Bodipy-VAD-Fmk, a useful tool to study yeast peptide *N*-glycanase activity†

Martin D. Witte, Carlos V. Descals, Sebastiaan V. P. de Lavoir, Bogdan I. Florea, Gijsbert A. van der Marel* and Herman S. Overkleeft*

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In this paper the development of a fluorescent activity based probe, Bodipy-VAD-Fmk, for visualization of yeast peptide *N*-glycanase is described. The activity based probe is used to assess the efficacy of known and new chitobiose-based electrophilic traps to bind yeast peptide *N*-glycanase.

Introduction

The majority of proteins in eukaryotes are synthesized by membrane bound ribosomes on the endoplasmic reticulum (ER). The newly synthesized proteins are inserted into the lumen of the ER *via* a specialized structure referred to as the translocon. In a co-translational process, oligosaccharyl transferase may glycosylate asparagine residues within the Asn-X-Ser/Thr (X cannot be Pro) consensus sequence.[1] The resulting $Glc_3Man_9GlcNHAc_2$ glycan is deglycosylated to $GlcMan_9GlcNHAc_2$ which is then recognized by calnexin and calrectulin. These chaperones retain glycoproteins in the ER until they are folded properly. Properly folded proteins progress through the ER and Golgi. A series of deglycosylation/glycosylation events transform the high mannose *N*-glycan into complex-type *N*-glycans. These glycans in turn help in guiding the glycoprotein to its final destination, such as the cell surface or the endocytic pathway.

The $Man_8GlcNAc_2$ glycan of misfolded *N*-linked glycoproteins is recognized by EDEM, a lectin-like protein of the endoplasmic reticulum associated degradation pathway, resulting 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] PNGase contains the characteristic catalytic Cys, His, Asp triad responsible for the cleavage of the β-aspartyl–glucosamine bond. 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 can 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 commercially available broad spectrum caspase inhibitor Z-VAD(OMe)-Fmk **2** or haloacetamidyl chitobiose inhibitor **3** (Fig. 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 RNase B by PNGase, followed by SDS-PAGE and visualization of digestion products with Coomassie blue. Before use as a substrate, commercially available RNase B requires further purification and denaturation.[6] Our group recently published an activity-based fluorescent proteasome probe, which enables direct in-gel detection of proteasome activity.[7] We reasoned that the availability of a fluorescent analogue of **2**, such as Bodipy TMR-Ahx-Val-Ala-Asp(OMe)-fluoromethyl ketone **1** (β-VAD-Fmk),[8] would allow straightforward screening of potential irreversible *N*-glycanase inhibitors in an analogous fashion. We here report the validity of this reasoning by the application of β-VAD-Fmk **1** in the identification of the two new chitobiose-based *N*-glycanase inhibitors, **4** and **5** (Fig. 1).

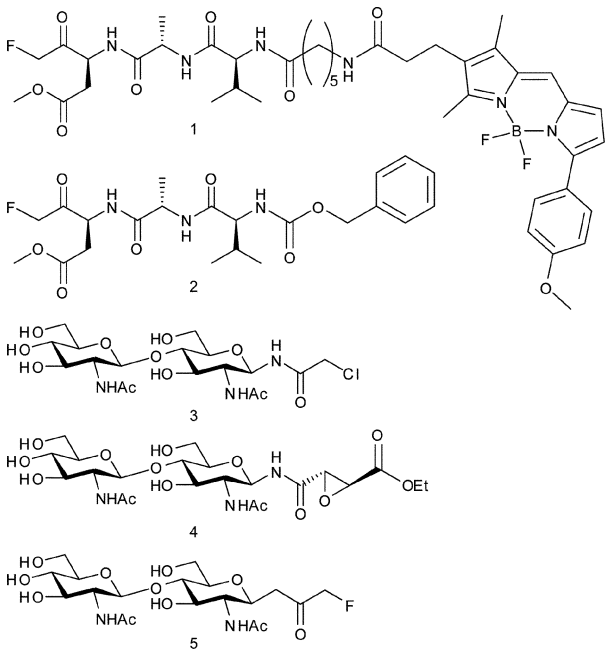

Fig. 1 Target compounds.

Results and discussion

The synthesis of β-VAD-Fmk **1** is depicted in Scheme 1. Boc-Asp-OBn **6** was converted to the fluoromethyl ketone *via* a modified procedure of the patented method by Palmer.[9] Hence,

Bioorganic Synthesis, Leiden Institute of Chemistry, Leiden University, P.O. Box 9502, 2300 RA Leiden, The Netherlands. E-mail: marel_g@chem.leidenuniv.nl, h.s.overkleeft@chem.leidenuniv.nl; Fax: 31 71 527 4307; Tel: 31 71 527 4342

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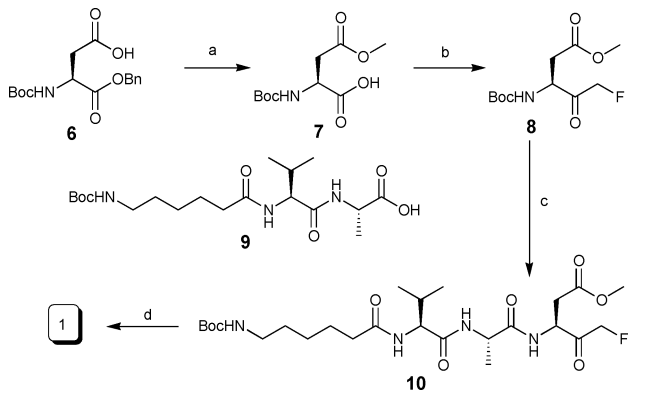


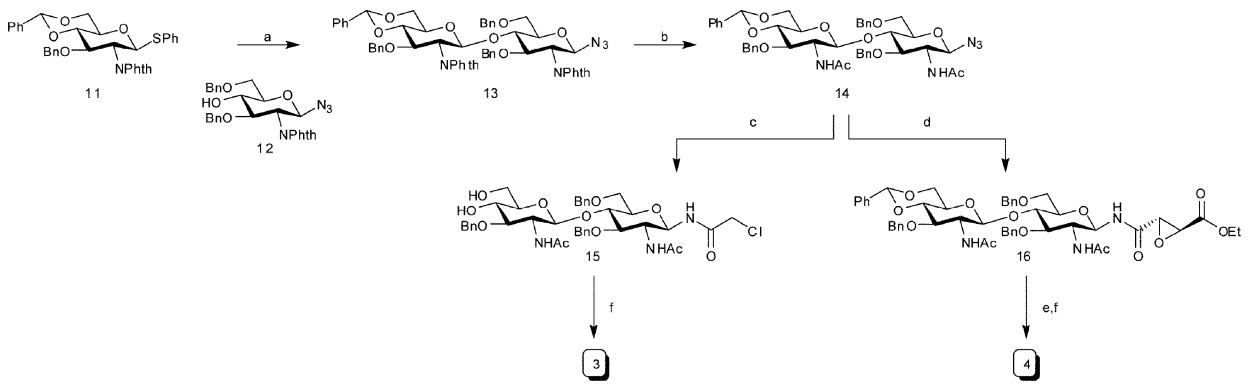
Scheme 1 *Reagents and conditions*: (a) (i) MeI, K_2CO_3, DMF; (ii) 10% Pd/C, H_2, EtOAc, quant.; (b) (i) CDI, THF, 1 h; (ii) monobenzyl fluoromalonate magnesium enolate, THF; (iii) 10% Pd/C, H_2, EtOAc, 68%; (c) (i) 4 M HCl–dioxane, 45 min; (ii) **9**, HCTU, DiPEA, DMF 68%; (d) (i) TFA–H_2O (95 : 5,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

Boc-Asp-OBn **6** was converted to the methyl ester, and ensuing reduction of the benzyl ester gave Boc-Asp(OMe)OH **7**. Treatment of the resulting acid with 1,1′-carbodiimidazole followed by reaction with the magnesium enolate of the monobenzyl-fluoromalonate and hydrogenation gave fluoromethylketone **8**. Removal of the Boc protective group in **8** and condensation of the resulting free amine with peptide **9** (see ESI†) 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.

With β-VAD-Fmk **1** in hand, our 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 azido group followed by coupling with either chloroacetic anhydride or epoxysuccinate monoethylester afforded the protected inhibitors **15** and **16**. Removal of protective groups furnished epoxide inhibitor **4**. In the case of inhibitor **3** partial reduction of the chloroacetamide moiety was observed.

Fluoromethyl ketone **5** was synthesized as follows (Scheme 3). *C*-glycoside **17**, prepared according to the literature procedure,[11] was treated with acidic methanol, followed by reaction with benzaldehyde dimethylacetal to give after benzylation of the 3-OH protected *C*-glycoside **18**. Reaction of the ethylene moiety in **18** with *m*CPBA afforded the desired epoxide **19** as a diastereomeric mixture. 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 **25** and **26**. Deprotection of the amine functionalities in **25** and **26**, ensuing acetylation of the resulting free amines and *O*-deacetylation furnished fluorohydrin **27**. During the removal of the phthaloyl protective groups of benzoylated disaccharide **26**, *O* to *N* benzoyl migration was observed. This problem was however overcome by synthesizing acetyl protected **25**. Oxidation of **27** afforded fully protected fluoromethyl ketone **28**. Global deprotection gave inhibitor **5** in a reasonable yield.

With probe **1** and inhibitors **2–5** in hand, we focused our attention on their biological evaluation. 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 1 h. Direct in-gel visualization with a fluorescent scanner revealed that probe **1** labeled yeast peptide *N*-glycanase in a concentration-dependent manner. From this image (Fig. 2(A)) it can be seen that the 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 YPng1 specifically (Fig. 2(A), (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 0.9 ng of purified YPng1 could be detected (Fig. 2(C)). The specificity of β-VAD-Fmk **1** was further investigated by incubating the probe with an *Escherichia coli* (*E. coli*) cell extract expressing YPng1. Exclusive labeling of YPng1 by probe **1** was observed by in-gel visualization. We next investigated the binding site of **1**. The reported X-ray structure of YPng1 co-crystallized with Z-VAD-Fmk **2** showed that Z-VAD-Fmk **2** binds to the catalytic Cys residue 191 of the active site of YPng1.[12] It is therefore reasonable to assume that **1**,



Scheme 2 *Reagents and conditions*: (a) **12**, Ph_2SO, TTBP, Tf_2O, DCM, −60 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, *p*-TsOH, 16 h, 47%; (d) (i) Lindlar's cat. H_2; (ii) epoxysuccinate monoethylester, HCTU, Et_3N, DMF, 39%; (e) 5% TFA–DCM, 70%; (f) 20% $Pd(OH)_2$/C, H_2, MeOH **3**: 14%, **4**: 67%.

just as lead-compound **2** binds to the same site. To verify this, catalytically inactive YPng(C191A) was expressed in *E. coli*. Incubation of YPng(C191A) with **1** followed by visualization revealed, as expected, no significant labeling of YPng(C191A) (Fig. 2(D), (E)).

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** (Fig. 2(F)). Therefore a similar competition assay was conducted without DTT. Non-specific labeling was minimized allowing us determine the concentration at which 50% of enzyme activity was inhibited (apparent IC_{50}) by quantification of the data 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. We are aware that our assay is not ideally suited for an accurate determination of IC_{50} values and is not suitable at all for the establishment of k_i values. Such detailed kinetic studies are executed best by making use of a substrate-based assay such as those reported by Ito and Ploegh.[5,6] We believe, however, that our assay is highly useful for the rapid identification of potential YPng1 inhibitors, and to give a first impression of its potency.

In order to further illustrate this point, 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. To our surprise, fluoromethyl ketone **5** proved to be a poor inhibitor of YPng1. Disaccharide **5** was preincubated with YPng1 for 2 h 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, fluoromethyl ketone **5** inhibits YPng1 in the high micromolar range (Fig. 2 (G)).

Conclusions

In summary, we have described the synthesis of β-VAD-Fmk **1** and have demonstrated its ability to covalently bind to the active site Cys191 of recombinant yeast peptide *N*-glycanase. β-VAD-Fmk **1** can now be used in an enzyme inhibitory assay that allows the rapid identification of potential YPng1 inhibitors as we validated for two known inhibitors (**2** and **3**) and two new chitobiose-based inhibitors (**4** and **5**). Current research is focused on the design of new inhibitors, both with respect to nature of the electrophilic trap and the length of the oligosaccharide position.

Experimental

All general experimental details, the synthesis of peptide **9**, monobenzyl fluoromalonate magnesium enolate, disaccharides **13** and **14** and acceptors **23** and **24**, the silver stained gels of YPng labeling in *E. coli* cell extracts and inhibitor-dose-response curves are available in the ESI.† TTBP (*tert*-butylpyrimidine), Bodipy TMR-OSu, tetrabutylammonium dihydrogen trifluoride ($TBA{\cdot}H_2F_3$) and epoxisuccinate monoethyl ester were synthesized as described.[7,14]

N-*tert*-Butoxycarbonylaspartyl(OMe) fluoromethyl ketone (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 16 h stirring, the reaction was diluted with EtOAc, washed with 1 M HCl, $NaHCO_3$ (sat. aq.), brine, dried ($MgSO_4$) and concentrated *in vacuo*. The resulting Boc-Asp(OMe)OBn

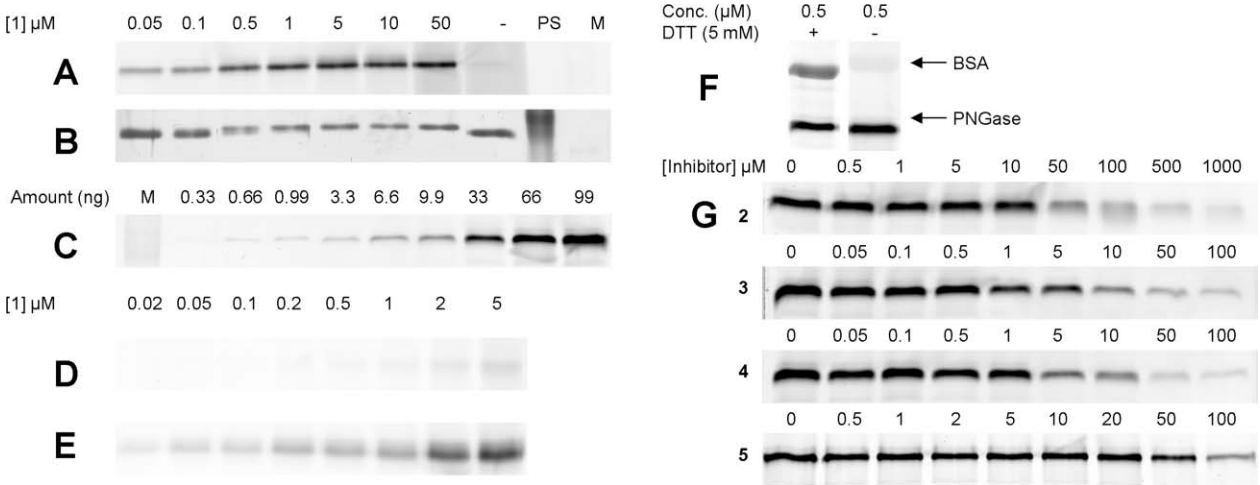


Fig. 2 (A) Fluorescent in-gel detection of YPng1 by labeling with the indicated concentration of **1**. "PS" represents the molecular marker (prestained marker purchased from Fermentas). "M" represents the dual color marker. "—" represents heat-inactivated YPng1, incubated with 50 μM **1** for 2 h at 37 °C. (B) Silver stained gel of labeled YPng1. (C) Determination of the sensitivity of probe **1** by incubating **1** (0.5 μM) with various amounts of YPng1. (D) Labeling of catalytically inactive YPng(C191A) (1 mg mL^{-1}) with a serial dilution of **1** in *E. coli* cell extracts. (E) Labeling of YPng1 (1 mg mL^{-1}) with **1** in *E. coli* cell extracts. (F) Non-specific labeling of BSA by β-VAD-Fmk **1** 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 1 h. Fluoromethyl ketone **5** was preincubated with YPng1 for 2 h followed by incubation with **1** for 30 min.

was dissolved in EtOAc before being reduced under the agency of palladium (10%) on charcoal under H_2 atmosphere. TLC-analysis showed complete reduction of the benzyl ester after 3 h. Filtration and ensuing concentration afforded Boc-Asp(OMe)OH **7** (1.36 g, 5 mmol) which was co-evaporated with toluene and dissolved in THF (25 mL). The solution was cooled to 0 °C, carbodiimidazole (851 mg, 5.25 mmol) was added and the reaction was stirred for 1 h at 0 °C. The mixture was cooled to −20 °C before monobenzyl fluoromalonate magnesium enolate (see ESI†) (6.25 mmol, 1.5 equiv.) was added dropwise. After 45 min stirring at −20 °C and 3.5 h additional stirring at room temperature, the reaction was poured into 1 M 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). ^{1}H NMR (600 MHz, $CDCl_3$) δ/ppm 5.54 (d, J = 7.9 Hz, 1H, NH), 5.23 (dd, J = 47.0, 16.4 Hz, 1H, CH_2F), 5.12 (dd, J = 47.3, 16.5 Hz, 1H, CH_2F), 4.63 (td, J = 9.2, 5.2, 5.2 Hz, 1H, H-α), 3.70 (s, 3H, CH_3 OMe), 3.08 (dd, J = 17.3, 4.1 Hz, 1H), 2.84 (dd, J = 17.3, 4.5 Hz, 1H, H-β), 1.46 (s, 9H, CH_3 tBu). ^{13}C NMR (150 MHz, $CDCl_3$) δ/ppm 203.17 (d, J = 16.4 Hz, C=O ketone), 171.69 (C=O COOtBu), 155.19 (C=O Boc), 84.14 (d, J = 183.4 Hz, CH_2F), 80.76 (C_q tBu), 53.49 (CH-α), 52.21 (CH_3 OMe), 35.36 (CH_2-β), 28.20 (CH_3 tBu). $[\alpha]_D^{23}$ −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, 861.3, 780.3, 631.8. HRMS: (M − Boc + H$^+$) calc. for $C_6H_{11}FNO_3$ 164.07175, found 164.07166.

Bodipy-Ahx-Val-Ala-Asp(OMe) fluoromethyl ketone (1)

Fluoromethyl ketone **8** (40 mg, 0.15 mmol, 1.25 equiv.) was dissolved in 4 M HCl in dioxane (2 mL). After 45 min, TLC analysis showed complete conversion to a very polar product. The solution was concentrated *in vacuo*, co-evaporated 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 consumption after 2 h. The reaction was diluted with CH_2Cl_2, washed with $NaHCO_3$ (sat. aq.), 1 M 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 − 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 co-evaporation 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 1 M 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). 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)$^+$. RP-HPLC: R_t 2.8 cv (column volume = 250 × 10.00 mm, particle size is 5); linear gradient 45 → 48.5% in 3 cv. Silica gel chromatography ($CHCl_3$ → 3% MeOH–$CHCl_3$) gave both diastereomers. Diastereomer 1: ^{1}H 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, 836.6, 799.1, 721.9, 662.6, 609.8.

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, H arom), 5.06 (d, J = 11.6, CH_2 Bn), 5.02 (d, J = 10.4 Hz, 1H, H-1) 4.90 (d, J = 11.5 Hz, 1H, CH_2 Bn), 4.72–4.58 (m, 5H, 4 × CH_2 Bn, H-1′), 4.12 (t, J = 9.2, 9.2 Hz, 1H, H-4), 4.02 (d, J = 6.3 Hz, 1H, CH_2Cl), 3.99 (t, J = 10.0, 10.0 Hz, 1H, H-2), 3.83–3.75 (m, 4H, H-2′, H-6, H-6a′), 3.64 (dd, J = 9.9, 9.1 Hz, 1H, H-3), 3.56–3.46 (m, 2H, H-3′, H-5), 3.42 (dd, J = 9.7, 8.8 Hz, 1H, H-6b′), 3.21 (ddd, J = 9.5, 7.1, 2.1 Hz, 1H, H-5′), 1.90 (s, 3H, CH_3 NHAc), 1.90 (s, 3H, CH_3 NHAc). ^{13}C NMR (125 MHz, MeOD) δ/ppm 173.75 (C=O), 173.52 (C=O), 169.85 (C=O), 140.29 (C_q Bn), 139.67 (C_q Bn), 139.51 (C_q Bn), 129.64–128.57 (CH arom), 101.14 (C-1′), 83.87 (C-3′), 83.00 (C-3), 80.48 (C-1), 78.72 (C-5′), 78.35 (C-5), 77.00 (CH_2 Bn), 75.83 (CH_2 Bn), 75.75 (C-4), 74.36 (CH_2 Bn), 72.67 (C-4′), 69.40 (C-6), 62.96 (C-6′), 57.30 (C-2′), 54.92 (C-2), 43.10 (CH_2Cl), 23.17 (CH_3 NHAc), 22.82 (CH_3 NHAc). FT-IR: ν_{max}(neat)/cm^{-1} 3277.8, 1651.8, 1557.8, 1455.5, 1372.5, 1312.7, 1050.9, 737.6, 696.0. $[\alpha]_D^{23}$ −2.4 (c = 0.74, MeOH). HRMS: (M + H$^+$) calc. 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 4 h, 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 gel filtration (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, H-1), 4.60 (d, $J = 8.5$ Hz, 1H, H-1′), 4.15 (d, $J = 14.3$ Hz, 1H, CH_2Cl), 4.11 (d, $J = 14.3$ Hz, 1H, CH_2Cl), 3.96–3.44 (m, 12H), 2.07 (s, 3H, CH_3 NHAc), 2.00 (s, 3H, CH_3 NHAc). ^{13}C NMR (150 MHz, D_2O) δ/ppm 174.31 (C=O), 174.06 (C=O), 169.91 (C=O), 100.83 (C-1′), 78.21, 78.19 (C-1), 75.77, 75.33, 72.85, 71.98, 69.12, 59.95 (C-6 or C-6′), 59.31 (C-6 or C-6′), 55.01 (C-2′), 53.12 (C-2), 41.46 (CH_2Cl), 21.53 (CH_3 NHAc), 21.35 (CH_3 NHAc).

(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 1 M 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) δ/ppm 8.72 (d, $J = 9.0$ Hz, 1H, NH), 8.24 (d, $J = 8.7$ Hz, 1H, NH), 8.21 (d, $J = 8.9$ Hz, 1H, NH), 7.52–7.24 (m, 20H, H arom), 5.73 (s, 1H, CHPh), 5.12 (t, $J = 9.3$, 9.3 Hz, 1H), 5.00 (d, $J = 11.1$ Hz, 1H, CH_2 Bn), 4.92 (d, $J = 7.6$ Hz, 1H, H-1′), 4.84 (d, $J = 11.9$ Hz, 1H, CH_2 Bn), 4.72 (d, $J = 11.0$ Hz, 1H, CH_2 Bn), 4.70 (d, $J = 11.8$ Hz, 1H, CH_2 Bn), 4.67 (d, $J = 11.8$ Hz, 1H, CH_2 Bn), 4.62 (d, $J = 11.8$ Hz, 1H, CH_2 Bn), 4.26–4.19 (m, 2H, CH_2 OEt), 4.11 (dd, $J = 10.2$, 5.0 Hz, 1H, H-6a), 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, CH_3 NHAc), 1.90 (s, 3H, CH_3 NHAc), 1.25 (t, $J = 7.1$, 7.1 Hz, 3H, CH_3 OEt). ^{13}C NMR (150 MHz, DMSO) δ/ppm 169.48 (C=O), 169.32 (C=O), 166.78 (C=O), 165.42 (C=O), 138.92 (C_q Bn or Ph), 138.63 (C_q Bn or Ph), 138.44 (C_q Bn or Ph), 137.47 (C_q Bn or Ph), 128.67–125.87 (CH arom), 100.40 (C-1′), 99.93 (CHPh), 80.85, 80.68, 78.31, 76.01, 75.10, 73.36 (CH_2 Bn), 73.15 (CH_2 Bn), 71.81 (CH_2 Bn), 68.22 (C-6 or C-6′), 67.68 (C-6 or C-6′), 65.50, 61.49 (CH_2 OEt), 53.24, 52.67, 51.13, 22.88 (CH_3 NHAc), 22.67 (CH_3 NHAc), 13.79 (CH_3 OEt). FT-IR: ν_{max}(neat)/cm^{-1} 3277.7, 1651.9, 1538.3, 1455.4, 1371.6, 1205.0, 1069.8, 735.8, 694.4. $[\alpha]_D^{23}$ +11.2 ($c = 0.66$, DMF). HRMS: (M + H^+) calc. 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-glucopyranosyl)-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosylcarbamoyl)-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 16 h. 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 two steps, 11.06 mg, 19.6 μmol). ^{1}H NMR (600 MHz, D_2O) δ/ppm 5.09 (d, $J = 9.6$ Hz, 1H, H-1), 4.59 (d, $J = 8.4$ Hz, 1H, H-1′), 4.28 (q, $J = 7.1$, 7.1, 7.1 Hz, 2H, CH_2 OEt), 3.96–3.43 (m, 14H), 2.06 (s, 3H, CH_3 NHAc), 2.01 (s, 3H, CH_3 NHAc), 1.29 (t, $J = 7.2$, 7.2 Hz, 1H, CH_3 OEt). ^{13}C NMR (150 MHz, D_2O) δ/ppm 174.26 (C=O), 174.06 (C=O), 168.31 (C=O), 167.98 (C=O), 100.83 (C-1′), 78.16, 77.85 (C-1), 75.79, 75.33, 72.87, 72.85, 71.92, 69.12, 62.90 (CH_2 OEt), 59.95 (C-6 or C-6′), 59.30 (C-6 or C-6′), 55.01, 53.19 (C-2), 52.72 (C-2′), 52.05, 21.53 (CH_3 NHAc), 21.38 (CH_3 NHAc), 12.58 (CH_3 OEt). 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, 896.9. $[\alpha]_D^{23}$ +19.2 ($c = 0.24$, H_2O). HRMS: (M + H^+) calc. for $C_{22}H_{36}N_3O_{14}$ 566.21918, found 566.21904.

(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 (25)

Before being dissolved in freshly distilled CH_2Cl_2, known donor **11** (454 mg, 0.78 mmol, 1.2 equiv.), diphenyl sulfoxide (174 mg, 0.86 mmol, 1.3 equiv.) and TTBP (487 mg, 1.96 mmol, 3.0 equiv.) were co-evaporated 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 pre-activation, acceptor **23** (427 mg, 0.65 mmol) was added, followed by stirring at −60 °C for 1 h. The temperature was slowly raised to 0 °C over a period of 4 h, 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 **26** as a colorless oil (97%, 668 mg, 0.63 mmol) which was directly used for the next reaction. ^{1}H NMR (600 MHz, $CDCl_3$) δ/ppm 8.03–6.79 (m, 28H, H arom), 5.53–5.50 (m, 1H, CHPh), 5.39–5.34 (m, 1H, H-1″), 5.22–5.00 (m, 1H, H-2″), 4.85–4.76 (m, 2H, CH_2 Bn), 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, CH_3 OAc), 1.71–1.51 (m, 2H, H-1′). ^{13}C NMR (150 MHz, $CDCl_3$) δ/ppm 170.31 (C=O Ac), 170.09 (C=O Ac), 167.86 (C=O phth), 167.73 (C=O phth), 167.70 (C=O phth), 167.61 (C=O phth), 138.53 (C_q Bn or Ph), 138.45 (C_q Bn or Ph),

138.22 (C_q Bn or Ph), 138.04 (C_q Bn or Ph), 137.85 (C_q Bn or Ph), 137.30 (C_q Bn or Ph), 137.28 (C_q Bn or Ph), 133.86 (CH phth), 133.78 (CH phth), 131.40 (C_q phth), 128.93–126.01 (CH arom), 123.35 (CH phth), 123.25 (CH phth), 101.13 (CHPh), 97.58 (C-1″), 97.39 (C-1″), 83.97 (d, $J = 173.7$ Hz, C-3′), 83.14, 83.13, 83.09 (d, $J = 172.6$ Hz, C-3′), 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, C-2′), 68.85 (d, $J = 19.0$ Hz, C-2′), 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, C-1′), 31.50 (d, $J = 6.7$ Hz, C-1′), 20.87 (CH_3 OAc), 20.83 (CH_3 OAc). 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, 874.0, 794.3, 736.0, 719.7, 695.8, 661.5. HRMS: (M + Na$^+$) calc. 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 (26)

Acceptor **24** (574 mg, 0.88 mmol) was condensed with donor **11** as described for disaccharide **25**. Purification by silica gel chromatography (Tol → 7.5% EtOAc–Tol) gave disaccharide **26** in 51% (500 mg, 0.45 mmol) as a colorless oil.

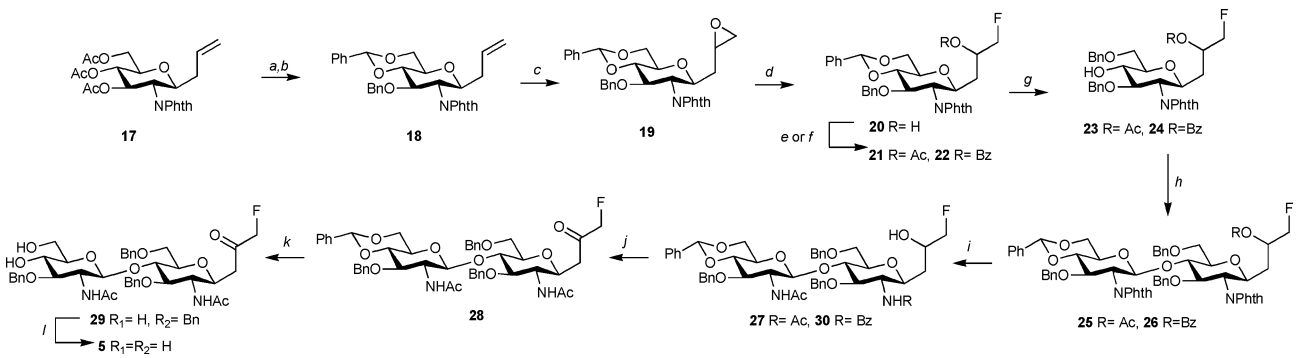
(2*R*/*S*)-3-*C*-(*O*-(2-Acetamido-3-*O*-benzyl-4,6-*O*-benzylidene-2-deoxy-β-D-glucopyranosyl)-(1→4)-2-acetamido-3,6-di-*O*-benzyl-2-deoxy-β-D-glucopyranosyl)-1-fluoro-2-hydroxypropane (27)

Starting from **26**: Disaccharide **26** (500 mg, 0.45 mmol) was dissolved in *n*-BuOH–ethylenediamine (10 : 1 v/v, 11 mL), before being stirred at 90 °C for 8 h. The mixture was cooled to rt, concentrated *in vacuo*, co-evaporated with toluene and used for 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 1 M 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 1 h, after which it was neutralized with AcOH and concentrated. Silica gel column chromatography (CH_2Cl_2 → 2% MeOH–CH_2Cl_2) furnished benzoyl migrated compound **30** (95 mg, 0.105 mmol, 23%, Scheme 3) and title compound **27** (211 mg, 0.250 mmol, 56%).

Starting from **25**: disaccharide **25** (286 mg, 0.27 mmol) was converted to fluorohydrin **27** as depicted above. After silica gel purification (CH_2Cl_2 → 2% MeOH–CH_2Cl_2) title compound **27** was obtained as a white solid (67%, 152 mg, 0.180 mmol). ^{1}H NMR (600 MHz, DMSO) δ/ppm 8.11 (d, $J = 8.4$ Hz, 1H, NH), 7.96–7.93 (m, 1H, NH), 7.43–7.23 (m, 20H, H arom), 5.67 (s, 1H, CHPh), 5.04–4.92 (m, 1H), 4.86–4.82 (m, 1H, CH_2 Bn), 4.75–4.71 (m, 2H, CH_2 Bn, H-1″), 4.65–4.54 (m, 4H, CH_2 Bn), 4.43–4.15 (m, 2H, H-3′), 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, CH_3 NHAc), 1.54–1.21 (m, 2H, H-1′). ^{13}C NMR (150 MHz, DMSO) δ/ppm 169.31 (C=O NHAc), 169.28 (C=O NHAc), 169.16 (C=O NHAc), 139.29 (C_q Bn or Ph), 139.27 (C_q Bn or Ph), 138.77 (C_q Bn or Ph), 138.74 (C_q Bn or Ph), 138.70 (C_q Bn or Ph), 137.60 (C_q Bn or Ph), 128.77–125.99 (CH arom), 100.91 (C-1″), 100.07 (CHPh), 87.29 (d, $J = 168.4$ Hz, C-3′), 86.50 (d, $J = 167.1$ Hz, C-3′), 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, C-2′), 65.59, 65.49 (d, $J = 18.9$ Hz, C-2′), 54.17, 54.06, 35.01 (d, $J = 7.8$ Hz, C-1′), 34.85 (d, $J = 6.5$ Hz, C-1′), 22.98 (CH_3 NHAc), 22.92 (CH_3 NHAc). 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, 746.9, 694.4, 668.1, 615.9. HRMS: (M + H$^+$) calc. 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 (28)

Fluorohydrin **27** (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 5 h, TLC analysis showed complete consumption of the starting material and the reaction was quenched with 10% aq. $NaHCO_3$ (10 mL) and 2 M aq. NaS_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 fluoromethyl ketone **28** (162 mg, 0.193 mmol) in 77% yield as a white solid. ^{1}H NMR (600 MHz, DMSO) δ/ppm 8.08 (d,



Scheme 3 *Reagents and conditions*: (a) (i) Amberlite H$^+$, MeOH, reflux, 16 h, quant; (ii) $PhCH(OMe)_2$, *p*TsOH, MeCN, 91%; (b) BnBr, NaH, TBAI, DMF, 6 h, 68%; (c) *m*CPBA, CH_2Cl_2, reflux, 4 h, 88%; (d) TBA·H_2F_3, Tol, microwave, 180 °C, 20 min, 84%; (e) Ac_2O, pyr, 96%; (f) BzCl, DMAP, pyr, 92%; (g) TES, TfOH, DCM, −78 °C, 45 min, 79–85%; (h) **11**, Ph_2SO, Tf_2O, TTBP, DCM, −60 to 0 °C, **25**: 97% **26**: 51%; (i) (i) $(H_2NCH_2)_2$–*n*-BuOH (1 : 10), 90 °C; (ii) Ac_2O, pyr; (iii) NaOMe, MeOH, starting from **25**: 67%, starting from **26**: 56%; (j) Dess–Martin periodinane, DCM, 77%; (k) 5% TFA–DCM, H_2O, 0 °C, 87%; (l) 20% $Pd(OH)_2$/C, H_2, MeOH, 45%.

J = 8.4 Hz, 1H, NH), 7.97 (d, J = 8.8 Hz, 1H, NH), 7.42–7.24 (m, 20H, H arom), 5.67 (s, 1H, CHPh), 5.06 (ddd, J =46.8, 30.1, 16.7 Hz, 2H, H-3′), 4.83 (d, J = 11.0 Hz, 1H, CH_2 Bn), 4.74–4.69 (m, 2H, CH_2 Bn, H-1″), 4.61–4.52 (m, 4H, CH_2 Bn), 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, CH_3 NHAc), 1.80 (s, 3H, CH_3 NHAc). ^{13}C NMR (150 MHz, DMSO) δ/ppm 203.14 (d, J = 14.8 Hz, C-2′), 169.31 (C=O NHAc), 169.20 (C=O NHAc), 139.05 (C_q Bn), 138.64 (C_q Bn), 138.56 (C_q Bn), 137.48 (C_q Bn), 128.66–125.86 (CH arom), 100.71 (C-1″), 99.91 (CHPh), 85.12 (d, J = 179.8 Hz, C-3′), 81.46, 80.81, 78.17, 75.94, 74.13, 73.26 (CH_2 Bn), 73.12 (CH_2 Bn), 71.72 (CH_2 Bn), 68.51 (C-6 or C-6′), 67.67 (C-6 or C-6′), 65.47, 55.33 (C-2 or C-2′), 53.63 (C-2 or C-2′), 40.71 (C-1′), 22.86 (CH_3 NHAc), 22.72 (CH_3 NHAc). 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, 733.9, 694.1. $[\alpha]_D^{23}$ −5 (c = 0.28, DMF). HRMS: (M + H$^+$) calc. 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 (29)

The benzylidene of fully protected **28** (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 **29** (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, H arom), 5.00 (d, J = 10.9 Hz, 1H, CH_2 Bn), 4.89 (d, J = 47.2 Hz, 1H, H-3′), 4.88 (d, J = 11.5 Hz, 1H, CH_2 Bn), 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, H-1a′), 2.58–2.52 (m, 1H, H-1b′), 1.89 (s, 3H, CH_3 NHAc), 1.87 (s, 3H, CH_3 NHAc). ^{13}C NMR (150 MHz, DMSO) δ/ppm 203.20 (d, J = 14.9 Hz, C-2′), 169.32 (C=O NHAc), 169.12 (C=O NHAc), 139.25 (C_q Bn), 139.17 (C_q Bn), 138.54 (C_q Bn), 128.14–126.95 (CH arom), 99.99 (C-1″), 85.15 (d, J = 179.9 Hz, C-3′), 82.37, 81.77, 78.27, 76.78, 75.23, 74.22, 73.26 (CH_2 Bn), 72.85 (CH_2 Bn), 71.86 (CH_2 Bn), 70.09, 68.76 (C-6), 60.80 (C-6′), 55.00 (C-2 or C-2′), 53.55 (C-2 or C-2′), 40.80 (C-1′), 22.91 (CH_3 NHAc), 22.73 (CH_3 NHAc). 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, 741.9, 695.9, 634.0, 602.0. $[\alpha]_D^{23}$ +23.3 (c = 0.42, MeOH). HRMS: (M + H$^+$) calc. 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 fluoromethyl ketone **29** (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 8 h, 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 gel filtration (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, H-3′), 4.57 (d, J = 8.5 Hz, 1H, H-1″), 3.96–3.41 (m, 13H), 2.77–2.68 (m, 2H), 2.06 (s, 3H, CH_3 NHAc), 2.00 (s, 3H, CH_3 NHAc). ^{13}C NMR (150 MHz, D_2O) δ/ppm 207.24 (d, J = 15.3 Hz, C-2′), 174.57 (C=O NHAc), 174.53 (C=O NHAc), 101.41 (C-1″), 85.35 (d, J = 180.1 Hz, C-3′), 79.45, 78.03, 75.88, 73.52, 73.42, 69.66, 60.49 (C-6 or C-6″), 60.11 (C-6 or C-6″), 55.56 (C-2″), 54.41 (C-2), 40.09 (C-2′), 22.09 (CH_3 NHAc), 22.04 (CH_3 NHAc). 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, 893.7. $[\alpha]_D^{23}$ −11.1 (c = 0.36, H_2O). HRMS: (M + H$^+$) calc. for $C_{19}H_{32}FN_2O_{11}$ 483.19846, found 483.19824.

Expression of YPng1 and YPng(C191A)

BL21/DE3 *E. coli* transformed with either pET28a-YPng1 or pET28a-YPng(C191A) were cultured overnight at 37 °C in 50 mL LB media containing kanamycin (50 μg mL^{-1}). The culture was transferred to 450 mL fresh LB media and cultured at 37 °C until OD_{600} = 0.8. Subsequently, IPTG (5 mL, 0.1 M) was added and the cells were further incubated for 3 h 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 incubate on ice for 30 min followed by 20 min sonication (90% maximum power, 5 s pulse and 3 s 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^{-1}) 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^{-1}. To assess the labeling properties of Bodipy TMR-Ahx-Val-Ala-Asp(OMe)-Fmk **1**, 9 μL of enzyme solution (11.1 ng μL^{-1}) was labeled for 2 h 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 Thyphoon Variable Mode Imager (Amersham Biosciences). Afterwards the protein amount was quantified by silverstaining. Non-specific labeling of YPng1 was evaluated: by treating heat inactivated YPng1 (9 μL, 11.1 ng μL^{-1} 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 1 h 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^{-1}) overexpressing either YPng1 or C191A were incubated with a serial dilution of β-VAD-Fmk **1** for 1 h. After denaturation, resolving on 10% SDS-PAGE, labeling

was visualized as described for purified YPng1. The total protein amount was quantified using silver staining (see ESI†).

Competition experiments

For competition experiments, 9 μL of YPng1 (11.1 ng μL^{-1}) in reaction buffer (1 μg μL^{-1} 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 1 h. 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 (see ESI†). To analyze the inhibitory potential of fluoromethyl ketone **5**, 1 μL of **5** was preincubated with 9 μL of YPng1 (11.1 ng μL^{-1}) in reaction buffer for 2 h 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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