Activity-based profiling of glycoconjugate processing enzymes

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7 Summary, work in progress and future prospects

The research described in this thesis aims at the synthesis and application of novel molecular tools to study enzymes involved in glycoconjugate degradation. An overview of the enzymes involved in the metabolism of glycoconjugates and their inhibitors/activity-based probes is given in **Chapter 1**.

Peptide *N*-glycanase (PNGase) is responsible for the hydrolysis of the glycosyl-protein linkage of misfolded *N*-linked glycoproteins. In **Chapter 2**, the synthesis of an activity-based probe for this enzyme is described. BODIPY TMR-VAD-Fmk **1** (Figure 1), a fluorescent analogue of Z-VAD-Fmk, labeled peptide *N*-glycanase selectively. The BODIPY in **1** enables rapid identification of new potential inhibitors by competition experiments.

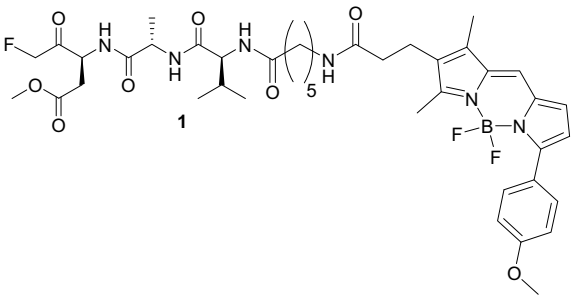


Figure 1. Structure of BODIPY-VAD-Fmk **1**.

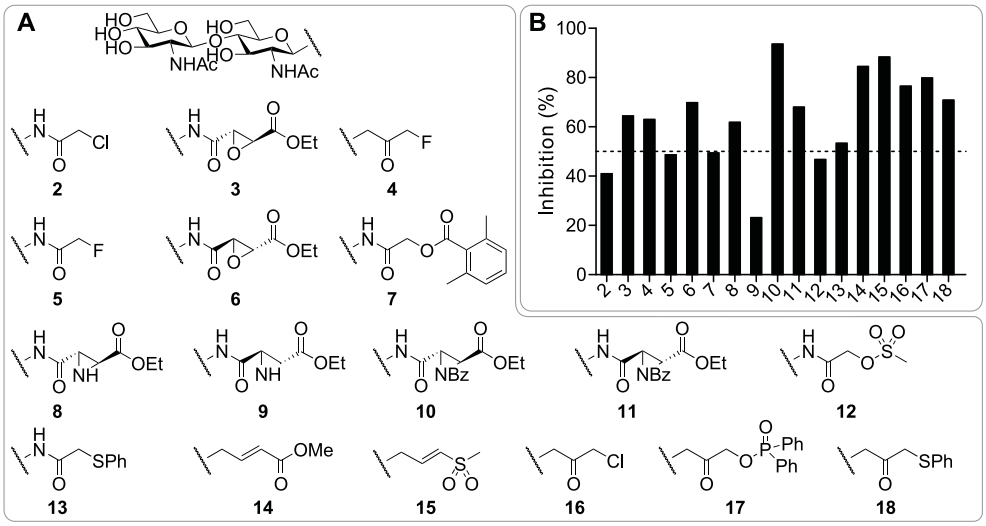


Figure 2. (A) Structures of compounds **2-18**. (B) Inhibition of human chitinase by compounds **2-18**. A solution of compounds **2-18** (12.5 µL, 1 mM in McIlvain) was incubated with chitinase (12.5 µL in McIlvain) at 37°C for 30 min followed by incubation with the substrate (0.25 mM, 100 µL) for 20 min at 37°C. The percentage of inhibition was determined by measuring the residual fluorescence.

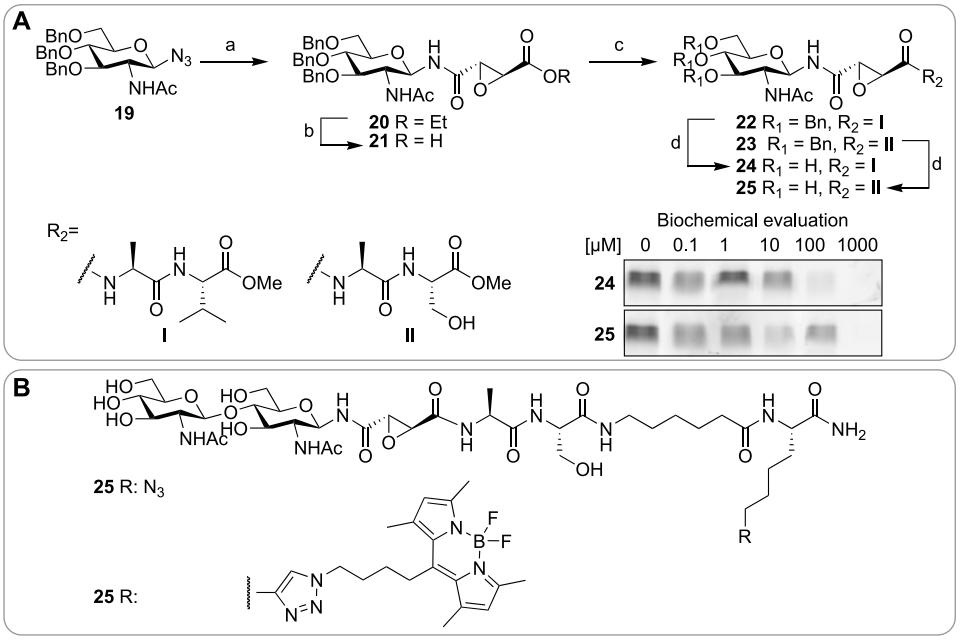
Chapter 3 deals with the employment of this strategy to investigate the inhibitory potential of chitobiose derived compounds **2-18 (**Figure 2A). It was established that the nature of the warhead has a decisive influence on the potency. Compounds containing a good leaving group, such as a chloride or a mesyloxy are better inhibitors of PNGase than inhibitors having a poor leaving group. Additionally, the importance of the stereochemistry of oxirane/aziridine inhibitors was shown, with the *S,S*-isomer being the most potent.

An initial attempt has been made to study the selectivity of the compound-library reported in Chapter 3. The effect of compounds **2-18** on chitinases was evaluated using a fluorogenic substrate assay. Human chitinase was preincubated with compounds **2-18** (0.5 mM), followed by incubation with the fluorogenic substrate (0.2 mM assay concentration) and measurement of the fluorescence. As can be seen in Figure 2B, all compounds showed considerable inhibitory activity (50-90%). It should be noted that at these high concentrations (0.1 mM assay concentration) the compounds can act as a reversible competitive inhibitor. The IC_{50} value was determined for the most potent inhibitors (**10**, **14-18**). Of these, aziridine **10** proved to be the best inhibitor of human chitinase with an IC_{50} below 30 µM. Furthermore, the profile of Michael acceptors **14** and **15** is interesting in that these compounds inhibit human chitinase and that they leave PNGase-activity untouched. Further research is needed to investigate the nature of inhibition of chitinases by these compounds.

As can be deduced from the crystal structure, the active site cleft of PNGase is divided in a carbohydrate binding site and a protein binding site.[1] It was therefore reasoned that combining a peptide and a carbohydrate recognition part would increase the selectivity of

inhibitors for PNGase. In a preliminary study, aziridine (not shown) and oxirane warheads were condensed with GlcNAc residue **19** (Scheme 1A). Hydrolysis of the ethyl ester in **20** and subsequent condensation of the resulting acid **21** to peptides **I** and **II** gave fully protected inhibitors **22** and **23**. Global deprotection furnished glycopeptides **24** and **25**. At 100-1000 μM, glycopeptides **24** and **25** completely abolished labeling of PNGase by β-VAD-Fmk. The potency of the compounds may be increased by replacing the monosaccharide for a disaccharide. Incorporation of an azido or fluorescent group as in **26** and **27** respectively would greatly facilitate the study towards the selectivity of these compounds in cell-lysates (Scheme 1B).

Scheme 1. (A) Synthesis and biological evaluation of bisubstrate inhibitors. (B) Potential new activity-based probes for PNGase containing a peptide and carbohydrate binding part.



Reagents and conditions: (a) i) Lindlar's cat., H_2; ii) epoxysuccinate monoethylester, HCTU, DiPEA, DMF, 68%; (b) LiOH, dioxane/H_2O, 90%; (c) H_2N-Ala-Val-OMe or H_2N-Ala-Ser(OBn)-OMe, HCTU, DiPEA, 48%; (d) H_2, $Pd(OH)_2$, dioxane/MeOH, 88%. Biochemical evaluation: the inhibitory potential of compounds **24** and **25** was tested in a competition experiment with BODIPY TMR-VAD-Fmk **1**. PNGase (100 ng) in phosphate buffer (20 mM, pH 7.2) was incubated with a concentration series of the inhibitors (**24** and **25**) for 2h at 37°C and subsequently non-reacted PNGase was labeled with BODIPY TMR-VAD-Fmk **1** (0.5 μM) for 30 min at 37°C.

Chapter 4 describes the use of activity-based probes to study degradation of *O*-GlcNAcylated proteins by the proteasome. *O*-GlcNAcylation is a major posttranslational modification in the nucleus and cytosol and it was therefore reasoned that the proteasome will encounter *O*-GlcNAcylated substrates.[2] Proteasome probes **28-31** (Figure 3A) equipped

with a carbohydrate not only blocked labeling by the fluorescent proteasome inhibitor MV151, but they did so with only slightly diminished potency compared to the parent compound epoxomicin. The covalent adduct could be visualized by Staudinger-Bertozzi modification, clearly demonstrating that deglycosylation prior to proteasome binding is not a prerequisite. It is therefore valid to ascertain that *O*-GlcNAcylated proteins are degraded by the proteasome. The resulting peptides may be presented by MHC I molecules. To obtain insight in the relevance of these complexes, a positional scan of Ser(*O*-GlcNAz) in epoxyketones resembling more closely actual proteasome products/MHC I peptides such as **32** and **33** can be helpful (Figure 3B). Additionally, these studies may elucidate the influence of the carbohydrate at the P2, P3 or P4 positions on binding to the proteasome.

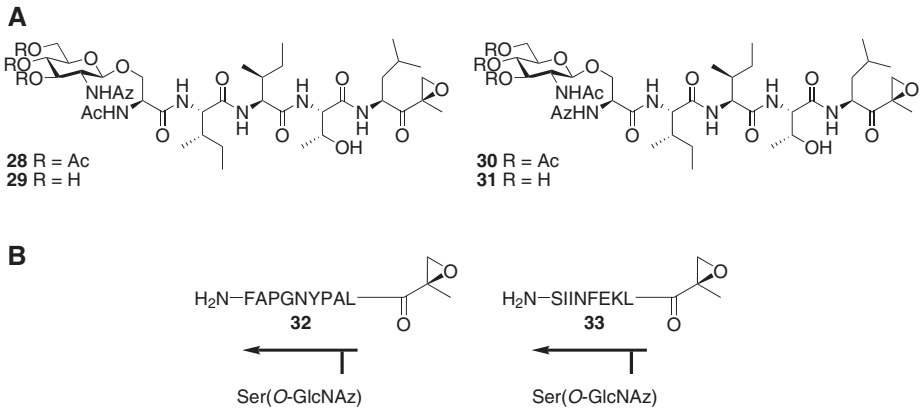


Figure 3. (A) Activity-based probes used to study the degradation of *O*-GlcNAc peptides. (B) Schematic overview of the positional scanning of epitopes. Incorporation of Ser(*O*-GlcNAz) at each position of the epitopes will be helpful to investigate the relevance of *O*-GlcNAcylation in potential MHC I epitopes

Chapter 5 discusses the syntheses of azidocyclophellitol, KY170 (**34**) and its fluorescent analogues, MDW933 (**35**) (Figure 4) and MDW941 (not shown) as well as their evaluation as activity-based probes for glucosidases. Determination of the IC_{50} value and kinetic data revealed that incorporation of an azido group did have a pronounced effect on the potency. KY170 (**34**) was applied in a two-step labeling experiment. Although purified GBA-1 could be labeled, significant non-specific labeling of BSA by the fluorophore was observed. In complex protein samples, low-abundant GBA-1 could not be visualized employing this strategy. The required labeling step of the two-step approach was circumvented by beforehand conjugation of the fluorescent reporter. It was shown that the potency of the resulting direct probes MDW933 (**35**) and MDW941 was increased. The BODIPY fluorophore probably binds to a lipophilic binding-pocket near the active site. GBA-1 could be labeled with both direct fluorescent probes. Labeling was extremely sensitive (0.3 ng could easily be visualized) and selective (exclusive labeling of GBA-1 in cell-lysates was observed). In **Chapter 6**, the versatility of probe **35** was further evaluated. Active glucocerebrosidase could be visualized with **35** in living cells. Mutant forms of GBA-1 could

be labeled with **35** as well. Finally, the effect of chaperones on the amount of active GBA-1 was established with probe **35**. Chaperones are known to restore trafficking of misfolded proteins to the Golgi, thereby increasing the levels of mutated proteins to nearly normal levels. Quantification of the activity using fluorogenic substrate based assay revealed that the levels of GBA-1 indeed increased upon stimulation with chaperones. The amount of GBA-1 that could be labeled with ABP **35** also increased. However, this increase was not completely proportional to the increase observed in the substrate-based assay. This finding indicates that part of the enzyme-activity may be blocked by the chaperone acting as an inhibitor.

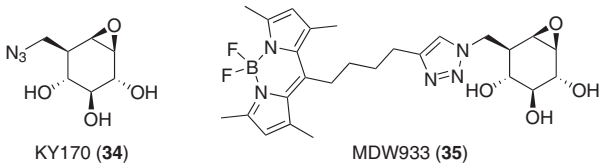
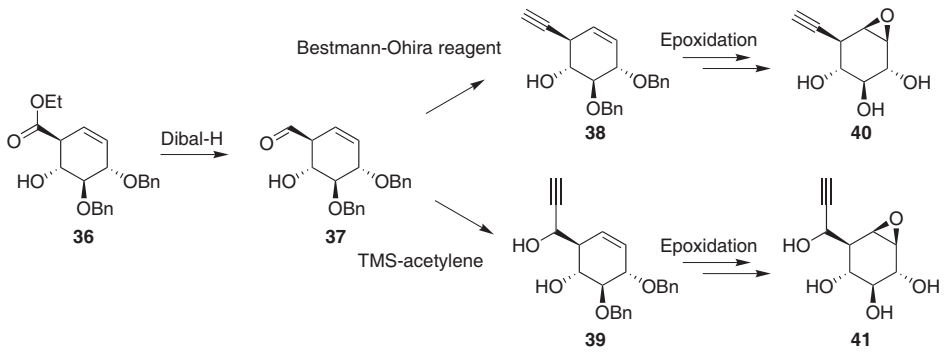


Figure 4. Activity-based probes for retaining β-glucosidases.

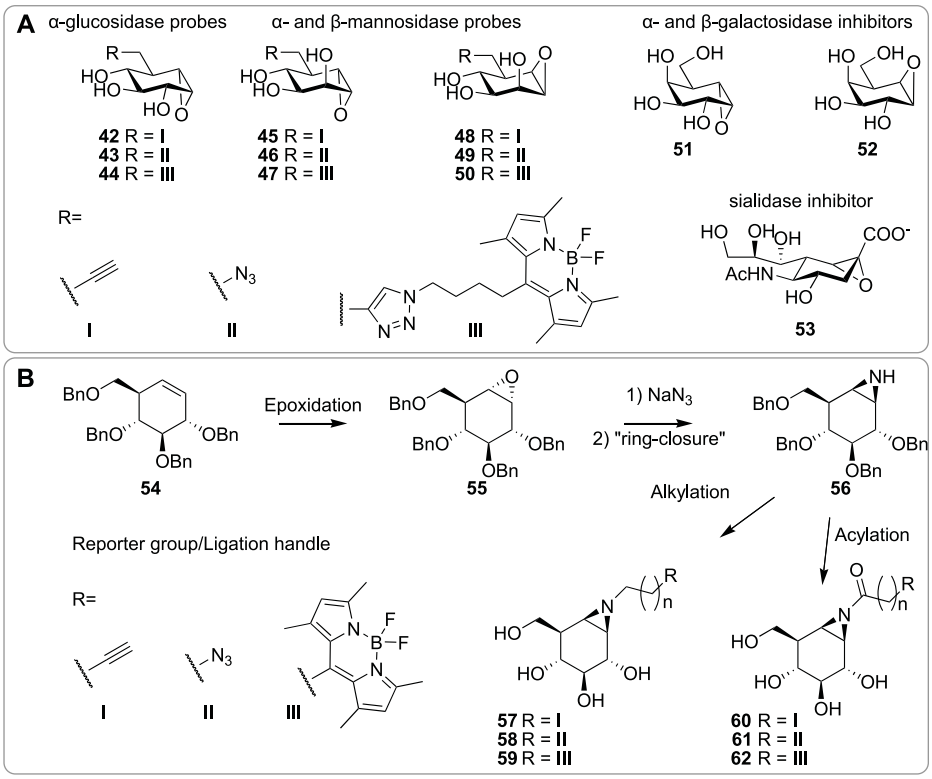
The promising results of labeling experiments with KY170 (**34**) and MDW933 (**35**) presented in Chapter 5 and 6 are an inspiration for the design of new glycosidase ABPs. One objective is the optimization of the two-step labeling strategy. Cravatt and co-workers performed a comparative study using alkyne and azido probes.[3] In this paper, it was shown that non-specific labeling could drastically be reduced by the use of probes containing an alkyne instead of an azido as ligation handle. To improve the two-step labeling strategy, second generation probes will therefore be equipped with alkyne group (**40** and **41**). Known ester **36** may serve as starting point for the synthesis of **40** and **41**. Conversion to aldehyde **37**, followed by treatment with TMS-acetylene/n-BuLi or the Bestmann-Ohira reagent[4] should afford alkynes **38** and **39**. Removal of the benzyl groups followed by benzoylation, epoxidation and deprotection should afford the alkyne probes **40** and **41** (Scheme 2).

Scheme 2. A route towards second generation two-step probes **40** and **41**.



The strategy used in Chapter 5 and 6 may also be exploited for retaining glycosidases with a different substrate preference such as mannosidases, galactosidases and sialidases. The α-mannose, β-mannose and α-glucose isomers of cyclophellitol have been reported to inhibit α-mannosidases, β-mannosidases and α-glucosidases respectively.[5] Incorporation of a fluorescent reporter group or a ligation handle such as an azide or alkyne in these molecules may afford potential probes for α-glucosidases (**42-44**), α-mannosidases (**45-47**) and β-mannosidases (**48-50**) (Scheme 3A). Analogues of cyclophellitol with the α- or β-galactoconfiguration (**51** and **52**) and sialic acid analogue **53** have not yet been reported. It may be interesting to synthesize these compounds and evaluate their biological activity. In case **51-53** inhibit galactosidases or sialidases, they may also be converted into ABPs. An initial attempt has been made for the α-glucosidases. Epi-azidocyclophellitol **43**, the α-glucose isomer of azidocyclophellitol was formed as a sideproduct during the synthesis of azidocyclophellitol **34** and was tested as ABP. From the fluorogenic substrate assay, it appeared that **43** in contrast to the parent compound epi-cyclophellitol did not inhibit retaining α-glucosidase of rice and yeast. This compound did however inhibit GBA-1 (IC_{50} 15 μM). Apparently, the azido group blocked binding to the active sites of α-glucosidases. These results clearly indicate that modification at the C-6 is not tolerated by all glycosidases.

Scheme 3. (A) Glycosidase probes based on cyclophellitol. (B) Potential ABPs containing a reporter group at the aglycone site.



To label exo-glycosidases that do not tolerated modifications at the C-6 position, the space at the aglycone site may be exploited for the introduction of a reporter group. By altering this position, all the hydroxyl groups that are crucial for binding to the enzyme will be maintained in these probes. The epoxide of cyclophellitol and conduritol B epoxide is located at the same position as the aglycone of the substrate. Since the epoxide is crucial for irreversible inhibition, cyclophellitol and conduritol B epoxide are not suitable leads for this class of probes. It is thought that aziridines, the nitrogen analogue of epoxides, allow introduction of the reporter group at the aglycon site by functionalisation of the ring nitrogen. Nakata demonstrated that aziridine analogues of cyclophellitol inhibited a variety of glycosidases.[6] Key aziridine **56** may be synthesized from alkene intermediate **54** by epoxidation followed by ring-opening with NaN_3 and subsequent ring-closure (Scheme 3B). The reporter group can then be introduced by condensation with an acylchloride or alkylation with an alkylbromide affording glycosidase probes **57**-**62**.

For identification of the labeled glycosidase by mass spectrometry, activity-based probes containing an affinity label would be beneficial. A first attempt was made by conjugating biotin to a mixture of epoxides (**34** and **42**) using the copper catalyzed click reaction. Incorporation of biotin, as in **63** resulted in complete loss of biological-activity as was evidenced by competition experiment versus **35** (Scheme 4). No significant labeling of α- or β-glucosidases was observed both in experiments using purified enzymes and in cell-lysates. The loss in activity was either caused by the hydrophilicity of biotin or by the introduction of an amide at the linker region.

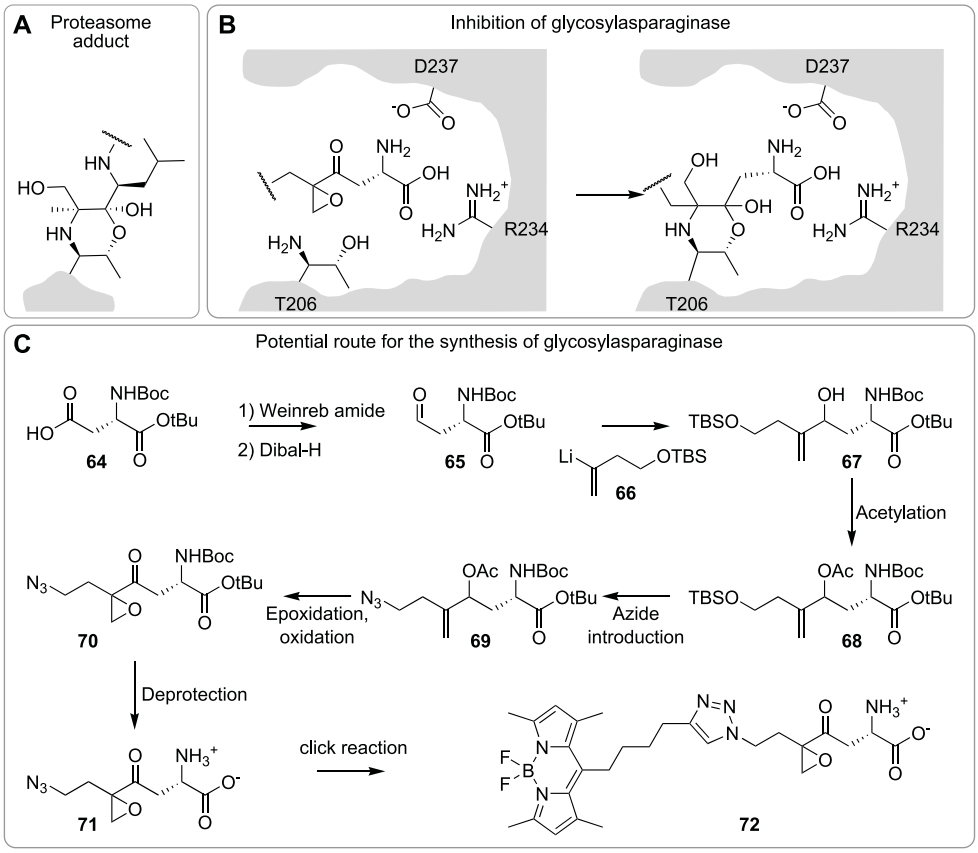
Scheme 4. Synthesis and biochemical evaluation of biotin equipped-cyclophellitol **63**.

Reagents and conditions: Biotin-alkyne, $CuSO_4$ (10 mol %), sodium ascorbate (15 mol %), tBuOH/H_2O/Tol, 80°C. The inhibitory potential of ABP **63** was evaluated in a competition experiment versus MDW933 (**35**). The indicated concentration of **63** was incubated with GBA-1 (20 ng) in McIlvain (9 μL, pH 5.2, 0.2% taurocholate, 0.1% Triton X-100, containing 0.1 μg/μL BSA) for 30 min at 37°C. The potency was determined by labeling with MDW933 (**35**) (0.2 μM final concentration), SDS-PAGE and subsequent fluorescent imaging.

Of the enzymes described in Chapter 1, no activity-based probes for glycosylasparaginase have appeared in literature so far. The catalytic mechanism of this nucleophilic N-terminal threonine hydrolase shares great similarities with that of the proteasome, a well studied protease (see also Chapter 4). Epoxyketones are mechanism based inhibitors which react selectively with the proteasome to form a stable morpholino adduct (Scheme 5A).[7] An

aspartic acid equipped with an epoxyketone may result in new inhibitors/probes for glycosylasparaginase (Scheme 5B). The inhibitors/probes may be synthesized as depicted in Scheme 5C. Boc-Asp-OtBu **64** is converted to β-aldehyde **65** by condensing the β-acid to *N*,*O*-dimethylhydroxylamine followed by reduction of the resulting Weinreb amide with Dibal-H. Grignard or organolithium addition of alkene **66** to aldehyde **65** should give allylic alcohol **67**. Suitable protection of the hydroxyl followed by deprotection of the primary alcohol and introduction of the azido group should afford **68**. Removal of acetyl group in **69**, ensuing epoxidation and oxidation of the alcohol should give epoxyketone **70**. After global deprotection, probe **71** may be used as such or may be conjugated to a BODIPY giving **72**.

Scheme 5. (A) Morpholino-adduct formed by the proteasome inhibitor epoxomicin. (B) Mechanism by which epoxyketone inhibitors should inhibit glycosylasparaginase. (C) A possible route towards epoxyketones **72** and **73**.



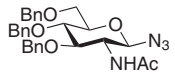
Experimental section

General procedure

All reagents were of commercial grade and were used as received unless stated otherwise. Diethyl ether (Et_2O), ethyl acetate (EtOAc), light petroleum ether (PE) and toluene (Tol) were purchased from Riedel-de Haën. Acetonitrile (MeCN), dichloroethane, dichloromethane, *N*,*N*-dimethylformamide (DMF), methanol, pyridine (pyr), tetrahydrofuran (THF) were obtained from Biosolve. THF was distilled over $LiAlH_4$ 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% sulfuric 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 10% sulfuric acid in water followed by charring at ~150°C. 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 Gilson automated HPLC system equipped with a semi-preparative Alltima C_{18} column was used. The applied buffers were A: 0.1% aq. TFA, B: MeCN. 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.

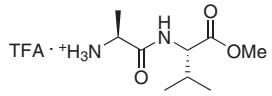
Chitinase inhibition

Inhibition of chitinase by chitobioses **2-18** was tested as follows: The inhibitor solution (12.5 μL, 1 mM in McIlvain (pH 5.2) containing 1 mg/mL BSA) was added to 12.5 μL of the enzyme solution (in McIlvain (pH 5.2) containing 1 mg/mL BSA). The solution was incubated for 30 min at 37°C followed by the addition of 100 μL 4-methylumbelliferyl-β-chitobiose (0.25 mM in McIlvain (pH 5.2)) and incubating for 20 min at 37°C. The reaction was stopped by the addition of glycine/NaOH (0.3 M, pH 10.6). The amount of hydrolyzed 4-MU substrate was determined using a Perkin Elmer Life Sciences Luminiscence Spectrometer LS-30.



(2-acetamido-3,4,6-tri-*O*-benzyl-2-deoxy-β-D-glucopyranosyl) azide (19)

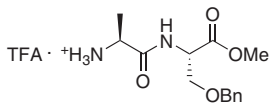
A stirred solution of (2-acetamido-2-deoxy-β-D-glucopyranosyl) azide[8] (2.95 g, 12 mmol) in absolute DMF (60 mL) was cooled to 0 °C before BaO (20 g, 120 mmol, 10 equiv.) and $BaOH{\cdot}8H_2O$ (8 g, 24 mmol, 8 equiv.) were added. After 10 min of stirring, benzylbromide (15 mL, 130 mmol, 11 equiv.) was added dropwise. After complete addition, the solution was allowed to come to room temperature and stirred for 2h. The solution was evaporated under reduced pressure and coevaporated thrice with H_2O. Extraction with EtOAc/H_2O gave a yellow oily liquid. Crystallization from CH_2Cl_2/MeOH and PE gave the title compound **19** (50%, 3.26 g, 6 mmol) as a brownish solid. ^1H NMR (500 MHz, $CDCl_3$) δ ppm 7.36-7.21 (m, 15H), 6.17 (d, *J* = 8.45 Hz, 1H), 4.83 (d, *J* = 2.91 Hz, 1H), 4.81 (d, *J* = 5.43 Hz, 1H), 4.76 (d, *J* = 10.85 Hz, 1H), 4.65 (d, *J* = 11.52 Hz, 1H), 4.60 (d, *J* = 12.19 Hz, 1H), 4.54 (dd, *J* = 11.45, 8.37 Hz, 2H), 3.88 (dd, *J* = 9.76, 8.90 Hz, 1H), 3.73-3.70 (m, 2H), 3.66 (d, *J* = 9.61 Hz, 1H), 3.63 (d, *J* = 11.07 Hz, 1H), 3.60-3.56 (m, 1H), 1.86 (s, 3H). ^{13}C NMR (150 MHz, $CDCl_3$) δ ppm 127.9, 88.06, 80.94, 77.25, 76.74, 74.72, 73.47, 55.69, 32.29. LC/MS: R_t 9.81 min; linear gradient 10→90% B in 13.5 min; ESI/MS: *m/z* = 517.13 $(M+H)^+$.



H_3N-Val-Ala-OMe·TFA (I)

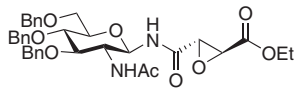
To a stirred solution of Boc-Ala-OH (950 mg, 5 mmol) in CH_2Cl_2 (1 mL) were added sequentially HCTU (2.5 g, 6 mmol, 1.2 equiv.) and

DiPEA (1.92 mL, 11 mmol, 2.2 equiv.). The mixture was allowed to stand for 15 min before H_2N-Val-OMe (787 mg, 6 mmol, 1.2 equiv.) was added, stirring was continued for 6h. Upon completion EtOAc was added, the organic layer was washed with 1M HCl (3×), saturated aqueous $NaHCO_3$ (3×) and brine (2×). *In vacuo* evaporation yielded a white solid wich was treated with TFA/CH_2Cl_2 (1:1, v/v) and evaporated under reduced presure to yield title compound **4** as a redish oil (80%, 1.3 g, 4.11 mmol). ^{1}H NMR (600 MHz, $CDCl_3$) δ ppm 4.54 (m, 1H), 4.40 (m, 1H), 3.73 (s, 3H), 2.16 (m, 1H), 1.35 (m, 3H), 0.93 (m, 6H). ^{13}C NMR (150 MHz, $CDCl_3$) δ ppm 56.49, 51.36, 48.80, 31.57, 30.45, 18.35. FT-IR: ν_{max} (neat)/cm^{-1} 2968.2, 1669.0, 1559.9, 1438.3, 1376.4 1270.7, 1144.3, 1045.3.



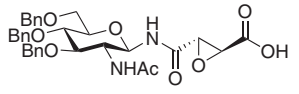
H_3N-Val-Ser(OBn)OMe·TFA (II)

Peptide **5** was synthetisized as described for **4**. H_2N-Ser(OBn)-OMe was used instead of H_2N-Val-OMe yielding a redish oil (90%, 3.6 g, 9 mmol).^{1}H NMR (200 MHz, $CDCl_3$) δ ppm 8.12 (s, 3H), 7.3-7.5 (m, 5H), 4.64 (m, 1H), 4.45 (d, *J* = 18.26, 1H), 4.40 (d, *J* = 18.26, 1H), 4.10 (m, 1H), 3.70 (s, 3H), 3.60 (m, 1H), 1.55 (m, 3H). FT-IR: ν_{max} (neat)/cm^{-1} 3067.7, 1740.2, 1669.3, 1559.8, 1456.8, 1438.1, 1363.6, 1139.7, 1052.0.



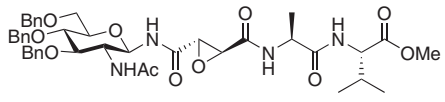
(2*S*,3*S*)-3-*N*-(2-acetamido-3,4,6-tri-*O*-benzyl-2-deoxy-β-D-gluco-pyranosylcarbamoyl) oxirane-2-carboxylic acid ethyl ester (20)

To a stirred solution of azide **19** (0.5 g, 1 mmol) in DMF (5 mL) was added Lindlar's catalyst (1 equiv.) and the mixture was stirred under a constant flow of H_2. The reaction was stirred until TLC showed complete conversion to a lower running spot. The reaction was quenched and the solution was filtered. To the filtered solution was sequentially added epoxysuccinate monoethyl ester[9] (1 equiv.), HCTU (1 equiv.) and DiPEA (2 equiv.). The reaction was stirred for 16h after which it was evaporated under reduced pressure yielding a yellow solid. The solid was dissolved in EtOAc, washed with saturated aqueous $NaHCO_3$ and brine. Silica gel column chromatography afforded (0.5 MeOH/CH_2Cl_2→4% MeOH/CH_2Cl_2) **20** (45%, 0.26 g, 0.41 mmol). ^{1}H NMR (400 MHz, $CDCl_3$/MeOD) δ ppm 7.37-7.20 (m, 15H), 4.95 (d, *J* = 9.69 Hz, 1H), 4.79 (d, *J* = 11.25 Hz, 1H), 4.74 (d, *J* = 10.69 Hz, 1H), 4.58 (d, *J* = 12.01 Hz, 1H), 4.52 (d, *J* = 10.79 Hz, 1H), 4.47 (d, *J* = 11.93 Hz, 1H), 4.2 (q, 2H), 3.94 (dd, *J* = 10.16, 9.42 Hz, 1H), 3.73-3.70 (m, 2H), 3.64 (dd, *J* = 8.89, 8.14 Hz, 1H), 3.62-3.57 (m, 1H), 3.56-3.49 (m, 1H), 1.85 (s, 3H), 1.28 (t, *J* = 15.12, 7.92 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$/MeOD) δ ppm 127.9, 82.90, 79.10, 76.73, 75.10, 73.58, 62.37, 54.22, 23.30, 13.68. FT-IR: ν_{max} (neat)/cm^{-1} 3286.9, 3206.3, 2996.1, 1737.1, 1689.3, 1655.7, 1549.9, 1495.8, 1453.6, 1388.1, 1358.1, 1309.1, 1286.6, 1259.3, 1208.0, 1145.6, 1098.4, 1069.7, 1027.9, 950.7, 924.9. $[\alpha]_D^{20}$ +214.4° (c = 0.5, dioxane/MeOH).



(2S,3S)-3-*N*-(2-acetamido-3,4,6-tri-*O*-benzyl-2-deoxy-β-D-gluco-pyranosylcarbamoyl) oxirane-2-carboxylic acid ethyl ester (21)

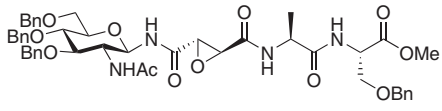
Epoxide **20** (0.33 g, 0.52 mmol) was dissolved in dioxane/H_2O (3:1, v/v, 3 mL). Anhydrous LiOH (10 equiv.) was added and the reaction was monitored by TLC. 1M HCl was added upon completion until pH 4. CH_2Cl_2 was added and the remaining aqeous layer was extracted with CH_2Cl_2 (3×). MeOH was added to the organic layer and the organic layer was dried with $MgSO_4$ and filtered. Evaporation *in vacuo* yielded epoxide **21** (100%, 0.31 g, 0.52 mmol) as a white solid. LC/MS: R_t 8.64 min; linear gradient 10→90% B in 13.5 min; ESI/MS: *m/z* = 605.40 $(M+H)^+$.



β-D-GlcNAc(3,4,6-tri-*O*-benzyl)-D-epoxysuccinate-Ala-Val-OMe (22)

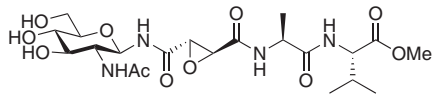
To a stirred solution of epoxide **21** (0.1 g, 0.16 mmol) in DMF (5 mL) was added peptide **I** (1.2 equiv.), HCTU (1.2 equiv.) and DIPEA (2 equiv.). The solution was stirred for 16h. *In vacuo* evaporation yielded a yellow oil. Silica gel column chromatography (5 MeOH/CH_2Cl_2→10% MeOH/CH_2Cl_2)

afforded **22** (82%, 0.10 g, 0.13 mmol). ^{1}H NMR (500 MHz, MeOD) δ ppm 7.42-7.21 (m, 15H), 4.98 (d, *J* = 9.72 Hz, 1H), 4.83 (d, *J* = 11.37 Hz, 1H), 4.78 (d, *J* = 10.77 Hz, 1H), 4.71 (d, *J* = 11.38 Hz, 1H), 4.61 (d, *J* = 11.94 Hz, 1H), 4.56 (d, *J* = 10.81 Hz, 1H), 4.40 (d, *J* = 5.59 Hz, 1H), 3.97 (dd, *J* = 10.28, 9.74 Hz, 1H), 3.72-3.70 (m, 2H), 3.69 (dd, *J* = 4.19, 3.76 Hz, 1H), 3.66 (dd, *J* = 5.62, 5.62 Hz, 1H), 3.58-3.52 (m, 1H), 3.49 (s, 3H), 2.17 (sext.d, J = 13.64, 6.79, 6.79, 6.79, 6.71, 6.71 Hz, 1H), 1.89 (s, 3H), 1.38 (s, 3H), 0.95 (dd, J = 6.87, 3.85 Hz, 6H). ^{13}C NMR (100 MHz, DMSO-*d*$_6$) δ ppm 138.12, 127.68, 82.65, 78.45, 77.74, 75.91, 74.70, 73.21, 68.63, 53.67, 51.65, 47.85, 29.76, 22.75, 18.90, 18.21. FT-IR: ν_{max} (neat)/cm^{-1} 3287.9, 2961.5, 1740.0, 1644.5, 1558.0, 1451.8, 1361.4, 1311.9, 1260.9, 1207.8, 1062.2, 1027.6. $[\alpha]_D^{20}$ +14.4° (c = 1, dioxane/MeOH).



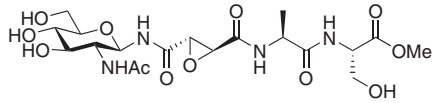
β-D-GlcNAc(3,4,6-tri-*O*-benzyl)-D-epoxysuccinate-Ala-SerOBn-OMe (23)

Epoxide **21** (0.1 g, 0.16 mmol) was converted to glycopeptide **23** as described for **22**. Silica gel column chromatography (2 MeOH/CH_2Cl_2→7% MeOH/CH_2Cl_2) afforded **23** (57%, 0.08 g, 0.09 mmol). ^{1}H NMR (600 MHz, $CDCl_3$/MeOD) δ ppm 7.42-7.24 (m, 20H), 4.90 (d, *J* = 9.68 Hz, 1H), 4.83 (d, *J* = 11.53 Hz, 1H), 4.78 (d, *J* = 10.69 Hz, 1H), 4.68 (d, *J* = 12.02 Hz, 1H), 4.62 (d, *J* = 11.99 Hz, 1H), 4.56 (d, *J* = 4.15 Hz, 1H), 4.54 (d, *J* = 5.69 Hz, 1H), 4.50 (d, *J* = 12.40 Hz, 1H), 4.48 (d, *J* = 8.95 Hz, 1H), 3.94 (dd, *J* = 10.71, 9.36 Hz, 1H), 3.89 (dd, *J* = 9.61, 3.81 Hz, 1H), 3.73-3.72 (m, 1H), 3.72-3.68 (m, 2H), 3.58 (dd, *J* = 10.14, 9.45 Hz, 1H), 3.55-3.50 (m, 1H), 3.50 (s, 3H), 1.84 (s, 3H), 1.33 (d, *J* = 6.54 Hz, 3H). ^{13}C NMR (125 MHz, DMSO-*d*$_6$) δ ppm 137.46, 127.82, 81.88, 78.93, 77.67, 76.43, 74.59, 73.01, 68.02, 53.61, 52.41, 22.37, 17.88. FT-IR: ν_{max} (neat)/cm^{-1} 3286.0, 1734.2, 1651.8, 1645.1, 1557.9, 1538.7, 1455.6, 1361.9, 1214.7, 1062.3, 893.5, 840.1, 741.0, 695.4, 668.0, 614.3 cm^{-1}. $[\alpha]_D^{20}$ +24.4° (c = 0.5, dioxane/MeOH).



β-D-GlcNAc-D-epoxysuccinate-Ala-Val-OMe (24)

Glycopeptide **22** (50 mg, 0.062 mmol) was dissolved in dioxane/MeOH. $Pd(OH)_2$ (1 equiv.) was added and the mixture was stirred under a continuous flow of H_2 until complete conversion to a very polar product was observed by TLC. Evaporation under reduced pressure followed by preparative HPLC gave PNGase-inhibitor **24** (quant, 32 mg, 0.062 mmol).^{1}H NMR (400 MHz, $CDCl_3$/MeOD) δ ppm 4.97 (d, *J* = 9.61 Hz, 1H), 4.47 (td, *J* = 7.46, 6.07, 6.07 Hz, 1H), 4.32 (m, 1H), 3.81 (dd, *J* = 11.43, 10.97 Hz, 1H), 3.71 (s, 3H), 3.66 (m, 1H), 3.56-3.48 (m, 3H), 3.48-3.43 (m, 1H), 2.16 (td, *J* = 13.09, 6.93, 6.93 Hz, 1H), 1.98 (s, 3H), 1.44-1.24 (m, 1H), 0.94 (d, *J* = 6.79 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$/MeOD) δ ppm 80.42, 79.91, 75.90, 71.70, 62.58, 59.31, 56.16, 54.57, 52.48, 50.27, 31.83, 22.74, 19.46, 18.44.



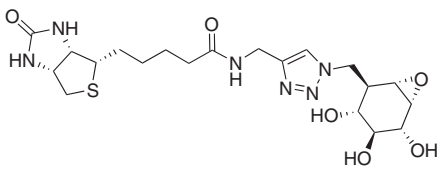
β-D-GlcNAc-D-epoxysuccinate-Ala-Ser-OMe (25)

The benzyl protective groups in epoxide **24** (50 mg, 0.058 mmol) were removed as described for **24** affording inhibitor **25** (quant, 29 mg, 0.058 mmol). ^{1}H NMR (400 MHz, $CDCl_3$/MeOD) δ ppm 5.03 (d, *J* = 9.62 Hz, 1H), 4.55-4.51 (m, 1H), 4.45 (dd, *J* = 13.88, 6.80 Hz, 1H), 3.93 (dd, *J* = 11.52, 4.73 Hz, 1H), 3.84 (dd, *J* = 11.26, 10.27 Hz, 1H), 3.76 (s, 3H), 3.74-3.63 (m, 1H), 3.70 (m, 1H), 3.44-3.39 (m, 1H), 2.01 (s, 3H), 1.42 (d, *J* = 7.06 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$/MeOD) δ ppm 78.77, 78.07, 74.08, 69.84, 61.12, 60.77, 54.84, 54.60, 53.32, 52.26, 49.23, 21.67,16.60.

Competition experiment of glycopeptides

The inhibitory potential of glycopeptides **24** and **25** was tested as follows. PNGase (9 µL, 11.1 ng/µL) in reaction buffer (pH 7.2, 20 mM sodium phosphate, 150 mM NaCl, BSA (1 µg/µL)), was incubated with a serial dilution of **24** and **25** (1 µL) for 2h at 37°C followed by incubation with BODIPY-VAD-Fmk **1** (0.5 µM) for 30 min. The reaction was quenched by the addition of 4× SDS-PAGE sample

buffer (5 µL) and boiling for 5 min. Resolving of the proteins on 10% SDS-PAGE gel was followed by measuring of the fluorescence in the wet gel slabs using the CY3/Tamra settings (λ_{ex} 532, λ_{em}560) on a Typhoon Variable Mode Imager (Amersham Biosciences).



Synthesis of MDW940 (63)

Azidocylcophellitol **34+42** (17 mg, 85 µmol) was dissolved in *tert*-BuOH/H_2O/Tol (1.8 mL 1/1/1 v/v/v). Biotin-acetylene (24 mg, 85 µmol), $CuSO_4$ (10 mol%) and sodium ascorbate (15 mol%) were added before the reaction was stirred at 80°C. TLC-analysis showed complete conversion after overnight stirring and the solution was concentrated. Purification by reverse phase HPLC (linear gradient 10→19% B in 14 min (B=MeOH)) afforded MDW940 (**63**) (36%, 15.09 mg, 31 µmol). ^{1}H NMR (400 MHz, MeOD) δ ppm 8.73 (s, 1H), 5.12 (t, *J* = 6.4, 6.4 Hz, 1H), 4.94 (dd, *J* = 12.9, 2.3 Hz, 1H), 4.86-4.80 (m, 1H), 4.56 (s, 2H), 4.50 (dd, *J* = 7.7, 4.8 Hz, 1H), 4.46 (dd, *J* = 5.7, 2.6 Hz, 1H), 4.30 (dd, *J* = 7.8, 4.5 Hz, 1H), 3.79 (dd, *J* = 7.4, 5.8 Hz, 1H), 3.60 (dd, *J* = 5.6, 2.7 Hz, 1H), 3.54 (dtd, *J* = 9.3, 6.9, 6.9, 2.4 Hz, 1H), 3.43 (dd, *J* = 9.6, 7.8 Hz, 1H), 3.21 (td, *J* = 9.2, 5.4, 5.4 Hz, 1H), 2.93 (dd, *J* = 12.8, 4.9 Hz, 1H), 2.72 (d, *J* = 12.8 Hz, 1H), 2.29 (t, *J* = 7.2, 7.2 Hz, 1H), 1.79-1.52 (m, 4H), 1.51-1.38 (m, 2H). ^{13}C NMR (100 MHz, MeOD) δ ppm 176.6, 166.1, 154.9, 128.3, 75.3, 74.7, 72.6, 68.5, 68.4, 63.3, 61.6, 57.0, 54.2, 49.6, 45.9, 41.0, 36.2, 36.1, 29.6, 29.4, 26.5. LC/MS: R_t 4.60, 4.66; linear gradient 0→50% B in 13.5 min; ESI/MS: *m/z* = 483.33 $[M+H]^+$. HRMS: $(M+H^+)$ calc. for $C_{20}H_{30}N_6O_6S$ 483.20203, found 483.20170.

Competition and labeling experiments with MDW940 (63)

For the competition experiments, a concentration serie of **63** was incubated with GBA-1 (20 ng) in McIlvain (9 µL, pH 5.2, 0.2% taurocholate, 0.1% Triton X-100, containing 0.1 µg/µL BSA) for 30 min at 37°C. Subsequently MDW933 (**35**) (1 µL, 0.2 µM final concentration) was added and the solution was incubated for an addititonal 30 min. The reaction was quenched by the addition of 4 µL sample buffer (4×) followed by boiling for 5 min. Resolving of the proteins with SDS-PAGE and subsequent fluorescent imaging allowed determination the potency of MDW940. Labeling experiments were performed similar to competition experiments. A serial dilution of **63** was incubated with GBA-1 and after 30 min the reaction was quenched. The protein samples were separated on a 7.5% SDS-PAGE gel and transferred to a PVDF-membrane. The membranes were blocked with 0.5% bovine serum albumin in TBS-TWEEN (0.1% TWEEN-20) for 30 min and incubated with Streptavidine-HRP (Amersham Bioscience 1:5000) for 30 min at ambient temperature. The membranes were briefly washed with TBS containing 0.1% TWEEN-20 and TBS followed by visualisation of the biotinylated proteins with an ECL+ Kit (Amersham Bioscience).

References and footnotes

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