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Ligand-Directed Chemistry on Glycoside Hydrolases – A Proof of Concept Study

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Selective covalent labelling of enzymes using small molecule probes has advanced the scopes of protein profiling. The covalent bond formation to a specific target is the key step of activity-based protein profiling (ABPP), a method which has become an indispensable tool for measuring enzyme activity in complex matrices. With respect to carbohydrate processing enzymes, strategies for ABPP so far involve labelling the active site of the enzyme, which results in permanent loss of activity. Here, we report in a proof of concept study the use of ligand-directed chemistry (LDC) for labelling glycoside hydrolases near – but not in – the active site. During the labelling process, the competitive inhibitor is cleaved from the probe, departs the active site and the enzyme maintains its catalytic activity. To this end, we designed a building block synthetic concept for small molecule probes containing iminosugar-based reversible inhibitors for labelling of two model β-glucosidases. The results indicate that the LDC approach can be adaptable for covalent proximity labelling of glycoside hydrolases.

Introduction

Many prevalent diseases such as diabetes, lysosomal storage disorders, Alzheimer's and Parkinson's diseases, have been linked to defective carbohydrate processing enzymes.[1–4] Thus, detecting which cell organelles hold active or inactive enzymes is vital for understanding and treating such diseases. In this regard, activity-based protein profiling (ABPP) has become an indispensable tool for the investigation of enzyme activity in complex matrices such as live cells and tissues. Providing information about the effective activity rather than abundance, ABPP reveals insights into enzyme-substrate interactions – information that is essential for diagnostic procedures as well as drug discovery.[5] Moreover, ABPP has been applied in optical imaging for diagnosis *in vivo*, to evaluate signalling pathways, and to find novel inhibitors for various enzyme classes including kinases, serine hydrolases, metalloproteases, phosphatases, and oxidoreductases.[6–8]

Carbohydrate processing enzymes have also been studied by ABPP.[9–16] In a number of approaches, Overkleeft and co-workers labelled different glycoside hydrolase families selectively. Besides others, they were successful in selective labelling human lysosomal acid β-glucocerebrosidase (GBA) in the presence of the other human retaining β-glucosidases (GBA3 and LPH).[17,18] These strategies for ABPP of glycoside hydrolases involve mechanism-based semi- or irreversible inhibitors forming a covalent bond in the active site of the target enzyme. Consequently, the activity of the labelled enzyme is permanently lost.

To maintain the activity of the enzyme after labelling an alternative approach has been reported by Hamachi and Tsukiji.[19] Ligand-directed chemistry (LDC) combines a reversible inhibitor and a cleavable electrophile for covalent labelling near – but not in – the active site. During the labelling process the reversible inhibitor is cleaved from the probe and, hence is able to depart from the active site thereby leaving the covalently modified – but still active – enzyme (see Figure 1). LDC has been applied for example, to human carbonic anhydrase CAII, FKBP12, congerin, and the ATP domain of Hsc70.[19–25] Scenarios where the enzyme activity is needed after labelling are described in the literature[23] such as ^{19}F-NMR biosensing for in cell kinetic measurements, live-cell FRET imaging or turn-on fluorescent biosensors.

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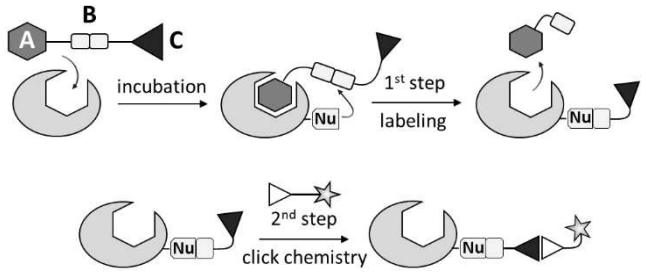


Figure 1. Schematic illustration of the LDC strategy resulting in chemical modification of the enzyme nearby – but not in – the active site, thereby preserving its activity. (A) Ligand, (B) linker with reactive cleavable site, (C) reporter tag, (Nu) nucleophilic amino acid.

For LDC the small molecule probe consists of three modules, as shown in Figure 1. (A) A reversible inhibitor specific for the enzyme of interest is used as ligand. (B) For covalent labelling, the probe is equipped with a reactive cleavable site within the linker region between the ligand and (C) a reporter tag. The tag is used for detection of the labelled enzyme.[26] This probe design establishes protein selectivity through the ligand whereas the actual labelling process occurs in proximity to the active site. By release of the cleaved off reversible inhibitor the enzyme is covalently labelled and still maintains its catalytic activity.

We herein report the proof of concept for using LDC to label glycoside hydrolases.

Results and Discussion

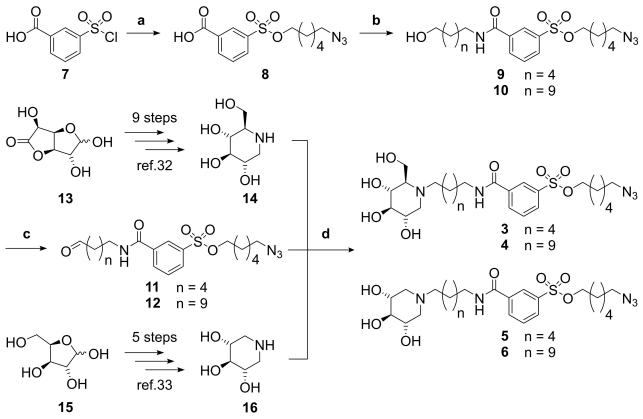
Probe design and synthesis

Applying the LDC probe format, we designed and synthesized small molecule iminosugar-based probes. As models two commercially available enzymes, β-glucosidases from almonds (*Prunus dulcis*, *Pd*GH1) and *Thermotoga maritima* (*Tm*GH1) were chosen.

The modules for our probes are as follows (Figure1): (A) As ligand we use the iminosugar 1-deoxynojirimycin (DNJ), a known potent competitive inhibitor for the enzyme class under consideration.[27] (B) As the cleavable reactive site, we first introduced a benzoate ester moiety (compounds **1** and **2**, synthesis SI Scheme S1), similar to the phenyl esters introduced for protein labelling of marinopyrrole A.[28] Aromatic esters are convenient to handle and are relatively stable, which should minimize unwanted unselective labelling. We anticipated such esters to react with amines of lysine side chains which are located in close proximity after binding of the probe to the active site. However, no labelling was observed with probes **1** and **2** (SI Figure S6) indicating that a more reactive moiety as part B is needed. We therefore introduced a sulfonate ester as the electrophilic species since this has been shown previously to effect covalent labelling of histidines, tyrosines, glutamic and aspartic acids as well as cysteines.[19,29] (C) To detect the labelled enzyme we use a two-step approach to avoid problems with solubility as well as possible steric hindrance between the bulky fluorophore and the target enzyme (Figure 1). To this end, the LDC probe was functionalized with a terminal azide that can undergo click chemistry with an alkyne-fluorophore in a second step, enabling visualization after the LDC labelling process.

The linker between the ligand and the cleavable electrophile determines the site selectivity of labelling.[19,30,31] By optimizing the linker length, the reactive moiety is positioned in close proximity to the amino acid residue to be labelled. This so-called proximity effect is vital for covalent labelling of the target enzyme. Therefore, we developed a building block synthetic concept which enables to tailor each module of the small molecule probe for the respective target enzyme (Scheme 1). To test the influence of the linker between the ligand and the cleavable electrophile, we synthesized probes of different linker lengths **1**–**6** (Figure 2).

Starting from 3-(chlorosulfonyl)benzoic acid **7**, sulfonate ester **8** is formed by esterifying with 6-azidohexan-1-ol under standard conditions, installing the terminal reporter tag (C, Figure 1) in the first step of the synthesis. To insert the alkyl linker, classical mixed-anhydride conditions were employed, where different lengths of amino alcohols can be introduced. This allows for tailoring the distance between the ligand (A) and the cleavable electrophile (B). In this case, C-6 and C-11 amino



Scheme 1. Synthesis of ligand-directed sulfonate ester (LDSE) probes **3**–**6**.[32,33] Reaction conditions: a) $N_3(CH_2)_6OH$, pyridine, CH_2Cl_2, 0 °C, 54%; b) $H_2N(CH_2)_nOH$ (n = 6,11), DIPEA, $ClCO_2i$-Bu, CH_2Cl_2, 0 °C, 80%; c) DMP, CH_2Cl_2, 80%; d) $NaBH_3CN$, AcOH, MeOH, 40–60%.

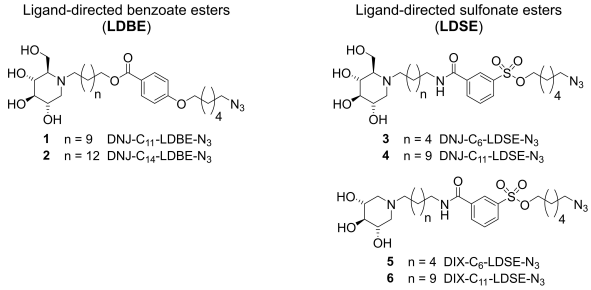


Figure 2. Ligand-directed benzoate ester probes: DNJ-C_{11}–LDBE-N_3 probe **1**, DNJ-C_{14}–LDBE-N_3 probe **2**. Ligand-directed sulfonate ester probes: DNJ-C_6–LDSE-N_3 probe **3**, DNJ-C_{11}–LDSE-N_3 probe **4** and DIX-C_6–LDSE-N_3 probe **5**, DIX-C_{11}–LDSE-N_3 probe **6**.

alcohols were used giving compounds **9** and **10**, respectively. Dess-Martin oxidation followed by reductive amination of aldehydes **11** and **12** with 1-deoxynojirimycin (DNJ) **14** or 1,5-dideoxy-1,5-imino-D-xylitol (DIX) **16** gave the desired probes **3**–**6** in yields of 40 to 60%, respectively (Scheme 1). Incorporation of the iminosugar ligand in the last step of the synthesis enables using the same building block B–C for targeting different enzymes by exchanging the ligand (A).

Biologic Evaluation – Proof of Concept

Evaluation of sulfonate ester probes DNJ-C_6–LDSE-N_3 **3**, DNJ-C_{11}–LDSE-N_3 **4** with the two model enzymes gave varying results, confirming the importance of the linker length. First, we assessed the initial inherent binding affinities of the probes prior to any covalent reaction by determining their inhibitory properties. All probes, benzoate esters **1** and **2** as well as sulfonate esters **3**–**6** proved to be competitive inhibitors as seen in the Lineweaver Burk plots (Table 1, details see SI, Fig S3–4). For DNJ-sulfonate ester probes **3** and **4** K_i-values in the low to sub-micromolar range are seen with higher affinity towards *Tm*GH1 compared to *Pd*GH1.

For the labelling studies, the enzymes were incubated with respective LDC probes **3** and **4** for 20 hours at room temperature in a ratio of enzyme to probe 1:10, corresponding to 100 μM of the respective probe. Formation of a covalent bond should take place through displacement of the sulfonate, followed by introduction of a fluorophore via a click-chemistry protocol (Figure 1, details see experimental section). Viewing of SDS-PAGE gels under UV irradiation revealed fluorescent protein bands from the respective enzymes in the case of *Pd*GH1 with DNJ-C_{11}–LDSE-N_3 probe **4** and of *Tm*GH1 with both probes **3** and **4** (Figure 3). The time- and concentration-dependence of labelling for each enzyme-probe combination were assessed by SDS PAGE analysis as shown in SI Figs S7–S14. In order to investigate the importance of the linker length, a concentration study for the labelling process has been performed. The most efficient labelling was seen with probe **4** and *Pd*GH1, for which we observed bright fluorescent protein bands at concentrations as low as 50 μM and after incubating for 1 h, while 300 μM of probe **3** was required with an incubation time of 20 h (SI Figure S9–10, S13–S14). Somewhat similar results were seen for *Tm*GH1 for which fluorescent bands were visible after incubating with 50 μM of probe **4** for 5 hours or with 50 μM of probe **3** after 20 h (SI Figure S7–S8, S11–S12).

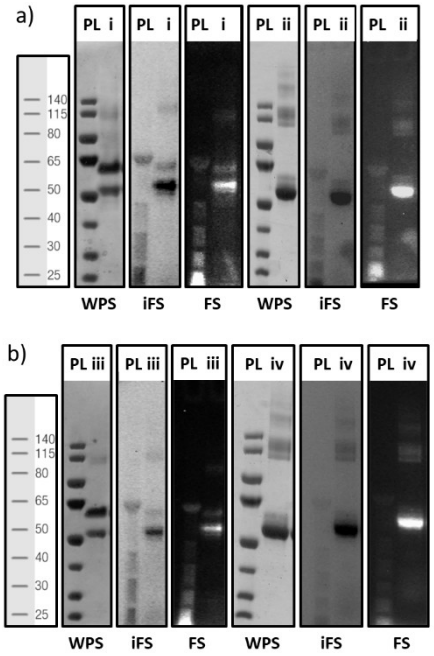


Figure 3. Labelling experiment with DNJ-based ligand-directed sulfonate ester probes (DNJ-LDSE). The enzyme was incubated with respective probes for 20 h at room temperature. The fluorophore was attached by using click chemistry followed by SDS PAGE analysis. a) Labelling of β-glucosidases by DNJ-LDSE probe **3**; PL (protein ladder); lane i (*Tm*GH1, 100 μM of **3**); lane ii (*Pd*GH1 400 μM of **3**). b) Labelling of β-glucosidases by DNJ-LDSE probe **4**; lane iii (*Tm*GH1, 100 μM of **4**); lane iv (*Pd*GH1, 100 μM of **4**). WPS = whole protein stain; iFS = inverted fluorescence signal; FS = fluorescence signal.

Table 1. K_i-values of ligand-directed probes **1**–**6** and parent iminosugars DNJ **14** and DIX **16** for β-glucosidases from almonds (*Prunus dulcis*, *Pd*GH1) and *Thermotoga maritima* (*Tm*GH1).

K_i-values [μM]		*Tm*GH1	*Pd*GH1
DNJ	**14**	6.8 ± 1.7	37.3 ± 4.0
DIX	**16**	18.7 ± 1.9	16.4 ± 1.0
DNJ-C_{11}–LDBE-N_3	**1**	0.98 ± 0.09	928 ± 102
DNJ-C_{14}–LDBE-N_3	**2**	5.0 ± 0.4	N.I.[a]
DNJ-C_6–LDSE-N_3	**3**	0.58 ± 0.03	1.9 ± 0.2
DNJ-C_{11}–LDSE-N_3	**4**	0.25 ± 0.02	12.5 ± 1.5
DIX-C_6–LDSE-N_3	**5**	17.4 ± 2.3	22.4 ± 1.9
DIX-C_{11}–LDSE-N_3	**6**	0.95 ± 0.11	139 ± 24

[a] N.I., no inhibition detected, due to solubility limits at 150 μM.

To investigate the importance of the enzyme specificity of the ligand we also synthesized and tested LDSE probes **5** and **6** with 1,5-dideoxy-1,5-imino-D-xylitol (DIX, **16**) as ligand (A). DIX **16** is another competitive inhibitor for β-glucosidases.[34–36] DIX-LDSE probes **5** and **6** showed lower affinity towards both model enzymes (Table 1). DIX-C_{11}–LDSE-N_3 probe **6** forms a fluorescent covalent complex with *Pd*GH1 after incubating for 20 h at 100 μM. However, no fluorescent protein bands were observed with *Tm*GH1 with either probe (SI Figure S15–16).

Incubating α-mannosidase from *Canavalia ensiformis* (*Ce*GH38) with probes **3**–**6** performing the same analysis gave no fluorescent protein bands for either probe (SI Figure S15–S16).

To verify that the labelling process is based on the ligand-enzyme interaction we performed a competitive labelling experiment. This was realised by pre-incubating the respective enzymes with conduritol B epoxide (CBE),[37,38] a known irreversible inhibitor, or isofagomine (IFG),[39,40] a more potent reversible inhibitor for glucoside hydrolases (K_i-values: *Tm*GH1 0.021 μM and *Pd*GH1 0.41 μM), followed by incubation with 100 μM of the actual LDC probes (details see experimental

section). Pre-incubating *Tm*GH1 with both IFG as well as CBE successfully prevents labelling of the enzyme with DNJ-C_6–LDSE-N_3 probe **3** (Figure 4).

These results verify that the labelling process is indeed largely dependent upon the ligand-enzyme interaction in the case of *Tm*GH1 with DNJ-C_6–LDSE-N_3 probe **3**. However, pre-incubation of *Tm*GH1 with CBE or IFG could not prevent labelling by DNJ-C_{11}–LDSE-N_3 probe **4**. This could be caused due to the longer linker of probe **4** compared to probe **3** and therefore reduced selectivity (SI Figure S18).

Finally, we tested the residual activity of the labelled enzyme. We incubated *Tm*GH1 with the most potent candidates DNJ-LDSE probes **3** and **4** for 20 h, removed remaining small molecules by washing, and measured Michaelis-Menten parameters for hydrolysis of *p*-nitrophenyl β-D-glucopyranoside. As shown in Figure 5 and Table 2, we observed significant activity of the enzyme (90% and 70% remaining activity in the best and worst case respectively) after labelling with similar kinetic parameters. These results highlight the applicability of ligand-directed chemistry for labelling carbohydrate processing enzymes where preserving activity is important.

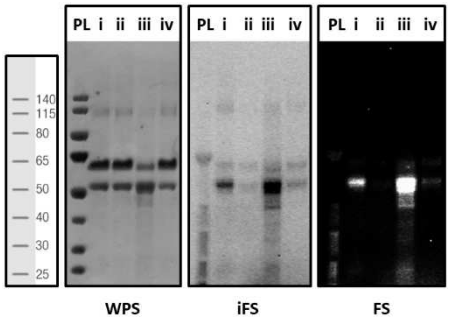


Figure 4. Competition labelling of *Tm*GH1 with DNJ-C_6–LDSE-N_3 probe **3**. WPS = whole protein stain; iFS = inverted fluorescence signal; FS = fluorescence signal; PL (protein ladder); lane i (10 min DMSO, 100 μM of **3**); lane ii (10 min 1000 μM IFG, 100 μM of **3**); lane iii (20 h DMSO, 100 μM of **3**); lane iv (20 h 1000 μM CBE, 100 μM of **3**).

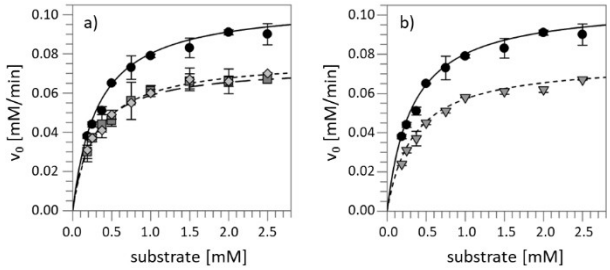


Figure 5. Determination of residual activity of covalently modified *Tm*GH1 after incubating with DNJ-LDSE probes. a) Control vs. DNJ-C_6–LDSE-N_3 probe **3**. ● control (incubation with 1v% DMSO for 20 h); ◇ probe **3** (100 μM for 20 h); ▫ probe **3** (400 μM for 20 h). b) Control vs. DNJ-C_{11}–LDSE-N_3 probe **4**, ● control (incubation with 1v% DMSO for 20 h);

Table 2. Kinetic parameters obtained in the determination of residual activity of *Tm*GH1 treated with DNJ-LDSE probes **3** and **4** for the hydrolysis of *p*-nitrophenyl β-D-glucopyranoside.

*Tm*GH1	k_{cat} [s^{-1}]	K_m [M]	k_{cat}/K_m [s^{-1} M^{-1}]
DMSO (Ref.)	0.78	3.20×10^{-4}	2.44×10^{3}
DNJ-C_6–LDSE-N_3 (3) 100 μM	0.56	2.57×10^{-4}	2.18×10^{3}
DNJ-C_6–LDSE-N_3 (3) 400 μM	0.59	3.00×10^{-4}	1.97×10^{3}
DNJ-C_{11}–LDSE-N_3 (4) 100 μM	0.58	3.36×10^{-4}	1.73×10^{3}

Conclusions

Herein, we designed a building block synthetic concept for iminosugar-based small molecule probes for covalent proximity labelling of glycoside hydrolases. In a proof of concept study synthesised probes **1**–**6** were biologically evaluated assessing the ligand-directed chemistry approach with two model β-glucosidases. Key to our approach was use of the sulfonate ester as the reactive, cleavable site for covalent labelling. Moreover, our results confirm that the linker length between the ligand and the electrophile plays a significant role in the labelling process. The DNJ-C_6–LDSE-N_3 probe **3** labels *Tm*GH1 at low concentrations, for labelling *Pd*GH1 higher concentrations are necessary. Hence, the length of the spacer can be used as another selectivity factor, when designing these probes for labelling specific enzymes in future applications.

Furthermore, we showed that the labelling process is driven by interaction of the ligand with the active site. This confirmed the fact that if the active site is blocked by a more potent reversible inhibitor (IFG) or an irreversible inhibitor (CBE), no labelling of *Tm*GH1 with DNJ-C_6–LDSE-N_3 probe **3** was observed. The results reveal that the choice of ligand and the linker length are crucial for labelling. Therefore, these modules must be tailored individually for a respective enzyme to optimize the probe.

Importantly, we demonstrated that catalytic activity is maintained after the labelling process. Taken together, our results show the applicability of the LDC approach for labelling carbohydrate processing enzymes where preserving activity is important.

Having achieved this proof of concept we are now ready to transfer the method of LDC labelling onto several glycoside hydrolases, such as human lysosomal enzymes. In principle, the LDC labelling can be transferable to α- and β-glycosidases, as well as retaining and inverting or *exo*- and *endo*- glycoside hydrolases with a respective probe design tailored for the enzymes of interest.

Experimental Section

General methods

Optical rotations were measured at 20 °C on a Schmidt Haensch variPol C polarimeter at 589 nm with a path length of 5 cm. NMR spectra were recorded on a Bruker Ultrashield spectrometer at 300.36 MHz (1H) and 75.53 MHz (13 C), respectively. $CDCl_3$ was employed for protected compounds and CD_3OD for unprotected inhibitors. Chemical shifts are listed in δ employing residual, non-

deuterated solvent or residual H_2O (CD_3OD) as the internal standard.[41] $CDCl_3$: 7.26 ppm (^1H), 77.16 ppm (^{13}C); CD_3OD: 4.87 ppm (^1H), 49.0 ppm (^{13}C). Signals were unambiguously assigned by COSY (correlation spectroscopy) and HSQC (heteronuclear single-quantum correlation spectroscopy) analysis. The signals of the aromatic groups are located in the expected regions and are not listed explicitly. MALDI-TOF was performed on a Micromass TofSpec 2E Time-of-Flight mass spectrometer. All reactions were monitored by thin-layer chromatography (TLC) performed on pre-coated aluminium plates silica gel 60 F254 and detected with UV light (254 nm). For staining, a solution of vanillin (9 g) in a mixture of H_2O/EtOH/H_2SO_4 (950 mL/750 mL/120 mL) or ceric ammonium molybdate ($(NH_4)_6Mo_7O_{24}\cdot 4\ H_2O$ (100 g)/$Ce(SO_4)_2\cdot 4\ H_2O$ (8 g) in 10% H_2SO_4 (1 L)) were employed, followed by heating on a hotplate.

Purification of the desired products by column chromatography was performed with the stated solvent systems on silica gel 60 (Acros Organics or Macherey-Nagel). Selected products were purified by flash chromatography using a Biotage® Selekt system and prepacked silica gel 20 columns.

General Procedure A: Mitsunobu reaction

A 10% solution of the respective starting material (1.0 equiv.) in THF, Ph_3P (1.0–1.1 equiv.), diisopropyl azodicarboxylate (DIAD, 1.0–1.1 equiv.) and the respective alcohol (1.0–1.1 equiv.) was stirred until complete conversion of the reactants was detected by TLC. Subsequently, the reaction mixture was diluted with CH_2Cl_2 and washed consecutively with aqueous HCl (2 *N*) and saturated $NaHCO_3$. After drying over Na_2SO_4, the filtrate was concentrated in vacuo to provide the corresponding crude product.

General Procedure B: Reductive amination employing $NaBH_3CN$

A 20% solution of the respective aldehyde (1.0 equiv.) and iminosugar (1.0–1.2 equiv.) in MeOH (containing a catalytic amount of AcOH) was stirred for 15 min before $NaBH_3CN$ (1.5–3.0 equiv.) was added. After complete conversion of the starting materials was detected by TLC, the reaction mixture was concentrated under reduced pressure to provide the corresponding crude title compound.

11-Bromoundecyl 4-((tetrahydro-2H-pyran-2-yl)oxy)benzoate (18)

Following general procedure A, compound **17**[42] (895 mg, 4.03 mmol) was treated with 11-bromoundecan-1-ol (1.01 g, 4.03 mmol), Ph_3P (1.06 g, 4.03 mmol) and DIAD (791 μL, 4.03 mmol). Purification on silica gel (cyclohexane/EtOAc 20:1, v/v) provided compound **18** (1.40 g, 3.07 mmol, 76.3%, $R_f = 0.7$, cyclohexane/EtOAc 3:1, v/v) as colourless syrup.

^{1}H-NMR (300 MHz, $CDCl_3$) δ = 7.97 (d, 2 H, Ar), 7.06 (d, 2 H, Ar), 5.49 (dd, 1 H, THP), 4.27 (t, 2 H, H-1′), 3.85 (m, 1 H, THP), 3.61 (m, 1 H, THP), 3.39 (t, 2 H, H-11′), 2.05–1.60 (po, 10 H, H-2′, H-10′, 3x THP), 1.45–1.25 (po, 14 H, H-3′, H-4′, H-5′, H-6′, H-7′, H-8′, H-9′).

^{13}C-NMR (75.5 MHz, $CDCl_3$) δ = 166.5 (OC=O), 160.9 (ipso), 131.5 (Ar), 123.8 (ipso), 115.9 (Ar), 96.2 (THP), 64.9 (C-1′), 62.1 (THP), 34.1 (C-11′), 32.9, 30.2, 29.6, 29.5, 29.5, 29.4, 28.9, 28.9, 28.3, 26.1, 25.2 (C-2′, C-3′, C-4′, C-5′, C-6′, C-7′, C-8′, C-9′, C-10′, 2x THP), 18.7 (THP).

MS (MALDI): Calculated for $[C_{23}H_{35}BrO_4Na]$: m/z $[M+Na]^+$477.1617; found $[M+Na]^+$477.1621.

11-Bromoundecyl 4-hydroxybenzoate (19)

A solution of **18** (1.22 g, 2.68 mmol) in dioxane/H_2O (40 mL, 3:1, v/v) was treated with acidic ion exchange resin (Amberlite® IR-120H$^+$) at 45 °C. After TLC confirmed complete conversion of the starting material (12 h), the reaction mixture was filtered off, and the solvents were removed under reduced pressure. Purification on silica gel (cyclohexane/EtOAc 10:1, v/v) provided compound **19** (909 mg, 2.45 mmol, 91.3%, $R_f = 0.3$, cyclohexane/EtOAc 3:1) as colourless solid.

^{1}H-NMR (300 MHz, $CDCl_3$) δ = 7.94 (d, 2 H, Ar), 7.14 (s, 1 H, OH), 6.90 (d, 2 H, Ar), 4.29 (t, 2 H, H-1′), 3.39 (t, 2 H, H-11′), 1.87–1.70 (po, 4 H, H-2′, H-10′), 1.45–1.25 (po, 14 H, H-3′, H-4′, H-5′, H-6′, H-7′, H-8′, H-9′).

^{13}C-NMR (75.5 MHz, $CDCl_3$) δ = 167.4 (OC=O), 160.7 (ipso), 132.0 (Ar), 122.4 (ipso), 115.4 (Ar), 65.3 (C-1′), 34.2 (C-11′), 32.9 (C-10′), 29.6, 29.5, 29.5, 29.3, 28.9, 28.8, 28.3, 26.1 (C-2′, C-3′, C-4′, C-5′, C-6′, C-7′, C-8′, C-9′).

MS (MALDI): Calculated for $[C_{18}H_{27}BrO_3Na]$: m/z $[M+Na]^+$393.1041; found $[M+Na]^+$393.1040.

11-Bromoundecyl 4-((6-azidohexyl)oxy)benzoate (20)

Following general procedure A, compound **19** (426 mg, 1.15 mmol) was reacted with 6-azidohexan-1-ol[43] (164 mg, 1.15 mmol), Ph_3P (302 mg, 1.15 mmol) and DIAD (226 μL, 1.15 mmol). Silica gel chromatography (cyclohexane/EtOAc 20:1, v/v) provided structure **20** (530 mg, 1.07 mmol, 92.8%, $R_f = 0.7$, cyclohexane/EtOAc 3:1) as colourless wax.

^{1}H-NMR (300 MHz, $CDCl_3$) δ = 7.98 (d, 2 H, Ar), 6.89 (d, 2 H, Ar), 4.27 (t, 2 H, H-1′), 4.01 (t, 2 H, H-1′′), 3.40 (t, 2 H, H-11′), 3.28 (t, 2 H, H-6′′), 1.88–1.71 (po, 6 H, H-2′′, H-2′, H-10′), 1.63 (m, 2 H, H-5′′), 1.52–1.26 (po, 18 H, H-3′′, H-4′′, H-3′, H-4′, H-5′, H-6′, H-7′, H-8′, H-9′).

^{13}C-NMR (75.5 MHz, $CDCl_3$) δ = 166.6 (OC=O), 162.9 (ipso), 131.7 (Ar), 123.0 (ipso), 114.1 (Ar), 68.0 (C-1′′), 64.9 (C-1′), 51.5 (C-6′′), 34.2 (C-11′), 33.0 (C-10′), 29.6, 29.6, 29.5, 29.4, 29.1, 28.9, 28.9, 28.3, 26.6, 26.2, 25.8 (C-2′′, C-3′′, C-4′′, C-5′′, C-2′, C-3′, C-4′, C-5′, C-6′, C-7′, C-8′, C-9′).

MS (MALDI): Calculated for $[C_{24}H_{38}BrN_3O_3Na]$: m/z $[M+Na]^+$ 518.1994; found $[M+Na]^+$518.1993.

11-Oxoundecyl 4-((6-azidohexyl)oxy)benzoate (21)

A 10% solution of compound **20** (682 mg, 1.37 mmol) in DMSO was treated with $NaHCO_3$ (462 mg, 5.49 mmol). The reaction mixture was stirred at 120 °C until complete conversion of the starting material was detected by TLC (4 h). After cooling the reaction mixture to room temperature, the mixture was diluted with CH_2Cl_2 and washed with H_2O. The combined organic phases were dried over Na_2SO_4, filtered off, and concentrated under reduced pressure. Purification on silica gel (cyclohexane/EtOAc 12:1, v/v) gave compound **21** (270 mg, 0.63 mmol, 45.7%, $R_f = 0.6$, cyclohexane/EtOAc 3:1, v/v) as colourless syrup.

^{1}H-NMR (300 MHz, $CDCl_3$) δ = 9.74 (s, 1 H, HC=O), 7.97 (d, 2 H, Ar), 6.88 (d, 2 H, Ar), 4.26 (t, 2 H, H-1′), 4.00 (t, 2 H, H-1′′), 3.27 (t, 2 H, H-6′′), 2.40 (m, 2 H, H-10′), 1.85–1.70 (po, 4 H, H-2′′, H-2′), 1.67–1.56 (po, 4 H, H-5′′, H-9′), 1.51–1.26 (po, 16 H, H-3′′, H-4′′, H-3′, H-4′, H-5′, H-6′, H-7′, H-8′).

^{13}C-NMR (75.5 MHz, CDCl3) δ = 203.0 (HC=O), 166.5 (OC=O), 162.9 (ipso), 131.6 (Ar), 122.9 (ipso), 114.1 (Ar), 68.0 (C-1′′), 64.9 (C-1′), 51.5

(C-6″), 44.0 (C-10′), 29.5, 29.4, 29.3, 29.2, 29.1, 28.9, 26.6, 26.1, 25.7, 22.2 (C-2″, C-3″, C-4″, C-5″, C-2′, C-3′, C-4′, C-5′, C-6′, C-7′, C-8′, C-9′).

MS (MALDI): Calculated for $[C_{24}H_{37}N_3O_4Na]$: m/z $[M+Na]^+$454.2682; found $[M+Na]^+$454.2681.

N-(11-((4-((6-Azidohexyl)oxy)benzoyl)oxy)undecyl)-1,5-dideoxy-1,5-imino-D–glucitol (DNJ-C_{11}–LDBE 1)

Following general procedure B, aldehyde **21** (107 mg, 0.25 mmol) was treated with iminosugar **14**[32] (40.3 mg, 0.25 mmol) and $NaBH_3CN$ (23.3 mg, 0.37 mmol). Purification on silica gel (EtOAc/MeOH 10:1, v/v) provided title compound 1 (83.4 mg, 0.14 mmol, 58.3%, R_f = 0.3, EtOAc/MeOH 3:1, v/v) as colourless syrup.

^{1}H-NMR (300 MHz, CD_3OD) δ = 7.94 (d, 2 H, Ar), 6.96 (d, 2 H Ar), 4.26 (t, 2 H, H-11′), 4.04 (t, 2 H, H-1″), 3.85 (m, 2 H, H-6a, H-6b), 3.48 (ddd, 1 H, $J_{1a,2}$ 4.8 Hz, $J_{1b,2}$ 10.2 Hz, H-2), 3.37 (dd, 1 H, $J_{4,5}$ 9.2 Hz, H-4), 3.28 (po, 2 H, H-6″), 3.14 (dd, 1 H, $J_{2,3}=J_{3,4}$ 9.0 Hz, H-3), 3.01 (dd, 1 H, $J_{1a,1b}$ 11.1 Hz, H-1a), 2.82 (m, 1 H, H-1′), 2.61 (m, 1 H, H-1′) 2.20 (po, 2 H, H-1b, H-5), 1.86–1.70 (po, 4 H, H-10′, H-2″), 1.62 (m, 2 H, H-5″), 1.53–1.26 (po, 20 H, H-2′, H-3′, H-4′, H-5′,H-6′, H-7′, H-8′, H-9′, H-3″, H-4″).

^{13}C-NMR (75.5 MHz, CD_3OD) δ = 168.0 (OC=O), 164.6 (ipso), 132.5 (Ar), 123.6 (ipso), 115.3 (Ar), 80.4 (C-3), 71.8 (C-4), 70.5 (C-2), 69.2 (C-1″), 67.4 (C-5), 65.9 (C-11′), 59.2 (C-6), 57.5 (C-1), 53.8 (C-1′), 52.4 (C-6″), 30.7, 30.6, 30.4, 30.1, 29,8, 28.6, 27.6, 27.1, 26.7, 25.2 (C-2′, C-3′, C-4′, C-5′, C-6′, C-7′, C-8′, C-9′, C-10′, C-2″, C-3″, C-4″, C-5″).

MS (MALDI): Calculated for $[C_{30}H_{50}N_4O_7Na]$: m/z $[M+Na]^+$601.3577; found $[M+Na]^+$601.3581. $[\alpha]_D^{20}$: −7.2 (c = 1.04, MeOH).

Methyl 4-(6-azidohexyloxy) benzoate 23

Following general procedure A, *p*-hydroxybenzoic acid methyl ester **22** (1.40 g, 9.20 mmol) was treated with 6-azidohexan-1-ol[43] (1.19 g, 8.31 mmol), Ph_3P (2.42 g, 9.22 mmol), and DIAD (1.9 mL, 9.22 mmol). Purification on silica gel (cyclohexane/EtOAc 25:1, v/v) provided compound **23** (1.01 g, 3.64 mmol, 43.8%, R_f = 0.7, cyclohexane/EtOAc 2:1, v/v) as colourless oily liquid.

^{1}H-NMR (300 MHz, $CDCl_3$) δ = 7.97 (d, 2 H, Ar), 6.89 (d, 2 H, Ar), 4.00 (t, 2 H, H-1″), 3.87 (s, 3 H, CH3–H-1′), 3.28 (t, 2 H, H-6″), 1.80 (m, 2 H, H-2″), 1.63 (m, 2 H, H-5″), 1.46 (m, 4 H, H-3″, H-4″).

^{13}C-NMR (75.5 MHz, $CDCl_3$) δ = 167.0 (OC=O), 163.0 (ipso), 131.7 (Ar), 122.6 (ipso), 114.2 (Ar), 68.0 (C-1″), 51.9 (C-1′), 51.5 (C-6″), 29.1 (C-2″), 28.9 (C-5″), 26.6, 25.7 (C-3″, C-4″).

4-(6-Azidohexyloxy) benzoic acid (24)

To methyl benzoate **23** (1.01 g, 3.64 mmol) in dioxane/H_2O (40 mL, 1:1 v/v), NaOH (3 *N*) was added to adjust pH 10. The reaction mixture was stirred until TLC confirmed complete consumption of the starting material (20 h). The reaction was carefully acidified by addition of HCl (2 *N*) causing the precipitation of **24**. After filtration, the precipitate was dissolved in EtOAc and washed with H_2O. The combined organic phases were dried over Na_2SO_4, filtered off, and the solvents were removed under reduced pressure providing product **24** (0.93 g, 3.53 mmol, 98.5%, R_f = 0.2, cyclohexane/EtOAc 2:1, v/v) as colourless powder.

^{1}H-NMR (300 MHz, CD_3OD) δ = 7.94 (d, 2 H, Ar), 6.94 (d, 2 H, Ar), 4.03 (t, 2 H, H-1″), 3.30 (m, 2 H, H-6″), 1.78 (m, 2 H, H-2″), 1.61 (m, 2 H, H-5″), 1.48 (m, 4 H, H-3″, H-4″).

^{13}C-NMR (75.5 MHz, CD_3OD) δ = 169.8 (OC=O), 164.5 (ipso), 132.8 (Ar), 123.9 (ipso), 115.2 (Ar), 69.1 (C-1″), 52.4 (1 C, C-6″), 30.1 (C-2″), 29.9 (C-5″), 27.6, 26.7 (C-3″, C-4″).

14-Bromotetradecyl 4-(6-azidohexyloxy)-benzoate (25)

Following general procedure A, benzoic acid **24** (181.0 mg, 0.69 mmol) was treated with Ph_3P (184 mg, 0.70 mmol), DIAD (0.15 mL, 0.76 mmol) and 14-bromotetradecan-1-ol[44] (188.7 mg, 0.68 mmol). Flash chromatography employing a Biotage® Selekt system and prepacked silica gel 20 columns provided compound **25** (249 mg, 0.46 mmol, 70.2%, R_f = 0.8, cyclohexane/EtOAc 2:1) as colourless solid.

^{1}H-NMR (300 MHz, $CDCl_3$) δ = 7.97 (d, 2 H, Ar), 6.89 (d, 2 H, Ar), 4.26 (t, 2 H, H-14′), 4.00 (t, 2 H, H-1″), 3.39 (t, 2 H, H-1′), 3.27 (t, 2 H, H-6″), 1.83 (bm, 4 H, H-2′, H-2″), 1.74 (bm, 2 H, H-13′), 1.63 (bm, 2 H, H-5″), 1.48–1.40 (bm, 4 H, H-3″, H-4″), 1.35–1.20 (bm, 20 H, H-3′; H-4′, H-5′, H-6′, H-7′, H-8′, H-9′, H-10′, H-11′, H-12′).

^{13}C-NMR (75.5 MHz, $CDCl_3$) δ = 166.5 (OC=O), 162.9 (ipso), 131.6 (Ar), 122.9 (ipso), 114.1 (Ar), 68.0, (C-1″), 64.9 (C-14′), 51.5 (C-6″), 34.1 (C-1′), 32.9, 29.7, 29.6, 29.5, 29.4, 29.1, 28.9, 28.3, 26.6, 26.2, 25.7 (C-2′, C-3′, C-4′, C-5′, C-6′, C-7′, C-8′, C-9′, C-10′, C-11′, C-12′, C-13′, C-2″, C-3″, C-4″, C-5″).

MS (MALDI): Calculated for $[C_{27}H_{44}N_3O_3BrNa]$: m/z $[M+Na]^+$ 560.2464; found $[M+Na]^+$560.2465.

N-(4-Azidohexyloxy)phenylcarbonyloxytetradecyl)-1,5-dideoxy-1,5-imino-D–glucitol (DNJ-C_{14}–LDBE 2)

To a solution of iminosugar 1-deoxynojirimycin[32] **14** (22.1 mg, 0.14 mmol) in DMF (5 mL), benzoate **25** (77.5 mg, 0.15 mmol) dissolved in DMF (2 mL) and $NaHCO_3$ (33.2 mg, 0.40 mmol) were added and the reaction mixture was heated to 65 °C. After TLC confirmed complete consumption of starting material 25 (2 d), the reaction mixture was concentrated under reduced pressure. Purification on silica gel (EtOAc/MeOH 25:1, v/v) provided DNJ–C14–LDBE probe **2** (14 mg, 23 μmol, 16.6%, R_f = 0.6, $CHCl_3$/MeOH 3:1 + 1vol% NH_4OH (25%), v/v) as colourless solid.

^{1}H-NMR (300 MHz, CD_3OD) δ = 7.95 (d, 2 H, Ar), 6.97 (d, 2 H, Ar), 4.27 (t, 2 H, H-14′), 4.06 (t, 2 H, H-1″), 3.90–3.79 (m, 2 H, H-6a, H-6b), 3.48 (ddd, 1 H, $J_{1a,2}$ 4.9 Hz, $J_{1b,2}$ 10.4 Hz, H-2), 3.37–3.24 (m, 3 H, H-4, H-6″), 3.14 (dd, 1 H, $J_{2,3}=J_{3,4}$ 9.1 Hz, H-3), 3.01 (dd, 1 H, $J_{1a,1b}$ 11.2 Hz, H-1a), 2.90–2.73 (m, 1 H, H-1′), 2.68–2.53 (m, 1 H, H-1′), 2.22 (po, 2 H, H-1b, H-5), 1.88–1.70 (po, 4 H, H-2″, H-13′), 1.69–1.57 (m, 2 H, H-5″), 1.55–1.40, 1.38–1.22 (po, 26 H, H-2′, H-3′, H-4′, H-5′, H-6′, H-7′, H-8′, H-9′, H-10′, H-11′, H-12′, H-3″; H-4″).

^{13}C-NMR (75.5 MHz, CD_3OD) δ = 168.1 (OC=O), 164.6, 132.5, 123.7, 115.3 (Ar), 80.4 (C-3), 71.9 (C-4), 70.6 (C-2), 67.4 (C-5), 69.2, 65.9 (C-1″, C-14′), 59.2 (C-6), 57.5 (C-1), 53.8 (C-1′), 52.4 (C-6″), 30.7, 30.6, 30.3, 30.1, 29.9, 29.8, 28.6, 27.6, 27.1, 26.7, 25.2 (C-2′ C-3′, C-4′, C-5′, C-6′, C-7′, C-8′, C-9′, C-10′, C-11′, C-12′, C-13′, C-2″, C-3″, C-4″, C-5″).

MS (MALDI): Calculated for $[C_{33}H_{56}O_7N_4H]$: m/z 621.4227 $[M+H]^+$; found $[M+H]^+$621.4224. $[\alpha]_D^{20}$: −3.6 (c = 0.90, MeOH).

3-(((6-Azidohexyl)oxy)sulfonyl)benzoic acid (8)

To an ice-cooled solution of commercially available 3-(chlorosulfonyl)benzoic acid **7** (646 mg, 2.93 mmol) in dry CH_2Cl_2 (20 mL), 6-azidohexan-1-ol[43] (482 mg, 3.37 mmol, dissolved in 5 mL dry CH_2Cl_2) and pyridine (2.40 mL, 29.3 mmol) were added. After complete conversion of the starting material was detected by TLC

(25 min), the reaction mixture was washed with HCl (2 *N*). The combined organic phases were dried over Na_2SO_4, filtered off, and the solvent was removed under reduced pressure. Purification on silica gel (cyclohexane/EtOAc 5:1, v/v) provided structure **8** (516 mg, 1.58 mmol, 53.8%, $R_f = 0.3$, cyclohexane/EtOAc 1:1, v/v) as colourless wax.

^{1}H-NMR (300 MHz, $CDCl_3$) $\delta = 8.96$ (bs, 1 H, COOH), 8.64 (s, 1 H, Ar), 8.39 (d, 1 H, Ar), 8.16 (d, 1 H, Ar), 7.72 (t, 1 H, Ar), 4.12 (t, 2 H, H-1''), 3.24 (t, 2 H, H-6''), 1.70 (m, 2 H, H-2''), 1.55 (m, 2 H, H-5''), 1.42–1.30 (po, 4 H, H-3'', H-4'').

^{13}C-NMR (75.5 MHz, $CDCl_3$) $\delta = 170{,}0$ (COOH), 137.4 (ipso), 135.2 (Ar), 132.7 (Ar), 130.8 (ipso), 129.9, 129.6 (Ar), 71.2 (C-1''), 51.3 (C-6''), 28.8, 28.7 (C-2'', C-5''), 26.2, 25.1 (C-3'', C-4'').

MS (MALDI): Calculated for $[C_{13}H_{16}N_3NaO_5SNa]$: m/z $[M+Na]^+$ 372.0606; found $[M+Na]^+$372.0608. The structure was found as sodium carboxylate.

6-Azidohexyl 3-((6-hydroxyhexyl)carbamoyl)benzenesulfonate (9)

Carboxylic acid **8** (240 mg, 0.73 mmol) dissolved in CH_2Cl_2 (20 mL) was first activated with isobutyl chloroformate (ClCOO*i*-Bu, 105 μL, 0.81 mmol) and diisopropylethylamine (DIPEA, 140 μL, 0.81 mmol) at 0 °C. After 10 min, 6-amino-1-hexanol (85.8 mg, 0.73 mmol) and DIPEA (140 μL, 0.81 mmol) were added and the reaction mixture was stirred until complete conversion of the reactants was detected by TLC (35 min at 0 °C). After washing with aqueous HCl (2 *N*) and saturated $NaHCO_3$ consecutively, combined organic layers were dried over Na_2SO_4, filtered off, and concentrated under reduced pressure. Purification on silica gel (cyclohexane/EtOAc 2:1, v/v) provided compound **9** (248 mg, 0.58 mmol, 79.4%, $R_f = 0.4$, cyclohexane/EtOAc 1:3, v/v) as colourless syrup.

^{1}H-NMR (300 MHz, $CDCl_3$) $\delta = 8.22$ (s, 1 H, Ar), 8.11 (d, 1 H, Ar), 8.02 (d, 1 H, Ar), 7.65 (t, 1 H, Ar), 6.35 (s, 1 H, NH), 4.08 (t, 2 H, H-1''), 3.66 (t, 2 H, H-1'), 3.48 (m, 2 H, H-6'), 3.24 (t, 2 H, H-6''), 1.70–1.55 (po, 8 H, H-2', H-5', H-2'', H-5''), 1.47–1.31 (po, 8 H, H-3', H-4', H-3'', H-4'').

^{13}C-NMR (75.5 MHz, $CDCl_3$) $\delta = 165{,}6$ (NC=O), 136.9, 136.3 (ipso), 132.8, 130.4, 130.0, 126.0 (Ar), 71.2 (C-1''), 62.8 (C-1'), 51.4 (C-6''), 40.3 (C-6'), 32.5, 29.6, 28.9, 28.7 (C-2', C-5', C-2'', C-5''), 26.7, 26.2, 25.4, 25.1 (C-3', C-4', C-3'', C-4'').

MS (MALDI): Calculated for $[C_{19}H_{30}N_4O_5SNa]$: m/z $[M+Na]^+$49.1835; found $[M+Na]^+$49.1837.

6-Azidohexyl 3-((11-hydroxyundecyl)carbamoyl)benzenesulfonate (10)

Carboxylic acid **8** (87.4 mg, 0.27 mmol) dissolved in CH_2Cl_2/DMF (1:1, v/v) was first activated with ClCOO*i*-Bu (38.2 μL, 0.29 mmol) and DIPEA (50.0 μL, 0.29 mmol) at 0 °C. After 10 min, 11-amino-1-undecanol[45] (50.0 mg, 0.27 mmol) and DIPEA (50.0 μL, 0.29 mmol) were added and the reaction mixture was allowed to reach room temperature and stirred until complete conversion of the reactants was detected by TLC (3 h). After washing with aqueous HCl (2 *N*) and saturated $NaHCO_3$ consecutively, combined organic layers were dried over Na_2SO_4, filtered off, and the solvent removed under reduced pressure. Silica gel chromatography (cyclohexane/EtOAc 5:1, v/v) gave compound **10** (105.7 mg, 0.21 mmol, 79.7%, $R_f = 0.3$, cyclohexane/EtOAc 1:1, v/v) as white solid.

^{1}H-NMR (300 MHz, $CDCl_3$) $\delta = 8.23$ (s, 1 H, Ar), 8.07 (d, 1 H, Ar), 7.94 (d, 1 H, Ar), 7.58 (t, 1 H, Ar), 6.90 (s, 1 H, NH), 4.01 (t, 2 H, H-1''), 3.57 (t, 2 H, H-11'), 3.39 (m, 2 H, H-1'), 3.18 (t, 2 H, H-6''), 2.31 (s, 1 H, OH), 1.63–1.46 (po, 8 H, H-2', H-10', H-2'', H-5''), 1.33–1.20 (po, 18 H, H-3', H-4', H-5', H-6', H-7', H-8', H-9', H-3'', H-4'').

^{13}C-NMR (75.5 MHz, $CDCl_3$) $\delta = 165{,}5$ (NC=O), 136.5, 136.2 (ipso), 132.6, 130.1, 129.7, 126.1 (Ar), 71.1 (C-1''), 62.9 (C-1'), 51.2 (C-6''), 40.5 (C-11'), 32.7, 29.5, 29.5, 29.5, 29.4, 29.3, 28.7, 28.6, 27.0, 26.1, 25.7, 24.9 (C-2', C-3', C-4', C-5', C-6', C-7', C-8', C-9', C-10', C-2'', C-3'', C-4'', C-5'').

MS (MALDI): Calculated for $[C_{24}H_{40}N_4O_5SNa]$: m/z $[M+Na]^+$ 519.2617; found $[M+Na]^+$519.2617.

6-Azidohexyl 3-((6-oxohexyl)carbamoyl)benzenesulfonate (11)

A 10% solution of alcohol **9** (160 mg, 0.37 mmol) in CH_2Cl_2 was reacted with Dess-Martin periodinane (175 mg, 0.41 mmol). After TLC confirmed complete conversion of the starting material (20 min), the reaction mixture was carefully quenched with saturated $NaHCO_3$. After separation, the organic layer was dried over Na_2SO_4, filtered off, and the solvent was removed under reduced pressure. Silica gel chromatography (cyclohexane/EtOAc 3:1, v/v) gave compound **11** (127 mg, 0.30 mmol, 79.7%, $R_f = 0.65$, cyclohexane/EtOAc 1:3, v/v) as a colourless syrup.

^{1}H-NMR (300 MHz, $CDCl_3$) $\delta = 9.74$ (s, 1 H, HC=O), 8.25 (s, 1 H, Ar), 8.10 (d, 1 H, Ar), 7.98 (d, 1 H, Ar), 7.62 (t, 1 H, Ar), 6.73 (s, 1 H, NH), 4.04 (t, 2 H, H-1''), 3.45 (m, 2 H, H-6'), 3.21 (t, 2 H, H-6''), 2.45 (m, 2 H, H-2'), 1.67–1.49 (po, 8 H, H-3', H-5', H-2'', H-5''), 1.43–1.28 (po, 6 H, H-4', H-3'', H-4'').

^{13}C-NMR (75.5 MHz, $CDCl_3$) $\delta = 202.7$ (HC=O), 165,6 (NC=O), 136.7, 136.2 (ipso), 132.7, 130.3, 129.8, 126.1 (Ar), 71.2 (C-1''), 51.3 (C-6''), 43.7 (C-5'), 40.1 (C-1'), 29.3, 28.8, 28.7, 21.6 (C-3', C-5', C-2'', C-5''), 26.4, 26.1, 25.0 (C-4', C-3'', C-4'').

MS (MALDI): Calculated for $[C_{19}H_{28}N_4O_5SNa]$: m/z $[M+Na]^+$47.1678; found $[M+Na]^+$47.1678.

6-Azidohexyl 3-((11-oxoundecyl)carbamoyl)benzenesulfonate (12)

Alcohol **10** (88.7 mg, 0.18 mmol) in CH_2Cl_2 (8 mL) was reacted with Dess-Martin periodinane (91.0 mg, 0.21 mmol). After complete conversion of the starting material was confirmed by TLC (20 min), the reaction was quenched with saturated $NaHCO_3$. The combined organic phases were dried over Na_2SO_4, filtered off, and concentrated under reduced pressure. Purification on silica gel (cyclohexane/EtOAc 6:1, v/v) provided compound **12** (71.8 mg, 0.15 mmol, 81.1%, $R_f = 0.50$, cyclohexane/EtOAc 1:1, v/v) as colourless wax.

^{1}H-NMR (300 MHz, $CDCl_3$) $\delta = 9.76$ (s, 1 H, HC=O), 8.22 (s, 1 H, Ar), 8.10 (d, 1 H, Ar), 8.02 (d, 1 H, Ar), 7.65 (t, 1 H, Ar), 6.23 (s, 1 H, NH), 4.08 (t, 2 H, H-1''), 3.47 (m, 2 H, H-11'), 3.24 (t, 2 H, H-6''), 2.42 (m, 2 H, H-2'), 1.70–1.54 (po, 8 H, H-3', H-10', H-2'', H-5''), 1.42–1.25 (po, 16 H, H-4', H-5', H-6', H-7', H-8', H-9', H-3'', H-4'').

^{13}C-NMR (75.5 MHz, $CDCl_3$) $\delta = 203.1$ (HC=O), 165,5 (NC=O), 136.9, 136.4 (ipso), 132.7, 130.4, 129.9, 126.0 (Ar), 71.2 (C-1''), 51.4 (C-6''), 44.0 (C-2'), 40.6 (C-11'), 29.7, 29.5, 29.4, 29.3, 29.2, 28.9, 28.8, 27.1, 26.2, 25.1, 22.2 (C-3', C-4', C-5', C-6', C-7', C-8', C-9', C-10', C-2'', C-3'', C-4'', C-5'').

MS (MALDI): Calculated for $[C_{24}H_{38}N_4O_5SNa]$: m/z $[M+Na]^+$ 517.2460; found $[M+Na]^+$517.2460.

N-(6-(3-(((6-Azidohexyl)oxy)sulfonyl)benzamido)hexyl)-1,5-dideoxy-1,5-imino-D–glucitol (DNJ-C_6–LDSE probe 3)

Following general procedure B, aldehyde **11** (129 mg, 0.30 mmol) was reacted with iminosugar **14**[32] (49.6 mg, 0.30 mmol) and $NaBH_3CN$ (28.7 mg, 0.46 mmol) Purification on silica gel (CH_2Cl_2/MeOH 10:1, v/v) gave title compound **3** (72.0 mg, 0.13 mmol, 41.4%, R_f = 0.4, CH_2Cl_2/MeOH 3:1, v/v) as colourless wax.

^{1}H-NMR (300 MHz, CD_3OD) δ = 8.36 (s, 1 H, Ar), 8.18 (d, 1 H, Ar), 8.08 (d, 1 H, Ar), 7.76 (t, 1 H, Ar), 4.12 (t, 2 H, H-1''), 3.88 (m, 2 H, H-6a, H-6b), 3.51 (ddd, 1 H, $J_{1b,2}$ 10.3 Hz, H-2), 3.45–3.36 (po, 3 H, H-4, H-6'), 3.25 (t, 2 H, H-6''), 3.17 (dd, 1 H, $J_{2,3} = J_{3,4}$ 9.0 Hz, H-3), 3.07 (dd, 1 H, $J_{1a,1b}$ 11.2 Hz, $J_{1a,2}$ 4.6 Hz, H-1a), 2.90 (m, 1 H, H-1'), 2.68 (m, 1 H, H-1'), 2.28 (po, 2 H, H-1b, H-5), 1.72–1.62 (po, 4 H, H-5', H-2''), 1.60–1.50 (po, 4 H, H-2', H-5''), 1.46–1.30 (po, 8 H, H-3', H-4', H-3'', H-4'').

^{13}C-NMR (75.5 MHz, CD_3OD) δ = 167.8 (NC=O), 138.3, 137.3 (ipso), 133.5, 131.5, 131.0, 127.7 (Ar), 80.2 (C-3), 72.5 (C-1''), 71.6 (C-4), 70.3 (C-2), 67.4 (C-5), 58.9 (C-6), 57.3 (C-1), 53.8 (C-1'), 52.3 (C-6''), 41.1 (C-6'), 30.3, 29.7, 29.7, 28.1, 27.9, 27.1, 26.0, 25.1 (C-2', C-3', C-4', C-5', C-2'', C-3'', C-4'', C-5'').

MS (MALDI): Calculated for $[C_{25}H_{41}N_5O_8SH]$: m/z $[M+H]^+$572.2754; found $[M+H]^+$572.2752. $[\alpha]_D^{20}$: −7.6 (c = 0.86, MeOH).

N-(11-(3-(((6-Azidohexyl)oxy)sulfonyl)benzamido)undecyl)-1,5-dideoxy-1,5-imino-D–glucitol (DNJ-C_{11}–LDSE-probe 4)

Following general procedure B, aldehyde **12** (84.3 mg, 0.17 mmol) was reacted with iminosugar **14**[32] (27.8 mg, 0.17 mmol) and $NaBH_3CN$ (16.0 mg, 0.26 mmol). After TLC indicated complete consumption of the starting materials (7 h), the reaction mixture was concentrated under reduced pressure. Purification on silica gel (EtOAc/MeOH 12:1, v/v) gave probe **4** (56.0 mg, 87.3 μmol, 51.3%, R_f = 0.3, EtOAc/MeOH 3:1, v/v) as colourless wax.

^{1}H-NMR (300 MHz, CD_3OD) δ = 8.36 (s, 1 H, Ar), 8.17 (d, 1 H, Ar), 8.07 (d, 1 H, Ar), 7.75 (t, 1 H, Ar), 4.12 (t, 2 H, H-1''), 3.88 (m, 2 H, H-6a, H-6b), 3.52 (ddd, 1 H, $J_{1a,2}$ 4.8 Hz, $J_{1b,2}$ 10.4 Hz, H-2), 3.44–3.36 (po, 3 H, H-4, H-11'), 3.24 (t, 2 H, H-6''), 3.18 (dd, 1 H, $J_{2,3} = J_{3,4}$ 9.1 Hz, H-3), 3.08 (dd, 1 H, $J_{1a,1b}$ 11.3 Hz, H-1a), 2.89 (m, 1 H, H-1'), 2.69 (m, 1 H, H-1'), 2.31 (po, 2 H, H-1b, H-5), 1.70–1.61 (po, 4 H, H-10', H-2''), 1.57–1.48 (po, 4 H, H-2', H-5''), 1.40–1.28 (po, 18 H, H-3', H-4', H-5', H-6', H-7', H-8', H-9', H-3'', H-4'').

^{13}C-NMR (75.5 MHz, CD_3OD) δ = 167.8 (NC=O), 138.2, 137.3 (ipso), 133.5, 131.5, 131.0, 127.7 (Ar), 80.1 (C-3), 72.5 (C-1''), 71.5 (C-4), 70.2 (C-2), 67.4 (C-5), 58.7 (C-6), 57.2 (C-1), 53.8 (C-1'), 52.3 (C-6''), 41.2 (C-11'), 30.6, 30.6, 30.6, 30.5, 30.4, 30.4, 29.7, 29.6, 28.4, 28.1, 27.1, 26.0, 25.0 (C-2', C-3', C-4', C-5', C-6', C-7', C-8', C-9', C-10', C-2'', C-3'', C-4'', C-5'').

MS (MALDI): Calculated for $[C_{30}H_{51}N_5O_8SH]$: m/z $[M+H]^+$642.3536; found $[M+H]^+$642.3536. $[\alpha]_D^{20}$: −5.9 (c = 1.01, MeOH).

N-(6-(3-(((6-Azidohexyl)oxy)sulfonyl)benzamido)hexyl)-1,5-dideoxy-1,5-imino-D–xylitol (DIX-C_6–LDSE probe 5)

Following general procedure B, aldehyde **11** (167.7 mg, 0.40 mmol) was reacted with iminosugar **16**[33] (55.0 mg, 0.41 mmol) and $NaBH_3CN$ (37.0 mg, 0.59 mmol). After TLC confirmed complete consumption of the starting materials, the reaction mixture was concentrated under reduced pressure. Purification on silica gel (EtOAc/MeOH 3:1, v/v) gave probe **5** (145.0 mg, 0.27 mmol, 67.8%, R_f = 0.4, EtOAc/MeOH 2:1, v/v) as colourless wax.

^{1}H-NMR (300 MHz, CD_3OD) δ = 8.36 (s, 1 H, Ar), 8.18 (d, 1 H, Ar), 8.08 (d, 1 H, Ar), 7.77 (t, 1 H, Ar), 4.13 (t, 2 H, H-1''), 3.52 (m, 2 H, $J_{1a,2}/J_{4,5a}$ 4.7 Hz, $J_{1b,2}/J_{4,5b}$ 10.1 Hz, H-2/H-4), 3.42 (t, 2 H, H-6'), 3.25 (t, 2 H, H-6''), 3.12 (dd, 1 H, $J_{2,3}/J_{3,4}$ 8.6 Hz, H-3), 3.01 (m, 2 H, $J_{1a,1b}/J_{5a,5b}$ 10.7 Hz, H-1a/H-5a), 2.45 (t, 1 H, H-1'), 1.96 (m, 2 H, H-1b/H-5b), 1.74–1.61 (po, 4 H, H-5', H-2''), 1.60–1.49 (po, 4 H, H-2', H-5''), 1.48–1.38, 1.36–1.27 (po, 8 H, H-3', H-4', H-3'', H-4'').

^{13}C-NMR (75.5 MHz, CD_3OD) δ = 167.8 (NC=O), 138.3, 137.3 (ipso), 133.5, 131.5, 131.0, 127.7 (Ar), 79.6 (C-3), 72.5 (C-1''), 71.1 (C-2/C-4), 59.1 (C-1/C-5), 58.8 (C-1'), 52.3 (C-6''), 41.1 (C-6'), 30.3, 29.7, 29.7, 28.1, 27.9, 27.1, 26.0, 25.1 (C-2', C-3', C-4', C-5', C-2'', C-3'', C-4'', C-5'').

MS (MALDI): Calculated for $[C_{24}H_{39}N_5O_7SNa]$: m/z $[M+Na]^+$ 564.2468; found $[M+Na]^+$564.2468.

N-(11-(3-(((6-Azidohexyl)oxy)sulfonyl)benzamido)undecyl)-1,5-dideoxy-1,5-imino-D–xylitol (DIX-C_{11}–LDSE-probe 6)

Following general procedure B, aldehyde **12** (83.1 mg, 0.17 mmol) was reacted with iminosugar **16**[33] (23.5 mg, 0.18 mmol) and $NaBH_3CN$ (13.7 mg, 0.22 mmol). After complete consumption of the starting materials was detected by TLC (16 h), the reaction mixture was concentrated under reduced pressure. Purification on silica gel (EtOAc/MeOH 7:1, v/v) provided probe **6** (50.4 mg, 82.4 μmol, 49.0%, R_f = 0.4, EtOAc/MeOH 2:1, v/v) as colourless wax.

^{1}H-NMR (300 MHz, CD_3OD) δ = 8.37 (s, 1 H, Ar), 8.18 (d, 1 H, Ar), 8.08 (d, 1 H, Ar), 7.76 (t, 1 H, Ar), 4.13 (t, 2 H, H-1''), 3.53 (m, 2 H, $J_{1a,2}/J_{4,5a}$ 4.6 Hz, $J_{1b,2}/J_{4,5b}$ 9.6 Hz, H-2/H-4), 3.41 (t, 2 H, H-11'), 3.25 (t, 2 H, H-6''), 3.14 (dd, 1 H, $J_{2,3}/J_{3,4}$ 8.7 Hz, H-3), 3.02 (m, 2 H, $J_{1a,1b}/J_{5a,5b}$ 10.8 Hz, H-1a/H-5a), 2.46 (t, 1 H, H-1'), 2.01 (m, 2 H, H-1b/H-5b), 1.73–1.62 (po, 4 H, H-10', H-2''), 1.58–1.48 (po, 4 H, H-2', H-5''), 1.40–1.27 (po, 18 H, H-3', H-4', H-5', H-6', H-7', H-8', H-9', H-3'', H-4'').

^{13}C-NMR (75.5 MHz, CD_3OD) δ = 167.8 (NC=O), 138.3, 137.3 (ipso), 133.5, 131.5, 131.0, 127.7 (Ar), 80.0 (C-3), 72.5 (C-1''), 71.2 (C-2/C-4), 59.2 (C-1/C-5), 59.0 (C-1'), 52.3 (C-6''), 41.3 (C-11'), 30.6, 30.6, 30.6, 30.5, 30.4, 30.4, 29.8, 29.7, 28.5, 28.1, 27.6, 27.1, 26.0 (C-2', C-3', C-4', C-5', C-6', C-7', C-8', C-9', C-10', C-2'', C-3'', C-4'', C-5'').

MS (MALDI): Calculated for $[C_{29}H_{49}N_5O_7SH]$: m/z $[M+H]^+$612.3431; found $[M+H]^+$612.3431.

Biology

General methods and materials

Solvents and components of buffering systems used were all commercially available and were used without further purification. The substrate *p*-nitrophenyl-β-D-*gluco*-pyranoside (*p*NP-β-Glc) and its hydrolysis product *p*-nitrophenol were purchased from Merck KGaA (EMD Millipore Corp. USA). Enzymes used in this proof of concept study were purchased and used without further purification. The β-glucosidase from *Thermotoga maritima* (*Tm*GH1, LOT: 151102a) is commercially available from Megazyme (Headquarter in Bray, Ireland since 1996) as ammonium sulphate suspension with a specified activity of 460 U/ml. The β-glucosidase from sweet almonds (*Prunus dulcis*, *Pd*GH1, LOT: BCCC7765) and α-mannosidase from *Canavalia ensiformis* (*Ce*GH38, LOT: 016H9555) were purchased from Sigma Aldrich as lyophilized powder and a specified lot activity

of 4.5 U/mg and 19 U/mg, respectively. Enzymes yielded more than one single band (at pH = 8.0, TRIS-HCl 50 mM) in sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) with different molecular weights of approx. 51 kDa for *Tm*GH1 and 62 kDa for *Pd*GH1 compared to 54 kDa and 135 kDa as specified by the supplier, respectively. Protein bands of 51 kDa for *Tm*GH1 and 62 kDa for *Pd*GH1 have been found to exhibit hydrolytic activity towards *p*NP-β-Glc and therefore were identified as the target enzymes.

Protein quantification (Bradford assay)

Protein quantification was done following the standard Bradford assay setup using the ROTI® Quant reagent from Carl Roth GmbH and Co. KG following instructions of the supplier. The calibration curve for protein quantification was determined in 96-well plates measuring the absorbance of the Coomassie Brilliant Blue Dye-G250 at 595 nm using calibration standards of BSA in the range between 0 to 100 μg/mL protein (details see SI Figure S19).

Labelling experiments

The enzyme (0.46 mg/mL) was incubated with the respective LDC probe (**1**–**6**) in a total volume of 200 μL including a final DMSO concentration of 2v%. Standard incubation conditions are found to be 100 μM of the ligand-directed chemistry (LDC) probe for 20 hours at room temperature, corresponding to an approximate molar ratio of 1 : 10 enzyme to probe. Consecutive removal of residual probe and small molecules by washing three times with 500 μL TRIS-HCl 50 mM pH = 8.0 and thereby exchanging the buffering system is performed by the use of Vivaspin® 500 centrifugal concentrator spin-columns from SARTORIUS (MWCO 10.000 PES membranes). Concentration of the protein solution yields approximately 20–25 μL of 4 mg/mL protein in TRIS-HCl 50 mM pH = 8.0. This solution is used for the second step, the introduction of the fluorescent tag via click chemistry.

Click chemistry protocol

Visualization of azide-functionalized enzyme was achieved by attaching the fluorescent dye AlexaFluorTM 594 alkyne (invitrogen, ThermoFisher Scientific) using the Click iT®™ Protein Reaction Buffer Kit (invitrogen, ThermoFisher Scientific). The fluorophore was introduced via copper-catalyzed click chemistry following the instructions of the manufacturer. The procedure was optimized to low volume reactions with the need for only 5 μL of the chemically modified enzyme (4 mg/mL, in TRIS-HCl 50 mM pH = 8.0). The volume ratios of the reagents and order of the click iT®™ protocol are maintained (details see SI Figure S20). After the addition of the last reagent the reaction was stirred for 20 min at 1000 rpm at room temperature. SDS-PAGE analysis was performed without an additional wash protocol.

SDS-PAGE analysis

SDS-PAGE analysis was performed on a NuPAGE™ 4–12% Bis–Tris Gel (1.0 mm×10/15 well; invitrogen ThermoFisher Scientific) using Mini Cell (invitrogen by ThermoFisher Scientific) together with a XCell SureLock™ mA700 Essential Power Supply (MERCK Millipore®). After running the gel with NuPAGE® MOPS SDS Running Buffer (20x) (novex® by Life Technologies) for 50–55 min at 200 V analysis of fluorescent protein bands was done using a BioRad Gel Doc 2000 combined with the Quantity One 4.6.9 (Basic)-GelDoc program, followed by whole protein staining of the gel using (1) staining solution: Brilliant Blue R 250 (0.5 g) in EtOH (200 mL), acetic acid (50 mL) and ddH_2O (250 mL) and (2) destaining solution: EtOH/acetic acid/ddH_2O (3 : 1 : 6).

Enzyme kinetics

The hydrolytic activity of β-glucosidases from *Thermotoga maritima* (*Tm*GH1) and sweet almonds (*Prunus dulcis*, *Pd*GH1) were assayed spectrophotometrically. The release of *p*-nitrophenol at the expense of *p*NP-β-Glc was measured at 405 nm over 2–5 minutes using 96-well plates from SARSTEDT on a Spark® Multimode Microplate Reader (TECAN Group AG, Switzerland). Kinetic parameters K_m, v_{max}, and k_{cat} were determined using constant amounts of enzyme together with 12 different substrate concentrations. Results and enzyme specific conditions are given in the supporting information Table S1 and Figure S2. Determination of inhibition constants (K_i-values) and IC_{50} values was performed by measuring residual hydrolytic activities after pre-incubation of β-glucosidases with the inhibitors at 7 different inhibitor concentrations and one control sample without the inhibitor, reflecting 100% of activity at the specified conditions. The reaction was started by addition of three different substrate concentrations ($[S] < K_m$, $[S] = K_m$, $[S] > K_m$). Details on inhibitor and substrate concentrations are given in the SI Table S2. Initial rates for Michaelis Menten kinetics and inhibition kinetics were calculated with Excel. Lineweaver-Burk plots were constructed to validate the use of competitive or mixed type inhibition models and to assess the fit of the data (see SI Figures S3–S4). The data were then fit to the Michaelis Menten model or a competitive/mixed type inhibition model using non-linear regression analysis with Grafit 7.0.3. (Erithacus Software, details see SI equation 1–3). For the IC_{50} values the series of initial rates obtained at 7 different inhibitor concentrations and one control sample at $[S] = K_m$ were analysed using non-linear regression with Grafit 7.0.3 (Erithacus Software, details in SI).

Competition labelling study

The enzyme was first incubated with an excess of IFG (1000 μM) for 10–15 min before incubation with the actual LDC probe (100 μM) for ongoing 20 hours at room temperature as seen before. As reference sample the enzyme was also pre-incubated with DMSO (1v% in the final solution) to prevent any adverse effects of the DMSO addition before adding the LDC probe. For the competition labelling study using CBE, the enzyme was pre-incubated with an excess of CBE (1000 μM) for 20 hours. Measurement of residual hydrolytic activity result in no observable hydrolysis of *p*NP-β-Glc. After initial 20 h pre-incubation the enzyme was incubated with the LDC probe (100 μM) for another 20 hours at room temperature following the same procedure as described previously. Again, as reference the enzyme was pre-incubated with DMSO only (1v% in final solution) for 20 h before incubation with the LDC probe to see any adverse effects caused by DMSO (summarized conditions for the competition labelling are highlighted in the SI Table S4 and Figures S17–18).

Activity assay of labelled TmGH1

Evaluation of the enzymatic activity of the labelled β-glucosidase *Tm*GH1 after incubation with the most potent candidates DNJ-C_6–LDSE-N_3 probe **3** and DNJ-C_{11}–LDSE-N_3 probe **4** was accomplished as follows: The enzyme was incubated with either probe **3** or **4** at distinct concentrations for 20 hours at room temperature as seen before for the initial labelling study. The reaction solution was transferred into a Vivaspin® 500 centrifugal concentrator spin-column and small molecules were removed by washing 3 times with sodium maleate 50 mM pH 6.5 working buffer. After concentration to obtain approximately 200 μL of the labelled enzyme, protein concentration was determined following the standard protocol for Bradford assay. After dilution the Michaelis-Menten kinetics were performed following the protocol described earlier, tracking the release of *p*-nitrophenol from *p*NP-β-Glc at 405 nm on a Spark® Multimode Microplate Reader (TECAN Group AG, Switzerland). Initial rates were calculated in Excel and kinetic parameters obtained by fitting the calculated initial rates as a function of the substrate concentration to a variation of the Michaelis-Menten model using non-linear regression with Grafit 7.0.3 (Erithacus Software, equation 6, SI).

Supporting Information

The authors have cited additional references within the Supporting Information.[46–51]

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: β-glucosidases · covalent enzyme labelling · glycoside hydrolases · iminoalditol probes · ligand-directed chemistry

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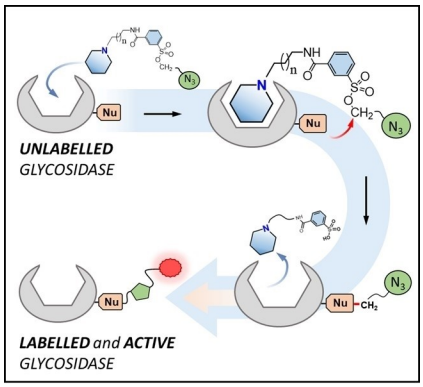
RESEARCH ARTICLE

We report ligand-directed chemistry for covalent proximity labelling of two model β-glucosidases employing small molecule iminosugar based probes in a proof of concept study. Successful enzyme labelling with respective designed and synthesized probes was determined by fluorescent readout of SDS page. Catalytic enzyme activity was maintained after the labelling process.



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Ligand-Directed Chemistry on Glycoside Hydrolases – A Proof of Concept Study