Activity-based profiling of glycoconjugate processing enzymes

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2 BODIPY-VAD-Fmk, a useful tool to study yeast peptide *N*-glycanase activity

Introduction

In eukaryotes, *N*-linked glycoproteins are synthesized by ribosomes bound to the membrane of the endoplasmic reticulum (ER). The translocon inserts the newly synthesized proteins into the lumen of the ER, where asparagine residues of newly synthesized proteins may be glycosylated by oligosaccharyl transferase in a co-translational process.[1] The resulting $Glc_3Man_9GlcNAc_2$ glycan is deglucosylated to $GlcMan_9GlcNAc_2$ which is then recognized by calnexin and calrectulin. Glycoproteins are retained in the ER by these chaperones until properly folded. Properly folded proteins progress through the ER and Golgi, where they are transformed into complex-type *N*-glycans by a series of deglycosylation/glycosylation events. These glycans in turn help in guiding the glycoprotein to its final destination, such as the cell surface or the endocytic pathway.

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The trimmed glycans of misfolded *N*-linked glycoproteins are recognized by lectin-like proteins of the endoplasmic reticulum associated degradation pathway such as OS9 which eventually results in dislocation of misfolded proteins from the ER to the cytosol. Upon arrival the misfolded proteins are ubiquitinated and are then directed to the proteasome for degradation.[2]

In this pathway, the amidase peptide *N*-glycanase (PNGase) is responsible for deglycosylation of most *N*-glycosylated proteins.[3] The β-aspartyl-glucosamine bond is cleaved by the characteristic catalytic Cys, His, Asp triad. PNGase can associate with the proteasome as well as with ER-bound proteins, suggesting that PNGase has multiple modes of action.[4] For fundamental studies on the role of PNGase *in vivo*, specific inhibitors will be beneficial. However, only few such inhibitors are available to date and little is known about their efficacy and selectivity. PNGase activity can be blocked by either the commercially available broad spectrum caspase inhibitor Z-VAD-Fmk **2** or haloacetamidyl chitobiose inhibitor **3** (Figure 1).[5]

In the search for new inhibitors, a sensitive and straightforward assay would be of great use. The *N*-glycanase activity assay currently in use entails the deglycosylation of a substrate, namely the glycoprotein RNase B, by PNGase. Subsequently, the digestion products are resolved by SDS-PAGE and visualized with Coomassie brilliant blue. Before use as a substrate, commercially available RNase B requires further purification and denaturation.[6] Verdoes *et al*. recently published an activity-based fluorescent proteasome probe, which enables direct in-gel detection of proteasome activity.[7] By applying this probe in competition experiments, the inhibitory potential of proteasome inhibitors could be determined. It was reasoned that the availability of a fluorescent analogue of **2**, such as BODIPY TMR-Ahx-Val-Ala-Asp(OMe)-fluoromethylketone **1** (β-VAD-Fmk)[8], would allow for straightforward screening of potential irreversible *N*-glycanase inhibitors in an analogous fashion. In this chapter, the validity of this reasoning is shown by the application of β-VAD-Fmk **1** in the identification of the two new chitobiose-based *N*-glycanase inhibitors, **4** and **5** (Figure 1).

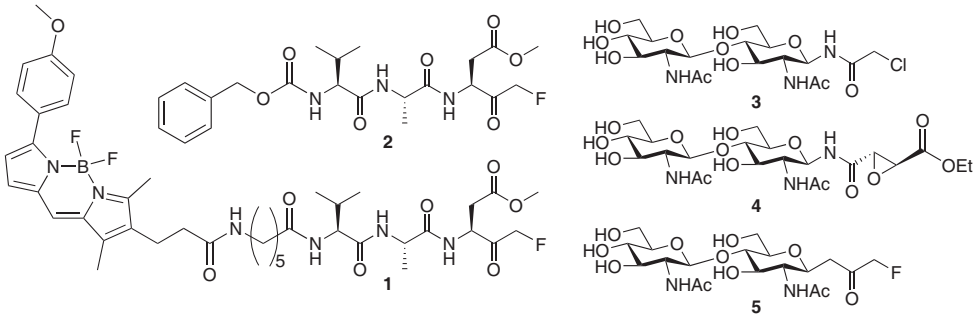
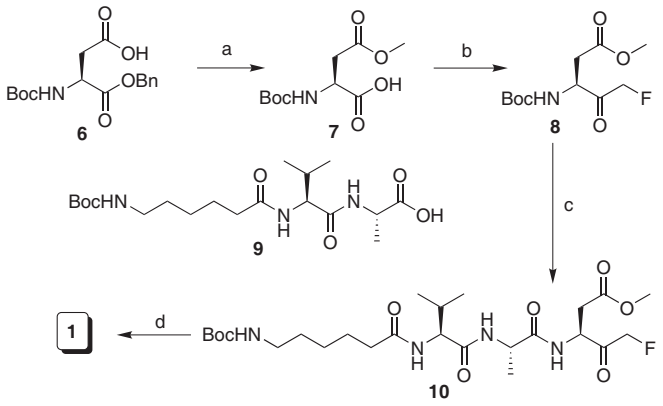


Figure 1. Target compounds.

Results and Discussion

The synthesis of β-VAD-Fmk **1** is depicted in Scheme 1. Boc-Asp-OBn **6** was converted to the fluoromethylketone via a modified procedure of the by Palmer patented method.[9] Hence, Boc-Asp(OMe)OH **7** was synthesized from Boc-Asp-OBn **6** by esterification of the γ-carboxylic acid in **6** using methyl iodide and K_2CO_3, and ensuing reduction of the benzyl ester. Treatment of the resulting acid with 1,1'-carbonyldiimidazole followed by reaction with the magnesium enolate of monobenzyl-fluoromalonate and consecutive hydrogenation gave fluoromethylketone **8**. Removal of the Boc protective group in **8** and condensation of the resulting free amine with peptide **9** was followed by *N*-Boc deprotection and treatment with BODIPY TMR-OSu to give target compound **1**. β-VAD-Fmk **1** was obtained as a diastereomeric mixture due to epimerization of the alanine α-carbon during the block coupling.

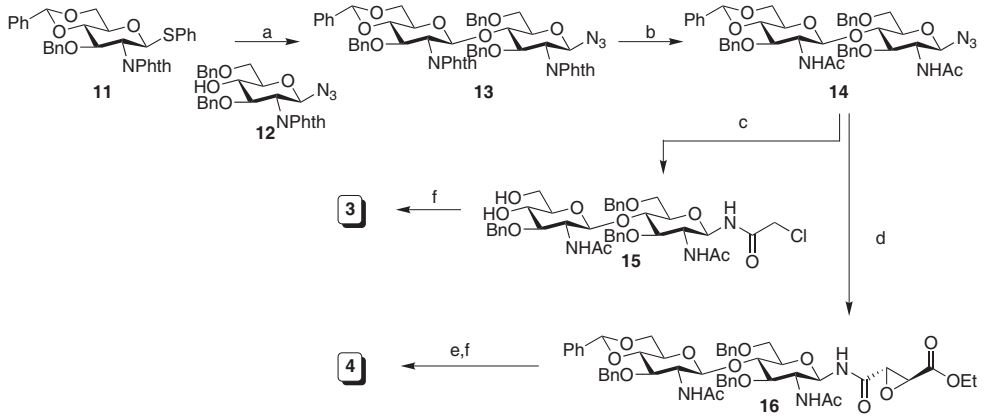
Scheme 1. Synthesis of fluorescent analog of Z-VAD-Fmk.



Reagents and conditions: (a) MeI, K_2CO_3, DMF; ii) 10% Pd/C, H_2, EtOAc, quant.; (b) i) CDI, THF, 1h; ii) monobenzyl fluoromalonate magnesium enolate, THF; iii) 10% Pd/C, H_2, EtOAc, 68%; (c) i) 4M HCl/dioxane, 45 min; ii) **9**, HCTU, DiPEA, DMF, 68%; (d) i) TFA/H_2O (95/5, v/v), 30 min; ii) BODIPY TMR-OSu, DiPEA, 16h, 41%.

With β-VAD-Fmk **1** in hand, attention was focused on the synthesis of reference compound **3** and epoxysuccinate inhibitor **4** (Scheme 2). Known donor **11** was condensed with acceptor **12** under the agency of Ph_2SO/Tf_2O giving disaccharide **13**.[10] Removal of the phthaloyl group and subsequent acetylation afforded protected chitobiose **14**. Reduction of the azide in **14** followed by coupling with either chloroacetic anhydride or epoxysuccinate monoethyl ester afforded the protected inhibitors **15** and **16**. Global deprotection furnished epoxide inhibitor **4** in good yield. In the case of inhibitor **3** however partial reduction of the chloroacetamide moiety was observed after deprotection.

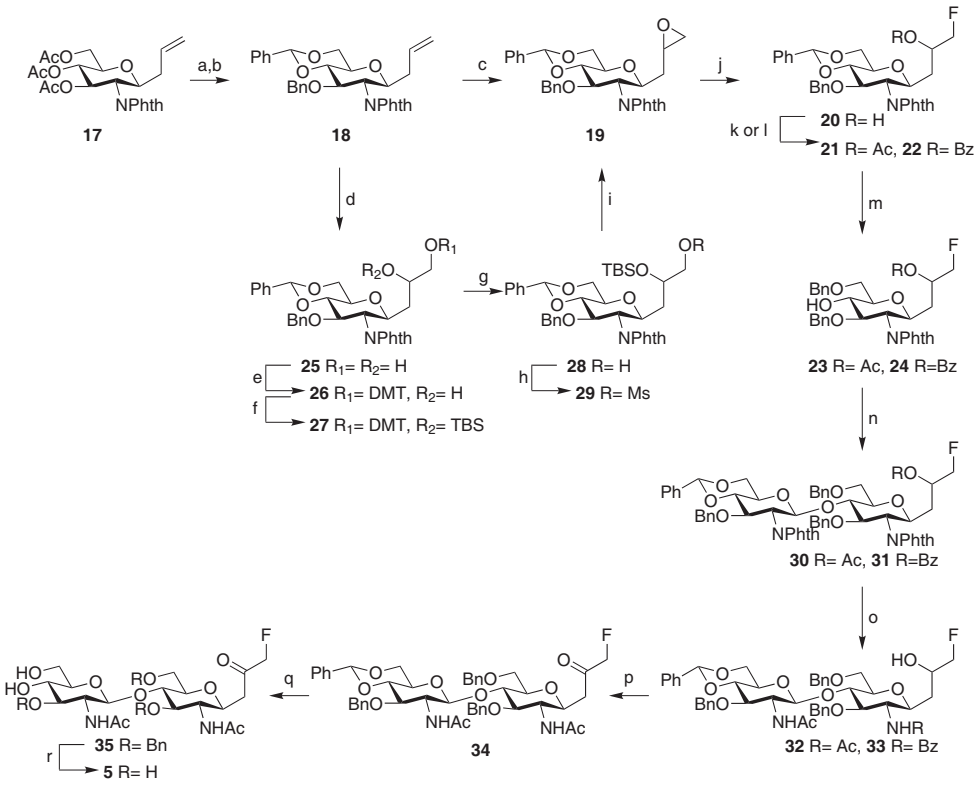
Scheme 2. Synthesis of acetamide inhibitors **3** and **4**.



Reagents and conditions: (a) **12**, Ph_2SO, TTBP, Tf_2O, CH_2Cl_2, -60°C to 0°C, 85%; (b) i) $(H_2NCH_2)_2$/n-BuOH (1/10), 90°C; ii) Ac_2O, pyr, 81%; (c) i) Lindlar's cat. H_2, DMF; ii) $(ClCH_2CO)_2O$, Et_3N, DMF; iii) MeOH, pTsOH, 16h, 47%; (d) i) Lindlar's cat. H_2; ii) epoxysuccinate monoethylester, HCTU, Et_3N, DMF, 39%; (e) 5% TFA/CH_2Cl_2, 70%; (f) 20% $Pd(OH)_2$, H_2, MeOH **3**: 14%, **4**: 67%.

Fluoromethylketone **5** was synthesized as depicted in Scheme 3. First acceptors **23** and **24** were synthesized. *C*-glycoside **17**, prepared according to a literature procedure,[11] was deacetylated by treatment with acidic methanol. Subsequent protection of the 4,6 hydroxyl functionalities by reaction with benzaldehyde dimethylacetal and benzylation of the 3-OH gave suitably protected *C*-glycoside **18**. Reaction of the ethylene moiety in **18** with mCPBA afforded the desired epoxide **19** as a diastereomeric mixture. For analytic purposes diastereomerically pure epoxides **19a** and **19b** were synthesized from protected **18** as follows. Dihydroxylation of alkene **18** afforded a mixture of diols **25a** and **25b** which was separated by silica gel chromatography. Selective tritylation of the primary alcohol with 4,4'-dimethoxytrityl chloride followed by silylation of the remaining secondary alcohol using TBS-Cl and imidazole gave protected **27a** and **27b**. Deprotection of the primary alcohol by treatment with dichloroacetic acid followed by mesylation furnished **29a** and **29b**. The resulting silyl mesylates could be converted to diastereomerically pure epoxides **19a** and **19b** under the agency of TBAF in THF. Regioselective opening of the epoxide in **19** with tetrabutylammonium dihydrogen trifluoride furnished fluorohydrin **20**. Acetylation or benzoylation of the resulting alcohol followed by opening of the benzylidene to the C6 position afforded acceptors **23** and **24**, respectively. Condensation of donor **11** with either acceptor **23** or **24** by treatment with Ph_2SO/Tf_2O gave disaccharides **30** and **31** in good yield. Deprotection of the amine functionalities in **30** and **31**, ensuing acetylation of the resulting free amines and *O*-deacetylation furnished fluorohydrin **32**. During the removal of the phthaloyl protective groups of disaccharide **31**, migration of the benzoyl protective group to the nitrogen was observed to give unwanted **33**. This problem could be overcome

Scheme 3. Assembly of fluoromethylketone **5**.



Reagents and conditions: (a) i) Amberlite H^+, MeOH, reflux, quant; ii) $PhCH(OMe)_2$, pTsOH, MeCN, 91%; (b) BnBr, NaH, TBAI, DMF, 68%; (c) mCPBA, CH_2Cl_2, reflux, 88%; (d) K_2OsO_4, NMO, THF/H_2O (6/1, v/v); (e) DMT-Cl, Et_3N, CH_2Cl_2, 3h, **a**: 97%, **b**: 94%; (f) TBS-Cl, Et_3N, imidazole, DMF, **a**: 90%, **b**: 85%; (g) 2% dichloroacetic acid in CH_2Cl_2, TES, **a**: 73%, **b**: 82%; (h) MsCl, Et_3N, DMAP, CH_2Cl_2, **a**: 89%, **b**: 93%; (i) TBAF (1M in THF), THF, **a**: 48%, **b**: 67%; (j) TBA·H_2F_3, Tol, microwave, 180°C, 20 min, 84%; (k) Ac_2O, pyr, 96%; (l) BzCl, DMAP, pyr, 92%; (m) TES, TfOH, CH_2Cl_2, -78°C, 45 min, 79-85%; (n) **11**, Ph_2SO, Tf_2O, TTBP, CH_2Cl_2, -60°C to 0°C, **30**: 97%, **31**: 51%; (o) i) $(H_2NCH_2)_2$/n-BuOH (1/10), 90°C; ii) Ac_2O, pyr; iii) NaOMe, MeOH, starting from **30**: 67%, starting from **31**: 56%; (p) Dess-Martin periodinane, CH_2Cl_2, 77%; (q) 5% TFA/CH_2Cl_2, H_2O, 0°C, 87%; (r) 20% $Pd(OH)_2$, H_2, MeOH, 45%.

by using *O*-acetyl protective groups. Although migration of the acetyl still occurred in acetylated **30**, this resulted in the formation of the desired product **32**. Oxidation of **32** afforded fully protected fluoromethylketone **34**. Global deprotection gave inhibitor **5** in a reasonable yield.

With probe **1** and inhibitors **2**-**5** in hand, their biological activity was evaluated. First, the capability of β-VAD-Fmk **1** to label PNGase was examined. To this end, purified recombinant yeast peptide *N*-glycanase (YPng1) was incubated with various concentrations of **1** for 1h. Direct in-gel visualization with a fluorescent scanner revealed that probe **1**

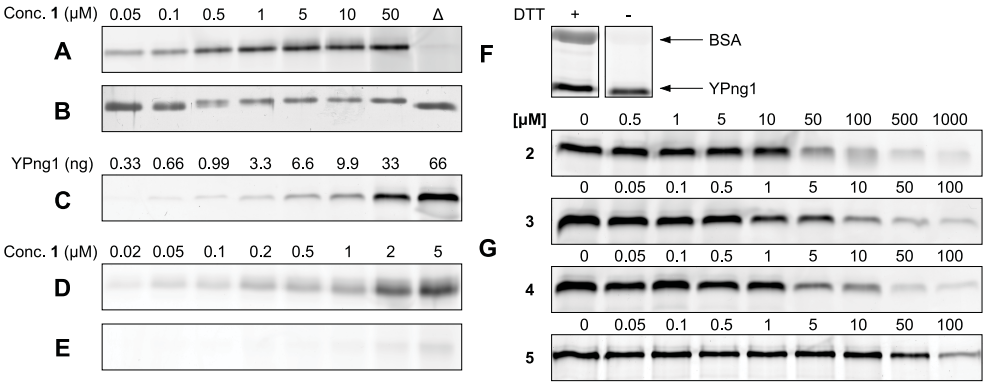


Figure 2. (A) Fluorescent in-gel detection of YPng1 by labeling with the indicated concentration of **1**. Δ represents heat-inactivated YPng1, incubated with 50 μM **1** for 2h at 37°C. (B) Silver stained gel of labeled YPng1. (C) Determination of the sensitivity of probe **1** by incubating **1** (0.5 μM) with increasing amounts of YPng1. (D) Labeling of wild-type YPng1 in *E. coli* cell extracts (1 mg/mL) with **1**. (E) Labeling of catalytically inactive YPng(C191A) in *E. coli* cell extracts (1 mg/mL) with a serial dilution of probe **1**. (F) Non-specific labeling of BSA by β-VAD-Fmk **1** (0.5 μM) in the presence of 5 mM DTT. (G) Competition assay. The indicated amount of inhibitors **2-4** in the presence of **1** (0.5 μM) was incubated with YPng1 (100 ng) and BSA (9 μg) in PBS (20 mM sodium phosphate, 150 mM NaCl, pH 7.2) for 1h. Fluoromethylketone **5** was preincubated with YPng1 for 2h followed by incubation with **1** for 30 min.

labeled yeast peptide *N*-glycanase in a concentration-dependent manner. From Figure 2A, it can be seen that saturation of the enzyme is reached at a concentration between 1 and 5 μM. Moreover, heat inactivated enzyme was not labeled by β-VAD-Fmk **1** suggesting that probe **1** labeled catalytically active YPng1 specifically (Figure 2A, B). The sensitivity was tested by labeling a serial dilution of YPng1 with **1** (0.5 μM). The labeling proved to be very sensitive since as little as 0.7 ng of purified YPng1 could be detected (Figure 2C). The specificity of β-VAD-Fmk **1** was further investigated by incubating the probe with an *E.coli* cell extract expressing YPng1. Exclusive labeling of YPng1 by probe **1** was observed by in-gel visualization (Figure 2D). Parent compound Z-VAD-Fmk **2** binds to the catalytic cysteine residue 191 of the active site of YPng1 as was shown in the reported X-ray structure of YPng1 co-crystallized with Z-VAD-Fmk **2**.[12] To verify if **1** binds to the same residue as lead compound **2**, catalytically inactive YPng(C191A) was expressed in *E.coli*. Incubation of YPng(C191A) with **1** followed by fluorescent imaging revealed, as expected, no significant labeling of YPng(C191A) (Figure 2E). Having assessed the active-site dependent labeling-ability of **1**, its application in the identification of the inhibitory potential of irreversible inhibitors was investigated. A solution of YPng1 and BSA was incubated with serial dilutions of the known active-site binding inhibitors **2** and **3**, followed by incubation with **1**.[13] Remarkably, labeling of YPng1 was still observed at 1 mM inhibitor concentration when the experiment was conducted in the presence of 5 mM DTT. In addition, non-specific labeling of BSA was detected, suggesting that reduction of disulfide bonds by DTT caused non-specific labeling by β-VAD-Fmk **1** (Figure 2F). Therefore a similar competition assay was conducted without DTT. Non-specific labeling was minimized allowing determination

of the concentration at which 50% of enzyme activity was inhibited (apparent IC_{50}) by quantificiation of the intensity of the bond with imaging software. The apparent IC_{50} of **2** was determined at 22 ± 5 μM and the IC_{50} of haloacetamide **3** was 1.6 ± 0.5 μM.

To further illustrate the usefulness of this assay, the inhibitory activity of potential chitobiose inhibitors **4** and **5** was examined *via* the competition assay. The apparent IC_{50} of epoxysuccinate **4** proved to be 1.6 ± 0.5 μM. Surprisingly, fluoromethylketone **5** proved to be a poor inhibitor of YPng1. Disaccharide **5** was preincubated with YPng1 for 2h allowing **5** to react with the active site of the enzyme. Next, unreacted active sites were labeled by treatment with **1** for 30 min. Even after preincubation, fluoromethylketone **5** inhibits YPng1 in the high micromolar range (Figure 2).

Conclusion

In summary, the synthesis of β-VAD-Fmk **1** is described and its ability to covalently bind to the active site Cys191 of recombinant yeast peptide *N*-glycanase has been demonstrated. β-VAD-Fmk **1** can be used in an enzyme inhibitory assay which is highly useful for the rapid identification of potential YPng1 inhibitors, as is demonstrated for two known inhibitors (**2** and **3**) and two new chitobiose-based inhibitors (**4** and **5**). This assay is not suitable for an accurate determination of IC_{50} values and establishment of k_i values since both the β-VAD-Fmk and the chitobiose based inhibitors bind in a time dependent fashion. IC_{50} values will therefore have to be interpreted as relative values. Furthermore, it cannot be excluded that weaker inhibitors may bind to the active site of yeast PNGase and induce the binding of β-VAD-Fmk *via* an induced fit.

Experimental section

General Procedures:

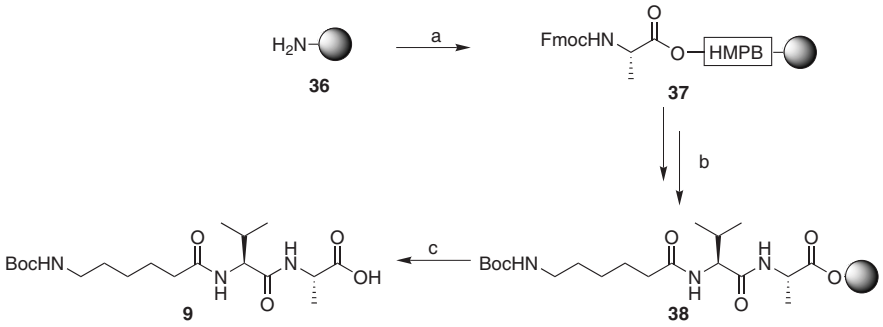
All reagents were of commercial grade and used as received, unless stated otherwise. Z-VAD-Fmk **2** was purchased from Biomol, international LP. Diethyl ether (Et_2O), ethyl acetate (EtOAc), light petroleum ether (PE) and toluene (Tol) were purchased from Riedel-de Haën. Acetonitrile (MeCN), dichloroethane, dichloromethane (CH_2Cl_2), *N*,*N*-dimethylformamide (DMF), methanol (MeOH), *N*-methylpyrrolidone (NMP), pyridine (pyr), tetrahydrofuran (THF) were obtained from Biosolve. THF was distilled over $LiAlH_4$ before use. Dichloromethane was boiled under reflux over CaH_2 for 2h and distilled prior to use. *n*-Butanol (n-BuOH) was refluxed over sodium for 2h, distilled and stored over 4Å MS. Trifluoromethanesulfonic anhydride (Tf_2O) was distilled from P_2O_5. Molecular sieves 4Å were flame dried *in vacuo* before use. All reactions were performed under an inert atmosphere of Argon unless stated otherwise. Solvents used for flash chromatography were of pro analysi quality. Flash chromatography was performed on Screening Devices silica gel 60 (0.04 – 0.063 mm). TLC-analysis was conducted on DC-alufolien (Merck, Kieselgel60, F254) with detection by UV-absorption (254 nm) were applicable and by spraying with 20% sulphuric acid in ethanol followed by charring at ~150°C or by spraying with a solution of $(NH_4)_6Mo_7O_{24}{\cdot}H_2O$ (25 g/L) and $(NH_4)_4Ce(SO_4)_4{\cdot}2H_2O$ (10 g/L) in aqueous 10% sulfuric acid followed by charring at ~150°C. ^1H and ^{13}C NMR spectra were recorded on a Bruker DMX-400 (400/100 MHz), a Bruker AV-400 (400/100 MHz), Bruker AV-500 (500/125 MHz) or a Bruker DMX-600 (600/150 MHz) spectrometer. Chemical shifts (δ) are given in ppm relative to the chloroform residual solvent peak or tetramethylsilane as internal standard. Coupling constants are given in Hz. All given ^{13}C spectra are proton decoupled. High resolution mass

spectra were recorded with a LTQ Orbitrap (Thermo Finnigan). LC/MS analysis was performed on a Jasco HPLC-system (detection simultaneously at 214 nm and 254 nm) equipped with an analytical Alltima C_{18} column (Alltech, 4.6 mmD × 50 mmL, 3µ particle size) in combination with buffers A: H_2O, B: MeCN and C: 1% aq. TFA and coupled to a Perkin Elmer Sciex API 165 mass instrument. For RP-HPLC purifications a BioCAD "Vision" automated HPLC system (PerSeptive Biosystems, inc.) equipped with a semi-preparative Alltima C_{18} column was used. The applied buffers were A: H_2O, B: MeCN and C: 1.0 % aq. TFA. Optical rotations were measured on a Propol automatic polarimeter (sodium D line, λ = 589 nm). FT-IR-spectra were recorded on a Paragon-PE 1000.

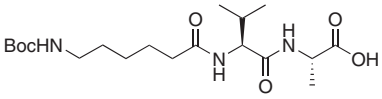
Synthesis of peptide 9

Peptide **9** was synthesized employing standard solid phase peptide synthesis. MBHA resin **36** was functionalized with a HMPB-linker before being loaded with Fmoc-Ala-OH. The resulting resin **37** was elongated furnishing resin-bound peptide **38**. Cleavage from the resin gave peptide **9** (Scheme 4).

Scheme 4. Assembly of peptide **9** using solid phase synthesis.



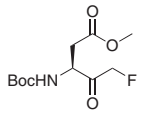
Reagents and conditions: (a) i) HMPB, HCTU, DiPEA; ii) Fmoc-Ala-OH, DIC, DMAP, CH_2Cl_2; (b) deprotection: piperidine/NMP (1/4, v/v); condensation: Fmoc-Val-OH or Boc-Ahx-OH, HCTU, DiPEA, NMP; (c) TFA/CH_2Cl_2 (1/99, v/v).



Boc-Ahx-Val-Ala-OH (9)

MBHA resin **36** (0.555 g, 0.46 mmol, 0.9 mmol/g) was solvated with NMP, before being reacted with HMPB (0.361 g, 1.5 mmol, 3 equiv.) in the presence of HCTU (0.62 g, 1.5 mmol, 3 equiv.) and diisoproylethylamine (0.532 mL, 3 mmol, 6 equiv.). The resin was shaken for 3h, after which it was filtered and washed with NMP (3× 5 mL) and CH_2Cl_2 (3× 5 mL). Next, the resin was coevaporated twice with dichloroethane and condensed with Fmoc-Ala-OH (429 mg, 1.38 mmol, 3 equiv.) under the agency of diisopropylcarbodiimide (DIC) (0.236 mL, 1.52 mmol, 3.3 equiv.) and DMAP (3 mg, 0.023 mmol, 0.05 equiv.) in CH_2Cl_2 for 2h. The resin was filtered, washed with CH_2Cl_2 (3× 5 mL) and subjected to a second condensation sequence. The obtained resin **37** was elongated by two cycles of Fmoc-solid phase synthesis. The consecutive steps of the cycles are as follows: (i) deprotection: piperidine in NMP (1/4, v/v, 15 min), (ii) wash with NMP (3× 5 mL), (iii) condensation: Fmoc-Val-OH (1.82 mmol, 4 equiv.) or Boc-Ahx-OH (1.82 mmol, 4 equiv.) was dissolved in NMP (7 mL). HCTU (0.753 g, 1.82 mmol, 4 equiv.) and diisopropylethylamine (0.643 mL, 3.64 mmol, 8 equiv.) were added. The resulting mixture was transferred to the reaction vessel and shaken for 90 min. (iv) Wash with NMP (3× 5 mL) and CH_2Cl_2 (3× 5 mL). Peptide **38** was liberated from the resin by treatment with TFA/CH_2Cl_2 (1/99, v/v, 4× 2 min). Subsequent addition of toluene followed by concentration *in vacuo* furnished crude peptide **9** (quant, 0.185 g, 0.46 mmol) which was

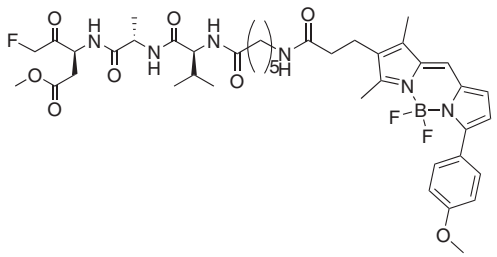
directly used for the condensation with **8**. LC/MS: R_t 5.32 min; linear gradient 10→90% B in 13.5 min; ESI/MS: *m/z* = 402.2 $(M+H)^+$, 302.2 $(M\text{-}Boc+H)^+$.



***N*-tert-butoxycarbonyl-aspartyl(OMe)-fluoromethylketone (8)**

Boc-Asp-OBn **6** (1.6 g, 5 mmol) was treated with iodomethane (0.622 mL, 10 mmol, 2 equiv.) and K_2CO_3 (0.69 g, 5 mmol) in DMF. After 16h stirring, the reaction was diluted with EtOAc, washed with 1M HCl, $NaHCO_3$ (sat. aq.), brine, dried ($MgSO_4$) and concentrated *in vacuo*. The resulting Boc-Asp(OMe)OBn was dissolved in EtOAc before being debenzylated with palladium (10%) on charcoal under H_2 atmosphere. TLC-analysis showed complete conversion of the benzyl ester after 3h. Filtration over celite and ensuing concentration afforded Boc-Asp(OMe)OH **7** (1.36 g, 5 mmol). Acid **7** was dissolved in THF (25 mL), cooled to 0°C, and carbonyldiimidazole (851 mg, 5.25 mmol) was added. The reaction was stirred for 1h at 0°C.

Monobenzyl-fluoromalonate (1.33 g, 6.25 mmol)[14] was dissolved in THF (2 mL/mmol) and cooled to 0°C before isopropylmagnesium chloride (2M in THF, 6.25 mL, 2 equiv.) was added. The white suspension was stirred for 1h and subsequently added dropwise to the precooled (-20°C) mixture of **7** and CDI. After 45 min of stirring at -20°C and 3.5h additional stirring at room temperature, the reaction was poured into 1M HCl, extracted with EtOAc, washed with $NaHCO_3$ (sat. aq.), brine, dried (Na_2SO_4) and concentrated *in vacuo*. The residue was redissolved in EtOAc, a catalytic amount of activated palladium (10%) on charcoal was added and the mixture was stirred overnight under H_2 atmosphere. Filtration over celite, concentration under reduced pressure followed by silica gel column chromatography (Tol→5% EtOAc/Tol) gave title compound **8** in 68% yield (0.893 g, 3.4 mmol). ^1H NMR (600 MHz, $CDCl_3$) δ ppm 5.54 (d, *J* = 7.9 Hz, 1H), 5.23 (dd, *J* = 47.0, 16.4 Hz, 1H), 5.12 (dd, *J* = 47.3, 16.5 Hz, 1H), 4.63 (td, *J* = 9.2, 5.2, 5.2 Hz, 1H), 3.70 (s, 3H), 3.08 (dd, *J* = 17.3, 4.1 Hz, 1H), 2.84 (dd, *J* = 17.3, 4.5 Hz, 1H), 1.46 (s, 9H). ^{13}C NMR (150 MHz, $CDCl_3$) δ ppm 203.17 (d, *J* = 16.4 Hz), 171.69, 155.19, 84.14 (d, *J* = 183.4 Hz), 80.76, 53.49, 52.21, 35.36, 28.20. $[\alpha]_D^{23}$ -5.6° (c = 1.26, $CHCl_3$). FT-IR: ν_{max}(neat)/cm^{-1} 3362.2, 2980.0, 1706.0, 1505.8, 1438.7, 1393.8, 1367.6, 1247.6, 1159.8, 1055.4, 1000.0. HRMS: $(M\text{-}Boc+H^+)$ calcd for $C_6H_{11}FNO_3$ 164.07175, found 164.07166.

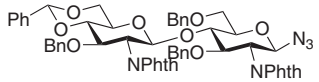


Bodipy-Ahx-Val-Ala-Asp(OMe) Fmk (1)

Fluoromethylketone **8** (40 mg, 0.15 mmol, 1.25 equiv.) was dissolved in 4M HCl in dioxane (2 mL). After 45 min, TLC analysis showed completed conversion to a very polar product. The solution was concentrated *in vacuo*, coevaporated thrice with toluene and dissolved in DMF (2 mL) before Boc-Ahx-Val-Ala-OH **9** (48 mg, 0.12 mmol), HCTU (62 mg, 0.15 mmol, 1.25 equiv.) and diisopropylethylamine (52 µL, 0.30 mmol, 2.5 equiv.) were added. LC/MS analysis showed complete conversion after 2 h. The reaction was diluted with CH_2Cl_2, washed with $NaHCO_3$ (sat. aq.), 1M HCl, brine, dried (Na_2SO_4) and concentrated. Purification by silica gel chromatography (CH_2Cl_2→1% MeOH/CH_2Cl_2) gave peptide **10** (68%, 45 mg, 82 µmol). LC/MS: R_t 7.10 min; linear gradient 10→90% B in 13.5 min; *m/z* = 547.2 $(M+H)^+$, 447.2 $(M\text{-}Boc+H)^+$.

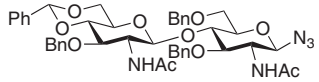
Intermediate **10** was dissolved in TFA/H_2O (2 mL, 95/5 v/v), stirred for 30 min and concentrated under reduced pressure. Residual traces of TFA were removed by coevaporation with toluene. Subsequently, the free amine was dissolved in DMF, treated with BODIPY TMR-OSu (41 mg, 82 µmol, 1 equiv.) and diisopropylethylamine (36 µL, 0.2 mmol, 2.5 equiv.) and stirred overnight. The solution was diluted with CH_2Cl_2, washed with 1M HCl, dried (Na_2SO_4) and concentrated. RP-HPLC yielded BODIPY TMR-Ahx-Val-Ala-Asp(OMe)-Fmk **1** as a diastereomeric mixture (41%, 28.3 mg, 34 µmol). RP-HPLC: R_t 2.8 cv; linear gradient 45%→48.5% in 3 cv. Silica gel chromatography ($CHCl_3$→3% MeOH/$CHCl_3$) gave both separated diastereomers. Diastereomer 1: ^1H NMR (600 MHz, $CDCl_3$) δ ppm 7.97-7.77 (m, 2H), 7.69-7.44 (m, 2H), 7.01-6.93 (m, 2H), 6.61-6.47 (m, 1H), 6.11-5.94 (m, 1H), 5.23-4.76 (m, 2H), 4.47-4.40 (m, 1H), 3.86 (s, 1H), 3.66 (s, 1H), 3.31-3.19 (m, 1H), 3.14-3.05

(m, 1H), 2.98-2.67 (m, 4H), 2.53 (s, 3H), 2.33-2.26 (m, 2H), 2.21 (s, 1H), 2.17-1.93 (m, 4H), 1.49-1.08 (m, 1H), 1.00-0.78 (m, 9H). Diastereomer 2: ^{1}H NMR (600 MHz, $CDCl_3$) δ ppm 7.90-7.84 (m, 2H), 7.14-7.05 (m, 1H), 6.98-6.94 (m, 2H), 6.61-6.52 (m, 1H), 6.16-5.96 (m, 1H), 5.16 (dd, *J* = 46.2, 16.0 Hz, 1H), 5.02 (dd, *J* = 47.0, 16.3 Hz, 1H), 4.89-4.84 (m, 1H), 4.46-4.36 (m, 1H), 4.14 (td, *J* = 15.0, 6.9, 6.9 Hz, 1H), 3.86 (s, 1H), 3.68 (s, 3H), 3.36-3.22 (m, 1H), 3.14-2.66 (m, 5H), 2.55 (s, 3H), 2.36-2.24 (m, 2H), 2.22 (s, 1H), 2.14-1.96 (m, 3H), 1.47-1.07 (m, 1H), 1.01-0.69 (m, 9H). FT-IR: ν_{max}(neat)/cm^{-1} 3267.9, 2940.0, 1602.3, 1526.8, 1461.8, 1435.9, 1293.8, 1255.0, 1232.7, 1199.7, 1176.9, 1135.2, 1056.5, 995.8, 942.1,. LC/MS: R_t 8.15 min; linear gradient 10→90% B in 13.5 min; *m/z* = 827.4 $(M+H)^+$, 807.3 $(M-F)^+$.



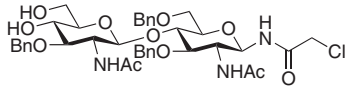
O-(3-O-benzyl-4,6-O-benzylidene-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-(1→4)-3,6-di-O-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl azide (13)[15]

Known donor **11** (1.36 g, 2.3 mmol, 1.1 equiv.), diphenylsulfoxide (0.512 g, 2.53 mmol, 1.3 equiv.) and TTBP (1.43 g, 5.75 mmol, 2.7 equiv.) were coevaporated thrice with toluene and dissolved in anhydrous CH_2Cl_2 (25 mL). Activated 4Å MS were added and the solution was stirred for 30 min before being cooled to -60°C. Tf_2O (0.406 mL, 2.415 mmol, 1.15 equiv.) was added. After 15 min stirring at -60°C, acceptor **12** (1.095 g, 2.13 mmol, 1 equiv.) was added in CH_2Cl_2 (5 mL). The temperature was raised to 0°C over 4h, after which the reaction was quenched with Et_3N, diluted with EtOAc, washed with $NaHCO_3$ (sat. aq.), brine, dried (Na_2SO_4), concentrated and purified by silica gel column chromatography (Tol→7.5% EtOAc/Tol) furnishing title compound **13** in 85% (1.79 g, 1.82 mmol) as a colorless oil. ^{1}H NMR (600 MHz, $CDCl_3$) δ ppm 7.92-7.48 (m, 9H), 7.45-7.40 (m, 2H), 7.34-7.18 (m, 9H), 7.00-6.79 (m, 11H), 5.44 (s, 1H), 5.32 (d, *J* = 8.4 Hz, 1H), 5.10 (d, *J* = 9.4 Hz, 1H), 4.74 (d, *J* = 12.4 Hz, 1H), 4.71 (d, *J* = 12.3 Hz, 1H), 4.45-4.34 (m, 5H), 4.19-4.10 (m, 4H), 3.97 (t, *J* = 9.7, 9.7 Hz, 1H), 3.64 (t, *J* = 9.1, 9.1 Hz, 1H), 3.49-3.43 (m, 2H), 3.36-3.27 (m, 3H). ^{13}C NMR (150MHz, $CDCl_3$) δ ppm 168.08, 168.06, 167.50, 167.46, 138.28, 137.97, 137.83, 137.28, 134.03, 134.01, 133.99, 133.86, 131.43, 128.96, 128.29, 128.23, 128.00, 127.95, 127.69, 127.58, 127.37, 127.31, 127.11, 126.02, 123.32, 123.30, 123.27, 123.24, 101.18, 97.65, 85.46, 83.10, 76.68, 76.50, 75.67, 74.45, 74.42, 74.08, 72.76, 68.65, 67.50, 65.74, 56.46, 55.08. FT-IR: ν_{max}(neat)/cm^{-1} 2870.0, 2114.6, 1992.1, 1776.3, 1710.2, 1615.4, 1496.6, 1468.8, 1454.6, 1385.6, 1310.8, 1254.2, 1197.2, 1173.5, 1145.5, 1067.9, 1027.5, 996.3, 969.0. $[\alpha]_D^{23}$ +17° (c = 0.43, $CHCl_3$). HRMS: $(M+Na^+)$ calcd for $C_{56}H_{49}N_5O_{12}Na$ 1006.32699, found 1006.32767.



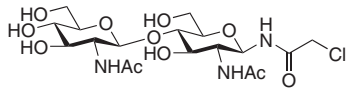
O-(2-acetamido-3-O-benzyl-4,6-O-benzylidene-2-deoxy-β-D-glucopyranosyl)-(1→4)-2-acetamido-3,6-di-O-benzyl-2-deoxy-β-D-glucopyranosyl azide (14)

Disaccharide **13** (1.79 g, 1.82 mmol) was dissolved in n-BuOH/ethylenediamine (10/1 v/v, 40 mL) followed by stirring overnight at 90°C. The reaction mixture was concentrated *in vacuo*, coevaporated with toluene, redissolved in pyridine (10 mL) and cooled to 0°C. Subsequently, acetic anhydride (2 mL) was added. After 5h stirring, the solution was concentrated, redissolved in CH_2Cl_2, extracted with 1M HCl, dried (Na_2SO_4) and concentrated. Purification over silica gel column chromatography (CH_2Cl_2→2% MeOH/CH_2Cl_2) gave title compound **14** in 81% (1.19 g, 1.48 mmol) as a white solid. ^{1}H NMR (600 MHz, DMSO-d_6) δ ppm 8.10 (d, *J* = 8.5 Hz, 1H), 8.07 (d, *J* = 9.2 Hz, 1H), 7.43-7.25 (m, 20H), 5.67 (s, 1H), 4.82 (d, *J* = 11.0 Hz, 1H), 4.75-4.70 (m, 2H), 4.67-4.51 (m, 5H), 4.03 (dd, *J* = 10.0, 4.6 Hz, 1H), 3.86-3.78 (m, 2H), 3.76-3.66 (m, 5H), 3.63 (dd, *J* = 9.5, 4.0 Hz, 1H), 3.60-3.52 (m, 2H), 3.18-3.12 (m, 2H), 1.84 (s, 3H), 1.83 (s, 3H). ^{13}C NMR (150MHz, DMSO-d_6) δ ppm 169.28, 169.19, 138.83, 138.63, 138.43, 137.47, 128.66, 128.17, 128.00, 127.98, 127.94, 127.32, 127.23, 127.19, 127.11, 127.01, 125.87, 100.76, 99.92, 87.78, 80.79, 80.07, 78.37, 76.17, 75.43, 73.26, 73.15, 71.87, 68.14, 67.64, 65.49, 55.23, 53.50, 22.87, 22.70. FT-IR: ν_{max}(neat)/cm^{-1} 3273.7, 2874.0, 2118.5, 1717.7, 1655.0, 1545.8, 1497.9, 1453.7, 1370.5, 1323.4, 1255.2, 1173.6, 1143.8, 1071.2, 1027.6, 1015.1, 960.5, 917.4, 747.4, 694.2. $[\alpha]_D^{23}$ -15° (c = 0.25, $CHCl_3$). HRMS: $(M+H^+)$ calcd for $C_{44}H_{50}N_5O_{10}$ 808.35522, found 808.35582.



N-(O-(2-acetamido-3-O-benzyl-2-deoxy-β-D-glucopyranosyl)-(1→4)-2-acetamido-3,6-di-O-benzyl-2-deoxy-β-D-glucopyranosyl) chloroacetamide (15)

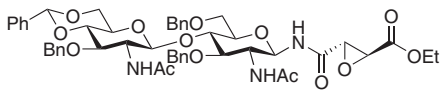
Azide **14** (225 mg, 0.3 mmol) was dissolved in DMF (2 mL), Lindlar's catalyst (50 mg) was added and the solution was stirred overnight under H_2 atmosphere. Subsequently, the mixture was purged with argon gas after which chloroacetic anhydride (77 mg, 0.45 mmol, 1.5 equiv.) and Et_3N (71 μL, 0.51 mmol, 1.7 equiv.) were added. The solution was stirred overnight, filtered, concentrated *in vacuo* and redissolved in MeOH (2 mL). *p*-Toluenesulfonic acid (6 mg, 30 μmol) was added. TLC analysis showed complete conversion to a polar product after overnight stirring. The reaction was quenched with Et_3N (0.1 mL), concentrated and applied to silica gel chromatography (CH_2Cl_2→2% MeOH/CH_2Cl_2) affording title compound **15** in 47% (109 mg, 0.14 mmol). ^{1}H NMR (500 MHz, MeOD) δ ppm 7.41-7.23 (m, 15H), 5.06 (d, *J* = 11.6, 1H), 5. 02 (d, *J* = 10.4 Hz, 1H) 4.90 (d, *J* = 11.5 Hz, 1H), 4.72-4.58 (m, 5H), 4.12 (t, *J* = 9.2, 9.2 Hz, 1H), 4.02 (d, *J* = 6.3 Hz, 1H), 3.99 (t, *J* = 10.0, 10.0 Hz, 1H), 3.83-3.75 (m, 4H), 3.64 (dd, *J* = 9.9, 9.1 Hz, 1H), 3.56-3.46 (m, 2H), 3.42 (dd, *J* = 9.7, 8.8 Hz, 1H), 3.21 (ddd, *J* = 9.5, 7.1, 2.1 Hz, 1H), 1.90 (s, 3H), 1.90 (s, 3H). ^{13}C NMR (125MHz, MeOD) δ ppm 173.75, 173.52, 169.85, 140.29, 139.67, 139.51, 129.64, 129.53, 129.41, 129.30, 129.03, 128.83, 128.76, 128.72, 128.57, 101.14, 83.87, 83.00, 80.48, 78.72, 78.35, 77.00, 75.83, 75.75, 74.36, 72.67, 69.40, 62.96, 57.30, 54.92, 43.10, 23.17, 22.82. FT-IR: ν_{max}(neat)/cm^{-1} 3277.8, 1651.8, 1557.8, 1455.5, 1372.5, 1312.7, 1050.9. $[\alpha]_D^{23}$ -2.4° (c = 0.74, MeOH). HRMS: (M+H$^+$) calcd for $C_{39}H_{49}ClN_3O_{11}$ 770.30501, found 770.30547.



N-(O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl) chloroacetamide (3)

Partially deprotected chloroacetamide **15** (30 mg, 39 μmol) was dissolved in MeOH (1 mL). 20% $Pd(OH)_2$ on activated charcoal (20 mg) was added. The resulting suspension was stirred under H_2 atmosphere. After 4h, the mixture was filtered and concentrated *in vacuo*. The crude product was acetylated by treatment with acetic anhydride (1 mL) and pyridine (2 mL). After stirring overnight, the reaction was concentrated and purified by silica gel chromatography (CH_2Cl_2→2% MeOH/CH_2Cl_2).

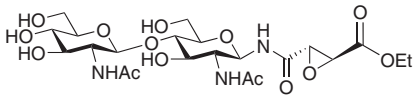
Fully acetylated haloacetamide was deprotected by stirring it under the agency of 30% NaOMe in MeOH (0.1 mL) in methanol (1 mL) until TLC showed complete conversion. Neutralization by amberlite IR-120 H$^+$ followed by concentration and purification over HW-40 gelfiltration (1% AcOH/H_2O) afforded title compound **3** as a white solid (14%, 2.69 mg, 5.4 μmol). ^{1}H NMR (600 MHz, D_2O) δ ppm 5.09 (d, *J* = 9.7 Hz, 1H), 4.60 (d, *J* = 8.5 Hz, 1H), 4.15 (d, *J* = 14.3 Hz, 1H), 4.11 (d, *J* = 14.3 Hz, 1H), 3.96-3.44 (m, 12H), 2.07 (s, 3H), 2.00 (s, 3H). ^{13}C NMR (150 MHz, D_2O) δ ppm 174.31, 174.06, 169.91, 100.83, 78.21, 78.19, 75.77, 75.33, 72.85, 71.98, 69.12, 59.95, 59.31, 55.01, 53.12, 41.46, 21.53, 21.35.



(2*S*,3*S*)-3-*N*-(*O*-(2-acetamido-3-*O*-benzyl-4,6-*O*-benzylidene-2-deoxy-β-D-glucopyranosyl)-(1→4)-2-acetamido-3,6-di-*O*-benzyl-2-deoxy-β-D-glucopyranosylcarbamoyl) oxirane-2-carboxylic acid ethyl ester (16)

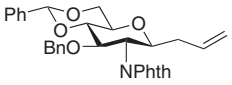
Azide **14** (225 mg, 0.3 mmol) was dissolved in DMF (2 mL), Lindlar's catalyst (50 mg) was added and the solution was stirred overnight under H_2 atmosphere. Subsequently, the mixture was purged with argon gas after which epoxisuccinate monoethyl ester (115 mg, 0.72 mmol, 2.4 equiv.), HCTU (323 mg, 0.78 mmol, 2.6 equiv.), Et_3N (0.216 mL, 1.56 mmol, 5.2 equiv) were added. After stirring overnight, the reaction was concentrated *in vacuo*, diluted with CH_2Cl_2, washed with aqueous 1M HCl, $NaHCO_3$ (sat. aq.) and brine, dried (Na_2SO_4) and evaporate to dryness. Silica gel chromatography (CH_2Cl_2→2% MeOH/CH_2Cl_2) furnished title compound **16** in 39% (110 mg, 0.119 mmol). ^{1}H NMR (500 MHz, DMF-d_7) δ ppm 8.72 (d, *J* = 9.0 Hz, 1H), 8.24 (d, *J* = 8.7 Hz, 1H), 8.21 (d,

J = 8.9 Hz, 1H), 7.52-7.24 (m, 20H), 5.73 (s, 1H), 5.12 (t, *J* = 9.3, 9.3 Hz, 1H), 5.00 (d, *J* = 11.1 Hz, 1H), 4.92 (d, *J* = 7.6 Hz, 1H), 4.84 (d, *J* = 11.9 Hz, 1H), 4.72 (d, *J* = 11.0 Hz, 1H), 4.70 (d, *J* = 11.8 Hz, 1H), 4.67 (d, *J* = 11.8 Hz, 1H), 4.62 (d, *J* = 11.8 Hz, 1H), 4.26-4.19 (m, 2H), 4.11 (dd, *J* = 10.2, 5.0 Hz, 1H), 4.01 (t, *J* = 9.0, 9.0 Hz, 1H), 3.97-3.90 (m, 3H), 3.88-3.83 (m, 2H), 3.82-3.77 (m, 2H), 3.74 (d, *J* = 1.8 Hz, 1H), 3.57-3.52 (m, 1H), 3.35-3.27 (m, 3H), 1.98 (s, 3H), 1.90 (s, 3H), 1.25 (t, *J* = 7.1, 7.1 Hz, 3H).^{13}C NMR (150 MHz, DMSO-d_6) δ ppm 169.48, 169.32, 166.78, 165.42, 138.92, 138.63, 138.44, 137.47, 128.67, 128.12, 128.01, 127.98, 127.94, 127.29, 127.23, 127.14, 127.10, 125.87, 100.40, 99.93, 80.85, 80.68, 78.31, 76.01, 75.10, 73.36, 73.15, 71.81, 68.22, 67.68, 65.50, 61.49, 53.24, 52.67, 51.13, 22.88, 22.67, 13.79. FT-IR: ν_{max}(neat)/cm^{-1} 3277.7, 1651.9, 1538.3, 1455.4, 1371.6, 1205.0, 1069.8. $[\alpha]_D^{23}$ +11.2° (c = 0.66, DMF). HRMS: (M +H$^+$) calcd for $C_{50}H_{58}N_3O_{14}$ 924.39133, found 924.39219.



(2*S*,3*S*)-3-*N*-(*O*-(2-acetamido-2-deoxy-β-D-gluco-pyranosyl)-(1→4)-2-acetamido-2-deoxy-β-D-gluco-pyranosylcarbamoyl) oxirane-2-carboxylic acid ethyl ester (4)

Epoxide **16** (27 mg, 29.2 μmol) was suspended in CH_2Cl_2 (1 mL), cooled to 0°C and treated with TFA (50 μL) and H_2O (5 μL). After 45 min stirring at 0°C, the reaction was quenched with $NaHCO_3$ (sat. aq.) followed by extraction. The organic layer was dried ($MgSO_4$) and concentrated. Silica gel chromatography purification (CH_2Cl_2→2% MeOH/CH_2Cl_2) afforded the partially protected epoxide (68%, 17 mg, 20.3 μmol). The remaining benzyl groups were removed by dissolving the epoxide in EtOH, followed by the addition of 20% $Pd(OH)_2$ on activated charcoal (cat.) and stirring under H_2 atmosphere for 16h. The solution was filtered, concentrated and applied to HW-40 gel filtration (1% AcOH/H_2O) furnishing epoxide inhibitor **4** as a white solid (67% over 2 steps, 11.06 mg, 19.6 μmol). ^{1}H NMR (600 MHz, D_2O) δ ppm 5.09 (d, *J* = 9.6 Hz, 1H), 4.59 (d, *J* = 8.4 Hz, 1H), 4.28 (q, *J* = 7.1, 7.1, 7.1 Hz, 2H), 3.96-3.43 (m, 14H), 2.06 (s, 3H), 2.01 (s, 3H), 1.29 (t, *J* = 7.2, 7.2 Hz, 3H). ^{13}C NMR (150 MHz, D_2O) δ ppm 174.26, 174.06, 168.31, 167.98, 100.83, 78.16, 77.85, 75.79, 75.33, 72.87, 72.85, 71.92, 69.12, 62.90, 59.95, 59.30, 55.01, 53.19, 52.72, 52.05, 21.53, 21.38, 12.58. FT-IR: ν_{max}(neat)/cm^{-1} 3275.0, 1737.5, 1651.0, 1539.8, 1410.3, 1374.5, 1309.5, 1205.3, 1159.8, 1023.7, 943.9. $[\alpha]_D^{23}$ +19.2° (c = 0.24, H_2O). HRMS: (M+H$^+$) calcd for $C_{22}H_{36}N_3O_{14}$ 566.21918, found 566.21904.

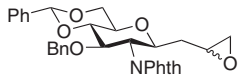


3-*C*-(3-*O*-benzyl-4,6-*O*-benzylidene-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-1-propene (18)

Known acetylated allyl glucosamine **17** (12.6 g, 27.4 mmol) was dissolved in MeOH. Amberlite IR-120 H$^+$ was added until pH 3. The reaction mixture was refluxed overnight after which TLC-analysis showed complete conversion of the starting material to a lower running spot. Subsequently, the solution was filtered, coevaporated thrice with anhydrous toluene and dissolved in MeCN. Benzaldehyde dimethylacetal (5.06 mL, 33.6 mmol, 1.2 equiv.) and pTsOH (521 mg, 2.74 mmol, 0.1 equiv.) were added. After 4h stirring, the reaction was quenched with Et_3N (5 mL) and concentrated *in vacuo*. Purification by silica gel column chromatography (5% EtOAc/PE→25% EtOAc/PE) gave the benzylidene protected glucosamine (91%, 10.57 g, 25 mmol). ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 7.88-7.68 (m, 4H), 7.51-7.32 (m, 5H), 5.74 (tdd, *J* = 17.1, 10.2, 6.9, 6.9 Hz, 1H), 5.55 (s, 1H), 4.95 (ddd, *J* = 17.2, 3.0, 1.4 Hz, 1H), 4.90 (ddd, *J* = 10.3, 2.9, 1.4 Hz, 1H), 4.62 (dd, *J* = 10.2, 9.1 Hz, 1H), 4.37-4.31 (m, 2H), 4.14 (t, *J* = 10.2, 10.2 Hz, 1H), 3.73 (dd, *J* = 10.3, 9.9 Hz, 1H), 3.60 (dt, *J* = 9.9, 9.5, 5.1 Hz, 1H), 3.52 (dd, *J* = 9.5, 9.1 Hz, 1H), 2.73 (s, 1H), 2.28-2.24 (m, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) δ ppm 168.26, 168.03, 137.06, 134.13, 134.09, 133.01, 131.59, 131.43, 129.21, 128.28, 126.25, 123.63, 123.24, 117.36, 101.78, 82.62, 75.17, 70.05, 69.04, 68.75, 56.36, 36.79. FT-IR: ν_{max}(neat)/cm^{-1} 3311.9, 2865.7, 1768.1, 1709.7, 1662.1, 1651.9, 1472.0, 1456.7, 1440.8, 1385.8, 1359.0, 1336.7, 1251.2, 1220.5, 1122.3, 1090.2, 1056.1, 1040.2, 997.9, 968.3, 915.5, 881.6, 795.5, 770.2, 723.2, 701.8, 679.0, 662.3. $[\alpha]_D^{23}$ +4° (c = 1.00, $CHCl_3$). HRMS: (M+H$^+$) calcd for $C_{24}H_{24}NO_6$ 422.15981, found 422.15865.

Next, the resulting 3-OH (10.96 g, 26 mmol) was protected. Hence, it was coevaporated thrice with dry toluene before being dissolved in DMF (125 mL). Subsequently, benzylbromide (9.3 mL, 78

mmol, 3 equiv.) and TBAI (1.92 g, 5.2 mmol, 0.2 equiv.) were added and the reaction was cooled to 0°C. Sodium hydride, 60% in mineral oil, (1.14 g, 28.6 mmol, 1.1 equiv.) was added portionwise over 2h. TLC analysis showed complete consumption of the starting material after 4h of additional stirring. The reaction mixture was poured into NH_4Cl (sat. aq.), extracted with EtOAc, washed with 1M $Na_2S_2O_3$, brine, dried (Na_2SO_4) and concentrated. Crystallization from EtOAc/PE furnished benzyl protected **18** (68%, 9.01 g, 17.6 mmol,). ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 7.84-7.63 (m, 4H), 7.55-7.35 (m, 5H), 7.00-6.85 (m, 5H), 5.71 (dddd, *J* = 17.1, 10.2, 6.9, 6.9 Hz, 1H), 5.62 (s, 1H), 4.93 (ddd, *J* = 17.2, 3.0, 1.4 Hz, 1H), 4.89 (ddd, *J* = 10.3, 2.7, 1.2 Hz, 1H), 4.80 (d, *J* = 12.3 Hz, 1H), 4.51 (d, *J* = 12.3 Hz, 1H), 4.45 (dd, *J* = 10.0, 9.0 Hz, 1H), 4.39 (dd, *J* = 10.4, 4.9 Hz, 1H), 4.32 (td, *J* = 10.5, 5.6, 5.6 Hz, 1H), 4.14 (t, *J* = 10.2, 10.2 Hz, 1H), 3.78 (t, *J* = 10.3, 10.3 Hz, 1H), 3.77 (t, *J* = 9.1, 9.1 Hz, 1H), 3.65 (dt, *J* = 9.9, 9.8, 4.9 Hz, 1H), 2.22 (tdd, *J* = 7.0, 5.7, 1.3, 1.3 Hz, 1H). ^{13}C NMR (125 MHz, $CDCl_3$) δ ppm 167.85, 167.77, 138.02, 137.44, 133.89, 133.81, 133.02, 131.55, 131.48, 128.91, 128.23, 127.96, 127.29, 126.02, 123.36, 123.27, 117.37, 101.19, 83.62, 75.24, 75.21, 74.04, 70.19, 68.89, 55.61, 36.97. FT-IR: ν_{max}(neat)/cm^{-1} 2854.5, 1783.6, 1713.3, 1497.9, 1458.1, 1430.4, 1409.9, 1382.1, 1363.7, 1301.9, 1207.8, 1170.9, 1143.2, 1118.9, 1099.0, 1066.7, 1050.5, 1012.1, 1001.7, 964.4, 919.8. $[\alpha]_D^{23}$ +73.2° (c = 1.00, $CHCl_3$). HRMS: (M+H$^+$) calcd for $C_{31}H_{30}NO_6$ 512.20676, found 512.20665.



(2*R*/*S*)-3-*C*-(3-*O*-benzyl-4,6-*O*-benzylidene-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-1,2-epoxypropane (19)

Compound **18** (7.67 g, 15 mmol) was dissolved in dichloromethane (150 mL). After the addition of *m*-chloroperoxybenzoic acid (8.51 g, 34.5 mmol, 2.3 equiv.) the reaction mixture was refluxed for 4h. Subsequently, the reaction was diluted with EtOAc before being washed with aqueous 1M $Na_2S_2O_3$, $NaHCO_3$ (sat. aq.) and brine. The organic layer was dried (Na_2SO_4) and concentrated. Silica gel column chromatography (20% EtOAc/PE→30% EtOAc/PE) gave a 2:3 mixture of diastereomers **19a** and **19b** of epoxide **19** in 88% (6.96 g, 13.2 mmol). ^{1}H NMR (600 MHz, $CDCl_3$) δ ppm 7.85-7.80 (m, 1H), 7.75-7.67 (m, 2H), 7.65-7.62 (m, 1H), 7.55-7.51 (m, 2H), 7.43-7.35 (m, 3H), 7.01-6.84 (m, 5H), 5.63 (s, 1H), 4.80 (d, *J* = 12.3 Hz, 1H), 4.53-4.48 (m, 2H), 4.47-4.42 (m, 1H), 4.42-4.37 (m, 1H), 4.17 (t, *J* = 10.3, 10.3 Hz, 0.4H), 4.12 (t, *J* = 10.2, 10.2 Hz, 0.6H), 3.83-3.75 (m, 2H), 3.73-3.65 (m, 1H), 3.06-2.99 (m, 1H), 2.71 (dd, *J* = 4.7, 4.2 Hz, 0.6H), 2.65 (dd, *J* = 4.9, 4.1 Hz, 0.4H), 2.37-2.33 (m, 1H), 1.85 (ddd, *J* = 15.1, 8.7, 5.5 Hz, 0.4H), 1.77 (ddd, *J* = 14.6, 8.7, 4.0 Hz, 0.6H), 1.50 (ddd, *J* = 14.9, 5.8, 3.0 Hz, 0.4H), 1.43 (ddd, *J* = 14.7, 7.5, 3.2 Hz, 0.6H). ^{13}C NMR (150 MHz, $CDCl_3$) δ ppm 167.93, 167.71, 167.64, 167.61, 137.92, 137.86, 137.35, 137.34, 134.02, 133.91, 133.89, 131.48, 131.36, 128.94, 128.23, 127.96, 127.92, 127.31, 126.00, 123.47, 123.39, 123.32, 101.20, 83.56, 83.49, 75.04, 74.09, 74.06, 74.04, 73.80, 70.32, 70.11, 68.81, 55.91, 55.64, 49.09, 48.75, 47.36, 46.33, 35.99, 35.15. FT-IR: ν_{max}(neat)/cm^{-1} 2941.8, 2853.7, 1781.9, 1710.2, 1496.0, 1467.8, 1411.5, 1381.3, 1305.2, 1292.0, 1256.1, 1208.5, 1170.7, 1140.2, 1100.1, 1087.6, 1067.2, 1047.3, 1012.4, 1000.4, 965.3, 944.4, 910.2. HRMS: (M+H$^+$) calcd for $C_{31}H_{30}NO_7$ 528.20168, found 528.20148.

C-Allylglucosamine **18** (9.01 g, 17.6 mmol) was dissolved in 160 mL THF/H_2O (6/1 v/v), treated with K_2OsO_4 (130 mg, 0.352 mmol, 0.02 equiv.) in the presence of 4-methylmorpholino-*N*-oxide (5.2 g, 44 mmol, 2.5 equiv.). TLC analysis showed complete conversion to lower running spot after overnight stirring. The solution was diluted with EtOAc, washed with 1M HCl, 1M $Na_2S_2O_3$, brine, dried ($MgSO_4$) and concentrated under reduced pressure. Purification by column chromathography (2% EtOH/CH_2Cl_2) yielded higher running diastereomer **25a** (1.72 g, 3.1 mmol), lower running diastereomer **25b** (4.09 g, 7.5 mmol) and a mixture of alcohols **25a** and **25b** (3.49 g, 6.4 mmol) furnishing **25** in 97% total yield. Diol **25a** and **b** were dissolved in anhydrous CH_2Cl_2 under Argon atm. The reaction mixture was cooled to 0°C, Et_3N (1.5 equiv.) and 4,4'-dimethoxytritylchloride (1.1 equiv.) were added. After 3h stirring, the reaction was quenched with $NaHCO_3$ (sat. aq.) and extracted with EtOAc. The organic layer was washed with brine, dried (Na_2SO_4) and concentrated *in vacuo*. Silica gel chromathography (10% EtOAc/PE(1%Et_3N)→30% EtOAc/PE(1%Et_3N)) furnished **26a** (2.62 g, 3.1 mmol, 97%) and **26b** (5.96 g, 7.0 mmol, 94%). Primary protected **26a** and **b** were dissolved in DMF, after which it was reacted with tert-butyldimethylsilyl chloride (2 equiv.) in the presence of

Et_3N (2 equiv.) and imidazole (6 equiv.). After overnight stirring, an additional portion of tert-butyldimethylsilyl chloride (0.5 equiv.) was added followed by 2h additional stirring. Next, the solution was diluted with Et_2O, washed with $NaHCO_3$ (sat. aq.), brine, dried ($MgSO_4$) and concentrated. Column chromatography (10% EtOAc/PE(1%Et_3N)→20% EtOAc/PE(1%Et_3N)) gave **27a** (2.70 g, 2.8 mmol, 90%) and **27b** (5.81 g, 6.0 mmol, 85%).

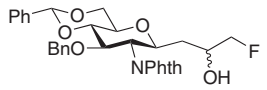
Dimethoxytrityl protected **27a** and **b** were treated with 2% dichloroacetic acid/CH_2Cl_2 (10mL/mmol) in the presence of triethylsilane (5 equiv.). After 1h, TLC analysis showed complete consumption of the starting material. The reaction was quenched with MeOH, extracted with $NaHCO_3$ (sat. aq.), dried (Na_2SO_4) and concentrated. Silica gel chromatography (5% EtOAc/PE(1%Et_3N)→40% EtOAc/PE(1%Et_3N)) afforded primary alcohol **28a** in 73% (1.35 g, 2.05 mmol) and **28b** in 82% (3.23 g, 4.9 mmol). Alcohols **28a** and **b** were coevaporated with toluene before being dissolved in anhydrous dichloromethane. Subsequently, the solution was cooled to 0°C, reacted with methanesulfonyl chloride (2.5 equiv.) under the agency of Et_3N (2.5 equiv.) and DMAP (0.1 equiv.). After stirring overnight, the reaction was diluted with EtOAc, washed with $NaHCO_3$ (sat. aq.), brine, dried (Na_2SO_4) and concentrated in vacuo. Purification by column chromatography (5% EtOAc/PE→30% EtOAc/PE) yielded mesylate **29a** (1.31 g, 1.78 mmol, 89%) and **29b** (3.359 g, 4.6 mmol, 93%). Mesylates **29a** and **b** were dissolved in THF. TBAF (1M in THF, 2.2 equiv.) was added, stirred for 2h, poured into $NaHCO_3$ (sat. aq.), extracted with EtOAc. The organic layer was washed with brine, dried (Na_2SO_4) and concentrated under reduced. The residue was purified by silica gel chromatography affording epoxides **19a** (1.12 g, 2.1 mmol, 48%) and **19b** (1.63 g, 3.10 mmol, 67%).

19a

^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 7.85-7.82 (m, 1H), 7.76-7.68 (m, 2H), 7.66-7.63 (m, 1H), 7.56-7.52 (m, 2H), 7.43-7.35 (m, 3H), 6.99-6.85 (m, 5H), 5.64 (s, 1H), 4.80 (d, *J* = 12.3 Hz, 1H), 4.52 (d, *J* = 12.3 Hz, 1H), 4.47-4.38 (m, 3H), 4.18 (t, *J* = 10.2, 10.2 Hz, 1H), 3.83-3.78 (m, 2H), 3.68 (dt, *J* = 10.0, 9.9, 4.9 Hz, 1H), 3.04-3.00 (m, 1H), 2.66 (t, *J* = 4.4, 4.4 Hz, 1H), 2.35 (dd, *J* = 4.9, 2.7 Hz, 1H), 1.86 (ddd, *J* = 14.4, 8.4, 5.3 Hz, 1H), 1.51 (ddd, *J* = 14.8, 5.8, 3.1 Hz, 1H). ^{13}C NMR (125 MHz, $CDCl_3$) δ ppm 167.68, 167.60, 137.86, 137.33, 133.99, 133.88, 131.36, 131.34, 128.89, 128.19, 127.93, 127.29, 125.97, 123.43, 123.27, 101.18, 83.46, 75.07, 74.03, 74.01, 70.31, 68.79, 55.63, 49.03, 46.29, 35.13. FT-IR: ν_{max}(neat)/cm^{-1} 2877.9, 1775.7, 1710.0, 1613.3, 1495.5, 1468.5, 1453.5, 1382.9, 1301.9, 1172.5, 1089.7, 996.2, 916.6. $[\alpha]_D^{23}$ +59° (c = 1.11, $CHCl_3$). HRMS: ($M+H^+$) calcd for $C_{31}H_{30}NO_7$ 528.20168, found 528.20003.

19b

^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 7.84-7.61 (m, 4H), 7.55-7.34 (m, 5H), 6.99-6.84 (m, 5H), 5.63 (s, 1H), 4.80 (d, *J* = 12.3 Hz, 1H), 4.50 (d, *J* = 12.3 Hz, 1H), 4.50 (dd, *J* = 9.8, 9.0 Hz, 1H), 4.45 (ddd, *J* = 10.4, 8.6, 3.2 Hz, 1H), 4.39 (dd, *J* = 10.3, 4.7 Hz, 1H), 4.12 (t, *J* = 10.2, 10.2 Hz, 1H), 3.81-3.75 (m, 2H), 3.69 (dt, *J* = 9.7, 9.6, 4.7 Hz, 1H), 3.03 (dtd, *J* = 6.9, 4.0, 4.0, 2.6 Hz, 1H), 2.70 (dd, *J* = 5.0, 4.0 Hz, 1H), 2.35 (dd, *J* = 5.0, 2.6 Hz, 1H), 1.77 (ddd, *J* = 14.6, 8.6, 4.0 Hz, 1H), 1.44 (ddd, *J* = 14.6, 6.9, 3.2 Hz, 1H). ^{13}C NMR (125 MHz, $CDCl_3$) δ ppm 167.85, 167.56, 137.89, 137.34, 133.85, 133.83, 131.46, 131.34, 128.89, 128.19, 127.93, 127.29, 125.97, 123.32, 123.26, 101.15, 83.51, 75.03, 74.03, 73.78, 70.09, 68.76, 55.88, 48.68, 47.27, 35.95. FT-IR: ν_{max}(neat)/cm^{-1} 2853.6, 1781.6, 1710.1, 1467.7, 1431.9, 1410.6, 1380.8, 1292.0, 1256.0, 1208.5, 1170.2, 1141.2, 1118.4, 1100.2, 1066.6, 1048.0, 1012.1, 1000.3, 964.2, 944.5, 913.7. $[\alpha]_D^{23}$ +55° (c = 1.00, $CHCl_3$). HRMS: ($M+H^+$) calcd for $C_{31}H_{30}NO_7$ 528.20168, found 528.20149.



(2*R*/*S*)-3-*C*-(3-*O*-benzyl-4,6-*O*-benzylidene-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-1-fluoro-2-hydroxypropane (20)

Epoxide **19** (264 mg, 0.5 mmol) and TBA·H_2F_3 (425 mg, 1.51 mmol, 3 equiv.) were suspended in toluene (2M), after which the reaction mixture was heated in the microwave to 180°C for 20 min. The resulting oil was diluted with EtOAc, washed with $NaHCO_3$ (sat. aq.), brine, dried ($MgSO_4$) and concentrated. Silica gel column chromatography purification (20% EtOAc/PE→30% EtOAc/PE) furnished fluorohydrin **20** in 84%

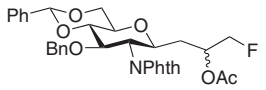
(226 mg, 0.41 mmol). ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 7.86-7.62 (m, 4H), 7.57-7.35 (m, 5H), 7.00-6.85 (m, 5H), 5.64 (s, 1H), 4.83-4.79 (m, 1H), 4.56-4.44 (m, 3H), 4.41-4.36 (m, 1H), 4.32-4.27 (m, 1H), 4.24-3.98 (m, 3H), 3.85-3.67 (m, 3H), 3.01 (s, 1H), 2.51 (s, 1H), 1.74-1.50 (m, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) δ ppm 167.88, 167.73, 167.63, 167.54, 137.87, 137.74, 137.29, 137.18, 134.02, 133.93, 133.90, 133.87, 131.41, 131.29, 131.25, 128.92, 128.88, 128.17, 127.92, 127.86, 127.32, 127.27, 125.96, 123.46, 123.37, 123.34, 101.18, 101.15, 86.65 (d, J = 169.4 Hz), 85.76 (d, J = 170.0 Hz), 83.46, 83.16, 75.32, 75.00, 74.03, 72.85, 70.26, 70.04, 68.83, 68.68, 68.50, 66.62 (d, J = 19.5 Hz), 55.79, 55.71, 34.42 (d, J = 6.5 Hz). FT-IR: ν_{max}(neat)/cm^{-1} 2871.1, 1775.6, 1709.9, 1615.4, 1496.4, 1455.4, 1385.5, 1173.2, 1087.1, 999.7, 963.0. HRMS: (M+H$^+$) calcd for $C_{31}H_{31}FNO_7$ 548.20791, found 548.20764.

20a

Diastereomerically pure epoxide **19a** (1.12 g, 2.1 mmol) was transformed to the fluorohydrin as previously depicted furnishing **20a** as a colorless oil in 62% (0.719 g, 1.31 mmol). ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 7.87-7.62 (m, 4H), 7.55-7.36 (m, 5H), 6.99-6.85 (m, 5H), 5.63 (s, 1H), 4.79 (d, J = 12.3 Hz, 1H), 4.50 (d, J = 12.3 Hz, 1H), 4.50-4.46 (m, 1H), 4.45 (dd, J = 9.9, 9.1 Hz, 1H), 4.38 (dd, J = 10.2, 4.6 Hz, 1H), 4.26 (dd, J = 47.3, 4.8 Hz, 2H), 4.17 (t, J = 10.2, 10.2 Hz, 1H), 4.08-3.98 (m, 1H), 3.83-3.76 (m, 2H), 3.71 (ddd, J = 10.1, 9.3, 4.6 Hz, 1H), 2.89 (s, 1H), 1.69-1.62 (m, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) δ ppm 167.80, 167.72, 137.83, 137.22, 134.11, 134.02, 131.40, 131.36, 129.04, 128.29, 128.01, 127.41, 126.02, 123.56, 123.45, 101.32, 85.83 (d, J = 170 Hz), 83.28, 75.63, 74.81, 74.15, 70.38, 69.00 (d, J = 20.4 Hz), 68.62, 55.89, 34.54 (d, J = 5.9 Hz). FT-IR: ν_{max}(neat)/cm^{-1} 3476.0, 2877.9, 1775.2, 1709.8, 1612.2, 1496.5, 1454.7, 1384.9, 1172.6, 1091.4, 1001.1, 962.5. $[\alpha]_D^{23}$ +57° (c = 0.27, $CHCl_3$). HRMS: (M+H$^+$) calcd for $C_{31}H_{31}FNO_7$ 548.20791, found 548.20612.

20b

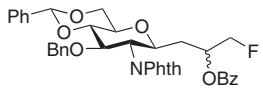
Diastereomerically pure epoxide **19b** (1.63 g, 3 mmol) was regioselectively opened as described for the diastereomeric mixture. Fluorohydrin **20b** was obtained in 77% (1.25 g, 2.3 mmol) ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 7.84-7.63 (m, 4H), 7.54-7.36 (m, 5H), 6.99-6.85 (m, 5H), 5.63 (s, 1H), 4.80 (d, J = 12.3 Hz, 1H), 4.54-4.48 (m, 3H), 4.38 (dd, J = 10.3, 4.5 Hz, 1H), 4.34 (ddd, J =47.3, 9.2, 3.0 Hz, 1H), 4.18 (ddd, J = 47.3, 9.4, 6.3 Hz, 1H), 4.16-4.05 (m, 2H), 3.78 (m, 2H), 3.69 (ddd, J = 10.2, 9.3, 4.7 Hz, 1H), 2.22 (s, 1H), 1.54 (dd, J = 6.4, 5.6 Hz, 1H). ^{13}C NMR (125 MHz, $CDCl_3$) δ ppm 167.94, 167.61, 137.95, 137.34, 134.00, 133.97, 131.52, 131.36, 128.99, 128.27, 128.00, 127.95, 127.36, 126.03, 123.47, 123.45, 101.29, 86.70 (d, J = 169.3 Hz), 83.57, 75.06, 74.15, 73.00, 70.17, 68.79, 66.83 (d, J = 19.6 Hz), 55.76, 34.46 (d, J = 6.6 Hz). FT-IR: ν_{max}(neat)/cm^{-1} 2876.0, 1775.3, 1709.9, 1496.4, 1455.1, 1385.4, 1172.8, 1086.1, 998.1, 963.9. $[\alpha]_D^{23}$ +23° (c = 1.00, $CHCl_3$). HRMS: (M+H$^+$) calcd for $C_{31}H_{31}FNO_7$ 548.20791, found 548.20765.



(2*R*/*S*)-3-*C*-(3-*O*-benzyl-4,6-*O*-benzylidene-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-1-fluoro-2-acetoxypropane (21)

Fluorohydrin **20** (4.737 g, 8.7 mmol) was dissolved in pyridine (100 mL), cooled to 0°C, before acetic anhydride (33 mL) was added. After stirring overnight, the reaction was quenched with MeOH, concentrated, diluted with EtOAc, washed with 1M HCl, $NaHCO_3$ (sat. aq.), brine, dried ($MgSO_4$) and concentrated under reduced pressure. Purification over silica gel chromatography (Tol→10% EtOAc/Tol) yielded acetylated fluorohydrin **21** (96%, 4.93 g, 8.35 mmol). ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 7.86-7.82 (m, 1H), 7.76-7.62 (m, 3H), 7.55-7.51 (m, 2H), 7.43-7.35 (m, 3H), 6.99-6.85 (m, 5H), 5.62 (s, 1H), 5.28-5.10 (m, 1H), 4.79 (d, J = 12.3 Hz, 1H), 4.54-4.32 (m, 5H), 4.28-4.21 (m, 1H), 4.14-4.07 (m, 1H), 3.80-3.72 (m, 2H), 3.68-3.58 (m, 1H), 2.02-2.00 (m, 3H), 1.86-1.62 (m, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) δ ppm 170.22, 170.05, 167.84, 167.72, 167.57, 167.51, 137.84, 137.79, 137.36, 137.30, 133.99, 133.93, 131.45, 131.36, 131.33, 128.93, 128.91, 128.20, 127.99, 127.94, 127.33, 126.00, 125.98, 123.45, 123.33, 123.28, 101.22, 83.85 (d, J = 173.8 Hz), 83.50, 83.38, 82.95 (d, J = 173.3 Hz), 74.91, 74.82, 74.03, 73.98, 72.58, 72.31, 70.21, 70.13, 69.62 (d, J = 19.4 Hz), 68.73 (d, J = 19.2 Hz), 68.69, 55.77, 55.70, 32.64 (d, J = 5.9 Hz), 31.72 (d, J = 6.5 Hz), 20.91, 20.82. FT-IR: ν_{max}(neat)/cm^{-1} 2877.0, 1775.7, 1738.4, 1710.3, 1613.2, 1495.9, 1468.9,

1454.2, 1427.7, 1383.5, 1371.9, 1233.0, 1172.5, 1097.8, 1073.6, 1013.0, 962.1, 916.5. HRMS: (M+H$^+$) calcd for $C_{33}H_{33}FNO_8$ 590.21847, found 590.21671.



(2*R*/*S*)-3-*C*-(3-*O*-benzyl-4,6-*O*-benzylidene-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-1-fluoro-2-benzoyloxypropane (22)

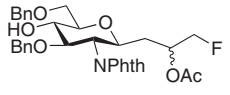
After coevaporation with anhydrous toluene, fluorohydrin **20** (0.826 g, 1.5 mmol) was dissolved in pyridine (7 mL), cooled to 0°C, treated with benzoylchloride (0.435 mL 3.75 mmol, 2.5 equiv.) and a catalytic amount of DMAP. TLC analysis showed complete conversion of the starting material to a higher running spot, after overnight stirring. The reaction mixture was concentrated, redissolved in EtOAc and washed with 1M HCl, $NaHCO_3$ (sat. aq.) and brine. The organic layer was dried ($MgSO_4$), concentrated and applied to silica gel column chromathography (5% EtOAc/PE→20% EtOAc/PE) affording benzoyl protected **22** in 92% (0.895 g, 1.4 mmol) as a colorless oil. ^{1}H NMR (600 MHz, $CDCl_3$) δ ppm 7.98-7.93 (m, 2H), 7.87-7.80 (m, 1H), 7.77-7.62 (m, 3H), 7.59-7.47 (m, 4H), 7.44-7.33 (m, 5H), 6.96-6.83 (m, 5H), 5.60-5.56 (m, 1H), 5.48-5.37 (m, 1H), 4.80-4.75 (m, 1H), 4.65-4.53 (m, 1H), 4.51-4.36 (m, 4H), 4.29 (dd, *J* = 10.5, 4.9 Hz, 1H), 4.18-4.09 (m, 1H), 3.79-3.67 (m, 2H), 3.65-3.53 (m, 2H), 2.01-1.75 (m, 2H). ^{13}C NMR (150 MHz, $CDCl_3$) δ ppm 167.91, 167.83, 167.52, 167.48, 165.80, 165.62, 137.87, 137.83, 137.34, 137.32, 134.02, 133.97, 133.94, 133.90, 133.14, 133.09, 131.46, 131.39, 131.33, 131.31, 129.76, 129.67, 129.64, 128.95, 128.92, 128.36, 128.28, 128.23, 128.21, 127.98, 127.96, 127.33, 126.00, 123.51, 123.44, 123.37, 123.35, 101.22, 83.86 (d, *J* = 174.1 Hz), 83.51, 83.36, 83.01 (d, *J* = 173.9 Hz), 74.90, 74.87, 74.05, 74.01, 73.13, 72.42, 70.39 (d, *J* = 19.6 Hz), 70.11, 69.39 (d, *J* = 19.3 Hz), 68.66, 68.60, 55.88, 55.81, 32.59 (d, *J* = 5.7 Hz), 32.11 (d, *J* = 6.1 Hz). FT-IR: ν_{max}(neat)/cm^{-1} 1775.4, 1710.1, 1452.4, 1383.5, 1267.2, 1175.1, 1096.0, 1013.4.

22a

Fluorohydrin **20a** (0.719 g, 1.31 mmol) was benzoylated as described above, giving title compound **22a** in 91% (0.778 g, 1.19 mmol) as a colorless oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 8.01-7.95 (m, 2H), 7.84-7.81 (m, 1H), 7.74-7.65 (m, 2H), 7.58-7.49 (m, 4H), 7.44-7.36 (m, 5H), 6.99-6.82 (m, 5H), 5.58 (s, 1H), 5.43 (dddd, *J* = 22.6, 10.4, 6.7, 4.1 Hz, 1H), 4.78 (d, *J* = 12.3 Hz, 1H), 4.60-4.47 (m, 4H), 4.44 (dd, *J* = 9.9, 8.9 Hz, 1H), 4.16-4.10 (m, 2H), 3.75 (t, *J* = 8.9, 8.9 Hz, 1H), 3.67-3.59 (m, 2H), 1.98-1.86 (m, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) δ ppm 167.85, 167.56, 165.65, 137.88, 137.36, 133.98, 133.92, 133.11, 131.38, 131.35, 130.13, 129.80, 129.68, 128.95, 128.29, 128.23, 127.97, 127.33, 126.01, 123.47, 123.37, 101.24, 83.02 (d, *J* = 174.0 Hz), 82.34, 74.94, 74.08, 73.18, 70.40 (d, *J* = 19.6 Hz), 70.15, 68.63, 55.93, 32.14 (d, *J* = 6.2 Hz). FT-IR: ν_{max}(neat)/cm^{-1} 1776.3, 1713.8, 1699.9, 1455.3, 1385.6, 1270.0, 1096.0, 1014.5. $[\alpha]_D^{23}$ +48° (c = 0.57, $CHCl_3$). HRMS: (M+Na$^+$) calcd for $C_{38}H_{34}FNO_8Na$ 674.21607, found 674.21425.

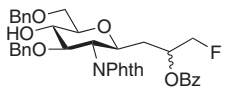
22b

Fluorohydrin **20b** (1.25 g, 2.3 mmol) was converted to benzoyl protected fluorohydrin **22b** as depicted for **22** giving benzoyl protected **22b** as a colorless oil in 92% yield (1.39 g, 2.13 mmol). ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 7.99-7.92 (m, 2H), 7.87-7.85 (m, 1H), 7.77-7.70 (m, 2H), 7.65-7.63 (m, 1H), 7.58-7.54 (m, 1H), 7.44-7.34 (m, 5H), 6.98-6.84 (m, 5H), 5.60 (s, 1H), 5.45 (dddd, *J* = 23.9, 9.5, 6.9, 3.4 Hz, 1H) 4.78 (d, *J* = 12.3 Hz, 1H), 4.67-4.37 (m, 5H), 4.30 (dd, *J* = 10.4, 4.9 Hz, 1H), 4.16 (t, *J* = 10.2, 10.2 Hz, 1H), 3.78 (t, *J* = 9.1, 9.1 Hz, 1H), 3.71 (t, *J* = 10.3, 10.3 Hz, 1H), 3.57 (dt, *J* = 9.8, 9.8, 4.9 Hz, 1H), 1.94 (ddd, *J* = 14.8, 9.5, 2.0 Hz, 1H), 1.81 (ddd, *J* = 14.8, 10.0, 3.4 Hz, 1H). ^{13}C NMR (125 MHz, $CDCl_3$) δ ppm 167.90, 167.49, 165.80, 137.90, 137.36, 134.01, 133.93, 133.13, 131.48, 131.41, 129.64, 128.91, 128.35, 128.20, 127.98, 127.95, 127.32, 126.01, 123.50, 123.37, 101.24, 83.86 (d, *J* = 174.1 Hz), 83.51, 74.95, 74.02, 72.45, 70.14, 69.41 (d, *J* = 19.3 Hz), 68.67, 55.83, 32.61 (d, *J* = 5.9 Hz). FT-IR: ν_{max}(neat)/cm^{-1} 2875.5, 1775.9, 1710.2, 1602.6, 1495.9, 1452.4, 1384.8, 1315.0, 1266.3, 1174.9, 1097.3, 1069.9, 1026.2, 1001.8, 962.8. $[\alpha]_D^{23}$ +198° (c = 1.00, $CHCl_3$). HRMS: (M+Na$^+$) calcd for $C_{38}H_{34}FNO_8Na$ 674.21607, found 674.21594.



(2*R*/*S*)-3-*C*-(3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-1-fluoro-2-acetoxypropane (23)

Acetyl protected fluorohydrin **21** (4.83 g, 8.18 mmol) was coevaporated thrice with toluene before being dissolved in freshly distilled dichloromethane. Activated 4Å MS and triethylsilane (4.36 mL, 27 mmol, 3.3 equiv.) were added and the reaction mixture was cooled to -78°C. Subsequently, trifluoromethanesulfonic acid (2.17 mL, 24.54 mmol, 3 equiv.) was added and the reaction was stirred for 45 min at -78°C. The reaction was quenched by addition of MeOH (5 mL) and Et_3N (5 mL), warmed to room temperature, extracted with $NaHCO_3$ (sat. aq.), brine, dried ($MgSO_4$) and concentrated. Purification by silicagel chromathography (10% EtOAc/PE→40% EtOAc/PE) gave building block **23** in 84% (4.52 g, 6.9 mmol) as colorless oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 7.81-7.76 (m, 1H), 7.71-7.63 (m, 3H), 7.38-7.28 (m, 5H), 7.03-6.99 (m, 2H), 6.94-6.90 (m, 3H), 5.30-5.10 (m, 1H), 4.80-4.73 (m, 1H), 4.62 (d, *J* = 12.0 Hz, 1H), 4.59-4.00 (m, 8H), 3.87-3.69 (m, 3H), 3.62-3.52 (m, 1H), 3.19-3.10 (m, 1H), 2.02-1.97 (m, 3H), 1.84-1.60 (m, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) δ ppm 170.19, 170.03, 167.82, 167.78, 167.70, 138.07, 137.98, 137.55, 133.84, 133.80, 131.28, 131.18, 128.32, 127.93, 127.90, 127.68, 127.64, 127.61, 127.56, 127.22, 127.19, 123.27, 123.19, 123.11, 83.82 (d, *J* = 173.6 Hz), 82.99 (d, *J* = 172.6 Hz), 79.30, 79.11, 77.68, 77.43, 74.21, 73.92, 73.53, 73.49, 71.54, 71.50, 70.28, 69.86 (d, *J* = 19.3 Hz), 68.93 (d, *J* = 19.0 Hz), 55.41, 55.24, 32.28 (d, *J* = 5.9 Hz), 31.38 (d, *J* = 6.6 Hz) 20.79, 20.73. FT-IR: ν_{max}(neat)/cm^{-1} 3475.9, 2871.9, 1774.1, 1738.5, 1709.8, 1496.8, 1454.1, 1384.0, 1233.9, 1074.2, 1026.1, 962.6,. HRMS: (M+H$^+$) calcd for $C_{33}H_{35}FNO_8$ 592.23412, found 592.23417.



(2*R*/*S*)-3-*C*-(3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-1-fluoro-2-benzoyloxypropane (24)

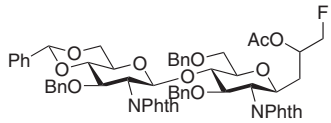
Benzoyl protected fluorohydrin **22** (0.895 g, 1.4 mmol) was converted to acceptor **24** as described for acetylated **23**. Silica gel purification (10% EtOAc/PE→30% EtOAc/PE) furnished **24** (80%, 0.730 g, 1.11 mmol) as a colorless oil. ^{1}H NMR (600 MHz, $CDCl_3$) δ ppm 7.99-7.92 (m, 2H), 7.84-7.77 (m, 1H), 7.74-7.62 (m, 3H), 7.58-7.53 (m, 1H), 7.45-7.27 (m, 7H), 7.03-6.89 (m, 5H), 5.50-5.38 (m, 1H), 4.78-4.70 (m, 1H), 4.67-4.34 (m, 5H), 4.33-4.27 (m, 1H), 4.25-4.20 (m, 1H), 4.10-4.05 (m, 1H), 3.87-3.80 (m, 1H), 3.78-3.74 (m, 1H), 3.70-3.66 (m, 1H), 3.61-3.47 (m, 1H), 2.98-2.83 (m, 1H), 1.99-1.69 (m, 2H). ^{13}C NMR (150 MHz, $CDCl_3$) δ ppm 167.97, 167.84, 167.79, 165.82, 165.61, 138.19, 137.55, 133.98, 133.92, 133.10, 131.48, 131.42, 129.66, 128.46, 128.33, 128.03, 127.84, 127.74, 127.67, 127.31, 123.46, 123.34, 83.96 (d, *J* = 172.5 Hz), 83.10 (d, *J* = 172.5 Hz), 82.53, 79.23, 77.20, 74.59, 74.32, 73.65, 72.21, 70.54, 69.64 (d, *J* = 19.5 Hz), 55.53, 55.50, 32.37 (d, *J* = 6.0 Hz). FT-IR: ν_{max}(neat)/cm^{-1} 3479.8, 3031.9, 2873.5, 1775.5, 1709.9, 1700.0, 1602.3, 1495.8, 1469.0, 1452.6, 1385.1, 1315.8, 1267.1, 1207.6, 1176.8, 1070.2, 1025.7, 964.5. HRMS: (M+H$^+$) calcd for $C_{38}H_{37}FNO_8$ 654.24977, found 654.24998.

24a

The benzylidene of diastereomerically pure **22a** (0.713 g, 1.1 mmol) was regioselectively opened as described for **23**. After silica gel purification (10% EtOAc/PE→30% EtOAc/PE) acceptor **24a** (79%, 0.570 g, 0.87 mmol) was obtained. ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 7.99-7.94 (m, 2H), 7.81-7.51 (m, 4H), 7.43-7.28 (m, 8H), 7.04-6.91 (m, 5H), 5.42 (dddd, *J* = 22.4, 12.0, 6.2, 3.2 Hz, 1H), 4.73 (d, *J* = 12.2 Hz, 1H), 4.56 (ddd, *J* = 47.6, 10.6, 5.0 Hz, 1H), 4.54 (d, *J* = 12.0 Hz, 1H), 4.54 (ddd, *J* = 47.6, 10.6, 3.2 Hz, 1H), 4.51 (d, *J* = 12.2 Hz, 1H) 4.46 (d, *J* = 12.0 Hz, 1H), 4.39 (td, *J* = 10.4, 6.0, 6.0 Hz, 1H), 4.25 (dd, *J* = 10.3, 8.6 Hz, 1H), 4.06 (t, *J* = 10.3, 10.3 Hz, 1H), 3.80 (t, *J* = 8.6, 8.6 Hz, 1H), 3.71-3.66 (m, 1H), 3.61-3.57 (m, 2H), 2.87 (s, 1H), 1.89 (t, *J* = 6.0, 6.0 Hz, 1H). ^{13}C NMR (125 MHz, $CDCl_3$) δ ppm 167.87, 165.62, 138.17, 137.55, 133.94, 133.88, 133.01, 131.44, 131.34, 129.89, 129.71, 128.49, 128.34, 128.29, 128.25, 128.09, 128.07, 127.86, 127.76, 127.74, 127.70, 127.36, 123.44, 123.29, 83.12 (d, *J* = 173.43 Hz), 79.26, 77.28, 74.54, 74.35, 73.67, 72.26, 70.64, 70.61 (d, *J* = 19.56 Hz), 55.52, 31.91 (d, *J* = 6.33 Hz). FT-IR: ν_{max}(neat)/cm^{-1} 1702.1, 1383.9, 1269.7, 1070.9. $[\alpha]_D^{23}$ +39° (c = 0.67, $CHCl_3$). HRMS: (M+H$^+$) calcd for $C_{38}H_{37}FNO_8$ 654.24977, found 654.24831.

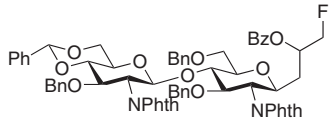
24b

Protected **22b** (1.32 g, 2.03 mmol) was converted to acceptor **24b** as depicted for **23**. Silica gel column chromatography gave title compound **24b** (85%, 1.13 g, 1.73 mmol). ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm 7.99-7.94 (m, 2H), 7.84-7.64 (m, 4H), 7.59-7.40 (m, 3H), 7.39-7.29 (m, 5H), 7.03-6.92 (m, 5H), 5.46 (qdd, *J* = 24.0, 9.8, 4.2, 3.7, 2.7 Hz, 1H), 4.76 (d, *J* = 12.2 Hz, 1H), 4.61 (ddd, *J* = 48.1, 10.4, 2.7 Hz, 1H), 4.60 (d, *J* = 12.2 Hz, 1H), 4.53 (d, *J* = 12.0 Hz, 1H), 4.51 (d, *J* = 12.0 Hz, 1H), 4.39 (ddd, *J* = 46.8, 10.4, 4.2 Hz, 1H), 4.31 (dt, *J* = 10.2, 10.2, 2.1 Hz, 1H), 4.24 (dd, *J* = 10.3, 8.5 Hz, 1H), 4.09 (t, *J* = 10.3, 10.2 Hz, 1H), 3.85 (dd, *J* = 9.3, 8.5 Hz, 1H), 3.77 (dd, *J* = 10.1, 4.3 Hz, 1H), 3.69 (dd, *J* = 10.1, 5.1 Hz, 1H), 3.51 (td, *J* = 9.4, 5.1, 4.3 Hz, 1H), 2.89 (s, 1H), 1.92-1.85 (m, 1H), 1.78 (ddd, *J* = 14.5, 10.2, 3.7 Hz, 1H). ^{13}C NMR (125 MHz, $CDCl_3$) δ ppm 167.94, 167.78, 165.80, 138.21, 137.56, 133.96, 133.90, 133.08, 131.49, 131.44, 129.72, 129.67, 128.46, 128.33, 128.04, 127.84, 127.74, 127.31, 123.45, 123.32, 83.95 (d, *J* = 173.9 Hz), 79.25, 77.18, 74.62, 74.31, 73.69, 71.72, 70.54, 69.65 (d, *J* = 19.1 Hz), 55.54, 32.38 (d, *J* = 5.8 Hz). FT-IR: ν_{max}(neat)/cm^{-1} 3474.9, 2923.0, 1775.3, 1709.9, 1699.9, 1602.4, 1496.1, 1452.7, 1385.4, 1266.9, 1176.8, 1070.2, 1025.7. $[\alpha]_D^{23}$ +63° (c = 1.24, $CHCl_3$). HRMS: (M+H$^+$) calcd for $C_{38}H_{37}FNO_8$ 654.24977, found 654.24836.



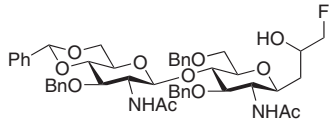
(2*R*/*S*)-3-*C*-(*O*-(3-*O*-benzyl-4,6-*O*-benzylidene-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-(1→4)-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-1-fluoro-2-acetoxypropane (30)

Before being dissolved in freshly distilled CH_2Cl_2, known donor **11** (454 mg, 0.78 mmol, 1.2 equiv.), diphenylsulfoxide (174 mg, 0.86 mmol, 1.3 equiv.) and TTBP (487 mg, 1.96 mmol, 3.0 equiv.) were coevaporated thrice with toluene. Subsequently, activated 4Å MS were added and the reaction mixture was cooled to -60°C. Tf_2O (145 μL, 0.86 mmol, 1.3 equiv.) was added. After 10 min preactivation, acceptor **23** (427 mg, 0.65 mmol) was added, followed by stirring at -60°C for 1h. The temperature was slowly raised to 0°C over a period of 4h, the reaction was quenched by addition of Et_3N, diluted with EtOAc, washed with $NaHCO_3$ (sat. aq.), brine, dried and concentrate *in vacuo*. Column chromatography (Tol→10% EtOAc/Tol) furnished title compound **30** as a colorless oil (97%, 668 mg, 0.63 mmol). ^{1}H NMR (600 MHz, $CDCl_3$) δ ppm 8.03-6.79 (m, 28H), 5.53-5.50 (m, 1H), 5.39-5.34 (m, 1H), 5.22-5.00 (m, 1H), 4.85-4.76 (m, 2H), 4.57-4.10 (m, 11H), 4.02-3.91 (m, 2H), 3.76-3.68 (m, 1H), 3.57-3.34 (m, 3H), 3.32-3.18 (m, 2H), 1.96-1.85 (m, 3H), 1.71-1.51 (m, 2H). ^{13}C NMR (150 MHz, $CDCl_3$) δ ppm 170.31, 170.09, 167.86, 167.73, 167.70, 167.61, 138.53, 138.45, 138.22, 138.04, 137.85, 137.30, 137.28, 133.86, 133.78, 131.40, 128.93, 128.28, 128.23, 128.20, 127.97, 127.92, 127.90, 127.89, 127.87, 127.80, 127.75, 127.55, 127.51, 127.34, 127.33, 127.26, 127.20, 127.02, 127.01, 126.01, 123.35, 123.25, 101.13, 97.58, 97.39, 83.97 (d, *J* = 173.7 Hz), 83.14, 83.13, 83.09 (d, *J* = 172.6 Hz), 78.25, 78.16, 77.27, 77.22, 77.16, 77.01, 76.79, 76.18, 75.93, 74.43, 74.40, 74.35, 74.31, 74.04, 72.61, 72.59, 71.58, 71.33, 69.97 (d, *J* = 19.4 Hz), 68.85 (d, *J* = 19.0 Hz), 68.70, 68.68, 68.17, 68.08, 65.69, 65.61, 56.46, 55.82, 55.67, 32.40 (d, *J* = 6.2 Hz), 31.50 (d, *J* = 6.7 Hz), 20.87, 20.83. FT-IR: ν_{max}(neat)/cm^{-1} 2873.8, 1774.7, 1739.9, 1710.2, 1612.1, 1496.1, 1468.1, 1454.0, 1383.9, 1233.5, 1173.0, 1144.7, 1070.8, 1027.4, 997.4, 967.8. HRMS: (M+Na$^+$) calcd for $C_{61}H_{57}FN_2O_{14}Na$ 1083.36860, found 1083.36897.



(2*R*/*S*)-3-*C*-(*O*-(3-*O*-benzyl-4,6-*O*-benzylidene-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-(1→4)-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-1-fluoro-2-benzoyloxypropane (31)

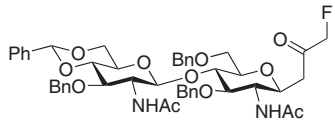
Acceptor **24** (574 mg, 0.88 mmol) was condensed with donor **11** as described for disaccharide **30**. Purification by silica gel chromatography (Tol→7.5% EtOAc/Tol) gave disaccharide **31** in 51% (500 mg, 0.45 mmol) as a colorless oil which was directly used for the next reaction.



(2*R*/*S*)-3-*C*-(*O*-(2-acetamido-3-*O*-benzyl-4,6-*O*-benzylidene-2-deoxy-β-D-glucopyranosyl)-(1→4)-2-acetamido-3,6-di-*O*-benzyl-2-deoxy-β-D-glucopyranosyl)-1-fluoro-2-hydroxy-propane (32)

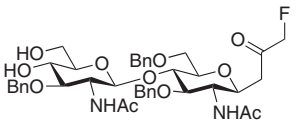
Starting from **31**: Disaccharide **31** (500 mg, 0.45 mmol) was dissolved in n-BuOH/ethylenediamine (10/1 v/v, 11 mL), before being stirred at 90°C for 8h. The mixture was cooled to rt, concentrated *in vacuo*, coevaporated with toluene and used directly in the next reaction. The mixture was suspended in pyridine (5 mL), cooled to 0°C, treated with Ac_2O (3 mL) and stirred overnight. After being quenched with MeOH, the mixture was concentrated *in vacuo*, redissolved in dichloromethane, washed with 1M HCl, dried ($MgSO_4$) and concentrated. The resulting off-white powder was dissolved in MeOH (2 mL), reacted with 30% NaOMe in MeOH (0.2 mL) for 1h, after which it was neutralized with AcOH and concentrated. Silicagel column chromathography (CH_2Cl_2→2% MeOH/CH_2Cl_2) furnished benzoyl migrated compound **33** (95 mg, 0.105 mmol, 23%, Scheme 3) and title compound **32** (56%, 211 mg, 0.250 mmol).

Starting from **30**: Disaccharide **30** (286 mg, 0.27 mmol) was converted to fluorohydrin **32** as depicted above. After silica gel purification (CH_2Cl_2→2% MeOH/CH_2Cl_2) title compound **32** was obtained as a white solid (67%, 152 mg, 0.180 mmol). ^{1}H NMR (600 MHz, DMSO-d_6) δ ppm 8.11 (d, *J* = 8.4 Hz, 1H), 7.96-7.93 (m, 1H), 7.43-7.23 (m, 20H), 5.67 (s, 1H), 5.04-4.92 (m, 1H), 4.86-4.82 (m, 1H), 4.75-4.71 (m, 2H), 4.65-4.54 (m, 4H), 4.43-4.15 (m, 2H), 4.06-4.00 (m, 1H), 3.94-3.81 (m, 1H), 3.79-3.29 (m, 12H), 3.19-3.08 (m, 1H), 1.88-1.79 (m, 6H), 1.54-1.21 (m, 2H). ^{13}C NMR (150 MHz, DMSO-d_6) δ ppm 169.31, 169.28, 169.16, 139.29, 139.27, 138.77, 138.74, 138.70, 137.60, 128.77, 128.26, 128.24, 128.12, 128.09, 128.02, 127.35, 127.21, 127.06, 125.99, 100.91, 100.07, 87.29 (d, *J* = 168.4 Hz), 86.50 (d, *J* = 167.1 Hz), 80.95, 78.13, 78.02, 76.32, 74.72, 73.29, 73.26, 71.96, 71.86, 68.82, 67.80, 66.55 (d, *J* = 18.6 Hz), 65.59, 65.49 (d, *J* = 18.9 Hz), 54.17, 54.06, 35.01 (d, *J* = 7.8 Hz), 34.85 (d, *J* = 6.5 Hz), 22.98, 22.92. FT-IR: ν_{max}(neat)/cm^{-1} 3275.4, 2870.2, 1652.0, 1538.6, 1453.7, 1371.7, 1319.7, 1072.8, 1011.3. HRMS: (M+H$^+$) calcd for $C_{47}H_{56}FN_2O_{11}$ 843.38627, found 843.38699.



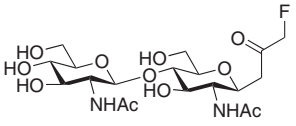
3-*C*-(*O*-(2-acetamido-3-*O*-benzyl-4,6-*O*-benzylidene-2-deoxy-β-D-glucopyranosyl)-(1→4)-2-acetamido-3,6-di-*O*-benzyl-2-deoxy-β-D-glucopyranosyl)-1-fluoro-2-propanone (34)

Fluorohydrin **32** (211 mg, 0.250 mmol) was dissolved in CH_2Cl_2 (5 mL) before being reacted with Dess-Martin periodinane (415 mg, 0.98 mmol, 3 equiv.). After 5h, TLC analysis showed complete consumption of the starting material and the reaction was quenched with 10% aq. $NaHCO_3$ (10 mL) and 2M aq. $Na_2S_2O_3$ (10 mL). The layers were separated, the organic layer was dried ($MgSO_4$) and concentrated. Purification over silica gel column chromatography (CH_2Cl_2→2% MeOH/CH_2Cl_2) gave fluoromethylketone **34** (162 mg, 0.193 mmol) in 77% as a white solid. ^{1}H NMR (600 MHz, DMSO-d_6) δ ppm 8.08 (d, *J* = 8.4 Hz, 1H), 7.97 (d, *J* = 8.8 Hz, 1H), 7.42-7.24 (m, 20H), 5.67 (s, 1H), 5.06 (ddd, *J* =46.8, 30.1, 16.7 Hz, 2H), 4.83 (d, *J* = 11.0 Hz, 1H), 4.74-4.69 (m, 2H), 4.61-4.52 (m, 4H), 4.03 (dd, *J* = 10.0, 4.7 Hz, 1H), 3.80-3.58 (m, 8H), 3.55 (t, *J* = 10.1, 10.1 Hz, 1H), 3.50 (t, *J* = 9.1, 9.1 Hz, 1H), 3.36 (dd, *J* = 9.2, 4.5 Hz, 1H), 3.13 (dt, *J* = 9.6, 9.4, 5.6 Hz, 1H), 1.84 (s, 3H), 1.80 (s, 3H). ^{13}C NMR (150MHz, DMSO-d_6) δ ppm 203.14 (d, *J* = 14.8 Hz), 169.31, 169.20, 139.05, 138.64, 138.56, 137.48, 128.66, 128.11, 128.00, 127.97, 127.92, 127.23, 127.14, 127.04, 126.92, 125.86, 100.71, 99.91, 85.12 (d, *J* = 179.8 Hz), 81.46, 80.81, 78.17, 75.94, 74.13, 73.26, 73.12, 71.72, 68.51, 67.67, 65.47, 55.33, 53.63, 40.71, 22.86, 22.72. FT-IR: ν_{max}(neat)/cm^{-1} 3274.2, 2863.9, 1727.9, 1657.8, 1651.9, 1557.8, 1497.1, 1454.4, 1371.4, 1319.4, 1174.3, 1085.8, 1028.1, 1016.2, 960.2, 917.5. $[\alpha]_D^{23}$ -5° (c = 0.28, DMF). HRMS: (M+H$^+$) calcd for $C_{47}H_{54}FN_2O_{11}$ 841.37062, found 841.37119.



3-*C*-(*O*-(2-acetamido-3-*O*-benzyl-2-deoxy-β-D-glucopyranosyl)-(1→4)-2-acetamido-3,6-di-*O*-benzyl-2-deoxy-β-D-glucopyranosyl)-1-fluoro-2-propanone (35)

The benzylidene of fully protected **34** (152 mg, 0.181 mmol) was removed as described in the first step of the synthesis of disaccharide **4**. Silica gel column chromatography (CH_2Cl_2→5% MeOH/CH_2Cl_2) furnished title compound **35** (118 mg, 0.157 mmol) in 87% as a white powder. ^{1}H NMR (500 MHz, MeOD) δ ppm 7.37-7.22 (m, 15H), 5.00 (d, *J* = 10.9 Hz, 1H), 4.89 (d, *J* = 47.2 Hz, 1H), 4.88 (d, *J* = 11.5 Hz, 1H), 4.66-4.53 (m, 6H), 4.00 (t, *J* = 9.2, 9.2 Hz, 1H), 3.86-3.65 (m, 7H), 3.53 (dd, *J* = 9.6, 9.0 Hz, 1H), 3.50-3.44 (m, 2H), 3.43-3.38 (m, 2H), 3.20 (ddd, *J* = 9.5, 7.2, 2.1 Hz, 1H), 2.70 (ddd, *J* = 16.2, 8.9, 2.1 Hz, 1H), 2.58-2.52 (m, 1H), 1.89 (s, 3H), 1.87 (s, 3H). ^{13}C NMR (150MHz, DMSO-d_6) δ ppm 203.20 (d, *J* = 14.9 Hz), 169.32, 169.12, 139.25, 139.17, 138.54, 128.22, 127.99, 127.91, 127.75, 127.45, 127.30, 127.16, 127.13, 127.04, 99.99, 85.15 (d, *J* = 179.9 Hz), 82.37, 81.77, 78.27, 76.78, 75.23, 74.22, 73.26, 72.85, 71.86, 70.09, 68.76, 60.80, 55.00, 53.55, 40.80, 22.91, 22.73. FT-IR: ν_{max}(neat)/cm^{-1} 3292.1, 1733.0, 1652.0, 1549.7, 1455.2, 1372.2, 1317.1, 1104.3, 1073.7, 1054.8, 1024.1. $[\alpha]_D^{23}$ +23.3° (c = 0.42, MeOH). HRMS: (M+H$^+$) calcd for $C_{40}H_{50}FN_2O_{11}$ 753.33931, found 753.33980.



3-*C*-(*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl)-1-fluoro-2-propanone (5)

Partly deprotected fluoromethylketone **35** (59 mg, 78.5 μmol) was dissolved in MeOH (1 mL), followed by the addition of 20% $Pd(OH)_2$ on activate charcoal (15 mg). The reaction mixture was stirred under H_2 atmosphere for 8h, after which TLC-analysis showed complete conversion. Argon gas was bubbled through, the solution was filtered over celite concentrated and purified over a HW-40 gelfiltration (1% AcOH/H_2O). Title compound **5** (16 mg, 33.2 μmol, 45%) was obtained as a white solid. ^{1}H NMR (600 MHz, D_2O) δ ppm 5.08 (d, *J* = 46.5 Hz, 1H), 4.57 (d, *J* = 8.5 Hz, 1H), 3.96-3.41 (m, 13H), 2.77-2.68 (m, 2H), 2.06 (s, 3H), 2.00 (s, 3H). ^{13}C NMR (150 MHz, D_2O) δ ppm 207.24 (d, *J* = 15.3 Hz), 174.57, 174.53, 101.41, 85.35 (d, *J* = 180.1 Hz), 79.45, 78.03, 75.88, 73.52, 73.42, 69.66, 60.49, 60.11, 55.56, 54.41, 40.09, 22.09, 22.04. FT-IR: ν_{max}(neat)/cm^{-1} 3270.1, 2872.1, 1732.6, 1652.1, 1558.1, 1406.9, 1372.9, 1306.3, 1204.1, 1162.8, 1105.6, 1077.5, 1025.8, 948.3. $[\alpha]_D^{23}$ -11.1° (c = 0.36, H_2O). HRMS: (M+H$^+$) calcd for $C_{19}H_{32}FN_2O_{11}$ 483.19846, found 483.19824.

Expression of YPng1 and YPng(C191A)

BL21/DE3 *E. coli* transfected with either pET28a-YPng1 or pET28a-YPng(C191A) were cultured overnight at 37°C in 50 mL LB media containing kanamycin (50 μg/mL). The culture was transferred to 450 mL fresh LB media and cultured at 37°C until OD_{600}=0.8. Subsequently, IPTG (5 mL, 0.1M) was added and the cells were further incubated for 3h at 37°C. Next, the cells were centrifuged at 6000 rpm for 20 min at 4°C. The resulting pellet was resuspended in 20 mM Tris (pH 8), 100 mM NaCl, 5% glycerol and 1% Triton X-100 The resulting suspension was incubated on ice for 30 min followed by 20 min sonication (90% maximum power, 5 sec pulse and 3 sec wait), the solution was centrifuged at 12000× *g* (10 min at 4°C) affording the cell extracts.

Labeling of purified YPNGase with BODIPY probe 1

Purified recombinant yeast peptide *N*-glycanase (4.7 mg/mL) was diluted with 20 mM sodium-phosphate buffer (pH 7.2), 150 mM NaCl, 5 mM DTT to a final enzyme concentration of 11.1 ng/μL. To assess the labeling properties of BODIPY TMR-Ahx-Val-Ala-Asp(OMe)-Fmk **1**, 9 μL of enzyme solution (11.1 ng/μL) was labeled for 2h at 37°C with increasing concentrations of β-VAD-Fmk **1** (1 μL). The reaction mixture was quenched by the addition of 4× SDS-PAGE sample buffer (5 μL), boiled for 3 min and separated on 10% SDS-PAGE. In-gel visualization of protein labeling was directly performed in the wet gel slabs by using the Cy3/Tamra settings (λ_{ex} 532, λ_{em} 560) on a

Typhoon Variable Mode Imager (Amersham Biosciences). Afterwards the total protein amount was quantified by silverstaining. Non-specific labeling of YPng1 was evaluated: by treating heat inactivated YPng1 (9 μL, 11.1 ng/μL boiled with 1% SDS for 3 min) with β-VAD-Fmk **1** (50 μM) or by treating a solution of 9 μL YPng1 (100 ng) and BSA (9 μg) with probe **1** (0.5 μM) in the presence or in the absence of 5 mM DTT. To assess the minimal amount of YPng1 which could be labeled and visualized with β-VAD-Fmk **1**, a serial dilution of YPng1 was incubated with 0.5 μM **1** for 1h after which direct in-gel visualization was performed.

Labeling of YPng1 and C191A with β-VAD-Fmk 1.

E.Coli cell extracts (1 mg/mL) overexpressing either YPng1 or C191A were incubated with a serial dilution of β-VAD-Fmk **1** for 1h. After denaturation, resolving on 10% SDS-PAGE, labeling was visualized as described for purified YPng1. The total protein amount was quantified using silver staining.

Competition experiments

For competition experiments, 9 μL of YPng1 (11.1 ng/μL) in reaction buffer (1 μg/μL BSA, 20 mM sodium-phosphate buffer (pH 7.2), 150 mM NaCl) was incubated with 1 μL of inhibitors **2-4** and BODIPY TMR-Ahx-Val-Ala-Asp(OMe)-Fmk **1** (0.5 μM) for 1h. The reaction was quenched by the addition of 4× SDS-PAGE sample buffer (5 μL) and boiling for 3 min. The samples were analyzed by SDS-PAGE as described above. Data was quantified with ImageQuant and analyzed with Graphpad Prism. To analyze the inhibitory potential of fluoromethylketone **5**, 1 μL of **5** was preincubated with 9 μL of YPng1 (11.1 ng/μL) in reaction buffer for 2h followed by labeling with β-VAD-Fmk **1** (0.5 μM) for 30 min. The reaction was quenched and analyzed as described above.

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