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**Title:** On the reactivity and selectivity of donor glycosides in glycochemistry and glycobiology  
**Date:** 2012-10-18
Chapter 9

2-Deoxy-2-fluoroglucosides as Activity-based Probes for Retaining β-Glucosidases

Introduction
The study of enzyme activity, and in particular of (retaining) glycosidases, has benefitted greatly from the development of activity-based inhibitors, as described in Chapter 8.¹ There it was revealed that cyclophellitol-based probes (A, Figure 1) were much more potent in activity-based profiling of acid β-glucosidase (GBA) than 2-deoxy-2-fluoroglucosides (B and C, Figure 1). While the fluorine atom is generally regarded to be a good mimic for the hydroxyl function at C-2, both in size and in polarity, its high electronegativity has a deactivating effect on the probe. To be used as an activity-based probe (ABP), the fluorine is most often introduced at the C-2 or C-5 position, the sites closest to the anomeric center, to retard glycosidic bond hydrolysis of the covalent enzyme-inhibitor adduct (for mechanistic details, see Chapter 8). To enable the glycosylation step to occur, a reactive anomeric group, generally a fluoride or nitrophenyl,² is installed. The inherently poorer affinity of the fluoroglucoside inhibitors for GBA may be attributed to the lower reactivity of the anomeric aglycones, as compared to the epoxide in the cyclitol-based inhibitors, on top of the deactivating effect of the fluorine at C-2. Therefore it was hypothesized that the 2-deoxy-2-fluoride probes could evolve into better inhibitors by tuning the leaving group capacity of the anomeric moiety.

Together with the development of electron-deprived glycoside probes as glycosidase inhibitors by Withers and co-workers in the 1980s, anomeric fluorides were introduced as good leaving groups (B). An important observation was that the anomeric fluoride did not need enzymatic protonation to be expelled, allowing the use of such probes for kinetic studies with (acid/base) mutant enzymes. With the increasing and effective use of fluoroglycoside probes in research on enzymatic mechanisms and active sites, the need for a chromogenic aglycone arose, which would allow for in situ fluorescence monitoring of the inhibition reaction. To this end, anomeric p-nitro- and 2,4-dinitrophenyl ethers (C) were installed on various fluoroglycosides and successfully used in activity-based enzymatic profiling studies.

Current state-of-the-art in activity-based protein profiling research makes use of one of the anomeric leaving groups mentioned above. However, when the design of a suitable ABP is approached from a synthetic carbohydrate chemistry viewpoint, several other anomeric leaving groups can be considered. Recently, Withers et al. have reported on a comparative study using different anomeric phosphates to tailor the specificity and reactivity of 2-deoxy-2-fluoroglycoside probes for GBA, both as inhibitors and as chaperones. Increasing the lipophilicity of the anomeric phosphate moiety caused a large increase in potency towards GBA, supposedly due to resemblance in polarity of the aglycone to the ceramide moiety of the natural substrate.

This Chapter describes the comparative survey of a set of 2-deoxy-2-fluoro probes bearing different anomeric leaving groups for their inhibitory potential and use in activity-based profiling of GBA. These probes were compared to the known anomeric fluoride and 2,4-dinitrophenyl probes, as described in Chapter 8. The 2-deoxy-2-fluoro carbohydrate core was decorated with a BODIPY fluorophore to allow fluorescence evaluation of binding efficiency.

**Results and Discussion**

The four different anomeric functionalities selected for this comparative study are depicted in Figure 2, and include, next to the common fluoride (1) and 2,4-dinitrophenyl (2), the anomeric (S)-tolyl 3, diastereomERICALLY pure yet stereoERICALLY unidentified sulfoxides 4 and 5, N-phenyl trifluoroacetimidate 6, and diphenylphosphate 7. These probes are equipped with a green-fluorescent BODIPY using ‘click’ chemistry.
Synthesis of the probes. The stereoselectivity of the electrophilic fluorination of D-glucal with Selectfluor® has been shown to depend greatly on the protecting group pattern.\(^8\) Whereas the per-acetylated D-glucal roughly produced a 1:1 epimeric gluco:manno mixture (see Chapter 8), the per-pivaloylated D-glucal 9 revealed a high preference for the gluco epimer.\(^8\) Therefore, this strategy was applied here in the synthesis of probes 3-7 as depicted in Scheme 1. Thus, commercially available 3,4,6-tri-O-acetyl-D-glucal was deacetylated using Zemplén conditions, and the triol was directly pivaloylated to give 9 in 60% over two steps. Fluorination using Selectfluor in MeNO\(_2\)/H\(_2\)O yielded 66% of the gluco epimer 10 after ensuing acetylation and column chromatography. Subsequent anomic bromination (HBr/AcOH) and direct substitution with p-thiocresol using phase-transfer conditions exclusively gave β-thioglucoside 12 in 96% over two steps. The pivaloyl esters were removed by prolonged treatment with NaOMe in MeOH (5 days) to produce triol 13. The azido functionality was introduced by selective tosylation of 6-OH (Ts-Cl, tetramethylethylenediamine) and substitution with NaN\(_3\) while heating at 80 °C overnight to yield product 14 in 63% over two steps. Compound 14 was used in the copper-catalyzed click reaction with alkyne 8 to produce direct probe 3 in 44% yield. To produce probes 4-7, compound 14 was first acetylated and subsequently treated with NBS in acetone/H\(_2\)O. Because it was observed before that the anomic thio functionality was readily oxidized with aqueous NBS (see Chapter 8), these conditions were applied in this synthetic scheme. In this way, sulfoxide 15 (mixture of diastereomers on sulfur) was obtained in 59% yield, next to hemiacetal byproduct (29%). Removal of the acetyl in 15 (NaOMe, MeOH) provided compound 16, which was coupled to the BODIPY-moiety to produce a diastereomeric mixture of sulfoxides 4/5. Using RP-HPLC the diastereomers were separated to give direct probes 4 and 5 in 20% and 18% yield, respectively. Sulfoxide 16 was efficiently hydrolyzed towards hemiacetal 17 (94%) by treatment with NBS for 3 h. To access the more labile anomic imidate probe 6 and phosphate probe 7, it was decided to install the BODIPY-moiety prior to anomic leaving group introduction. Thus, hemiacetal 17 was connected to alkyne 8 under the standardized click conditions to produce compound 18 in 53%. Subsequently, an anomic mixture of N-phenyl trifluoracetimidates was produced under mild basic conditions, which were resolved using RP-HPLC (NH\(_4\)OAc). Subsequent lyophilization afforded the pure β-anomer 6 in 15% and
α-anomer 19 in 10%. In a first attempt to obtain anomeric phosphate 7, the anomeric mixture of imidates was treated with diphenylphosphoric acid to give immediate and quantitative conversion to an anomeric mixture of phosphates. While this mixture was separable on RP-HPLC, the β-phosphate 7 did not withstand lyophilization in the presence of aqueous NH₄OAc. To circumvent this hydrolysis, pure α-imidate 19 was substituted by diphenylphosphate in an S₉₂-like reaction to yield β-phosphate 7, which was purified using flash column chromatography and subsequently lyophilized under neutral conditions.

Scheme 1. Synthesis of 2-fluoro β-glucoside probes 3-7

Reagents and conditions: a) NaOMe, MeOH; b) Piv-Cl, DMAP, pyridine (9: 60% two steps); c) i. Selectfluor®, MeNO₂/H₂O; ii. Ac₂O, pyridine, DCM (10: 66%); d) HBr/AcOH, DCM; e) TolSH, TBAB, KOH, CHCl₃/H₂O (12: 96%; two steps); f) NaOMe, MeOH (13: quant.); g) i. Ts-Cl, TMEDA, MeCN; ii. NaN₃, DMF, 80 ºC (14: 63% over two steps); h) BODIPY-alkyne 8, sodium ascorbate, CuSO₄, DMF, 80 ºC (3: 44%, 4: 20%, 5: 18%, 18: 53%); i) Ac₂O, pyridine; j) NBS, acetone/H₂O (15: 39% over two steps, 17: 94%); k) NaOMe, MeOH (16: quant.); l) CF₃C(NPh)Cl, K₂CO₃, acetone (6: 15%, 19: 10%); m) HOP(O)(OPh)₂, DCM (7: 59%).

Biological evaluation. The inhibitory potentials of probes 1-7 for GBA were (re-)established by determining their apparent IC₅₀ values (Table 1). This was accomplished by incubating recombinant GBA for 30 min with different concentrations of probes 1-7 (1 mM to 10 nM), followed by measuring the residual enzymatic activity using the fluorogenic
substrate 4-methylumbelliferyl β-D-glucopyranoside. The inhibition curves are shown in Figure 3 (left). While fluoride probe 1 inhibited GBA (Figure 3, □), it was not possible to determine an IC$_{50}$ value because the inhibition did not converge to zero. In this experiment, 2,4-dinitrophenyl probe 2 did not show significant inhibition of GBA, and thioether probe 3 and sulfoxide probes 4 and 5 all revealed no inhibition of GBA at all. In contrast, imidate probe 6 blocked all activity at the highest concentrations used (Figure 3, ★), and its IC$_{50}$ value was determined to be 5.5 µM, indicating that probe 6 is twice as potent as conduritol B epoxide (CBE) for GBA (9.49 µM). Phosphate probe 7 showed some enzyme inhibition at lower concentrations than fluoride probe 1, although its IC$_{50}$ value could not be determined accurately (Figure 3, ‡).

### Table 1. Apparent IC$_{50}$ values

<table>
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<tr>
<th>Probe</th>
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<td>0.0012$^b$</td>
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<tr>
<td>CBE</td>
<td>9.49$^b$</td>
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$^a$ Using probe concentrations up to 1 mM, no complete inhibition was observed. $^b$ Reported literature values.

(Note: all BODIPYs in this table are green-fluorescent)

To prove that the abolished enzyme activity was a result of inhibition of active enzyme via a covalent inhibitor-enzyme intermediate, 2 picomol of GBA was incubated with different concentrations of probes 1, 2, 6 and 7 for 30 min, followed by separation of the proteins on SDS-PAGE and visualization of the enzyme mixture after fluorescent scanning of slab gels. As shown in Figure 3 (right), fluorescently labeled enzyme could be observed for probes 1, 6 and 7, while 2,4-dinitrophenyl probe 2 showed no labeling even at 50 µM. Fluoride probe 1 could visualize GBA down to 5 µM, the same concentration as phosphate probe 7. The apparent IC$_{50}$ value obtained for imidate probe 6 is reflected in the detection limit and fluorescently labeled GBA could be visualized using as little as 500 nM of probe 6. While the gel depicted in Figure 3 reveals that imidate probe 6 is not as potent as cyclitol- and aziridine-based probes MDW941 and MDW1044 (labeling GBA in the picomolar range),
the minimal concentration for labeling is 100-fold lower than the concentration required for fluoride probe 1, and not comparable to probe 2 which did not bind at all. Having established that probes 1, 6 and 7 bind GBA in a covalent manner, the hypothesis of activity-based binding to the active site was validated. To this end, a solution of recombinant GBA was pre-incubated with known inhibitors (CBE, cyclophellitol, MDW941, and AMP-DNM) or denatured by heating, followed by incubation with probes 1, 6 and 7 (data not shown). Fluorescent scanning analysis of the slab gels after electrophoresis revealed no labeling in all cases, proving that active and intact enzyme is needed for labeling.

**Figure 3.** Inhibition curves and detection limit of fluoride 1 (◻), DNP 2 (▲), thioether 3 (×), imidate 6 (◆), and phosphate 7 (◇), as compared to the cyclitol (MDW941, ◆) and aziridine (MDW1044) analogs. Left: inhibition curves of GBA. Right: labeling of recombinant GBA (● = imiglucerase labeled with 1nM of MDW933 and MDW941)

Recombinant GBA was incubated with the probe at the indicated concentrations for 60 min, denatured, resolved by SDS-PAGE and visualized by scanning.

The results presented above indicate that probes 6 and 7 inhibit GBA in an equal fashion or better than fluoride probe 1. This difference may be explained by assuming a different inhibitory mechanism. Considering the proposed mechanism of enzymatic hydrolysis (see Chapter 8), in which the acid/base residue catalyzes the reaction while the nucleophile covalently traps the inhibitor, probes 1, 6 and 7 might display different mechanistic requirements. To investigate the intermediacy of the acid/base residue in the processing of
these probes, GBA was pre-incubated at different pH values, followed by labeling with either probe 1, 6 or 7 for 30 min at 37 °C. Analysis of the labeled enzyme using slab gel electrophoresis and ensuing fluorescent scanning revealed that the three probes all labeled GBA at pH values between 5.0 and 6.0, while imidate probe 6 and, to a higher extent, cyclitol-analogue MDW941 also labeled faintly at pH 7.0 (Figure 4). This similarity in pH-dependent labeling is an indication that probes 1, 6 and 7 at least need active GBA enzyme, since the optimal pH for enzyme activity is pH 5.2.

Figure 4. pH-dependent labeling

Recombinant GBA was incubated at the indicated pH for 30 min, followed by incubation with the probe (MDW941: 1 nM, 1: 50 µM, 6: 500 nM, 7: 5 µM) for 30 min, denatured, resolved by SDS-PAGE and visualized by scanning (● = imiglucerase labeled with 1nM of MDW933 and MDW941).

The requirement of probes 1, 6 and 7 for catalysis by the acid/base residue was evaluated using mutant GBA enzyme, in which the glutamic acid residue (E235) was substituted for a glycine (E235G) or a glutamine (E235Q). Homogenates of cells over-expressing wild-type or mutant mycHis-tagged GBA were incubated with probes 1, 6 and 7 for 2 h and 24 h, followed by pull-down of the (labeled) mutant GBA with nickel-agarose beads. As displayed in Figure 5 (left), labeling of the wild-type enzyme was observed with all probes upon incubation for 2 h. Interestingly, incubation with the probes for 24 h revealed a different behavior of the probes (Figure 5, right). Fluoride probe 1 labeled both GBA variants with the mutated acid/base residues, while imidate 6 and phosphate 7 were incapable of binding the mutant GBA enzymes. Aziridine-based probe MDW1044 evidently labeled the two mutant enzymes after 2 h, and epoxide-based probe MDW933 labeled the glutamine-mutant after 24 h incubation. It follows from these results that fluoride probe 1 does not require acid/base catalyzed protonation to bind covalently in the active site of GBA, similar to the aziridine probe, albeit with a markedly lower labeling velocity and decreased affinity considering the concentrations used (MDW933, MDW1044: 1 µM, probe 1: 100 µM). On the contrary, the labeling experiment with probes 6 and 7 confirmed that the presence of the acid/base catalyst was a prerequisite for their active binding, analogous to the synthetic activation of imidate and phosphate moieties under acidic conditions.
Figure 5. Labeling of wild-type and acid/base mutants of GBA after incubation for 2 hours (left) and 24 hours (right)

Homogenates over-expressing wild-type or mutant GBA were incubated with the probe (MDW1044, MDW933: 1 µM, 1: 100 µM, 6: 1 µM, 7: 10 µM) for 2 h or 24 h, denatured, either directly resolved by SDS-PAGE or subjected to Ni-beads pull-down prior to SDS-PAGE, and visualized by scanning (● = imiglucerase labeled with 1nM of MDW933 and MDW941).

The ability of probes 1, 6 and 7 to label GBA in living cells was also investigated. To this end, confluent human skin fibroblasts were grown in the presence of 1 or 10 µM of the fluoroglycoside probes (compared to 1 or 10 nM for MDW933 and MDW1044) for 2 hours and 24 hours (see Figure 6 and Appendix 4). After lysis of the cells, the lysates were treated with red-fluorescent MDW941 to label any free enzyme. Ensuing slab gel electrophoresis and fluorescent scanning provided the pictures in Figure 6A, and the quantification of residual labeling is shown in Figure 6B.

Figure 6. Labeling of GBA in human skin fibroblasts using green-fluorescent probes for 2 hours (A) (see Appendix 4 for a colored picture), and the percentage of residual labeling by red-fluorescent MDW941 (B)

Confluent fibroblasts were incubated with the probe for 2 h or 24 h and lysed, followed by incubation with MDW941 for 30 min. Proteins were denatured, resolved by SDS-PAGE and visualized by scanning (● = imiglucerase labeled with 1nM of MDW933 and MDW941).
Imidate 6 labeled GBA at a concentration of 1 µM after 2 h (left, green trace), allowing 49% of residual labeling by MDW941 (left, red trace). After labeling for 24 h, the residual labeling decreased to 15%. Incubating with 10 µM of probe 6 for 2 h resulted in complete covalent blocking of the enzyme (right, green trace), with only minimal residual labeling (6%), which did not decrease further after 24 h. Fluoride probe 1 only showed labeling with 10 µM, resulting in 70% residual labeling after 2 h, and 26% after 24 h. Phosphate probe 7 gave a significant amount of residual labeling (71%) after 24 h at the highest concentration (10 µM). In this last case, it may be argued whether the phosphate moiety is preserved in living cells before it reaches the lysosomal GBA, or that it is attacked by other (phosphatase) enzymes, or hydrolyzed.\textsuperscript{15}

**Conclusion**

In summary, a series of BODIPY-functionalized 2-deoxy-2-fluoro-β-glycosides was synthesized, bearing anomeric fluoride, 2,4-dinitrophenyl, (S)-tolyl, (S)\textsubscript{R/S}-sulfoxide, N-phenyl trifluoroacetimidate, and diphenylphosphate leaving groups. These compounds were tested for their inhibitory potential against glucocerebrosidase (GBA), revealing that only imidate probe 6 was able to fully block the enzyme activity, with a lower apparent IC\textsubscript{50} than conduritol B epoxide (CBE). Probe 6 labels GBA as an activity-based covalent inhibitor, enabling the use of 500 nM to visualize GBA on slab gels. Mutant GBA lacking the acid/base catalyst was not labeled by imidate 6, while fluoride probe 1 did reveal covalent binding to this mutant enzyme, although at a low kinetic rate. And finally, probe 6 labeled endogenous GBA in human skin fibroblasts already after 2 h using 1 µM concentration. This study thus revealed that novel imidate probe 6 is an excellent candidate to probe enzyme activity, and is a mechanism-based inhibitor. Although not as potent as cyclitol- or aziridine-based probes, its ease of synthesis regardless of carbohydrate configuration renders this probe highly suitable in the design of ABPs targeting other retaining glycosidases.

**Experimental Section**

**Probe 3.** Compound 14 (20 mg, 67 µmol) and BODIPY-alkyne 8 (24 mg, 73 µmol) were together dissolved in DMF (1.5 mL) and treated with sodium ascorbate (10 µL, 1M solution in H\textsubscript{2}O) and CuSO\textsubscript{4} (7 µL, 1M solution in H\textsubscript{2}O). The resulting mixture was stirred at 80 ºC for 2 days, during which time the addition of sodium ascorbate and CuSO\textsubscript{4} was repeated twice. The mixture was allowed to cool to RT and diluted with EtOAc and H\textsubscript{2}O. The organic phase was washed with sat. aq. NaCl, dried over Na\textsubscript{2}SO\textsubscript{4} and the product was obtained using flash column chromatography (silica gel, 4% MeOH in DCM) followed by lyophilization as an orange solid (Yield: 18.8 mg, 29.3 µmol, 44%). TLC: R\textsubscript{f} 0.32 (DCM/MeOH, 9/1, v/v); IR (neat, cm\textsuperscript{-1}): 894, 1065, 1508, 1551, 3394; \textsuperscript{1}H NMR (CDCl\textsubscript{3}/MeOH-d\textsubscript{4}, 400 MHz, HH-COSY, HSQC): δ 7.26 (d, 2H, J = 8.0 Hz, CH\textsubscript{arom}), 7.05 (d, 2H, J = 7.9 Hz, CH\textsubscript{arom}), 6.06 (s, 2H, CH pyrrole), 4.79 (dd, 1H, J = 2.1, 14.5 Hz, H-6), 4.57 (d, 1H, J = 9.3 Hz, H-1), 4.46 (dd, 1H, J = 7.0, 14.5 Hz, H-6), 3.94 (dt, 1H, J = 9.0, 49.6 Hz, H-2), 3.68 (dt, 1H, J = 7.7, 15.4 Hz, H-3), 3.50-3.60 (m, 1H, H-5), 3.09 (t, 1H, J = 9.4 Hz, H-4), 2.99 (dd, 2H, J = 6.6, 10.1 Hz, CH\textsubscript{2}), 2.74 (t, 2H, J = 7.5 Hz, CH\textsubscript{2}), 2.50 (s, 6H, CH\textsubscript{3}), 2.39 (s, 6H, CH\textsubscript{3}), 2.31 (s, 3H, CH\textsubscript{3}STol), 1.85-1.94 (m, 2H, CH\textsubscript{2}), 1.63-1.71 (m, 2H, CH\textsubscript{2}). \textsuperscript{13}C-APT NMR (CDCl\textsubscript{3}, 100 MHz, HSQC): δ 153.9, 147.3, 145.8, 140.2, O\textsubscript{F} HO HO STol N NN N B N F
Probes 4 and 5. Compound 16 (25 mg, 78 µmol) and BODIPY-alkyne 8 (28 mg, 85 µmol) were together dissolved in DMP (1 mL) and treated with sodium ascorbate (12 µL, 1M solution in H2O) and CuSO4 (8 µL, 1M solution in H2O). The resulting mixture was stirred at 80 °C for 2 days, during which time the addition of sodium ascorbate and CuSO4 was repeated twice. The mixture was allowed to cool to RT and diluted with EtOAc and H2O. The organic phase was washed with sat. aq. NaCl, dried over Na2SO4 and the product was isolated using flash column chromatography (silica gel, 10% MeOH in DCM). The two diastereomers were separated using RP-HPLC followed by lyophilization to yield 4 (Yield: 10.1 mg, 15% µmol, 20%) and 5 (Yield: 9.5 mg, 14.4 µmol, 18%) both as orange solids. TLC: Rf 0.45 (DCM/MeOH, 8.5/1.5, v/v); IR (neat, cm−1): 984, 1080, 1200, 1508, 1551, 3406. Spectroscopic data for product 4: 1H NMR (MeCN-d3, 600 MHz, HH-COSY, HSQC): δ 7.32 (d, 2H, J = 7.9 Hz, CHarom), 7.16 (d, 2H, J = 8.2 Hz, CHarom), 6.76 (s, 1H, CH triazole), 6.08 (bs, 2H, CH pyrrole), 4.57 (dt, 1H, J = 14.8 Hz, H-6), 4.47 (dt, 1H, J = 9.3, 50.4 Hz, H-2), 4.14 (dd, 1H, J = 14.8, 14.8 Hz, H-6), 4.01 (dd, 1H, J = 2.9, 9.7 Hz, H-1), 3.67 (dt, 1H, J = 8.9, 15.5 Hz, H-3), 3.44 (t, 1H, J = 8.3 Hz, H-5), 3.14 (t, 1H, J = 9.4 Hz, H-4), 2.89 (ddd, 2H, J = 5.0, 12.9, 13.0, 25.2 Hz, CH2), 2.41-2.59 (m, 2H, CH2), 2.36 (s, 12H, CH2Tol), 1.64-1.78 (m, 2H, CH2), 1.33-1.52 (m, 2H, CH2); 13C-APT NMR (MeCN-d3, 150 MHz, HSQC): δ 148.3, 147.8, 142.7, 136.7 (C2), 130.8, 126.2, 123.0, 122.6 (CHarom), 90.7 (d, J = 24 Hz, C-1), 88.6 (d, J = 183 Hz, C-2), 80.5 (C-5), 76.0 (d, J = 17 Hz, C-3), 71.4 (d, J = 8 Hz, C-4), 51.6 (C-6), 31.8, 30.4, 29.0, 25.8 (CH2), 21.7 (CH2Tol), 16.6, 14.6 (CH2); LC-MS: Rf 7.79 min (C18 column, linear gradient 10 → 90% B in 13.5 min); HRMS: [M+Na]+ calc for C24H24BF8N6O9SNa 680.26601, found 680.26583. Spectroscopic data for product 5: 1H NMR (MeCN-d3, 600 MHz, HH-COSY, HSQC): δ 7.46 (d, 2H, J = 11.7 Hz, CHarom), 7.34 (d, 2H, J = 8.1 Hz, CHarom), 6.17 (bs, 2H, CH pyrrole), 4.73 (dd, 1H, J = 2.1, 14.7 Hz, H-6), 4.40-4.51 (m, 3H, H-1, H-2, H-6), 3.75 (ddd, 1H, J = 2.1, 7.4, 9.6 Hz, H-5), 3.66-3.69 (m, 1H, H-3), 3.10 (t, 1H, J = 9.2 Hz, H-4), 3.03-3.07 (m, 2H, CH2), 2.78 (t, 2H, J = 7.3 Hz, CH2), 2.46 (s, 6H, CH3), 2.44 (s, 6H, CH3), 2.40 (s, 3H, CH3Tol), 1.89-1.95 (m, 2H, CH2), 1.67-1.72 (m, 2H, CH2); 13C-APT NMR (MeCN-d3, 150 MHz, HSQC): δ 130.6, 125.8, 123.7 (CHarom), 122.7 (CH pyrrole), 93.1 (d, J = 24 Hz, C-1), 87.8 (d, J = 185 Hz, C-2), 79.6 (C-5), 76.2 (d, J = 18 Hz, C-3), 71.1 (d, J = 8 Hz, C-4), 51.4 (C-6), 31.9, 30.5, 29.0, 25.9 (CH2), 20.3 (CH2Tol), 16.6 (CH2); LC-MS: Rf 8.00 min (C18 column, linear gradient 10 → 90% B in 13.5 min); HRMS: [M+Na]+ calc for C24H24BF8N6O9SNa 658.28407, found 658.28426.

Probe 6. A solution of compound 18 (17 mg, 32 µmol) in acetonitrile (2 mL) was cooled to 0 °C, followed by the addition of N-phenyl trifluoroacetimidoyl chloride (10 µL, 63 µmol) and K3CO3 (6 mg, 43 µmol). The reaction was stirred at RT overnight, after which the mixture was diluted with EtOAc. The organic phase was washed with sat. aq. NaCl, dried over Na2SO4 and concentrated in vacuo. Purification using flash column chromatography (silica gel, 87% EtOAc in PE) yielded an anomic mixture of imidates. The anomers were separated using RP-HPLC to give β-anomer 6 (Yield: 3.4 mg, 4.8 µmol, 15%) and α-anomer 9 (Yield: 2.2 mg, 3.0 µmol, 10%) both as orange solids. TLC: Rf 0.64 (DCM/MeOH, 8.5/1.5, v/v); IR (neat, cm−1): 986, 1082, 1161, 1202, 1510, 1551, 1719, 3383. Spectroscopic data for the β anomer 6: 1H NMR (MeCN-d3, 600 MHz, HH-COSY, HSQC, T = 335 K): δ 7.57 (s, 1H, CH triazole), 7.31 (t, 2H, J = 7.9 Hz, CHarom), 7.14 (t, 1H, J = 7.5 Hz, CHarom), 6.76 (d, 2H, J = 7.5 Hz, CHarom), 6.18 (s, 2H, CH pyrrole), 5.68 (bs, 1H, H-1), 4.81 (dd, 1H, J = 1.7, 14.6 Hz, H-6), 4.42 (dd, 1H, J = 8.2, 14.7 Hz, H-6), 4.33 (dt, 1H, J = 8.4, 51.5 Hz, H-2), 3.72-3.79 (m, 1H, H-3), 3.65-3.72 (m, 1H, H-3), 3.35 (t, 1H, J = 9.3 Hz, H-4), 3.01 (t, 2H, J = 8.8 Hz, CH2), 2.59-2.71 (m, 2H, CH2), 2.49 (s, 6H, CH3), 2.41 (s, 6H, CH3), 1.78-1.86 (m, 2H, CH2), 1.54-1.67 (m, 2H, CH2); 13C-APT NMR (MeCN-d3, 150 MHz, HSQC, T = 330 K): δ 154.9, 148.6, 148.5, 144.5, 142.6, 132.6 (C1), 130.2, 125.9 (CHarom), 123.5 (CH triazole), 122.9 (CH pyrrole), 120.3 (CHarom), 95.9 (d, J = 25 Hz, C-
Probe 7, α-Imidate (19) (2.2 mg, 3 µmol) was dissolved in dry DCM (1.5 mL) under an argon atmosphere. The resulting solution was cooled to 0 °C and treated with diphenyl phosphate (~ 1 mg, 3.5 µmol) for 20 min, after which time the reaction was halted by the addition of sat. aq. NaHCO₃ (2 mL). The mixture was diluted with EtOAc, the organic layer was washed with sat. aq. NaCl, dried over Na₂SO₄, and concentrated in vacuo. Purification using flash column chromatography (silica gel, 10% MeOH in EtOAc) and subsequent lyophilization afforded the title compound as an orange amorphous solid (Yield: 1.4 mg, 1.8 µmol; TLC: Rò.0.22 (EtOAc); IR (neat, cm⁻¹): 974, 1080, 1161, 1202, 1510, 1551, 2292, 3337; ¹H NMR (MeCN-d₃, 600 MHz, HH-COSY, HSQC): δ 7.38-7.45 (m, 4H, CH₃pyrrole), 7.25-7.31 (m, 2H, CH₁arom), 7.20-7.25 (m, 4H, CH₁arom), 6.17 (s, 2H, CH pyrrole), 5.49 (dd, 1H, J = 2.7, 7.3, 1.7 Hz, H-1), 4.73 (dd, 1H, J = 1.8, 14.7 Hz, H-6), 4.45 (dd, 1H, J = 7.5, 14.8 Hz, H-6), 4.20 (dt, 1H, J = 8.4, 51.3 Hz, H-2), 3.83-3.87 (m, 1H, H-5), 3.70-3.77 (m, 1H, H-3), 3.25 (t, 1H, J = 9.3 Hz, H-4), 2.95-2.99 (m, 2H, CH₂), 2.56-2.61 (m, 2H, CH₂), 2.46 (s, 6H, CH₃), 2.39 (s, 6H, CH₃), 1.74-1.80 (m, 2H, CH₂), 1.57-1.65 (m, 2H, CH₂); ¹³C-APT NMR (MeCN-d₃, 150 MHz, HSQC): δ 154.6, 148.3, 148.2, 142.4, 132.2 (C₁), 139.1, 130.1, 126.9, 123.8, 121.6 (CH₁arom, CH triazole), 122.6 (CH pyrrole), 121.1, 121.1 (CH₁arom), 97.7 (dd, J = 6, 25 Hz, C-1), 92.9 (dd, J = 9, 187 Hz, C-2), 76.4 (C-5), 74.7 (dd, J = 2, 17 Hz, C-3), 71.3 (d, J = 8 Hz, C-4), 51.1 (C-6), 31.9, 30.4, 29.0, 26.0 (CH₂), 16.6, 14.6 (CH₂); LC-MS: Rₚ. 9.44 min (C18 column, linear gradient 10 → 90% B in 13.5 min); HRMS: [M+H]⁺ calcd for C₁₆H₁₂BF₅N₄O₇, 707.29463, found 707.29459.

3,4,6-Tri-O-pivaloyl-D-glucal (9). 3,4,6-Tri-O-acetyl-D-glucal (13.6 g, 50.0 mmol) was dissolved in MeOH (500 mL) and treated with NaOMe (0.27 g, 5 mmol) overnight at RT. The mixture was neutralized by the addition of AcOH, and the solvents were evaporated. The residue was repeatedly co-evaporated with toluene. The crude triol (~24 mmol) was dissolved in pyridine (120 mL) and treated with NaOMe (0.27 g, 5 mmol) over night at RT. The mixture was neutralized by the addition of AcOH, and the solvents were evaporated. The residue was dissolved in EtOAc and washed with H₂O and sat. aq. NaCl. The organic phase was dried over Na₂SO₄ and concentrated in vacuo. Purification using flash column chromatography (silica gel, 100% PE) yielded the title compound as a colored oil (Yield: 5.77 g, 14.5 mmol, 60% over two steps). The spectroscopic data were in full accord with those reported previously.¹¹H NMR (CDCl₃, 400 MHz, HH-COSY, HSQC): δ 8.646 (dd, 1H, J = 1.2, 6.2 Hz, H-1), 5.30-5.33 (m, 1H, H-3), 5.28 (dd, 1H, J = 5.9, 7.4 Hz, H-4), 4.82 (dd, 1H, J = 3.1, 6.2 Hz, H-2), 4.33 (dd, 1H, J = 5.5, 11.7 Hz, H-6), 4.25-4.30 (m, 1H, H-5), 4.21 (dd, 1H, J = 2.5, 11.7 Hz, H-6), 1.23 (s, 9H, CH₃Bu), 1.19 (s, 9H, CH₃Bu), 1.18 (s, 9H, CH₃Bu); ¹³C-APT NMR (CDCl₃, 100 MHz, HSQC): δ 178.1, 177.7, 176.5 (C=O Piv), 145.6 (C-1), 99.0 (C-2), 74.1 (C-5), 67.5 (C-3), 66.6 (C-4), 61.3 (C-6), 38.8, 38.7, 38.7 (C₄Bu), 27.0, 27.0, 27.0 (CH₂Bu).
Acetyl 2-deoxy-2-fluoro-3,4,6-tri-O-pivaloyl-D-β-D-glucopyranoside (10). 3,4,6-Tri-O-pivaloyl-D-glucal 9 (5.77 g, 14.48 mmol) was dissolved in nitromethane/H2O (60 mL, 5/1, v/v), and Selectfluor (6.16 g, 17.38 mmol) was portion-wise added at RT. The resulting mixture was stirred for 2 days, followed by heating at reflux (95 °C) for 1 h. The mixture was cooled to RT and concentrated in vacuo. The residue was taken up in EtOAc and washed with sat. aq. NaHCO3 (2x) and sat. aq. NaCl (2x). The organic phase was dried over Na2SO4 and concentrated in vacuo. The residue was subsequently dissolved in DCM (50 mL) and treated with Ac2O (1.6 mL) and pyridine (2.1 mL) overnight. The mixture was concentrated in the presence of toluene, and the product was isolated using flash column chromatography (silica gel, 9% EtOAc in PE) as a colorless oil (Yield: 4.56 g, 9.56 mmol, 66%. α: β = 1 : 2). The spectroscopic data were in full accord with those reported previously.16 TCL: Rf 0.53 (PE/EtOAc, 5/1, v/v); 1H NMR (CDCl3, 300 MHz, HH-COSY, HSQC): δ 6.41 (d, 0.5H, J = 3.8 Hz, H-1α), 5.80 (dd, 1H, J = 3.0, 8.1 Hz, H-ββ), 5.59 (dd, 0.5H, J = 10.0, 21.0 Hz, H-3α), 5.44 (dt, 1H, J = 9.3, 14.2 Hz, H-3β), 5.10–5.18 (m, 0.5H, J = 4.5, 4.6, 10.0 Hz, H-5β), 2.20 (s, 1.5H, CH3), 1.17 (s, 3H, CH3, Ac-β). 13C-NMR (CDCl3, 75 MHz, HSQC): δ 177.8, 176.9, 176.4 (C=O Piv), 168.6 (C=O Ac), 91.2 (d, J = 24 Hz, C-1β), 88.5 (d, J = 191 Hz, C-2β), 88.3 (d, J = 22 Hz, C-1α), 86.6 (d, J = 194 Hz, C-2α), 73.0 (C-5β), 72.1 (d, J = 19 Hz, C-3β), 70.0 (C-5α), 69.9 (d, J = 19 Hz, C-3α), 66.9 (d, J = 7 Hz, C-6α), 66.5 (d, J = 7 Hz, C-4α), 61.3 (C-6β), 61.1 (C-6α), 38.7, 38.7 (C8tBu), 26.9, 26.9 (CH3tBu), 20.7 (CH3Ac-α), 20.6 (CH3Ac-β).

Tolyl 2-deoxy-2-fluoro-3,4,6-tri-O-pivaloyl-1-thio-D-glucopyranoside (12). A solution of compound 10 (0.93 g, 1.97 mmol) in dry DCM (3 mL) was cooled to 0 °C, and HBr/AcOH (33 wt%, 1.8 mL, 177.8 mmol) was added. The resulting solution was stirred at RT overnight, after which time it was poured in ice-water. The organic phase was diluted with EtOAc, washed with H2O, sat. aq. NaHCO3 and sat. aq. NaCl, dried over Na2SO4, and concentrated in vacuo in the presence of toluene. The crude anomic bromide 11 was used in the next reaction without further purification. TCL: Rf 0.80 (PE/EtOAc, 5/1, v/v); 1H NMR (CDCl3, 400 MHz, HH-COSY, HSQC): δ 6.52 (d, 1H, J = 4.2 Hz, H-1), 5.66 (dt, 1H, J = 9.6, 20.4 Hz, H-3), 5.15 (s, 1H, J = 10.0 Hz, H-4), 4.49 (ddd, 1H, J = 4.3, 9.4, 49.5 Hz, H-2), 4.32 (dt, 1H, J = 3.2, 10.4 Hz, H-5), 4.14–4.20 (m, 2H, H-6), 1.21 (s, 9H, CH3tBu), 1.18 (s, 9H, CH3tBu), 1.17 (s, 9H, CH3tBu), 13C-NMR (CDCl3, 100 MHz, HSQC): δ 177.4, 176.6, 176.1 (C=O Piv), 86.6 (d, J = 194 Hz, C-2), 85.5 (d, J = 21 Hz, C-1), 72.5 (C-5), 70.3 (d, J = 18 Hz, C-3), 65.6 (d, J = 7 Hz, C-4), 60.5 (C-6), 38.6, 38.6, 38.6 (C8tBu), 26.8, 26.8 (CH3 tBu). A solution of crude bromide 11 (~1.97 mmol) in CHCl3 (20 mL) was cooled to 0 °C, followed by the addition of p-thioresol (0.37 g, 2.96 mmol) and TAB (0.13 g, 0.39 mmol, dissolved in 3 mL H2O). A solution of KOH (0.22 g, 3.94 mmol) in H2O (3 mL) was drop-wise added, and the reaction was allowed to stir for 2 h. The mixture was diluted with EtOAc and washed with sat. aq. NaCl. The organic phase was dried over Na2SO4, and the title compound was obtained by flash column chromatography (silica gel, 9% EtOAc in PE) as a colorless oil (Yield: 1.02 g, 1.89 mmol, 96% over two steps). TCL: Rf 0.59 (PE/EtOAc, 5/1, v/v); 1H NMR (CDCl3, 400 MHz, HH-COSY, HSQC): δ 7.46 (d, 2H, J = 8.0 Hz, CH3tBu), 7.11 (d, 2H, J = 8.0 Hz, CH3tBu), 5.40 (dt, 1H, J = 9.3, 13.7 Hz, H-3), 4.99 (t, 1H, J = 9.9 Hz, H-4), 4.71 (d, 1H, J = 9.5 Hz, H-1), 4.04–4.25 (m, 3H, H-2, H-6, H-6), 3.78 (dd, 1H, J = 4.6, 10.1 Hz, H-5), 2.34 (s, 3H, CH3tStol), 1.21 (s, 3H, CH3tBu), 1.15 (s, 3H, CH3tBu), 1.14 (s, 3H, CH3tBu), 13C-NMR (CDCl3, 100 MHz, HSQC): δ 177.3, 176.8, 176.0 (C=O Piv), 138.4 (C4), 133.9, 129.5 (CH3tBu), 126.6 (C8tBu), 87.1 (d, J = 190 Hz, C-2), 84.1 (d, J = 23 Hz, C-1), 75.9 (C-3), 73.1 (d, J = 20 Hz, C-3), 66.7 (d, J = 7 Hz, C-4), 61.5 (C-6), 38.5, 38.4, 38.4 (C8tBu), 26.8, 26.7 (CH3tBu), 20.9 (CH3Stol); HRMS: [M+Na]+ calculated for C29H40FOSNa 563.24492, found 563.24459.

Tolyl 2-deoxy-2-fluoro-1-thio-D-glucopyranoside (13). A solution of compound 12 (0.84 g, 1.55 mmol) in MeOH (20 mL) was treated with NaOMe (cat.) and stirred at RT for 5 days. The mixture was quenched by the addition of Amberlite-H+, filtered off and concentrated in vacuo. The product was used in the next reaction without further purification. (Yield: 0.45 g, 1.54 mmol, quant.). The spectroscopic data were in full accord with those reported previously. TCL: Rf 0.46 (EtOAc); 1H NMR (CDCl3/MeOH-d4, 400 MHz, HH-COSY, HSQC):
\( \delta \) 7.45 (d, 2H, \( J = 8.0 \text{ Hz} \), CH\textsubscript{arom}), 7.14 (d, 2H, \( J = 8.0 \text{ Hz} \), CH\textsubscript{arom}), 4.64 (d, 1H, \( J = 9.6 \text{ Hz} \), H-1), 3.99 (dt, 1H, \( J = 9.2, 49.7 \text{ Hz} \), H-2), 3.87 (dd, 1H, \( J = 2.5, 12.2 \text{ Hz} \), H-6), 3.73 (dd, 1H, \( J = 4.7, 12.2 \text{ Hz} \), H-6), 3.63-3.70 (m, 1H, H-3), 3.32-3.39 (m, 2H, H-4, H-5), 2.35 (s, 3H, CH\textsubscript{STol}); \( ^{13} \text{C}-\text{APT NMR} \) (CDCl\(_3\)/MeOH-d\(_4\), 100 MHz, HSQC): \( \delta \) 138.4 (C\textsubscript{STol-CH\(_2\)}), 133.3, 129.5 (CH\textsubscript{arom}), 127.2 (C\textsubscript{STol}), 89.5 (d, \( J = 186 \text{ Hz} \), C-2), 84.3 (d, \( J = 24 \text{ Hz} \), C-1), 79.9 (C-5), 75.9 (d, \( J = 18 \text{ Hz} \), C-3), 69.4 (d, \( J = 8 \text{ Hz} \), C-4), 61.4 (C-H\textsubscript{STol}); LC: R\(_f\) 5.53 (C18 column, linear gradient 10 → 90% B in 13.5 min); TLC-MS: \( m/z \) = 311.1 (M+Na\(^+\)).

Tolyl 6-azido-2,6-di-deoxy-1-thioβ-D-glucopyranosyl (14). Triol 13 (0.72 g, 2.50 mmol) was co-evaporated with dry acetonitrile (2x) and dissolved in acetonitrile (25 mL) under an argon atmosphere. To the mixture Ts-Cl (0.71 g, 3.75 mmol) and TMEDA (0.57 mL, 3.75 mmol) were added. The reaction was stirred for 2 h, after which time the mixture was diluted with EtOAc and 1M aq. HCl. The organic phase was washed with sat. aq. NaCl, dried over Na\(_2\)SO\(_4\) and concentrated in vacuo. Purification using flash column chromatography (silica gel, 66% EtOAc in PE) yielded the 6-O-tosyl intermediate as a colorless oil (Yield: 0.77 g, 1.74 mmol, 70%). A solution of the tosylate (0.77 g, 1.74 mmol) and sodium azide (0.34 g, 5.22 mmol) in DMF (17 mL) was heated at 80 ºC overnight. The mixture was diluted with EtOAc, washed with sat. aq. NaHCO\(_3\) and 1M aq. HCl. The organic phase was washed with sat. aq. NaCl, dried over Na\(_2\)SO\(_4\) and concentrated in vacuo. Purification using flash column chromatography (silica gel, 66% EtOAc in PE) afforded the title compound as a white amorphous solid (Yield: 0.35 g, 0.86 mmol, 57%, A : B = 1.7 : 1), next to the hydrolyzed product (Yield: 0.13 g, 0.44 mmol, 29%). TLC: \( R_f \) = 0.8 (PE/AcO\(_2\) 1/1, v/v); IR (neat, cm\(^{-1}\)): 729, 1038, 1067, 1290, 2102, 3399; \( ^{1} \text{H NMR} \) (CDCl\(_3\), 400 MHz, HH-COSY, HSQC): \( \delta \) 7.46 (d, 2H, \( J = 8.4 \text{ Hz} \), CH\textsubscript{arom}), 7.36 (d, 2H, \( J = 9.6 \text{ Hz} \), CH\textsubscript{arom}), 7.34 (d, 2H, \( J = 8.3 \text{ Hz} \), CH\textsubscript{arom}), 5.30-5.46 (m, 1.6H, H-3A, H-3B), 4.94-5.00 (m, 0.9H, H-2B, H-4B), 4.91 (t, \( J = 9.6 \text{ Hz} \), H-4A), 4.80-4.85 (m, 0.8H, H-2A, H-2B), 4.71 (t, \( J = 8.9 \text{ Hz} \), H-2A), 4.52 (dd, 1H, \( J = 3.9, 9.2 \text{ Hz} \), H-1A), 4.19 (dd, 0.6H, \( J = 3.1, 9.7 \text{ Hz} \), H-1B), 3.77 (ddd, 1H, \( J = 3.2, 5.8, 9.8 \text{ Hz} \), H-5A), 3.54 (ddd, 1H, \( J = 4.2, 5.3, 9.5 \text{ Hz} \), H-5B), 3.40 (dd, 1H, \( J = 3.3, 13.9 \text{ Hz} \), H-6A), 3.36 (5.9, 13.8 \text{ Hz} \), H-6A), 3.23-3.28 (m, 1.2H, H-6B), 2.43 (s, 1.8H, CH\textsubscript{STol-B}), 2.42 (s, 3H, CH\textsubscript{Ac-A}), 2.10 (s, 1.8H, CH\textsubscript{Ac-B}), 2.05 (s, 3H, CH\textsubscript{Ac-A}), 2.02 (s, 3H, CH\textsubscript{Ac-A}), 2.01 (s, 1.8H, CH\textsubscript{Ac-B}); \( ^{13} \text{C}-\text{APT NMR} \) (CDCl\(_3\), 100 MHz, HSQC): \( \delta \) 169.9 (C=O Ac-B), 169.8, 169.3 (C=O Ac-A), 169.2 (C=O Ac-B), 142.4 (C\(_2\)), 142.4 (C\(_3\)), 134.7 (C\(_{STol-A}\)), 134.5 (C\(_{STol-B}\)), 129.8 (CH\textsubscript{arom-A}), 129.8 (CH\textsubscript{arom-A}), 125.2 (CH\textsubscript{arom-B}), 125.0 (CH\textsubscript{arom-A}), 92.1 (d, \( J = 23 \text{ Hz} \), C-1A), 90.1 (d, \( J = 23 \text{ Hz} \), C-1B), 85.0 (d, \( J = 190 \text{ Hz} \), C-2A), 83.9 (d, \( J = 189 \text{ Hz} \), C-2A), 77.6 (C-5A, C-5B), 73.2 (d, \( J = 20 \text{ Hz} \), C-3B), 73.1 (d, \( J = 20 \text{ Hz} \), C-3A), 68.5 (d, \( J = 7 \text{ Hz} \), C-4B), 68.2 (d, \( J = 7 \text{ Hz} \), C-4A), 50.9 (C-6B), 50.7 (C-6A), 21.4 (CH\textsubscript{STol-B}), 21.4 (CH\textsubscript{STol-A}), 20.5, 20.5, 20.4 (CH\textsubscript{Ac}); HRMS: [M+Na\(^+\)] calcd for C\(_17\)H\(_{20}\)F\(_3\)N\(_2\)O\(_5\)SNa 436.09491, found 436.09448.
6-Azido-2,6-dideoxy-1-thio-β-D-glucopyranosyl (S)hydroxide (16). Compound 15 (65 mg, 0.16 mmol) was dissolved in MeOH (2 mL) and treated with NaOMe (cat.) for 90 min. The mixture was neutralized by the addition of Amberlite-IR, filtered and concentrated in vacuo. The title compound was used in the next reaction without further purification (Yield: quant., A : B = 1.7 : 1). TLC: Rf 0.18 (PE/ETOAc, 1/3, v/v); IR (neat, cm⁻¹): 1003, 1032, 1065, 1078, 2102, 3333; ¹H NMR (MeOH-d₄, 400 MHz, HH-COSY, HSSQC): δ 7.56 (d, 1H, J = 8.2 Hz, CH₃), 7.35 (d, 0.6H, J = 3.7 Hz, CH₃), 3.68-3.69 (m, 1.2H, H-2B), 3.25-3.30 (m, 1.8H, H-4B, H-5B, H-6B). ¹³CAPT NMR (MeOH-d₄, 100 MHz, HSQC): δ 143.8 (C-1), 143.7 (C-3B), 136.0 (C-4), 135.6 (C-5), 130.9 (CH₃), 120.7 (CH₃), 126.6 (CH₃), 93.2 (d, J = 24 Hz, C-1A), 91.4 (d, J = 24 Hz, C-2B), 88.2 (d, J = 16 Hz, C-2B), 84.4 (d, J = 18 Hz, C-2A), 81.2 (C-5A), 81.1 (C-5B), 76.8 (d, J = 18 Hz, C-3A), 76.7 (d, J = 17 Hz, C-3B), 71.3 (d, J = 8 Hz, C-4B), 70.9 (d, J = 8 Hz, C-4A), 52.5 (C-6B), 52.4 (C-6A), 21.5 (CH₃), 21.5 (CH₃); LC-MS: Rf 0.19, found 330.09193.

6-Azido-2,6-dideoxy-2-fluoro-β-D-glucopyranose (17). A solution of compound 16 (53 mg, 0.16 mmol) in acetone/H₂O (2 mL, 3/1, v/v) was treated with NBS (85 mg, 0.48 mmol) for 3 h at RT. The reaction was quenched by the addition of sat. Na₂SO₄ (1 mL) and subsequently diluted with EtOAc and H₂O. The aqueous phase was extracted with EtOAc (2x), the combined organic layers were dried over Na₂SO₄ and concentrated in vacuo. Purification using flash column chromatography (silica gel, 75% EtOAc in PE) yielded the title compound as a colorless oil (Yield: 31 mg, 0.15 mmol, 94%, α : β = 1 : 1). TLC: Rf 0.35 (PE/ETOAc, 1/3, v/v); IR (neat, cm⁻¹): 816, 1001, 1051, 1177, 1290, 1694, 1771, 2104, 3329; ¹H NMR (MeOH-d₄, 300 MHz, HH-COSY, HSSQC): δ 5.25 (d, 1H, J = 3.7 Hz, H-1a), 4.68 (dd, 1H, J = 2.5, 7.7 Hz, H-1b), 4.17 (ddd, 1H, J = 3.7, 9.3, 49.8 Hz, H-2a), 3.78-4.02 (m, 2H, H-2b, H-3), 3.22-3.60 (m, 7H, H-3B, H-5a, H-5B, 2 x H-6a, 2 x H-6b). ¹³CAPT NMR (MeOH-d₄, 100 MHz, HSQC): δ 98.5 (d, J = 21 Hz, C-1B), 94.7 (d, J = 182 Hz, C-2B), 92.0 (d, J = 188 Hz, C-2a), 91.5 (d, J = 22 Hz, C-1a), 76.5 (C-5), 76.2 (d, J = 18 Hz, C-3B), 72.7 (d, J = 17 Hz, C-3a), 72.3 (d, J = 8 Hz, C-4a), 72.2 (d, J = 8 Hz, C-4b), 76.5 (C-5), 52.7, 52.7 (C-6a, C-6b); TLC-MS: Rf 0.26 (230.1 (M+Na)⁺).

BODIPY compound 18. Compound 17 (34 mg, 164 µmol) and BODIPY-alkyne 8 (59 mg, 180 µmol) were together dissolved in DMF (1.5 mL) and treated with sodium ascorbate (12 µL, 1M solution in H₂O) and CuSO₄ (8 µL, 1M solution in H₂O). The resulting mixture was stirred at 80 °C for 2 days, during which time the addition of sodium ascorbate and CuSO₄ was repeated twice. The mixture was allowed to cool to RT and diluted with EtOAc and H₂O. The organic phase was washed with sat. NaCl, dried over Na₂SO₄ and the product was obtained using flash column chromatography (silica gel, 15% MeOH in DCM) as an orange solid (Yield: 46 mg, 86 µmol, 53%, α : β = 1.1 : 1). TLC: Rf 0.59 (DCM/MeOH, 8:5:1.5, v/v); IR (neat, cm⁻¹): 984, 1061, 1200, 1508, 1551, 3429; ¹H NMR (MeOH-d₄, 400 MHz, HH-COSY, HSSQC): δ 6.09 (s, 2H, CH pyrrole), 5.21 (d, 1H, J = 3.7 Hz, H-1a), 4.78 (dd, 0.9H, J = 2.2, 14.4 Hz, H-6b), 4.71 (dd, 1H, J = 2.4, 14.3 Hz, H-6a), 4.64 (dd, 0.9H, J = 2.5, 7.8 Hz, H-1b), 4.50 (dd, 1H, J = 7.4, 14.0 Hz, H-6a), 4.47 (dd, 0.9H, J = 7.6, 14.1 Hz, H-6b), 4.10 (ddd, 1H, J = 3.7, 9.4, 49.8 Hz, H-2a), 4.10 (dd, 1H, J = 2.4, 7.3, 9.8 Hz, H-5c), 3.79-3.96 (m, 1.9H, H-2b, H-3a), 3.56-3.67 (m, 1.8H, H-3b), 3.15 (t, 0.9H, J = 9.4 Hz, H-4b), 2.86-2.94 (m, 3.8H, CH₂), 2.72 (t, 3.8H, J = 7.5 Hz, CH₃), 2.43 (s, 11.4H, CH₃), 2.33 (s, 11.4H, CH₃), 1.79-1.90 (m, 3.8H, CH₂), 1.55-1.66 (m, 3.8H, CH₃); ¹³CAPT NMR (MeOH-d₄, 100 MHz, HSQC): δ 154.9, 148.4, 148.3, 147.9, 142.2, 132.6 (C-1), 124.6 (CH triazole) 122.6 (CH pyrrole), 95.7 (d, J = 23 Hz, C-1), 94.5 (d, J = 184 Hz, C-2b), 91.7 (d, J = 187 Hz, C-2a), 91.5 (d, J = 22 Hz, C-1a), 76.0 (d, J = 18 Hz, C-3b), 75.8 (C-5b), 72.6 (d, J = 17 Hz, C-3a), 72.6 (d, J = 7 Hz, C-4), 72.4 (d, J = 8 Hz, C-4), 71.0 (C-5c), 52.2, 52.1 (C-6a, C-6b), 32.2, 30.8, 28.9, 25.9 (CH₃), 16.4, 14.5 (CH₃); LC-MS: Rf 0.86 min (C18 column, linear gradient 10 → 90% B in 13.5 min); HRMS: [M+H]⁺ calc'd for C₅H₁₃B₄N₅O₅S 356.26505, found 356.26523.
Determination of the IC\textsubscript{50}. Imiglucerase (12.5 µL, 20 nM) was prepared in 150 mM McIlvaine buffer (pH 5.2) containing 0.2% (w/v) taurocholate and 0.1% (v/v) Triton X-100. The enzyme was incubated with a range of probe concentrations (12.5 µL, 1 mM to 10 nM final concentration, DMSO) for 30 at 37 °C. Then 4MUGlc (100 µL, 3.75 mM) substrate in McIlvaine buffer (pH 5.2) containing 0.2% (w/v) taurocholate, 0.1% (v/v) Triton X-100, and 0.1% (w/v) BSA was added, and the resulting mixture was incubated for 15 min at 37 °C. The mixture was inactivated with 2.5 mL NaOH-Glycine (300 mM, pH 10.6), followed by measuring of the fluorescence of liberated 4MU (λ\text{ex}, 366 nm, λ\text{em}, 445 nm). IC\textsubscript{50} values were obtained by plotting of the residual fluorescence versus the concentration (GraphPad Prism 5).

Detection limit. Imiglucerase (10 µL, 100 nM) was prepared in 150 mM McIlvaine buffer (pH 5.2) containing 0.2% (w/v) taurocholate and 0.1% (v/v) Triton X-100. The enzyme was pre-incubated with CBE (10 µL, 20 mM in H\textsubscript{2}O), cyclophilin (10 µL, 2 mM in H\textsubscript{2}O), MDW941 (10 µL, 2 µM in H\textsubscript{2}O), or AMP-DNM (10 µL, 20 mM in H\textsubscript{2}O) for 30 min at 37 °C, or with 10 µL 2% (w/v) SDS and boiled for 4 min at 100 °C. The pre-incubated mixtures were labeled with MDW933 (10 µL, 30 nM in H\textsubscript{2}O), probe 1 (10 µL, 150 µM in H\textsubscript{2}O), probe 6 (10 µL, 1.5 µM in H\textsubscript{2}O), or probe 7 (10 µL, 15 µM in H\textsubscript{2}O) for 30 min at 37 °C. The sample was denatured with 10 µL Laemmli buffer (50% (v/v) 1M Tris-HCl, pH 6.8, 50% (v/v) 100% glycerol, 10% (w/v) DTT, 10% (w/v) SDS, 0.01% (w/v) bromophenol blue), boiled for 4 min at 100 °C, and separated by electrophoresis on 7.5% (w/v) SDS-PAGE gel running continuously at 90 V, followed by fluorescent scanning.

Competition for the active site. Imiglucerase (10 µL, 100 nM) was prepared in 150 mM McIlvaine buffer (pH 5.2) containing 0.2% (w/v) taurocholate and 0.1% (v/v) Triton X-100. The enzyme was pre-incubated with CBE (10 µL, 20 mM in H\textsubscript{2}O), cyclophilin (10 µL, 2 mM in H\textsubscript{2}O), MDW941 (10 µL, 2 µM in H\textsubscript{2}O), or AMP-DNM (10 µL, 20 mM in H\textsubscript{2}O) for 30 min at 37 °C, or with 10 µL 2% (w/v) SDS and boiled for 4 min at 100 °C. The pre-incubated mixtures were labeled with MDW933 (10 µL, 30 nM in H\textsubscript{2}O), probe 1 (10 µL, 150 µM in H\textsubscript{2}O), probe 6 (10 µL, 1.5 µM in H\textsubscript{2}O), or probe 7 (10 µL, 15 µM in H\textsubscript{2}O) for 30 min at 37 °C. The sample was denatured with 10 µL Laemmli buffer (50% (v/v) 1M Tris-HCl, pH 6.8, 50% (v/v) 100% glycerol, 10% (w/v) DTT, 10% (w/v) SDS, 0.01% (w/v) bromophenol blue), boiled for 4 min at 100 °C, and separated by electrophoresis on 7.5% (w/v) SDS-PAGE gel running continuously at 90 V, followed by fluorescent scanning.

pH-dependent labeling. Imiglucerase (10 µL, 10 nM) was prepared in 1.5 mM McIlvaine buffer (pH 5.2) containing 0.2% (w/v) taurocholate and 0.1% (v/v) Triton X-100, and incubated with 150 mM McIlvaine buffer of pH 2-9 (25 µL), containing 0.2% (w/v) taurocholate and 0.1% (v/v) Triton X-100, for 30 min at 37 °C. Pre-incubated enzyme was labeled with MDW941 (5 µL, 8 nM in H\textsubscript{2}O), probe 1 (5 µL, 400 µM), probe 6 (5 µL, 4 µM), or probe 7 (5 µL, 40 µM) for 30 min at 37 °C. The sample was denatured with 10 µL Laemmli buffer (50% (v/v) 1M Tris-HCl, pH 6.8, 50% (v/v) 100% glycerol, 10% (w/v) DTT, 10% (w/v) SDS, 0.01% (w/v) bromophenol blue), boiled for 4 min at 100 °C, and separated by electrophoresis on 7.5% (w/v) SDS-PAGE gel running continuously at 90 V, followed by fluorescent scanning.

Labeling of mutant GBA. All probe solutions were prepared in 150 mM McIlvaine buffer (pH 5.2) containing 0.2% (w/v) taurocholate, 0.1% (v/v) Triton X-100, and protease inhibitor cocktail (Roche). Homogeneous (20 µL) of cos-7 cells overexpressing wild-type and acid/base mutant (E235G and E235Q) GBA was incubated with MDW1044 (20 µL, 2 µM), MDW933 (20 µL, 2 µM), probe 1 (20 µL, 200 nM), probe 6 (20 µL, 2 µM), or probe 7 (20 µL, 20 nM) for either 2 h or 24 h at 37 °C. The samples were split in two, and one half (20 µL) was directly denatured etcetera (vide infra). The labeled homogenate (20 µL) was incubated with Ni-agarose beads (5 µL) and native lysis buffer (100 µL, pH 8.0) containing NaCl (300 mM) and imidazole (10 mM) while rotating for 1 h at 4 °C. The samples were centrifuged for 3 min at 800 rpm, cleaned with wash buffer (200 µL, pH 8.0) containing NaCl (300 mM) and imidazole (20 mM) for 10 min at 4 °C (repeated 3x). Then the nickel beads were pelleted by centrifugation for 10 min at 800 rpm and resuspended in McIlvaine buffer (20 µL, pH 5.2) containing 0.2% (w/v) taurocholate, 0.1% (v/v) Triton X-100. The sample was denatured with 10 µL Laemmli buffer (50% (v/v) 1M Tris-HCl, pH 6.8, 50% (v/v) 100% glycerol, 10% (w/v) DTT, 10% (w/v) SDS, 0.01% (w/v) bromophenol blue), boiled for 4 min at 100 °C, and separated by electrophoresis on 7.5% (w/v) SDS-PAGE gel running continuously at 90 V, followed by fluorescent scanning.
**Labeling in fibroblasts.** Wild-type human skin fibroblasts were grown to confluency (RPMI medium) for 3 days and cultured in the presence of MDW933 (0/1/10 nM), MDW1044 (0/1/10 nM), probe 1 (0/1/10 µM), probe 6 (0/1/10 µM), or probe 7 (0/0/10 µM) (probe solutions in PBS buffer) for 2 or 24 h at 37 ºC. The cells were lysed by scraping in KPi buffer (100 µL, 25 mM, pH 6.5) containing 0.1% (v/v) Triton X-100 and protease inhibitor cocktail. The protein concentration was determined using a BCA kit (Pierce), and 21 µg (2 h) or 27 µg (24 h) was loaded per lane. The homogenates (35 µL) were incubated with MDW941 (5 µL, 800 nM in McIlvaine buffer, pH 5.2, containing taurocholate, 0.1% (v/v) Triton X-10, and protease inhibitor cocktail) for 30 min at 37 ºC. The samples were denatured with 10 µL Laemmli buffer (50% (v/v) 1M Tris-HCl, pH 6.8, 50% (v/v) 100% glycerol, 10% (w/v) DTT, 10% (w/v) SDS, 0.01% (w/v) bromophenol blue), boiled for 4 min at 100 ºC, and separated by electrophoresis on 7.5% (w/v) SDS-PAGE gel running continuously at 90 V, followed by fluorescent scanning.

**Footnotes and References**


[13] To determine the stability of the probes at different pH values, probes 1, 2, 6 and 7 were added to McIlvaine buffers with pH 4.0, 5.2 and 7.0 (200 mM) at 37 ºC and analyzed at different time points. No significant hydrolysis of probes 1 and 2 was observed after 24 h at the three pH values. In contrast, analysis of imidate probe 6 revealed > 50% hydrolysis at pH 4, and only trace amounts of hydrolyzed product at pH 5.2 and 7.0 (after 24 h). Hydrolysis of phosphate probe 7 started immediately, leading to full conversion of the hemiacetal within 24 h at the three pH values.


