Activity-based protein profiling of glucosidases, fucosidases and glucuronidases

Jiang, J.

4

Comparing *N*-alkyl and *N*-acyl cyclophellitol aziridine and its isomer as activity-based glycosidase probes

Jianbing Jiang, Thomas J. M. Beenakker, Wouter W. Kallemeijn, Gijsbert A. van der Marel, Hans van den Elst, Jeroen D. C. Codée, Johannes M. F. G. Aerts and Herman S. Overkleeft, *Chemistry–A European Journal,* **2015**, *21*, 10861-10869.

4.1 Introduction

Glycosidases constitute a large family of hydrolytic enzymes expressed throughout all kingdoms of life and essential in a myriad of biological processes. From a substrate point of view glycosidases can be roughly classified as *endo*-glycosidases (cleaving within an oligo/polysaccharide to yield an oligosaccharide) and *exo*-glycosidases (recognizing a single monosaccharide at the non-reducing end of an oligosaccharide/glycoconjugate). All glycosidases hydrolyze their substrate glycosidic bonds with either retention or inversion of configuration with respect to the anomeric configuration of the released glycoside.[1] This difference in enzymatic hydrolysis, which is perhaps irrelevant with respect to the produced hemi-acetals that will undergo post-hydrolysis anomerization, is caused by the distinct mechanisms employed by the two enzyme families. Inverting glycosidases directly substitute, in an S_N2 fashion, the aglycon of the glycosidic bond and do so by simultaneously activating the anomeric leaving group (protonation by a catalytic acid/base residue present in the enzyme active site) and activation of a water molecule residing in the active site by a catalytic

base. Retaining glycosidases in contrast employ a two-step double displacement mechanism. In this mechanism, first proposed by Koshland,[2] S_N2 displacement of the aglycon (activated through protonation by the general acid/base residue) via attack of the enzyme catalytic nucleophile yields a covalent glycosyl-enzyme intermediate and concomitant expulsion of the aglycon. In a second step and after entry of a water molecule in the enzyme active site this enzyme-glycosyl intermediate is hydrolyzed to yield the carbohydrate hemi-acetal.

The occurrence of a covalent intermediate in the catalytic cycle of retaining glycosidases invites the development of tagged, covalent inhibitors, termed activity-based probes (ABPs), and thereby monitoring these enzymes in cell extracts, *in situ* and *in vivo* by means of activity-based protein profiling (ABPP) experiments. Indeed, the development of retaining glycosidase ABPs has met with considerable more success than identification of related probes for inverting glycosidases.[3] The first reported conceptual design for retaining glycosidase ABPs is from the laboratories of Withers, Vocadlo and Bertozzi, who employed electron-deficient, fluorine-modified carbohydrates (either substitution of the 2-OH or the 5-H for fluorine).[4] The work described in this chapter focused on the natural product mechanism-based β-glucosidase inhibitor, cyclophellitol.[5]

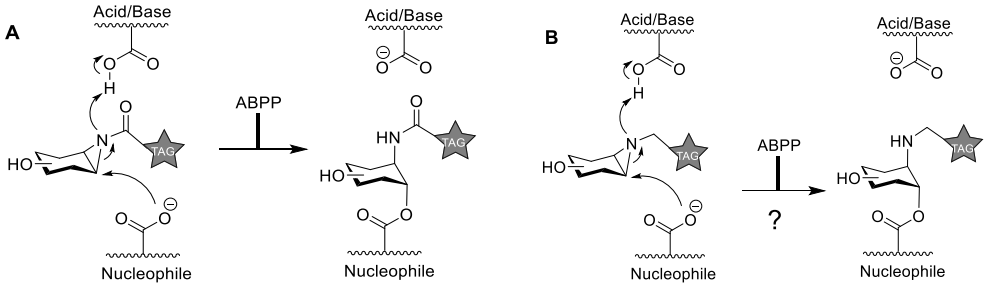


Figure 1. A) Covalent and irreversible inhibition of retaining glycosidases by *N*-acyl cyclophellitol aziridine ABPs. B) Proposed new retaining glycosidase ABP design based on *N*-alkyl cyclophellitol aziridines.

The first inroads into activity-based glycosidase profiling concerned installment of fluorophores at C6 (glucopyranose numbering) of cyclophellitol.[6] The resulting compounds proved highly potent and very specific for GH30 (CAZypedia nomenclature[7]) human acid glucosylceramidase (GBA) over the other three human retaining β-glucosidases (GBA2, GBA3 and LPH). They also proved more effective than the corresponding 2-deoxy-2-fluoroglucosides in subsequent studies, underscoring the usefulness of the cyclophellitol scaffold for ABP design for retaining glycosidases.[8] With the aim to arrive at in-class (with respect to the retaining glycosidase family at hand) broad spectrum ABPs, the epoxide in cyclophellitol was in subsequent studies substituted for aziridine and the reporter moiety was installed at the aziridine nitrogen through *N*-acylation. In this fashion, effective ABPs were obtained for GH30

β-glucosidases[9], GH27 α-galactosidases (GLA)[10] and GH29 α-fucosidases (FUCA).[11] In each case, as depicted in Figure 1A, after attack from the catalytic nucleophile present in the glycosidase active site of enzyme, the detectable probe-enzyme complex is formed. Probe specificity appears configuration dependent, with the configuration of cyclitol aziridine conferring selectively towards the corresponding retaining glycosidases.

In the studies on *N*-acyl aziridine-based glycosidase probes, it was observed that the *N*-acyl moiety are relatively hydrolysis-prone, which puts some strain on their synthesis, purification and handling. With the aim to establish whether *N*-alkyl cyclophellitol aziridines would be valid scaffolds for retaining glycosidase ABP design and following the literature precedent set by Tatsuma and co-workers[12], a set of cyclophellitol aziridine analogues were recently evaluated varying in *N*-substitution as inhibitors of human retaining β-glucosidases. It was found that *N*-pentanyl cyclophellitol aziridine inhibits GBA, GBA2 and GBA3 at least equally potent as the corresponding *N*-pentanoyl cyclophellitol aziridine.[13] Based on these observations the effectiveness, both in synthesis and in activity-based glycosidase profiling, of a set of *N*-alkyl aziridine and *N*-acyl aziridine probes targeting GH30 β-glucosidases and GH29 α-fucosidases (Figure 1B) was evaluated, The results of these studies are presented in this chapter and the structures of the compounds studied here are depicted in Figure 2.

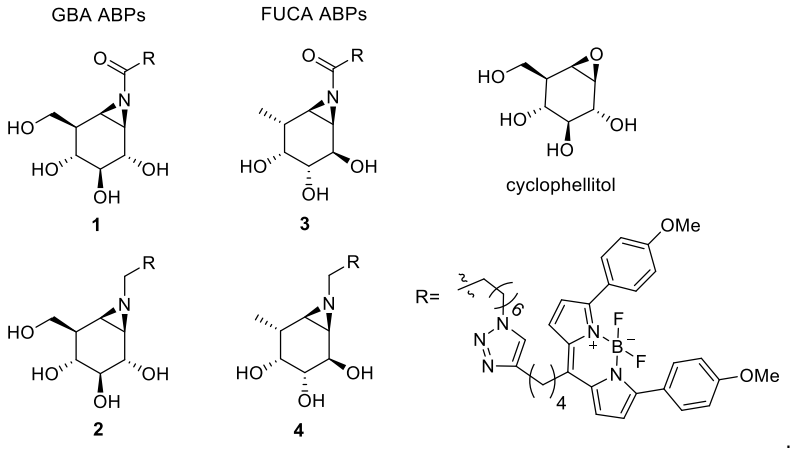
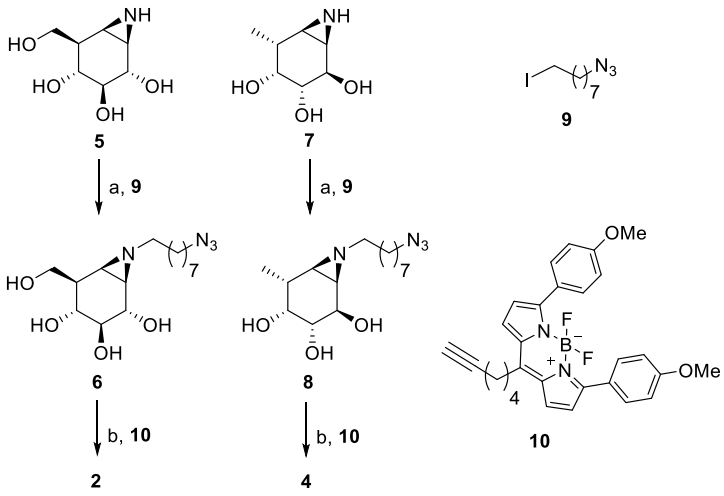


Figure 2. Structures of the natural product, cyclophellitol, and *N*-alkyl/acyl aziridines derived thereof and that are subject of the here presented studies.

4.2 Results and Discussion

The preparation of *N*-acyl cyclophellitol aziridine ABPs **1** and **3** (Figure 2) is described in previous literature reports on the development of these ABPs.[11,14] In order to produce the corresponding *N*-alkyl cyclophellitol aziridines **2** and **4** (Scheme 1), sufficient quantities of the unmodified aziridines **5** and **7** were prepared. These were condensed with

1-azido-8-iodooctane **9** in DMF with K_2CO_3 as base, giving azido-aziridine **6** and **8**. The target ABPs **2** and **4** were synthesized by conjugation of alkyl-BODIPY **10** followed by HPLC purification and lyophilization in moderate yields.



Scheme 1. Synthesis of alkyl aziridine ABPs **2** and **4**. (a) K_2CO_3, DMF, 80 °C, **6**: 49%, **8**: 36%; (b) $CuSO_4$ (1.0 M in H_2O), sodium ascorbate (1.0 M in H_2O), DMF, **2**: 30%, **4**: 9%.

At a first glance there appears not much difference between the efficiency (quantity, yield) of the synthesis of *N*-acyl aziridine **1** (5.8 mg, 6%)[14a] and its alkyl counterpart **2** (26 mg, 15%). The same holds true when comparing *N*-acyl aziridine **3** (15 mg, 4%)[11] and *N*-alkyl aziridine **4** (1.8 mg, 3%). However, the HPLC purification of cyclophellitol *N*-acyl aziridines has to be performed at neutral (H_2O, MeCN) or slightly basic (25 mM NH_4HCO_3 in H_2O/MeCN) pH, and hydrolysis of the *N*-acyl aziridine is always at risk during lyophilization and use in ABPP. The *N*-alkyl aziridines in contrast can be purified by standard silica gel column chromatography, and are stable during standard HPLC purification conditions (50 mM aqueous NH_4HCO_3) and LC-MS detection in the presence of 1.0 % TFA, as well as during evaporation or lyophilization.

After having the cyclophellitol aziridine ABPs **1**-**4** in hand, their inhibitory potency was determined towards GBA (**1**, **2**) and FUCA (**3**, **4**) as well as their potential as ABPs for these three retaining glycosidases in a set of head-to-head comparative experiments, focusing in each case on the difference between *N*-acyl- and *N*-alkyl substitution on the cyclophellitol aziridines of the same configuration. For this purpose, recombinant GBA and FUCA enzymes were expressed in COS-7 cells.

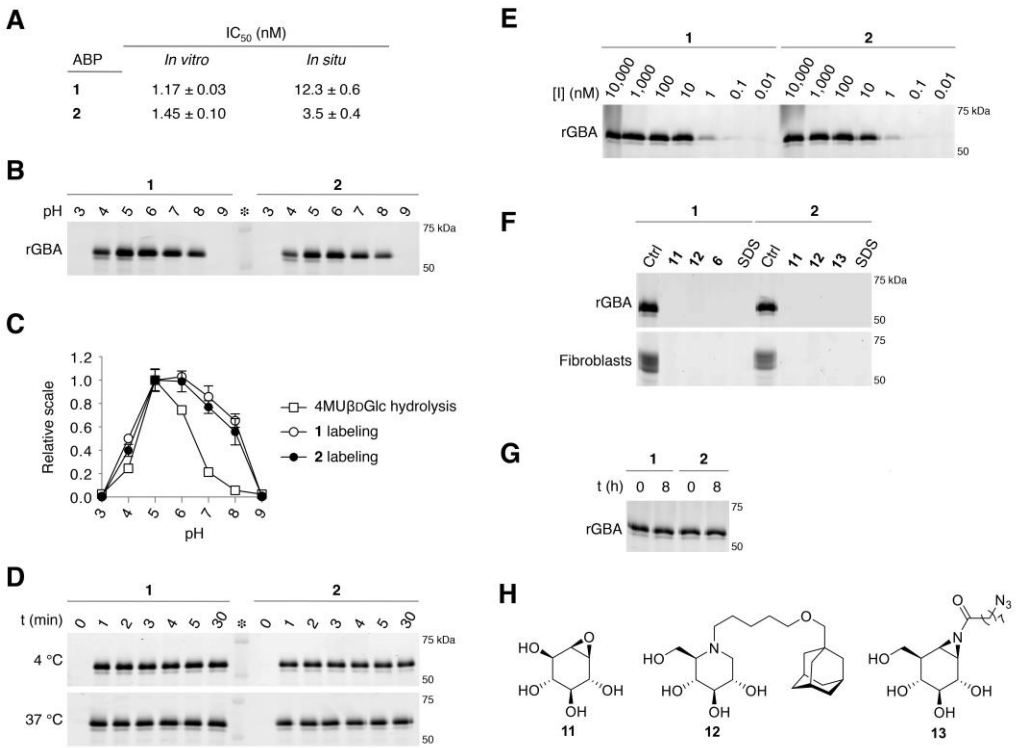


Figure 3. A) Apparent IC_{50} values of β-D-glucopyranose ABPs **1** and **2**, determined *in vitro* against rGBA and *in situ* in wild-type GBA fibroblasts. Data are averages of two separate experiments in duplo, and error bars depict standard deviation. B) ABP-labeling of rGBA at various pH. C) Quantification of ABP-labeling in (B) compared to hydrolysis of 4-methylumbelliferyl-β-D-glucopyranoside at varying pH. Data are averages of two separate experiments in duplo, and error bars depict standard deviation. D) Labeling of 100 fmol rGBA with 100 fmol ABP at 4 °C (top) and 37 °C (bottom). E) Detection limit of ABP-emitted fluorescence. rGBA (10 pmol) was labeled with varying concentrations of ABP **1** or **2**. F) ABP labeling of rGBA (top) or wild-type GBA fibroblast lysate (bottom) competed with inhibitors **6** (Scheme 1)**, 11**, **12**, **13** or SDS. G) Stability of ABP-rGBA nucleophile adducts after a 0 and 8 hour chase in the dark, with hourly extensive washing. H) Structure of compounds CBE **11**, **12** and **13**.

The first comparative study was performed on acyl aziridine ABP **1** and alkyl aziridine ABP **2** as inhibitors and ABPs of recombinant human acid β-glucoceramidase (GBA). Both aziridines inhibit GBA activity in the nanomolar range, both *in vitro* and *in situ* (IC_{50} values in Figure 3A). The observed values for the known *N*-acyl aziridine **1** correspond with those reported previously.[9] Both compounds exhibit a similar pH-dependent activity (Figure 3B) and effective labeling of recombinant purified GBA is observed at concentrations as low as 1 nanomolar (Figure 3E). Figure 3C depicts a quantification curve of GBA labeling with **1** or **2** as derived from the data presented in Figure 3B offset against GBA-mediated hydrolysis at various pH of the fluorogenic substrate, 4-methylumbelliferyl-β-D-glucopyranoside. These data show that

N-alkyl-aziridine **2**, as is *N*-acyl-aziridine **1**, is able to covalently modify GBA up to slightly basic conditions, whereas the pH optimum of GBA as reflected by substrate hydrolysis is at around pH 5. Labeling efficiency of *N*-acyl aziridine **1** and *N*-alkyl aziridine **2** at 100 femtomole were determined at both 4 °C and 37 °C (Figure 3D). Labeling of GBA was almost complete within one minute with both compounds, and thus the effective GBA labeling is too fast to allow accurate determination of kinetic constants. In competitive activity-based protein profiling experiment, recombinant GBA and wild type fibroblast were pre-incubated with the mechanism-based inhibitor, conduritol B epoxide (CBE, **11**), the competitive inhibitor, *N*-(adamantanemethyloxypentyl)- deoxynojirimycin **12,** as well as acyl aziridine (JJB103, **13**) and alkyl aziridine (JJB339, **6**), the latter two compounds being the non-fluorescent precursors towards the synthesis of compounds **1** and **2**, respectively. All compounds proved able to completely block the GBA activity in the conditions applied (Figure 3F). Finally, labeling intensity of GBA treated with either **1** or **2** did not change after an 8 hour chase (Figure 3G), which was performed in darkness with hourly continuous extensive washing and the control adducts were denatured and frozen at -20 °C. It was suggested that both **1** and **2** formed stable ABP-GBA nucleophile adducts. Based on these results, it can be concluded that *N*-alkyl aziridine **2** performs equal to *N*-acyl aziridine **1** in labeling GBA and is, based on the fact that it is easier to prepare and handle, perhaps the retaining β-glucosidase ABP of choice.

In a next set of experiments, recombinant human α-fucosidase (FUCA) was subjected to a similar analysis, now using *N*-acyl aziridine **3** and *N*-alkyl aziridine **4**. As can be seen in Figure 4A, *N*-alkyl aziridine **4** inhibits FUCA about 500-fold less potently for than *N*-acyl aziridine **3**. This result is consistent with the detection limit in FUCA labeling with these probes (Figure 4E). FUCA hydrolyses the fluorogenic substrate, 4-methylumbelliferyl-α-L-fucopyranoside (4-MUαFuc, Figure 4C) optimally at pH 5.0, at which pH also optimal labeling with **3** and (though less effective) **4** is observed (Figure 4B). Time-dependent FUCA labeling with ABP **3** and **4** were also difficult to analyze similar as GBA labeling due to the very fast inhibition rates, this result is backed up by the differences in labeling seen in Figure 4D. The *N*-alkyl aziridine inhibitor **8** (JJB349) blocked FUCA labeling with either **5** or **6** equally effective as non-tagged *N*-acyl aziridines **14** and **15**. As before, in-gel labeling intensity appeared unchanged between 0 and 8 hours of chase (Figure 4G). These results indicate that *N*-acyl aziridine ABP **5** is by far the more effective FUCA activity-based probe and the reagent of choice, even though *N*-alkyl aziridine **6** is the most user-friendly in terms of stability and handling.

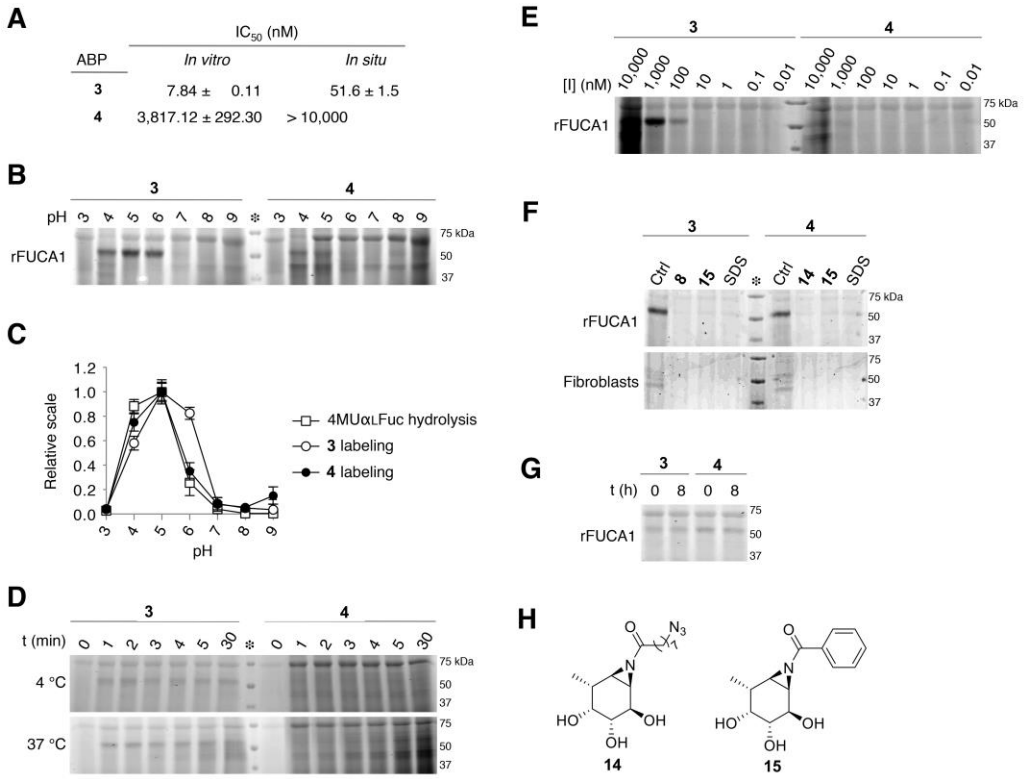


Figure 4. A) Apparent IC_{50} values of α-L-fucopyranose ABPs **3** and **4**, determined *in vitro* against rFUCA and *in situ* in wild-type FUCA fibroblasts. Data are averages of two separate experiments in duplo, and error bars depict standard deviation. B) ABP-labeling of rFUCA at varying pH. C) Quantification of ABP-labeling in (B) compared to hydrolysis of 4-methylumbelliferyl-α-L-fucopyranoside at varying pH. Data are averages of two separate experiments in duplo, and error bars depict standard deviation. D) Labeling of 100 fmol rFUCA with 100 fmol ABP at 4 °C (top) and 37 °C (bottom). E) Detection limit of ABP-emitted fluorescence. rFUCA (10 pmol) was labeled with varying concentrations of ABP. F) ABP labeling of rFUCA (top) or wild-type FUCA fibroblast lysate (bottom) competed with the irreversible inhibitors **8** (Scheme 1), **14** or **15** or SDS. G) Stability of ABP-rFUCA nucleophile adducts after 0 and 8 hour chase in the dark, with hourly extensive washing. (H) Structure of compounds **14** and **15**.

4.3 Conclusions

In conclusion, an in-depth study has been conducted on the efficacy of a new series of *N*-alkyl aziridine-based probes **2** and **4** as activity-based probes for retaining glycosidases. The efficacy of these probes were compared – relatively easy to synthesize and handle – with the previously reported set of *N*-acyl aziridine probes **1** and **3**, which were developed for the profiling of GBA and FUCA, respectively. Preparation of alkyl aziridine compounds is easier because of the intrinsically more stable (compared to *N*-acyl aziridines) *N*-alkyl aziridine electrophilic trap. Activity-based labeling of GBA proved equally effective with *N*-alkyl aziridine **2** as with *N*-acyl aziridine acyl **1**, but the corresponding *N*-alkyl analogues for FUCA proved to

perform less well. These intriguing results warrant future investigations using crystals of the various retaining glycosidases soaked with corresponding ABPs to render an explanation. It can be concluded that *N*-alkyl aziridines can be considered as scaffold to design activity-based probes directed at retaining glycosidases other than targeted to date, but when not active the corresponding *N*-acyl derivatives need be assessed as well before disregarding the cyclophellitol scaffold for ABPP targeting of the glycosidase at hand.

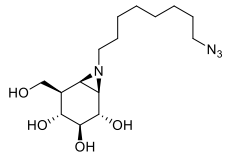
4.4 Experimental section

General synthesis

All reagents were of a commercial grade and were used as received unless stated otherwise. Dichloromethane (DCM), tetrahydrofuran (THF) and *N, N*-dimethylformamide (DMF) were stored over 4 Å molecular sieves, which were dried *in vacuo* 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 TLC analysis using Merck aluminium sheets pre-coated with silica gel 60 with detection by UV absorption (254 nm) and by spraying with a solution of $(NH_4)_6Mo_7O_{24}.H_2O$ (25 g/L) and $(NH_4)_4Ce(SO_4)_4.H_2O$ (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 using either Baker or Screening Device silica gel 60 (0.04 - 0.063 mm) in the indicated solvents. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on Bruker AV-850 (850/214 MHz), Bruker DMX-600 (600/150 MHz) and Bruker AV-400 (400/100 MHz) spectrometer in the given solvent. Chemical shifts are given in ppm relative to the deuterated chloroform or methanol residual solvent peak or tetramethylsilane (TMS) as internal standard. Coupling constants are given in Hz. All given ^{13}C-NMR spectra are proton decoupled. High-resolution mass spectra were recorded with a LTQ Orbitrap (Thermo Finnigan). Optical rotations were measured on Propol automatic polarimeter (Sodium D-line, λ = 589 nm). IR spectra were recorded on a Shimadzu FT-IR 83000 spectrometer. LC-MS analysis was performed on an LCQ Adventage Max (Thermo Finnigan) ion-trap spectrometer (ESI^+) coupled to a Surveyor HPLC system (Thermo Finnigan) equipped with a C_{18} column (Gemini, 4.6 mm x 50 mm, 3.0 µm particle size, Phenomenex) equipped with buffers A: H_2O, B: acetonitrile (MeCN) and C: 1% aqueous TFA. For reversed-phase HPLC purifications an Agilent Technologies 1200 series instrument equipped with a semi preparative Gemini C_{18} column (10 x 250 mm) was used. The applied buffers were A: 50 mM NH_4HCO_3 in H_2O, B: MeCN.

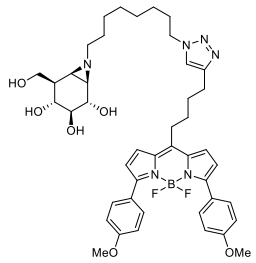
Synthesis of compounds 1 - 15

The β-D-glucoside acyl β-aziridine ABPs JJB75 **1** and JJB103 **13** were synthesized as described earlier.[9] CBE **11** was bought from Sigma and iminosugar **12** was synthesized as reported before.[15] The α-L-fucoside acyl α-aziridines JJB244 **3**, JJB237 **14** and JJB261 **15** were synthesized as described earlier.[11]

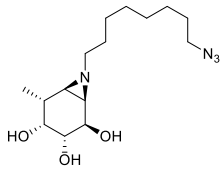


(1R,2S,3S,4R,5R,6R)-7-(8-azidooctyl)-5-(hydroxymethyl)-7-azabicyclo[4.1.0]heptane-2,3,4-triol (6): Unprotected cyclophellitol aziridine **7**[14a] (163 mg, 0.93 mmol, 1.0 eq.) was dissolved in DMF (4.0 mL). Compound 1-azido-8-iodooctane **9** (360 mg, 1.3 mmol, 1.4 eq.) and K_2CO_3 (448 mg, 4.0 mmol, 4.3 eq.) were added to the solution and the mixture

was stirred at 80 °C for 24 h. After the full conversion of the starting material, the resulting solution was filtered over a pad of celite in syringe and the volatiles were concentrated under reduced pressure. Then the crude product was purified by silica gel column chromatograph (linear gradient: 2%→20%, MeOH in DCM) resulting as a colorless oil **6** (150 mg, 0.46 mmol, 49%). TLC: R_f 0.41 (15%, v/v, MeOH in DCM); $[\alpha]_D^{20}$ +49.6 (*c* = 1, MeOH); ^{1}H-NMR (400 MHz, CD_3OD): *δ* ppm 3.99 (dd, *J* = 11.3, 4.5 Hz, 1H), 3.66 – 3.59 (m, 2H), 3.28 (t, *J* = 8.0 Hz, 2H), 3.15 – 3.02 (m, 2H), 2.40 – 2.33 (m, 1H), 2.18 – 2.11 (m, 1H), 2.01 – 1.98 (m, 1H), 1.95 – 1.85 (m, 1H), 1.66 (d, *J* = 6.2 Hz, 1H), 1.62 – 1.52 (m, 4H), 1.44 – 1.27 (d, *J* = 13.6 Hz, 8H); ^{13}C-NMR (100 MHz, CD_3OD): *δ* ppm 78.92, 73.79, 70.15, 63.76, 62.05, 52.40, 45.36, 45.34, 42.98, 30.45, 30.24, 30.14, 29.85, 28.25, 27.72; IR (neat, cm^{-1}): 3316, 2926, 2855, 2095, 1454, 1348, 1256, 1096, 1020, 818; LC-MS: R_t 4.41 min, linear gradient 10%→90% B in 12.5 min; ESI-MS: *m/z* = 329.20 $(M+H)^+$; HRMS: calculated for $C_{15}H_{28}N_4O_4$ $[M+H^+]$ 329.21833, found: 329.21809.

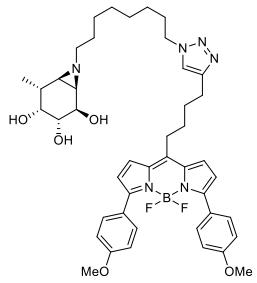


(1R,2S,3S,4R,5R,6R)-7-(8-(4-(4-(5,5-difluoro-3,7-bis(4-methoxyphenyl)-5H-4λ^4,5 λ^4-dipyrrolo[1,2-c:2',1'-*f*][1,3,2]diazaborinin-10-yl)butyl)-1H-1,2,3-triazol-1-yl)octyl)-5-(hydroxymethyl)-7-azabicyclo[4.1.0]heptane-2,3,4-triol (2): Azide compound **6** (36 mg, 0.11 mmol, 1 eq.) was dissolved in DMF (3.0 mL), red BODIPY compound **10** (53 mg, 0.11 mmol, 1.1 eq), $CuSO_4$ (1.0 M in H_2O, 60 µL, 0.060 mmol, 0.55 eq.) and sodium ascorbate (1.0 M in H_2O, 70 µL, 0.070 mmol, 0.64 eq.) was added to the solution under argon atmosphere. After stirring at room temperature overnight, the resulting mixture was concentrated under reduced pressure. Then the crude product was purified by semi-preparative reversed HPLC (linear gradient: 50%→56% B in A, 12 min, solutions used A: 50 mM NH_4HCO_3 in H_2O, B: MeCN) and lyophilized resulting in product **2** as a purple powder (26.5 mg, 0.032 mmol, 30%). ^{1}H-NMR (400 MHz, CD_3OD): *δ* ppm 7.86 – 7.81 (m, 4H), 7.63 (s, 1H), 7.41 (d, *J* = 4.4 Hz, 2H), 7.00 – 6.91 (m, 4H), 6.68 (d, *J* = 4.4 Hz, 2H), 4.29 (t, *J* = 7.0 Hz, 2H), 3.99 (dd, *J* = 10.0, 4.4 Hz, 1H), 3.83 (s, 6H), 3.62 – 3.57 (m, 2H), 3.13 – 3.08 (m, 1H), 3.03 – 2.98 (m, 3H), 2.75 (t, *J* = 6.7 Hz, 2H), 2.32 – 2.25 (m, 1H), 2.10 – 2.01 (m, 1H), 1.97 – 1.94 (m, 1H), 1.90 – 1.78 (m, 7H), 1.62 (d, *J* = 6.3 Hz, 1H), 1.55 – 1.45 (m, 2H), 1.35 – 1.15 (m, 8H); ^{13}C-NMR (100 MHz, CD_3OD): *δ* ppm 162.18, 158.75, 148.58, 146.75, 137.48, 132.15, 128.44, 126.49, 123.23, 121.05, 114.64, 79.02, 73.89, 70.12, 63.78, 62.09, 55.83, 51.22, 45.50, 45.42, 43.01, 34.14, 31.22, 30.97, 30.34, 30.23, 29.87, 28.16, 27.34, 25.77; LC-MS: R_t 6.85 min, linear gradient 10%→90% B in 12.5 min; ESI-MS: *m/z* = 813.27 $(M+H)^+$; HRMS: calculated for $C_{44}H_{55}BF_2N_6O_6$ $[M+H^+]$ 813.43245, found: 813.43125.



(1R,2R,3R,4R,5R,6R)-7-(8-azidooctyl)-5-methyl-7-azabicyclo[4.1.0]heptane-2,3,4-triol (8): Unprotected aziridine compound **7**[11] (44 mg, 0.28 mmol, 1 eq.) was dissolved in DMF (2 mL). Compound 1 -azido-8-iodooctane **9** (118 mg, 0.42 mmol, 1.5 eq.) and K_2CO_3 (174 mg, 1.3 mmol, 1.4 eq.) were added to the solution and the mixture was stirred at 80 °C for 24 h. After the full conversion of the starting material, the resulting solution was filtered over a pad of celite in syringe and the volatiles were concentrated under reduced pressure. Then the crude product was purified by silica gel column chromatography (linear gradient: 0%→10%, MeOH in DCM) resulting in **8** as colorless oil (31 mg, 0.10 mmol, 36%). TLC: R_f = 0.39 (10%, v/v, MeOH in DCM); $[\alpha]_D^{20}$ - 47.6 (*c* = 0.5, MeOH); ^{1}H-NMR (850 MHz, CD_3OD): *δ* ppm 3.99 (dd, *J* = 8.7, 4.3 Hz, 1H), 3.54 – 3.53 (m, 1H), 3.34 (dd, *J* = 9.4, 7.5 Hz,

1H), 3.28 (t, *J* = 6.9 Hz, 2H), 2.37 – 2.34 (m, 1H), 2.15 – 2.12 (m, 1H), 1.91 – 1.89 (m, 1H), 1.87 – 1.86 (m, 1H), 1.61 – 1.57 (m, 4H), 1.40 – 1.33 (m, 9H), 1.16 (d, *J* = 7.5 Hz, 3H); ^{13}C-NMR (214 MHz, CD_3OD): δ ppm 76.05, 75.13, 70.18, 62.33, 52.45, 46.45, 45.76, 36.95, 30.60, 30.57, 30.18, 29.89, 28.32, 27.75, 16.75; IR (cm^{-1}): 3370, 2927, 2854, 2092, 1456, 1348, 1253, 1105,1057, 814, 748; LC-MS: R_t 5.06 min, linear gradient 10%→90% B in 12.5 min; ESI-MS: *m/z* = 313.20 $(M+H)^+$; HRMS: calculated for $C_{15}H_{28}N_4O_3$ [$M+H^+$] 313.22342, found: 313.22387.



(1R,2R,3R,4R,5R,6R)-7-(8-(4-(4-(5,5-difluoro-3,7-bis(4-methoxyphenyl)-5H-4λ^4,5λ^4-dipyrrolo[1,2-c:2',1'-*f*][1,3,2]diazaborinin-10-yl)butyl)-1H-1,2,3-triazol-1-yl)octyl)-5-methyl-7-azabicyclo[4.1.0]heptane-2,3,4-triol (4): Azide compound **8** (8.3 mg, 0.0266 mmol, 1 eq.) was dissolved in DMF (0.8 mL). Red BODIPY **10** (13 mg, 0.033 mmol, 1.24 eq.), $CuSO_4$ (1.0 M in H_2O, 12 µL, 0.012 mmol, 0.45 eq.) and sodium ascorbate (1.0 M in H_2O, 13 µL, 0.013 mmol, 0.48 eq) were added to the solution under argon atmosphere. After stirring at room temperature for 12 h, the reaction volatiles were removed under reduced pressure. Then the crude product was purified by semi-preparative reversed HPLC (linear gradient: 58%→68% B in A, 12 min, solutions used A: 50 mM NH_4HCO_3 in H_2O, B: MeCN) and lyophilized resulting in product **4** as a purple powder (1.84 mg, 2.31 µmol, 9%). ^{1}H-NMR (600 MHz, CD_3OD): δ ppm 7.86 – 7.83 (m, 4H), 7.69 (s, 1H), 7.43 (d, *J* = 4.4 Hz, 2H), 6.98 – 6.96 (m, 4H), 6.69 (d, *J* = 4.3 Hz, 2H), 4.33 (t, *J* = 7.0 Hz, 2H), 3.99 (dd, *J* = 8.7, 4.3 Hz, 1H), 3.85 (s, 6H), 3.52 – 3.51 (m, 1H), 3.34 – 3.32 (m, 1H) 3.06 (t, *J* = 7.3 Hz, 2H), 2.78 (t, *J* = 6.8 Hz, 2H), 2.28 – 2.24 (m, 1H), 2.10 – 2.05 (m, 1H), 1.89 – 1.82 (m, 7H), 1.52 – 1.48 (m, 2H), 1.31 – 1.21 (m, 9H), 1.13 (d, *J* = 7.5 Hz, 3H); ^{13}C-NMR (150 MHz, CD_3OD): δ ppm 162.18, 158.76, 146.75, 137.48, 132.15, 128.42, 126.49, 123.25, 121.00, 114.61, 76.03, 75.08, 70.11, 62.26, 55.81, 51.24, 46.44, 45.69, 36.91, 34.13, 31.24, 30.96, 30.53, 30.45, 30.32, 29.91, 28.20, 27.34, 25.75, 16.79; LC-MS: R_t 6.96 min, linear gradient 10%→90% B in 12.5 min; ESI-MS: *m/z* = 797.07 $(M+H)^+$; HRMS: calculated for $C_{44}H_{55}BF_2N_6O_5$ [$M+H^+$] 796.44041, found: 796.44218.

Materials of biological assays.

Recombinant GBA was obtained from Genzyme (Cambridge, MA, USA). Fibroblast cell lines containing wild-type GBA and FUCA1 were cultured in HAMF12-DMEM medium (Invitrogen) supplied with 10% (v/v).

Molecular cloning and recombinant expression.

Confluent COS-7 cells were transfected with pcDNA3.1 empty vector (mock) or vector containing the coding sequence of *H. sapiens* FUCA1 (NCBI reference sequence XM_005245821.1; cloning described in conjunction with FuGENE (Roche).[11] After 72 hours, cells were harvested by scraping in potassium phosphate buffer (25 mM K_2HPO_4-KH_2PO_4, 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.

Enzyme activity assays and IC_{50} measurements.

The β-D-glucosidase activity of rGBA was assayed at 37 °C by incubating with 3.8 mM 4-methylumbelliferyl-β-D-glucopyranoside as substrate in 150 mM McIlvaine buffer, pH 5.2, supplemented with 0.2% (w/v) sodium taurocholate, 0.1% (v/v) Triton X-100 and 0.1% (w/v) BSA. The α-L-fucosidase activity of rFUCA1 was

determined by incubating with 1.5 mM 4-methylumbelliferyl-α-L-fucopyranoside in 150 mM McIlvaine buffer, pH 5.0, supplemented with 0.1% (w/v) BSA. The values obtained correspond to net α-L-fucosidase activity left after subtracting endogenous α-L-fucosidase activity present in lysate of mock-transfected COS-7 cells. To determine the apparent *in vitro* IC_{50} value, recombinant GBA or lysate of COS-7 cells, either mock or over-expressing FUCA1, was firstly pre-incubated with a range of inhibitor dilutions for 30 min at 37 °C, prior to addition of the substrate. 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 H_2O. The enzymatic reaction was quenched by adding excess NaOH-glycine (pH 10.6), after which fluorescence of liberated 4-methylumbelliferyl was measured with a fluorimeter LS55 (Perkin Elmer) using λ_{EX} 366 nm and λ_{EM} 445 nm. The *in situ* IC_{50} 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 K_2HPO_4-KH_2PO_4, 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. All IC_{50} values were determined by replicating each assay twice in duplo in two separate cell lines. Data was corrected for background fluorescence, then normalized to the untreated control condition and finally curve-fitted via one phase exponential decay function (GraphPad Prism 5.0). [8a,9,10,11]

***In vitro* labeling and SDS-PAGE analysis.**

All pre-incubations and ABP labeling-reactions occurred for 30 min at 37 °C, unless stated otherwise. The detection limit of each ABP was analyzed by labeling rGBA (10 pmol) or rFUCA (100 µg total protein in lysate of COS-7 cells over-expressing rFUCA1) with 10,000−0.01 nM ABP (**1**/**2**, **3**/**4**, respectively) for 30 min at 37 °C. Influence of pH on ABP labeling involved pre-incubation of the aforementioned enzyme/lysate at pH 3−10 for 30 min on ice, prior to addition of 100 nM ABP **1**/**2**, 1.0 µM ABP **5** or 10 µM ABP **6**, dissolved in Nanopure H_2O and incubating for 30 min at 37 °C. For ABPP, rGBA (10 pmol) or rFUCA (100 µg total protein in lysate of COS-7 cells over-expressing rFUCA1), or 100 µg total protein in lysate of human, wild-type GBA/ FUCA1 fibroblasts, was pre-incubated with compounds 10 mM AMP-DNM, 1.0 mM CBE, 100 µM JJB339, JJB103, JJB349, JJB261 or boiled for 4 min in 2% (w/v) SDS, prior to labeling with 100 nM ABP **1**/**2**, 1.0 µM ABP **5** or 10 µM ABP **6** (all dissolved in Nanopure H_2O) 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 whilst at appropriate McIlvaine conditions, where-after 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 appropriate McIlvaine buffer, over a new Zeba column. Samples were denatured with 5 × Laemmli buffer (50% (v/v) 1.0 M 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[8a, 9, 10, 11]. 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 red fluorescent ABPs **1**−**6**.

4.5 References

[1] a) D. J. Vocadlo, G. J. Davies, R. Laine and S. G. Withers, *Nature* **2001**, 412, 835-838; b) D. L. Zechel and S. G. Withers, *Acc. Chem. Res.* **2000**, *33*, 11-18; c) G. Davies and B. Henrissat, *Structure* **1995**, 3, 853-859.

[2] D. E. Koshland, *Biol. Rev.* **1953**, 28, 416-436.

[3] a) K. A. Stubbs, *Carbohydr. Res.* **2014**, 390, 9-19; b) L. I. Willems, J. Jiang, K. Y. Li, M. D. Witte, W. W. Kallemeijn, T. J. Beenakker, S. P. Schroder, J. M. Aerts, G. A. van der Marel, J. D. C. Codée and H. S. Overkleeft, *Chem. Eur. J.* **2014**, 20, 10864-10872; c) B. F. Cravatt, A. T. Wright and J. W. Kozarich, *Annu. Rev. Biochem.* **2008**, 77, 383-414.

[4] a) D. J. Vocadlo and C. R. Bertozzi, *Angew. Chem. Int. Ed.* **2004**, *43*, 5452-5456; b) K. A. Stubbs, A. Scaffidi, A. W. Debowski, B. L. Mark, R. V. Stick and D. J. Vocadlo, *J. Am. Chem. Soc.* **2008**, 130, 327-335; c) O. Hekmat, Y.-W. Kim, S. J. Williams, S. He and S. G. Withers, *J. Biol. Chem.* **2005**, 280, 35126-35135; d) J. D. McCarter and S. G. Withers, *J. Am. Chem. Soc.* **1996**, 118, 241-242; e) B. P. Rempel and S. G. Withers, *Glycobiology* **2008**, 18, 570-586.

[5] S. Atsumi, K. Umezawa, H. Iinuma, H. Naganawa, H. Nakamura, Y. Iitaka and T. Takeuchi, *J. Antibiot.* **1990**, *43*, 49-53; b) S. Atsumi, H. Iinuma, C. Nosaka and K. Umezawa, *J. Antibiot.* **1990**, *43*, 1579-1585.

[6] M. D. Witte, W. W. Kallemeijn, J. Aten, K.-Y. Li, A. Strijland, W. E. Donker-Koopman, A. M. C. H. van den Nieuwendijk, B. Bleijlevens, G. Kramer, B. I. Florea, B. Hooibrink, C. E. M. Hollak, R. Ottenhoff, R. G. Boot, G. A. van der Marel, H. S. Overkleeft and J. M. F. G. Aerts, *Nat. Chem. Biol.* **2010**, 6, 907-913.

[7] V. Lombard, H. Golaconda Ramulu, E. Drula, P. M. Coutinho and B. Henrissat, *Nucleic Acids Res.* **2014**, 42, D490-D495.

[8] a) M. D. Witte, M. T. Walvoort, K. Y. Li, W. W. Kallemeijn, W. E. Donker-Koopman, R. G. Boot, J. M. Aerts, J. D. C. Codée, G. A. van der Marel and H. S. Overkleeft, *ChemBioChem* **2011**, *12*, 1263-1269; b) M. T. Walvoort, W. W. Kallemeijn, L. I. Willems, M. D. Witte, J. M. Aerts, G. A. van der Marel, J. D. C. Codée and H. S. Overkleeft, *Chem. Commun.* **2012**, 48, 10386-10388.

[9] a) W. W. Kallemeijn, K. Y. Li, M. D. Witte, A. R. Marques, J. Aten, S. Scheij, J. Jiang, L. I. Willems, T. M. Voorn-Brouwer, C. P. van Roomen, R. Ottenhoff, R. G. Boot, H. van den Elst, M. T. Walvoort, B. I. Florea, J. D. C. Codée, G. A. van der Marel, J. M. Aerts and H. S. Overkleeft, *Angew. Chem. Int. Ed.* **2012**, *51*, 12529-12533; b) B. Chandrasekar, T. Colby, A. Emran Khan Emon, J. Jiang, T. N. Hong, J. G. Villamor, A. Harzen, H. S. Overkleeft and R. A. van der Hoorn, *Mol. Cell. Proteomics* **2014**, *13*, 2787-2800.

[10] L. I. Willems, T. J. M. Beenakker, B. Murray, S. Scheij, W. W. Kallemeijn, R. G. Boot, M. Verhoek, W. E. Donker-Koopman, M. J. Ferraz, E. R. van Rijssel, B. I. Florea, J. D. C. Codée, G. A. van der Marel, J. M. F. G. Aerts and H. S. Overkleeft, *J. Am. Chem. Soc.* **2014**, *136*, 11622-11625.

[11] J. Jiang, W. W. Kallemeijn, D. W. Wright, A. M. C. H. van den Nieuwendijk, V. C. Rohde, E. C. Folch, H. van den Elst, B. I. Florea, S. Scheij, W. E. Donker-Koopman, M. Verhoek, N. Li, M. Schurmann, D. Mink, R. G. Boot, J. D. C. Codée, G. A. van der Marel, G. J. Davies, J. M. F. G. Aerts and H. S. Overkleeft, *Chem. Sci.* **2015**, *6*, 2782-2789.

[12] a) K. Tatsuta, Y. Niwata, K. Umezawa, K. Toshima and M. Nakata, *J. Antibiot.* **1991**, *44*, 912-914; b) M. Nakata, C. Chong, Y. Niwata, K. Toshima and K. Tatsuta, *J. Antibiot.* **1993**, *46*, 1919-1922; c) K. Tatsuta, *Pure Appl. Chem.* **1996**, *68*, 1341-1346.

[13] K. Y. Li, J. Jiang, M. D. Witte, W. W. Kallemeijn, W. E. Donker-Koopman, R. G. Boot, J. M. F. G. Aerts, J. D. C. Codée, G. A. van der Marel and H. S. Overkleeft, *Org. Biomol. Chem.* **2014**, 12, 7786-7791.

[14] a) K. Y. Li, J. Jiang, M. D. Witte, W. W. Kallemeijn, H. van den Elst, C. S. Wong, S. D. Chander, S. Hoogendoorn, T. J. M. Beenakker, J. D. C. Codée, J. M. F. G. Aerts, G. A. van der Marel and H. S. Overkleeft, *Eur. J. Org. Chem.* **2014**, 6030-6043; b) L. I. Willems, T. J. M. Beenakker, B. Murray, B. Gagestein, H. van den Elst, E. R. van Rijssel, J. D. C. Codée, W. W. Kallemeijn, J. M. F. G. Aerts, G. A. van der Marel and H. S. Overkleeft, *Eur. J. Org. Chem.* **2014**, 6044-6056.

[15] T. Wennekes, R. J. van den Berg, W. Donker, G. A. van der Marel, A. Strijland, J. M. Aerts and H. S. Overkleeft, *J. Org. Chem*. **2007**, 72, 1088-1097.