, H-1), 4.92 (d, *J* = 4.6 Hz, 2H, 2xOH), 4.88 (d, *J* = 4.6 Hz, 1H, OH), 4.48 (t, *J* = 5.8 Hz, 1H, 6'OH), 4.18 (d, *J* = 7.8 Hz, 1H, H-1'), 3.98 (dd, *J* = 11.7, 1.7 Hz, 1H, H-6a), 3.70 – 3.58 (m, 3H, H-5', H-6'a and H-6b), 3.42 (dt, *J* = 11.7, 6.0 Hz, 1H, H-6'b), 3.31 – 3.26 (m, 2H, H-2 and H-3), 3.21 (td, *J* = 9.0, 5.3 Hz, 1H, H-4'), 3.12 – 2.94 (m, 4H, H-2', H-3', H-4 and H-5), 2.41 (d, *J* = 1.2 Hz, 3H, $CH_3$-Um); $^{13}$C NMR (126 MHz, DMSO) $\delta$ 160.2, 160.1, 154.3, 153.4 (4x$C_q$-Um), 126.6 (CH-Um), 114.2 ($C_q$-Um), 113.2, 111.7, 103.5 (3xCH-Um), 103.4 (C-1'), 99.9 (C-1), 77.0, 76.8, 76.4 (C-3', C-3 and C-5), 75.7 (C-5'), 73.5 (C-2'), 73.1 (C-2), 70.1 (C-4), 69.5 (C-4'), 68.5 (C-6), 61.0 (C-6'), 18.2 ($CH_3$-Um); HRMS: calcd. For $C_{22}H_{28}O_{13}$ $[M+Na]^+$: 523.14221, Found: 523.14300

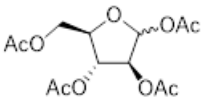
**(3*S*,4*R*,5*R*)-5-(Hydroxymethyl)tetrahydrofuran-2,3,4-triyl triacetate (12)**



D-Arabinose (2 g, 13.32 mmol) was dissolved in dry pyridine (27 mL) under inert atmosphere. TBDPSCl (4.4 g, 16 mmol) was added and the mixture was stirred at 55 °C. After 18 hours acetic anhydride (6.93 mL, 73.3 mmol) was added to the reaction at room temperature and after stirring the reaction mixture for 4 hours work up was performed. The reaction was quenched by addition of 6 mL of MeOH after which the mixture was diluted with $CH_2Cl_2$ and washed with 1 M HCl (3x). The organic layer was dried with $MgSO_4$, filtered and concentrated *in vacuo*. TBAF (16 mL, 16 mmol, 1.0 M THF) was added dropwise to a stirred solution of the crude furanoside in anhydrous THF (33 mL) at 0 °C under nitrogen. The mixture was allowed to reach rt and was stirred for 18 h. The reaction mixture was diluted with EtOAc and washed with sat. aq. $NH_4Cl$ and brine. The organic layer was dried using $MgSO_4$,

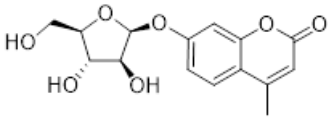
filtered and concentrated *in vacuo*. The crude compound was purified using silica column chromatography to afford the product (1.89 g, 6.83 mmol, 51% over 3 steps) as a colourless oil. α-anomer: $^{1}$H NMR (400 MHz, $CDCl_3$) $\delta$ 6.18 (s, 1H, H-1), 5.25 (d, $J$ = 1.8 Hz, 1H, H-2), 5.12 (ddd, $J$ = 5.2, 1.9, 0.7 Hz, 1H, H-3), 4.24 (dt, $J$ = 5.1, 3.8 Hz, 1H, H-4), 3.90 (dd, $J$ = 12.3, 3.4 Hz, 1H, H-5a), 3.81 (dd, $J$ = 12.3, 4.1 Hz, 1H, H-5b), 2.17 – 2.10 (m, 9H, 3x$CH_3$-OAc). $^{13}$C NMR (101 MHz, $CDCl_3$) $\delta$ 170.5, 169.7, 169.6 (3x$C_q$-OAc), 99.4 (C-1), 85.1 (C-4), 81.1 (C-2), 76.8 (C-3), 61.8 (C-5), 21.2, 20.9, 20.8 (3x$CH_3$-OAc). β-anomer: $^{1}$H NMR (400 MHz, $CDCl_3$) $\delta$ 6.13 (d, $J$ = 0.9 Hz, 1H, H-1), 5.49 (d, $J$ = 3.4 Hz, 1H, H-2), 5.20 (dd, $J$ = 10.6, 3.5 Hz, 1H, H-3), 4.80 (dd, $J$ = 5.4, 2.3 Hz, 1H, H-4), 4.41 (dd, $J$ = 8.2, 2.9 Hz, 1H, H-5a), 4.29 – 4.26 (m, 1H, H-5b), 2.17 – 2.10 (m, 9H, 3x$CH_3$-OAc). $^{13}$C NMR (101 MHz, $CDCl_3$) $\delta$ 170.5, 169.7, 169.3 (3x$C_q$-OAx), 102.1 (C-1), 91.1 (C-2), 80.3 (C-4), 68.7 (C-3), 63.3 (C-5), 21.2, 21.1, 21.0 (3x$CH_3$-OAc); HRMS: calcd. for $C_{11}H_{16}O_8$ $[M+Na]^+$: 299.07374, Found: 299.07364.

**(3*S*,4*R*,5*R*)-5-(Acetoxymethyl)tetrahydrofuran-2,3,4-triyl triacetate (13)**




The sugar (722 mg, 2.61 mmol) was dissolved in dry pyridine (13 mL) after which acetic anhydride (742 µL, 7.84 mmol) was added at 0 °C. The reaction mixture was left to stir overnight after which the mixture was diluted with $CH_2Cl_2$ and washed with 1 M HCl (3x). The organic layer was dried with $MgSO_4$, filtered concentrated *in vacuo*. The resulting crude was purified over silica column chromatography yielding the product (361 mg, 1.13 mmol, 43%). α-anomer: $^{1}$H NMR (400 MHz, $CDCl_3$) $\delta$ 6.09 (d, $J$ = 1.4 Hz, 1H, H-1), 5.11 (d, $J$ = 1.6 Hz, 1H, H-2), 4.95 (dd, $J$ = 4.3, 2.0 Hz, 1H, H-3), 4.29 (dtt, $J$ = 9.9, 5.1, 2.7 Hz, 2H, H-4 and H-5a), 4.12 (tdd, $J$ = 7.1, 4.1, 2.2 Hz, 1H, H-5b), 2.08 – 1.99 (m, 12H, 4x$CH_3$-OAc). $^{13}$C NMR (101 MHz, $CDCl_3$) $\delta$ 170.4, 169.8, 169.3, 169.1 (4x$C_q$-OAc), 99.1 (C-1), 82.3 (C-4), 80.3 (C-2), 76.6 (C-3), 62.9 (C-5), 20.8, 20.6, 20.54, 20.51 (4x$CH_3$-OAc). β-anomer: $^{1}$H NMR (400 MHz, $CDCl_3$) $\delta$ 5.58 (dd, $J$ = 6.9, 1.1 Hz, 1H, H-1), 5.29 (dt, $J$ = 3.2, 1.5 Hz, 1H, H-4), 5.23 – 5.15 (m, 1H, H-2), 5.03 (ddd, $J$ = 9.0, 3.5, 1.0 Hz, 1H, H-3), 4.02 – 3.90 (m, 1H, H-5a), 3.77 – 3.67 (m, 1H, H-5b), 2.07 – 1.99 (m, 12H, 4x$CH_3$-OAc). $^{13}$C NMR (101 MHz, $CDCl_3$) $\delta$ 170.1, 170.0, 169.7, 169.0 (4x$C_q$-OAc), 91.9 (C-1), 69.7 (C-3), 67.9 (C-2), 67.0 (C-4), 62.6 (C-5), 20.7, 20.54, 20.51, 20.41 (4x$CH_3$-OAc); HRMS: calcd. for $C_{13}H_{18}O_9$ $[M+Na]^+$: 341.08430, Found: 341.08425.

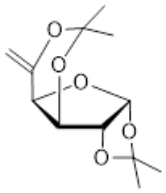
**7-(((2*S*,3*S*,4*S*,5*R*)-3,4-Dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)oxy)-4-methyl-2*H*-chromen-2-one (3)**




The peracetylated furanoside **13** (335 mg, 1.05 mmol) was dissolved in $CH_2Cl_2$ (11 mL) and cooled to 0 °C. HBr (2.6 mL, 10.5 mmol, 30% in AcOH) was added dropwise

and the reaction mixture was stirred in the dark for 4 h. The mixture was diluted with $CH_2Cl_2$ and washed with ice water (3x), sat. aq. $NaHCO_3$ (50 mL) and water again (50 mL). The water layers were extracted with $CH_2Cl_2$ and the combined organic layers were dried ($MgSO_4$), filtered and concentrated *in vacuo* to afford the crude bromo sugar. In parallel, 4MU (371 mg, 2.11 mmol) was added to a solution of NaOH (80 mg, 2 mmol) in $H_2O$ (10.5 mL). To this solution was added tetrabutylammonium bromide (339 mg, 1.05 mmol) followed by the crude brominated furanoside (357 mg, 1.05 mmol) dissolved in acetone (10.5 mL). The mixture was stirred in the dark for 18 h at room temperature. After reaching completion the reaction was diluted with $CH_2Cl_2$ and washed with 1 M NaOH (2x) and brine. The water layers were extracted with $CH_2Cl_2$ and the combined organic layers were dried ($MgSO_4$), filtered and concentrated *in vacuo*. The product was crudely purified over a silica plug after which the acetyl protected furanoside (36 mg, 83 µmol) was dissolved in MeOH/$CH_2Cl_2$ (1.6 mL, 1:1) followed by the addition of sodium methoxide (30% in MeOH, 2 drop). The mixture was stirred for 4 h at room temperature. After reaching completion the reaction was quenched with amberlite, and subsequently filtered off and washed with MeOH. The solution was concentrated *in vacuo* yielding the crude product which was purified by silica column chromatography to afford the product (12 mg, 39 µmol, 4% yield over 3 steps) as a white solid. $^1$H NMR (400 MHz, DMSO) $\delta$ 7.70 (d, $J$ = 9.4 Hz, 1H, CH-Um), 7.02 (dq, $J$ = 5.4, 2.4 Hz, 2H, 2xCH-Um), 6.25 (d, $J$ = 1.3 Hz, 1H, CH-Um), 5.31 (d, $J$ = 5.1 Hz, 1H, 2-OH), 5.03 (d, $J$ = 6.9 Hz, 1H, H-1), 4.91 (d, $J$ = 5.5 Hz, 1H, 3-OH), 4.71 (d, $J$ = 3.9 Hz, 1H, 5-OH), 3.76 – 3.66 (m, 3H, H-4 and H-5a,b), 3.63 (ddd, $J$ = 8.7, 6.8, 5.0 Hz, 1H, H-2), 3.48 (ddd, $J$ = 8.6, 5.3, 3.1 Hz, 1H, H-3), 2.40 (d, $J$ = 1.3 Hz, 3H, $CH_3$-Um); $^{13}$C NMR (101 MHz, DMSO) $\delta$ 160.1, 160.0, 154.4, 153.4 (4x$C_q$-Um), 126.5 (CH-Um), 114.0 ($C_q$-Um), 113.4, 111.7, 103.1 (3xCH-Um), 100.3 (C-1), 72.4 (C-3), 70.1 (C-2), 67.5 (C-4), 65.8 (C-5), 18.2 ($CH_3$-Um); HRMS: calcd. for $C_{15}H_{16}O_7$ $[M+H]^+$ : 309.09688, Found: 309.09670.

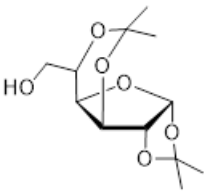
**(3*R*,3*S*,7*S*,8*R*)-2,2,5,5-Tetramethyl-7-methylenetetrahydro-7*H*-[1,3]dioxolo[4',5':4,5] furo[3,2][1,3]dioxine (14)**



To a solution of diacetone-D-glucose (4.00 g, 15.4 mmol) in anhydrous toluene (44 mL) was added triphenylphosphine (6.85 g, 26.1 mmol) and *N*-Bromosuccinimide (4.10 g, 23.1 mmol) at room temperature under nitrogen atmosphere. The reaction flask was heated at 90 °C for 45 min after which DBU (16 mL, 108 mmol) was added and the mixture was stirred at 90 °C for 2 hours. After cooling to room temperature, the solids were filtered over Celite followed by washing with pentane. The organic layer was washed with $H_2O$ and the aqueous phase was extracted with pentane (3x). The combined organic layers were washed with brine, dried over $MgSO_4$, filtered, and

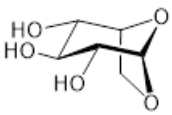
concentrated *in vacuo*. The residue was purified by silica column chromatography to afford the product (1.66 g, 6.84 mmol, 45%) as a colourless oil. $^{1}$H NMR (400 MHz, $CDCl_3$) $\delta$ 5.99 (d, $J$ = 3.7 Hz, 1H, H-1), 4.77 (d, $J$ = 0.9 Hz, 1H, H-6a), 4.70 (d, $J$ = 0.9 Hz, 1H, H-6b), 4.57 (d, $J$ = 3.7 Hz, 1H, H-2), 4.37 (d, $J$ = 2.3 Hz, 1H, H-3), 4.35 (d, $J$ = 2.3 Hz, 1H, H-3), 1.53 (s, 3H, $CH_3$-ketal), 1.48 (s, 3H, $CH_3$-ketal), 1.40 (s, 3H, $CH_3$-ketal), 1.33 (s, 3H, $CH_3$-ketal).; $^{13}$C NMR (101 MHz, $CDCl_3$) $\delta$ 150.4 ($C_q$-C-5), 112.0 ($C_q$-ketal), 105.4 (C-1), 101.7 ($CH_2$-C-6), 100.7 ($C_q$-ketal), 84.4 (C-2), 74.8 (C-4), 72.6 (C-3), 28.2, 26.9, 26.27, 21.4 (4x$CH_3$-ketal); HRMS: calcd. for $C_{12}H_{18}O_5$ $[M+Na]^+$ : 265.10464, Found: 265.10462.

**((3*R*,3*S*,7*R*,8*R*)-2,2,5,5-Tetramethyltetrahydro-7*H*-[1,3]dioxolo[4',5':4,5]furo[3,2-d][1,3]dioxin-7-yl)methanol (15)**



To a mixture of the intermediate **14** (1.45 g, 5.97 mmol) in THF (20 mL) was added $BH_3$•THF (6 mL, 1 M, 6 mmol) at room temperature under nitrogen atmosphere. After stirring for 3 h, the reaction flask was cooled in an ice-bath, and a mixed solution of 30% $H_2O_2$ (6 mL) and 3 M NaOH (6 mL) was slowly added to the mixture. The aqueous phase was extracted with EtOAc (3x), and the combined organic layers were washed with brine, dried over $MgSO_4$, filtered, and concentrated *in vacuo*. The crude was purified using silica column chromatography to yield the product (1.38 g, 89%) as a colourless oil. $^{1}$H NMR (400 MHz, $CDCl_3$) $\delta$ 5.96 (d, $J$ = 3.7 Hz, 1H, H-1), 4.50 (d, $J$ = 3.7 Hz, 1H, H-2), 4.32 (d, $J$ = 2.3 Hz, 1H, H-3), 4.13 (ddd, $J$ = 6.8, 4.6, 2.1 Hz, 1H, H-5), 4.02 (t, $J$ = 2.1 Hz, 1H, H-4), 3.89 (ddd, $J$ = 10.0, 7.1, 2.6 Hz, 1H, H-6a), 3.79 (ddd, $J$ = 11.9, 8.3, 4.5 Hz, 1H,H-6b), 2.32 – 2.22 (m, 1H, 6-OH), 1.49 (s, 3H, $CH_3$-ketal), 1.46 (s, 3, $CH_3$-ketal H), 1.41 (s, 3H, $CH_3$-ketal), 1.32 (s, 3H, $CH_3$-ketal); $^{13}$C NMR (101 MHz, $CDCl_3$) $\delta$ 111.9 ($C_q$-ketal), 105.4 (C-1), 98.4 ($C_q$-ketal), 84.0 (C-2), 74.0 (C-3), 71.9 (C-4), 69.2 (C-5), 63.3 (C-6), 29.3, 26.8, 26.2, 19.3 (4x$CH_3$-ketal); HRMS: calcd. for $C_6H_{10}O_5$ $[M+Na]^+$ : 283.11521, Found: 283.11512.

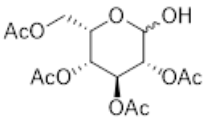
**(1*S*,2*S*,3*S*,4*R*,5*S*)-6,8-dioxabicyclo[3.2.1]octane-2,3,4-triol (16)**



Intermediate **15** (1.5 g, 5.76 mmol) was dissolved in HCl•EtOH (29 mL, 0.2 M, 484 µL 37% HCl in EtOH) and was stirred at 95°C for 18 h. After cooling down to room temperature, the reaction was neutralized with $Ag_2CO_3$ (1000 mg), and the resulting mixture was filtered through Celite to remove AgCl. The filtrate was concentrated in vacuo, and the crude residue was purified by silica column chromatography to afford the product (699 mg, 75%) as a white solid. $^{1}$H NMR (400 MHz, Acetone) $\delta$ 5.17 (d, $J$ = 1.8 Hz, 1H, H-1), 4.55 (d, $J$ = 41.5 Hz, 2H, 2xOH), 4.39 – 4.31 (m, 1H, H-5), 4.27 (s, 1H, OH), 3.97 (dd, $J$ = 7.4, 0.9 Hz, 1H, H-6a), 3.66 (ddd, $J$ = 8.2, 4.3, 1.1 Hz, 1H, H-4), 3.59 (ddd, $J$ = 7.6, 5.1, 1.1 Hz, 1H,

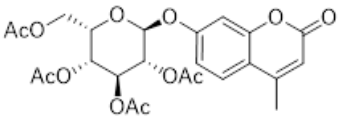
H-6b), 3.50 (t, *J* = 8.2 Hz, 1H, H-3), 3.36 (dd, *J* = 8.1, 1.8 Hz, 1H, H-2); $^{13}$C NMR (101 MHz, Acetone) δ 103.1 (C-1), 76.5 (C-5), 76.3 (C-3), 76.3 (C-2), 72.6 (C-4), 65.4 (C-6); HRMS not found.

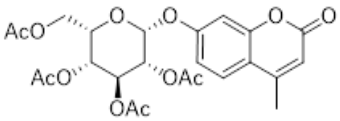
**(2*S*,3*R*,4*S*,5*R*)-2-(Acetoxymethyl)-6-hydroxytetrahydro-2*H*-pyran-3,4,5-triyl triacetate (17)**



To a solution of the unprotected sugar (699 mg, 4.31 mmol) in acetic anhydride (14.4 mL) , trifluoroacetic acid (2.84 mL, 37.1 mmol) was added at room temperature under nitrogen atmosphere. After stirring for 24 h, hydrogen bromide (30% in AcOH, 10.3 mL, 51.7 mmol) was added and the mixture was stirred at room temperature for another 6 h. The reaction was quenched while cooling on an ice bath with saturated aqueous $NaHCO_3$ and the whole mixture was extracted with EtOAc (3x). The combined organic layers were consecutively washed with saturated aqueous $NaHCO_3$ (2x) followed by brine. The organic phase was dried over $MgSO_4$, filtered, and concentrated *in vacuo*, and the resulting crude was purified by silica column chromatography yield the product (725 mg, 2.08 mmol, 90%) as a colourless oil. β-anomer: $^1$H NMR (400 MHz, $CDCl_3$) *δ* 5.23 (t, *J* = 3.9 Hz, 1H, H-2), 5.16 (dd, *J* = 8.2, 1.9 Hz, 1H, H-1), 4.89 – 4.81 (m, 2H, H-3 and H-5), 4.31 – 4.15 (m, 3H, H-4 and H-6a,b), 4.01 (d, *J* = 8.2 Hz, 1H, 1-OH), 2.18 – 2.06 (m, 12H, 4x$CH_3$-OAc); $^{13}$C NMR (101 MHz, $CDCl_3$) *δ* 170.9, 170.1, 169.6, 168.5 (4x$C_q$-OAc), 91.9 (C-1), 71.4 (C-4), 67.3 (C-3), 67.2 (C-2), 65.8 (C-5), 62.7 (C-6), 20.89, 20.85, 20.79, 20.71 (4x$CH_3$-OAc). α-anomer: $^1$H NMR (400 MHz, $CDCl_3$) *δ* 5.22 – 5.19 (m, 1H, H-1), 5.12 – 5.07 (m, 1H, H-3), 4.95 (dd, *J* = 4.4, 2.8 Hz, 1H, H-4), 4.89 – 4.81 (m, 1H, H-2), 4.60 (ddd, *J* = 6.9, 5.4, 2.8 Hz, 1H, H-5), 4.29 – 4.17 (m, 2H, H-6a,b), 3.73 (d, *J* = 5.5 Hz, 1H, 1-OH), 2.17 – 2.07 (m, 12H, 4x$CH_3$-OAc); $^{13}$C NMR (101 MHz, $CDCl_3$) *δ* 170.8, 169.9, 169.8, 169.4 (4x$C_q$-OAc), 92.5 (C-1), 68.4 (C-2), 67.5 (C-3), 67.1 (C-4), 65.1 (C-5), 62.3 (C-6), 20.93, 20.89, 20.85, 20.79 (4x$CH_3$-OAc); HRMS: calcd. for $C_{14}H_{20}O_{10}$ $[M+Na]^+$ : 371.09487, Found: 371.09473.

**(2*S*,3*R*,4*S*,5*R*,6*S*)-2-(Acetoxymethyl)-6-((4-methyl-2-oxo-2*H*-chromen-7-yl)oxy)tetrahydro-2*H*-pyran-3,4,5-triyl triacetate (α-Idose) (19)** and **(2*S*,3*R*,4*S*,5*R*,6*R*)-2-(acetoxymethyl)-6-((4-methyl-2-oxo-2*H*-chromen-7-yl)oxy)tetrahydro-2*H*-pyran-3,4,5-triyl triacetate (β-Idose) (18)**



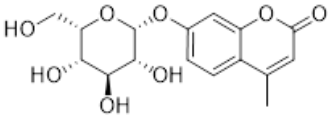


To a solution of diethyl azodicarboxylate (40% in toluene, 0.96 mL, 0.47 mmol) in THF (1 mL) was added the sugar intermediate **17** (120 mg, 0.34 mmol), $PMe_3$ (1 M in THF, 0.55 mL, 0.55 mmol), and 4MU (121 mg, 0.69 mmol) in THF

(1.5 mL) at 0 °C under nitrogen atmosphere. The reaction was allowed to reach room temperature and stirred overnight. The reaction was quenched with saturated aqueous $NaHCO_3$. The solution was diluted with EtOAc, and the mixture was washed with NaOH (0.2 M, 3x), $H_2O$ and brine. The organic layer was dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification of the residue through silica column chromatography yielded the desired products as a mixture (98 mg, 194 µmol, 56%, β:α 1:7). The α- and β-isomer were separated by prep-HPLC of a fraction of the columned material to afford the pure compounds.

α-anomer: $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 7.52 (d, *J* = 8.8 Hz, 1H, CH-Um), 7.07 (d, *J* = 2.4 Hz, 1H,CH-Um), 6.96 (dd, *J* = 8.8, 2.4 Hz, 1H, CH-Um), 6.19 (d, *J* = 1.5 Hz, 1H, CH-Um), 5.58 (s, 1H, H-1), 5.10 (td, *J* = 3.5, 1.0 Hz, 1H, H-3), 5.08 – 5.04 (m, 1H, H-2), 4.94 (t, *J* = 2.8 Hz, 1H, H-4), 4.50 (td, *J* = 6.4, 2.1 Hz, 1H, H-5), 4.18 (d, *J* = 6.4 Hz, 2H, H-6a,b), 2.41 (d, *J* = 1.3 Hz, 3H, $CH_3$-Um), 2.18 (s, 3H, $CH_3$-OAc), 2.16 (s, 3H, $CH_3$-OAc), 2.15 (s, 3H, $CH_3$-OAc), 1.92 (s, 3H, $CH_3$-OAc); $^{13}$C NMR (101 MHz, $CDCl_3$) $\delta$ 170.6, 169.8, 169.4, 169.1 (4x$C_q$-OAc), 161.0, 158.7, 155.0, 152.3 (3x$C_q$-Um), 125.7 (CH-Um), 115.3 ($C_q$-Um), 113.6, 113.2, 104.7 (CH-Um), 96.0 (C-1), 66.8 (C-3), 66.6 (C-2), 66.2 (C-4), 65.5 (C-5), 62.0 (C-6), 20.98, 20.95, 20.83, 20.70 (4x$CH_3$-OAc), 18.8 ($CH_3$-Um). β-anomer: $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 7.52 (d, *J* = 8.8 Hz, 1H, CH-Um), 7.05 (d, *J* = 2.5 Hz, 1H, CH-Um), 6.97 (dd, *J* = 8.8, 2.5 Hz, 1H, CH-Um), 6.20 (d, *J* = 1.3 Hz, 1H, CH-Um), 5.59 (d, *J* = 2.5 Hz, 1H, H-1), 5.44 (t, *J* = 5.5 Hz, 1H, H-3), 5.15 (dd, *J* = 5.7, 2.5 Hz, 1H, H-2), 5.02 (dd, *J* = 5.3, 3.5 Hz, 1H, H-4), 4.47 (ddd, *J* = 7.5, 5.3, 3.5 Hz, 1H, H-5), 4.30 (dd, *J* = 11.8, 7.6 Hz, 1H, H-6a), 4.24 (dd, *J* = 11.7, 5.4 Hz, 1H, H-6b), 2.41 (d, *J* = 1.2 Hz, 3H, $CH_3$-Um), 2.17 (s, 3H, $CH_3$-OAc), 2.15 (s, 3H, $CH_3$-OAc), 2.12 (s, 3H, $CH_3$-OAc), 2.01 (s, 3H, $CH_3$-OAc); $^{13}$C NMR (101 MHz, $CDCl_3$) $\delta$ 170.6, 169.8, 169.7, 168.8 ($C_q$-OAc), 161.0, 159.4, 155.0, 152.3 (4x$C_q$-Um), 125.8 (CH-Um), 115.4 ($C_q$-Um), 114.0, 113.3, 104.0 (3xCH-Um), 95.6 (C-1), 71.9 (C-5), 67.6 (C-2), 67.3 (C-3), 67.0 (C-4), 62.8 (C-6), 20.88, 20.75 (4x$CH_3$-OAc), 18.8 ($CH_3$-Um); HRMS-α: calcd. for $C_{24}H_{26}O_{12}$ $[M+H]^+$: 507.14970, Found: 507.14971; HRMS-β: calcd. for $C_{24}H_{26}O_{12}$ $[M+H]^+$: 507.14970, Found: 507.14990.

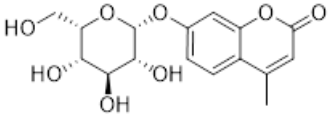
**4-Methyl-7-(((2*R*,3*R*,4*S*,5*S*,6*S*)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yl)oxy)-2*H*-chromen-2-one (4)**



The protected β-sugar **18** (4 mg, 7.9 µmol) was dissolved in MeOH/$CH_2Cl_2$ (0.5 mL, 1:1) followed by the addition of sodium methoxide (30% in MeOH, 1 drop). The mixture was stirred for 4 h at room temperature. After reaching completion the reaction was quenched with amberlite, and subsequently filtered off and washed with MeOH. The solution was concentrated *in vacuo* yielding the crude product which was purified by silica column chromatography and further prep-

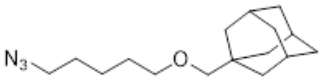
HPLC to afford the desired substrate (2.38 mg, 7 µmol, 89%) as a white solid. $^{1}$H NMR (400 MHz, DMSO) $\delta$ 7.71 (d, $J$ = 8.5 Hz, 1H, CH-Um), 7.08 – 6.99 (m, 2H, 2xCH-Um), 6.26 (d, $J$ = 1.4 Hz, 1H, CH-Um), 5.51 (d, $J$ = 3.7 Hz, 1H, OH), 5.39 (s, 1H, H-1), 5.15 (d, $J$ = 7.1 Hz, 1H, OH), 4.85 (d, $J$ = 7.6 Hz, 1H, OH), 4.72 (t, $J$ = 5.6 Hz, 1H, OH), 4.12 (q, $J$ = 5.2 Hz, 1H, OH), 4.00 (td, $J$ = 6.2, 5.7, 1.5 Hz, 1H, H-5), 3.89 (q, $J$ = 3.3 Hz, 1H, H-3), 3.69 (t, $J$ = 5.3 Hz, 1H, H-2), 3.61 (dt, $J$ = 10.9, 5.4 Hz, 1H, H-6a), 3.52 (dt, $J$ = 10.1, 5.4 Hz, 2H, H-4 and H-6b), 2.41 (d, $J$ = 1.3 Hz, 3H, $CH_3$-Um); $^{13}$C NMR (101 MHz, DMSO) $\delta$ 160.1, 160.0, 154.4, 153.4 (4x$C_q$-Um), 126.6 (CH-Um), 114.1 ($C_q$-Um), 113.2, 111.7, 103.1 (3xCH-Um), 97.0 (C-1), 75.8 (C-5), 69.7, (C-2 and C-3), 67.9 (C-4), 60.5 (C-6), 18.2 ($CH_3$-Um); HRMS: calcd. for $C_{16}H_{18}O_8$ $[M+Na]^+$: 361.08939, Found: 361.08916.

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



The protected α-sugar **19** (18 mg, 36 µmol) was dissolved in MeOH/$CH_2Cl_2$ (1.5 mL, 1:1) followed by the addition of sodium methoxide (30% in MeOH, 1 drop). The mixture was stirred for 4 h at room temperature. After reaching completion the reaction was quenched with amberlite, and subsequently filtered off and washed with MeOH. The solution was concentrated *in vacuo* yielding the crude product which was purified by silica column chromatography and a subsequent prep-HPLC to afford the desired substrate **5** (9.11 mg, 30 µmol, 76%) as a white solid. $^{1}$H NMR (400 MHz, DMSO) $\delta$ 7.69 (d, $J$ = 8.8 Hz, 1H, CH-Um), 7.18 (d, $J$ = 2.4 Hz, 1H, CH-Um), 7.08 (dd, $J$ = 8.8, 2.4 Hz, 1H, CH-Um), 6.24 (d, $J$ = 1.3 Hz, 1H, CH-Um), 5.40 – 5.37 (m, 2H, H-1 and OH), 5.16 (d, $J$ = 4.1 Hz, 1H, OH), 4.96 (d, $J$ = 5.4 Hz, 1H, OH), 4.74 (t, $J$ = 5.3 Hz, 1H, OH), 3.96 (ddd, $J$ = 8.4, 4.9, 3.0 Hz, 1H, H-5), 3.66 – 3.55 (m, 4H, H-3, H-4 and H-6a,b), 3.53 – 3.46 (m, 1H, H-2), 2.40 (d, $J$ = 1.3 Hz, 3H, $CH_3$-Um); $^{13}$C NMR (101 MHz, DMSO) $\delta$ 160.2, 160.0, 154.4, 153.4 ($C_q$-Um), 126.4 (CH-Um), 114.0 ($C_q$-Um), 113.7, 111.6, 103.5 (3xCH-Um), 98.6 (C-1), 73.0 (C-5), 71.8, 71.6, 69.8 (C-2, C-3 and C-4), 58.7 (C-6), 18.2 ($CH_3$-Um); HRMS: calcd. for $C_{16}H_{18}O_8$ $[M+H]^+$: 339.10744, Found: 399.10725.

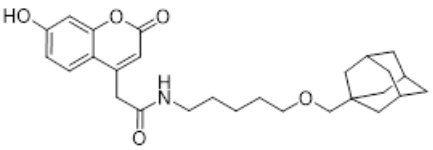
**(3*R*,5*R*,7*R*)-1-(((5-Azidopentyl)oxy)methyl)adamantane (21)**



Sodium azide (120 mg, 1.85 mmol) was added to an anhydrous solution of 5-(adamantan-1-yl-methoxy)pentyl iodide (516 mg, 1.42 mmol) in DMSO (2.85 mL). The reaction mixture was stirred for 20 h at room temperature. The mixture was diluted with $Et_2O$ and washed successively with $H_2O$ and brine. The organic phase was dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude residue was purified by silica column chromatography to yield the product (385 mg, 1.39 mmol, 97% yield) as a yellow oil. $^{1}$H NMR (400 MHz, $CDCl_3$) $\delta$ 3.38 (t, $J$ = 6.3 Hz, 2H, H-5'), 3.27 (t, $J$ =

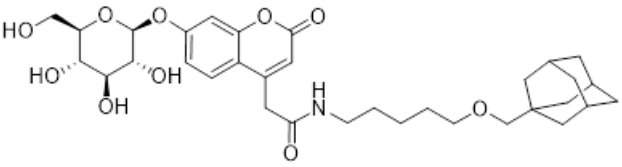
7.0 Hz, 2H, H-1'), 2.95 (s, 2H, H-6'), 1.98 – 1.93 (m, 3H, 3xCH-Ada), 1.74 – 1.55 (m, 10H, H-4', H-2' and 3x$CH_2$-Ada), 1.53 (d, *J* = 3.0 Hz, 6H, 3x$CH_2$-Ada), 1.48 – 1.39 (m, 2H, H-3'). $^{13}$C NMR (101 MHz, $CDCl_3$) *δ* 82.1 (C-6'), 71.4 (C-5'), 51.6 (C-1'), 39.9 (3x$CH_2$-Ada), 37.4 (3x$CH_2$-Ada), 34.2 ($C_q$-Ada), 29.2, 28.8 (C-2'and C-4'), 28.4 (3xCH-Ada), 23.6 (C-3'); HRMS not found.

**_N_-(5-(((3_R_,5_R_,7_R_)-adamantan-1-yl)methoxy)pentyl)-2-(7-hydroxy-2-oxo-2_H_-chromen-4-yl)acetamide (23)**



To a solution of 7-hydroxycoumarinyl-4-acetic acid (377 mg, 1.71 mmol) and HOBt (386 mg, 2.85 mmol) in acetonitrile (7 mL) and $CH_2Cl_2$ (3 ml) at 0 °C under nitrogen atmosphere, EDC (547 mg, 2.85 mmol) was added. After stirring for 10 minutes, the solution was treated with 5-(adamantan-1-yl-methoxy)pentyl azide (396 mg, 1.43 mmol) dissolved in 4 mL of $CH_2Cl_2$. Tri-*n*-butylphosphine (535 µl, 2.14 mmol) was added and the mixture was stirred in the dark for 18 h. After the reaction was completed, the mixture was diluted with $CH_2Cl_2$ and washed with 1 M NaOH (2x) and brine. The organic phase was dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude was purified by silica column chromatography yielding the desired product (434 mg, 0.957 mmol, 67%) as an off-white solid. $^1$H NMR (400 MHz, Acetone) *δ* 7.66 (d, *J* = 8.7 Hz, 1H, CH-Um), 6.83 (dd, *J* = 8.7, 2.4 Hz, 1H, CH-Um), 6.73 (d, *J* = 2.4 Hz, 1H, CH-Um), 6.18 (d, *J* = 1.0 Hz, 1H, CH-Um), 5.62 (s, 1H, NH), 3.71 (d, *J* = 0.9 Hz, 2H, $CH_2$-Um), 3.30 (t, *J* = 6.4 Hz, 2H, H-5), 3.20 (td, *J* = 7.0, 5.8 Hz, 2H, H-1), 2.92 (s, 2H, H-6), 1.92 (p, *J* = 3.2 Hz, 3H, 3xCH-Ada), 1.75 – 1.60 (m, 6H, 3x$CH_2$-Ada), 1.56 – 1.45 (m, 10H, 3x$CH_2$-Ada, H-2 and H-4), 1.34 (tdd, *J* = 8.1, 4.8, 2.4 Hz, 2H, H-3). $^{13}$C NMR (101 MHz, Acetone) *δ* 168.4 ($C_q$-CONH), 162.0, 161.0, 156.5, 151.4 (4x$C_q$-Um), 127.6, 113.5, 113.3 (CH-Um), 113.0 ($C_q$-Um), 103.4 (CH-Um), 82.4 (C-11), 71.8 (C-10), 40.44, 40.40, 40.1 (C-5, C-6 and 3x$CH_2$-Ada), 37.9 (3x$CH_2$-Ada), 30.0 (C-7 and C-9), 29.2 (3xCH-Ada), 24.2 (C-8); HRMS: calcd. for $C_{27}H_{35}NO_5$ $[M+H]^+$ : 454.25880, Found: 454.25865.

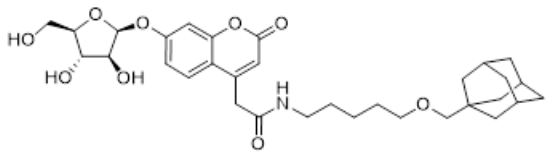
**_N_-(5-(((3_R_,5_R_,7_R_)-Adamantan-1-yl)methoxy)pentyl)-2-(2-oxo-7-(((2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2_H_-pyran-2-yl)oxy)-2_H_-chromen-4-yl)acetamide (6)**



The benzoyl protected glucose (200 mg, 285 µmol) was dissolved in $CH_2Cl_2$ (2.85 mL) and cooled to 0 °C. HBr (700 µL, 2.85 mmol,

30% in AcOH) was added dropwise and the reaction mixture was stirred in the dark for 3 h. The mixture was diluted with $CH_2Cl_2$ and washed with ice water (3x), sat. aq. $NaHCO_3$ and water again. The water layers were extracted with $CH_2Cl_2$ and the combined organic layers were dried ($MgSO_4$), filtered and concentrated *in vacuo*. Subsequently, Ada4MU (101 mg, 223 µmol) was added to a solution of NaOH (18 mg, 445 µmol) in $H_2O$ (2.2 mL). To this solution tetrabutylammonium bromide (72 mg, 223 µmol) was added and the bromo sugar (176 mg, 267 µmol) was dissolved in Acetone (2.2 mL). The mixture was stirred in the dark for 18 h at room temperature. After reaching completion, the reaction was diluted with $CH_2Cl_2$ and washed with 1 M NaOH (2x) and brine. The water layers were extracted with $CH_2Cl_2$ and the combined organic layers were dried ($MgSO_4$), filtered and concentrated *in vacuo*. The product was crudely purified over a silica plug after which the acetyl protected sugar (142 mg, 138 µmol) was dissolved in MeOH/$CH_2Cl_2$ (2.8 mL, 1:1) followed by the addition of sodium methoxide (30% in MeOH, 3 drops). The mixture was stirred for 4 h at room temperature and subsequently quenched with amberlite, filtered off and washed with MeOH. The solution was concentrated *in vacuo* yielding the crude product which was purified by silica column chromatography the product (31 mg, 50 µmol, 23% over 3 steps) as a white solid. $^1$H NMR (400 MHz, DMSO) $\delta$ 8.20 (t, *J* = 5.6 Hz, 1H, NH), 7.69 (d, *J* = 8.8 Hz, 1H, CH-Um), 7.06 (d, *J* = 2.4 Hz, 1H, CH-Um), 7.02 (dd, *J* = 8.8, 2.5 Hz, 1H, CH-Um), 6.27 (s, 1H, CH-Um), 5.41 (d, *J* = 4.6 Hz, 1H, OH), 5.16 (d, *J* = 4.3 Hz, 1H, OH), 5.09 (d, *J* = 5.3 Hz, 1H, OH), 5.03 (d, *J* = 7.1 Hz, 1H, H-1), 4.60 (t, *J* = 5.6 Hz, 1H, OH), 3.73 – 3.65 (m, 3H, H-6a and $CH_2$-Um), 3.48 – 3.41 (m, 2H, H-4 and H-6b), 3.29 (t, *J* = 6.5 Hz, 4H, H-2, H-3 and H-5'), 3.15 (qd, *J* = 7.5, 6.6, 4.2 Hz, 1H, H-5), 3.05 (q, *J* = 6.5 Hz, 2H, H-1'), 2.90 (s, 2H, H-6'), 1.91 (t, *J* = 3.1 Hz, 3H, 3xCH-Ada), 1.72 – 1.55 (m, 6H, 3x$CH_2$-Ada), 1.52 – 1.35 (m, 10H, H-2', H-4' and 3x$CH_2$-Ada), 1.27 (qd, *J* = 8.7, 5.4 Hz, 2H, H-3'). $^{13}$C NMR (101 MHz, DMSO) $\delta$ 167.3 ($C_q$-CONH), 160.13, 160.07, 154.6, 151.1 (4x$C_q$-Um), 126.5 (CH-Um), 113.6 ($C_q$-Um), 113.3, 113.0, 103.4 (3xCH-Um), 100.0 (C-1), 81.0 (C-6'), 77.2 (C-4), 76.5, 73.1 (C-2 and C-3), 70.6 (C-5'), 69.6 (C-5), 60.6 (C-6), 39.94 (3x$CH_2$-Ada), 39.3 ($CH_2$-Um), 38.7 (C-1'), 36.7 (3x$CH_2$-Ada), 28.76, 28.73 (C-2' and C-4'), 27.6 (3xCH-Ada), 23.1 (C-3'). ; HRMS: calcd. for $C_{33}H_{45}NO_{10}$ $[M+H]^+$ : 616.31162, Found: 616.31136.

**_N_-(5-(((3_R_,5_R_,7_R_)-adamantan-1-yl)methoxy)pentyl)-2-(7-(((2_S_,3_S_,4_S_,5_R_)-3,4-dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)oxy)-2-oxo-2_H_-chromen-4-yl)acetamide (7)**



The acetyl protected furanoside (300 mg, 943 µmol) was dissolved in $CH_2Cl_2$ (9.43 ml) and cooled to 0 °C. HBr (2.31 mL, 9.42 mmol, 30% in AcOH) was added dropwise and the reaction mixture was stirred in the dark for 4 h. The mixture was diluted with $CH_2Cl_2$ and washed with ice water (3x), sat. aq. $NaHCO_3$ and water again. The water layers were extracted with $CH_2Cl_2$ and the combined organic layers were dried ($MgSO_4$), filtered and concentrated *in vacuo*. Subsequently, Ada4MU (145 mg, 320 µmol) was added to a solution of NaOH (26 mg, 640 µmol) in $H_2O$ (3.2 mL). To this solution tetrabutylammonium bromide (103 mg, 320 µmol) was added and the bromo furanoside (217 mg, 640 µmol) dissolved in acetone (3.2 mL). The mixture was stirred in the dark for 18 h at room temperature. After reaching completion the reaction was diluted with $CH_2Cl_2$ and washed with 1 M NaOH (2x) and brine. The water layers were extracted with $CH_2Cl_2$ and the combined organic layers were dried ($MgSO_4$), filtered and concentrated *in vacuo*. The product was crudely purified over a silica plug after which the protected acetyl sugar (35 mg, 49 µmol) was dissolved in MeOH/$CH_2Cl_2$ (1 mL, 1:1), followed by the addition of sodium methoxide (30% in MeOH, 1 drop). The mixture was stirred for 4 h at room temperature and subsequently quenched with amberlite, filtered off and washed with MeOH. The solution was concentrated *in vacuo* yielding the crude product which was purified by silica column chromatography the product (9 mg, 15 µmol, 11% over 3 steps) as a white solid. $^1$H NMR (600 MHz, DMSO) $\delta$ 8.28 (t, $J$ = 5.6 Hz, 1H, NH), 7.71 (d, $J$ = 8.8 Hz, 1H, CH-Um), 7.02 (d, $J$ = 2.4 Hz, 1H, CH-Um), 7.00 (dd, $J$ = 8.8, 2.5 Hz, 1H, CH-Um), 6.26 (s, 1H, CH-Um), 5.29 (s, 1H, 2-OH), 5.03 (d, $J$ = 6.8 Hz, 1H, H-1), 4.91 (s, 1H, 3-OH), 4.69 (s, 1H, 5-OH), 3.76 – 3.71 (m, 1H, H-5a), 3.71 (s, 1H, H-4), 3.70 – 3.65 (m, 3H, $CH_2$-Um and H-5b), 3.66 – 3.60 (m, 1H, H-2), 3.48 (d, $J$ = 8.3 Hz, 1H, H-3), 3.28 (t, $J$ = 6.5 Hz, 2H, H5'), 3.05 (q, $J$ = 6.6 Hz, 2H, H1'), 2.90 (s, 2H, H6'), 1.90 (s, 3H, 3xCH-Ada), 1.69 – 1.56 (m, 6H, 3x$CH_2$-Ada), 1.49 – 1.37 (m, 10H, 3x$CH_2$-Ada and H2'+ H4'), 1.27 (q, $J$ = 8.4 Hz, 2H, H3'); $^{13}$C NMR (151 MHz, DMSO) $\delta$ 167.3 ($C_q$-CONH), 160.03, 159.95, 154.6, 151.1 (4x$C_q$-Um), 126.5 (CH-Um), 113.5 ($C_q$-Um), 113.3, 113.1, 103.2 (3xCH-Um), 100.2 (C-1), 81.0 (C-6'), 72.4 (C-3), 70.6 (C-5'), 70.1 (C-2), 67.4 (C-4), 65.8 (C-5), 39.8 (3x$CH_2$-Ada), 38.8 ($CH_2$-Um), 38.7 (C-1'), 36.7 (3x$CH_2$-Ada), 28.74, 28.72 (C-2' and C-4'), 27.6 (3x$CH_2$-Ada), 23.1 (C-3'); HRMS: calcd. for $C_{32}H_{43}NO_9$ $[M+H]^+$ : 586.30106, Found: 586.30164

## 5.7 References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