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# Nanomolar affinity, iminosugar-based chemical probes for specific labeling of lysosomal glucocerebrosidase

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ABSTRACT

Three different photoprobes were synthesized to label β-glucosidases; one probe was based on glucose, two probes on the iminosugar deoxynojirimycin. The affinity of the probes for three different β-glucosidases was determined. Furthermore, their labeling efficiencies, binding specificities through competition with deoxynojirimycin, and binding specificities in the presence of cell lysate, were evaluated. Especially one showed very high affinity towards non-lysosomal glucoceramidase ($IC_{50}$ = 20 nM).



## 1. Introduction

The glucosidases are a large class of hydrolases that occur in plants, fungi, mammals and microorganisms. The glucosidases catalyze the hydrolysis of glycosidic bonds, and are therefore involved in many important processes such as the biosynthesis and degradation of glycoproteins.[1,2] In mammalian systems, two well-studied types of β-glucosidases are the cytosolic and lysosomal β-glucosidases.[3] Cytosolic β-glucosidases are mainly present in the liver, the kidneys and the intestine. Gopolan et al. suggested that cytosolic β-glucosidases play a key role in the detoxification of plant β-glucosides,[4] which seems to correspond to the significant quantities of this enzyme present in especially the organs containing detoxification enzymes, namely the liver and the intestine.

The Lysosomal β-glucosidase cleaves the substrate glucosylceramide (glucocerebroside) into ceramide and glucose. Therefore, the enzyme is also called lysosomal glucocerebrosidase or glucosylceramidase (E.C. 3.2.1.45).[3] Lysosomal β-glucosidase is present in most tissues and cell types with various levels of activity. The enzyme is partially defective in Gaucher disease patients. The disorder is characterized by the accumulation of glucosylceramide in tissue macrophages (Gaucher cells).[5,6]

To study glycosidases and capture them selectively in the presence of complex protein mixtures, a chemical probe, based on a substrate or inhibitor of the enzyme can potentially be a very useful tool. Romaniouk et al. synthesized the first photoaffinity derivative of the iminosugar deoxynojirimycin.[7] With this [$^{125}$I]-labeled probe, it was possible to identify the peptide that is part the active site of the protein glucosidase I. The group of Withers[8,9] synthesized a probe to profile retaining β-endoglycosidases in complex proteomes. Their probe consisted of a mechanism-based inactivator of the enzyme. A number of peptides were identified, and they all proved to be derived from proteins belonging to the family of retaining glycanases. In a different approach, a fluorescent dansyl moiety was coupled to 1-amino-1,2,5-trideoxy-2,5-imino-D-mannitol by Hermetter et al.[10] with two powerful β-glucosidase probes as a result. In one compound the dansyl group was coupled directly to the sugar while the other probe contained a spacer in between the sugar and the fluorescent group. Both compounds were highly potent inhibitors of *Agrobacterium* sp. β-glucosidase, with $K_i$-values of 2 nM. With one probe it was possible to fluorescently label β-glucosidase selectively in a mixture with three other related proteins and visualize the labeled enzyme on a native PAGE.[10] Within this context we here describe our efforts in the synthesis of chemical probes containing a photolabel (benzophenone and acetophenone) for glucosidase capture. An alkyne moiety is part of the probe to enable the attachment via 'click' chemistry of a reporter tag, such as a fluorescein-azide for visualization in a gel. As the recognition moiety glucose and 1-deoxynojirimycin were used which exhibit greatly different inhibitory potencies for the enzymes targeted in our studies: lysosomal glucocerebrosidase (GBA), non-

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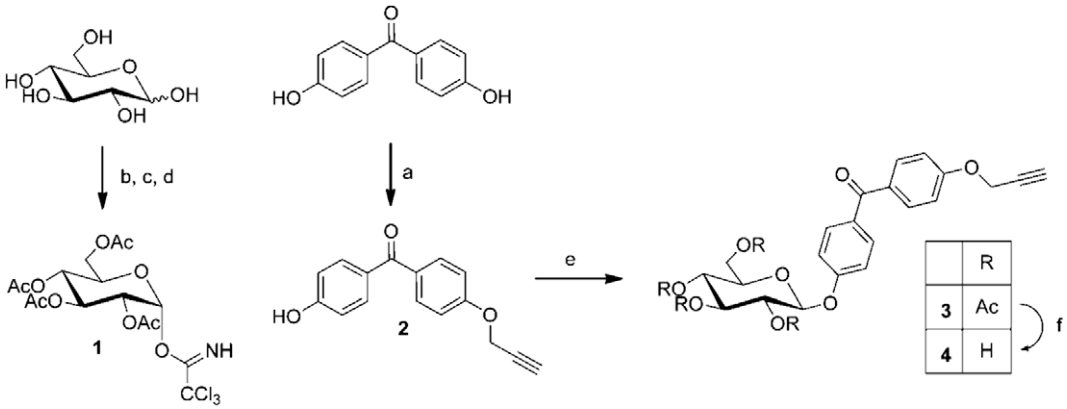


**Scheme 1.** Synthesis of glucose-benzophenone (**4**). Reagents and conditions: (a) propargyl bromide, $K_2CO_3$, acetone, 16 h, 31%; (b) $Ac_2O$, NaOAc, Δ, 30 min, quant.; (c) $NH_2CH_2CH_2NH_2$ (1.2 equiv), AcOH (1.4 equiv), THF, 18 h, 71%; (d) $Cl_3CCN$ (2.5 equiv), DBU (0.2 equiv), $CH_2Cl_2$, 0 °C to rt, 4 h, 89%; (e) TMSOTf (0.2 equiv), $CH_2Cl_2$, −20 °C to rt, 16 h; (f) **3**, NaOMe, MeOH, 10 min, 23% over two steps.

lysosomal glucoceramidase (GBA2), and almond β-glucosidase. The abilities of the probes to specifically capture the enzymes in the presence of a human cell lysate were evaluated.

## 2. Results and discussion

### 2.1. Syntheses

#### 2.1.1. Synthesis of the glucose-based probe (4)

D-Glucose was acetylated and converted to imidate-donor **1** according to known procedures.[11] Commercially available 4,4′-dihydroxybenzophenone was mono-alkylated with propargyl bromide and an excess of base to give **2** (Scheme 1). Subsequently, the photolabel was coupled to the sugar under standard coupling conditions (cat. TMSOTf in dry $CH_2Cl_2$).[11] Deprotection of the sugar using Zemplen's method yielded the glucose-based chemical probe **4**.

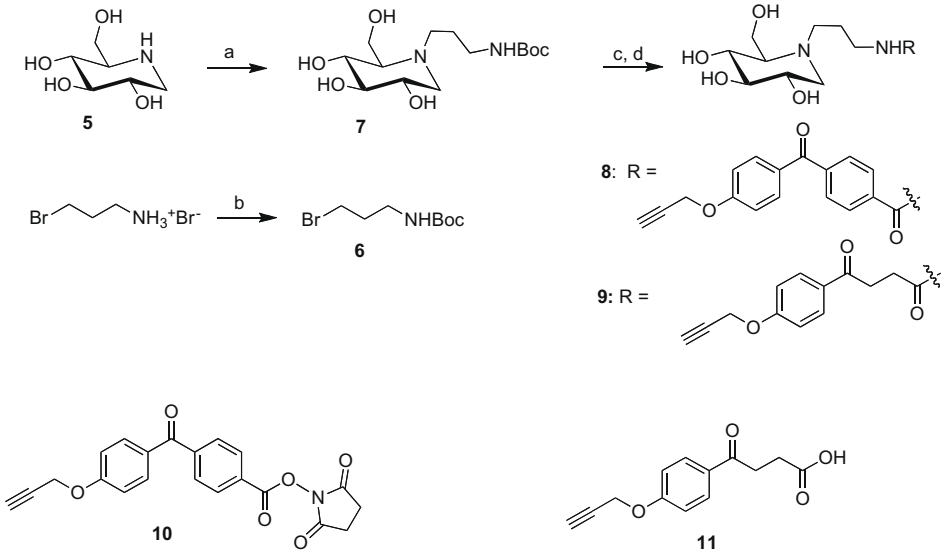
#### 2.1.2. Synthesis of iminosugar-based probes

The synthesis of the two iminosugar-based probes started with the alkylation of the hydrochloride salt of 1-deoxynojirimycin **5**[13] by bromide **6**. After removal of the Boc-group with TFA, the purified OSu ester **10**[12] was used to prepare **8**. For the synthesis of **9** acetophenone derivative **11**[12] was successfully coupled using EDC/NHS. After a final preparative HPLC purification benzophenone probe **8** and acetophenone probe **9** were obtained in 13% and 39% isolated yield, respectively (Scheme 2).

### 2.2. Evaluation of the probes

#### 2.2.1. Enzyme inhibition experiments

The probes were tested for their ability to inhibit three different β-glucosidases: lysosomal glucocerebrosidase (Cerezyme®, recombinant GBA, Ceredase®, human placental GBA), non-lysosomal glucoceramidase (GBA2), and almond β-glucosidase. The assays for the first two enzymes were based on a competition of the probe with the substrate 4-methylumbelliferyl (4-MU) β-glucoside. The 4-MU part is cleaved from β-glucose by the enzyme, and its amount can be measured with a fluorometer. The inhibitory potency is expressed as the inhibitor concentration resulting in 50% inhibition ($IC_{50}$-values).[14] The assay for the third enzyme is based on a competition of the probe with the substrate *para*-nitrophenol β-glucoside (PNPG), whose liberated PNP group was quantified by UV spectroscopy at 405 nm. Measurements were performed with different substrate concentrations, from which a $K_i$-value was



**Scheme 2.** Synthesis of the iminosugar-based probes. Reagents and conditions: (a) **6**, $K_2CO_3$, DMF, 85 °C, 18 h; (b) $Boc_2O$, dioxane/$H_2O$/NaOH, 1 h, 0 °C, 91%; (c) TFA/$CH_2Cl_2$ 1:1 (v/v), 1 h, 0 °C; (d) for **8**:**10**, DMF, 16 h, 0 °C to rt, 13%; for **9**:**11**, EDC, NHS, DMF, 16 h, 0 °C to rt, 39%.

determined. Results of our measurements are shown in Table 1. The affinity of DNM **5** and probe **4** for almond β-glucosidase are given for comparison. Especially the affinities of **8** and **9** for the membrane-bound glucosidase GBA2 were very high ($IC_{50}$ 20 and 75 nM, respectively). The best inhibitor thus far described in the literature has a $IC_{50}$-value of 1.7 nM,[14] which is only a factor of 10 lower than the value obtained for compound **8**. For a monosaccharide-protein interaction this is exceptional. The other $K_i$-values given in Table 1 are all in the low micromolar or high nanomolar range, so both **8** and **9** bind with high affinities.

### 2.2.2. Labeling of β-glucosidase with (4)

Probe **4** was evaluated first on its ability to label β-glucosidase. A general procedure, developed by Speers and Cravatt[15] that was also previously used in our group was followed.[16–18] During incubation of the enzyme with **4** for 30 min, the sample was irradiated with UV light. Fluorescein-$N_3$ was 'clicked' on overnight and the samples were resolved on SDS–PAGE. The results are shown in Figure 1. The probe was able to label the enzyme. It turned out that the fluorescence intensity depends on several factors such as the concentration of the probe, the amount of enzyme, the reaction time of the click reaction, the irradiation time and the extensiveness of washing the gel (which lowers the background) before fluorescence scanning. Clearly, UV light irradiation is necessary to fluorescently label the protein.

### 2.2.3. Profiling sugar binding and processing proteins with 4

In a second experiment we turned to cell lysates, to find out if it was possible to profile sugar binding proteins and sugar processing enzymes (e.g., glycosidases, glycosyltransferases) in such a complex mixture. The result is shown in Figure 2A, in which the fluorescence scan and the silver stain of one gel are compared.

It is clear that only a small percentage of all the proteins present were labeled with the probe. After excision of the fluorescent bands from the gel, trypsin digestion and MALDI-TOF analysis were performed. In this fashion the glucose-binding proteins tryptophanase, α-galactosidase, elongation factor Tu, outer membrane protein and OmpF porin were identified with high confidence (100% C.I.) according to Mascot (see Supplementary data).[19] Especially OmpF is an interesting result, because this protein is important in nutrient (including glucose) regulation.[20] Since this protein is a membrane protein, it is often lost in common 2D-gel MS identification techniques.[21,24]

Figure 2B shows an important control experiment, which is the profile of benzophenone-labeled proteins. From this experiment, we can conclude that the influence of the photolabel is dominant in the chemical probe; there are only small differences in the gel profile between the glucose-probe **4** and benzophenone **2**.

An explanation is probably the large size of the photolabel compared to the rest of the probe, as well as its hydrophobicity. Carbohydrate–protein interactions are usually weak,[25] which makes these probes more of a 'water-soluble photolabel' rather than a targeted probe. Despite this result, some glucose specific labeled proteins were obtained by subtracting the benzophenone-labeled proteins from the glucose-probe labeled proteins using suitable software.[26]

**Table 1**
Affinity measurements of the probes on three different enzymes

| Compds | GBA $IC_{50}$[a] (μM) | GBA2 $IC_{50}$[a] (μM) | Almond β-glucosidase $K_i$[a] (μM) |
|---|---|---|---|
| **9** | 1.65 (±0.15) | 0.075 (±0.03) | 6.4 (±0.1) |
| **8** | 0.35 (±0.05) | 0.02 (±0.005) | 1.3 (±0.1) |
| **5** | 506[c] | 28.80[c] | 167 (±1) |
| **4** | n.d. | n.d. | 588 (±133)[b] |

[a] Values are means of two experiments, standard deviation is given in parentheses (n.d. = not determined).
[b] Probe **4** can be considered as an inhibitor even though it is a slowly converting substrate.
[c] Values from Ref. 12 are given for comparison.

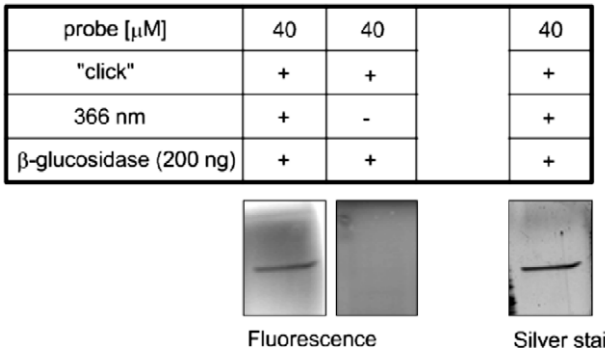



**Figure 1.** Almond β-glucosidase is labeled by probe **4**. Without light irradiation, no covalent bond is formed between the probe and the protein, and no fluorescent labeling occurs.

### 2.2.4. Labeling of lysomal glucocerebrosidase with the iminosugar-based probes (8) and (9)

Lysosomal glucocerebrosidase was used to test the difference in labeling efficiency of the probes. The iminosugar probes **8** and **9** were compared with the glucose-probe **4**. Concentration ranges were made of the probes, from 10 down to 0.1 μM. The result is shown in Figure 3A. It turned out that clear labeling could only be observed at the 10 μM probe concentration. At this concentration, there is a large difference in efficiency between the compounds. Probe **8** labeled the best, and the labeling by probe **9** was only negligible. The difference in affinity of both compounds for this enzyme was about a factor 5 (Table 1), which probably influences the labeling efficiency. However, the difference in reactivity between acetophenone and benzophenone is likely of greater influence, considering their absorptions in near UV.[12] Since compounds **4** and **8** differ roughly a factor of 100 in their binding constant (Table 1), the difference in labeling efficiency between these compounds is most likely a matter of affinity.

To test if **4**, **8** and **9** bind the enzyme specifically in the binding pocket, a competition experiment with **5** was performed. The result is shown in Figure 3B.

In this experiment, the labeling efficiency of **8** is again higher than the efficiency of **9**. For all molecules it is clear that they compete with **5**: the fluorescent signal decreases with an increase in concentration of **5**. From this picture we can conclude that both probes **8** and **9** bind specifically in the binding pocket of lysosomal glucocerebrosidase, and this seems to be the case also for glucose-probe **4**.

### 2.2.5. Specificity of probe 8 for labeling GBA in the presence of human cell lysate

The final experiment was set up to find out if probe **8** was specific for GBA in the presence of other proteins. The chosen probe concentration was relatively low, to avoid non-specific interactions. However, since the labeling efficiency of this compound is not that high, the samples had to contain a considerable amount (300 ng) of the enzyme. A concentration range of a human cell lysate was prepared up to 3 μg of total proteins/sample. The results are shown in Figure 4.

Probe **8** seems to be highly specific for GBA, as we can conclude from the fluorescence picture. Surprisingly, the fluorescent signal does not decrease with an increasing amount of other proteins present, which is in contrast to another highly selective probe to

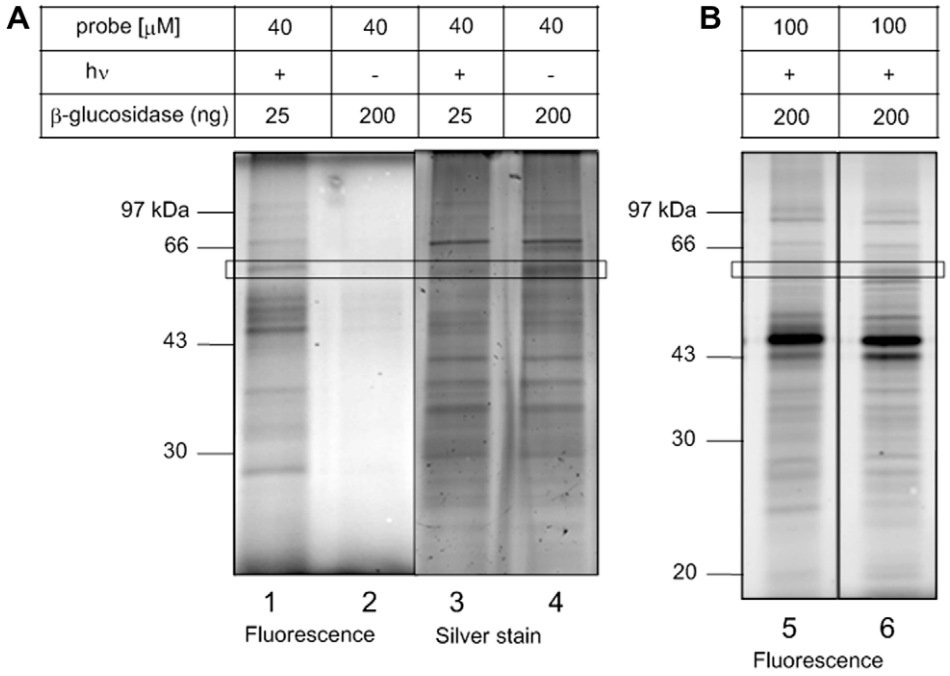



**Figure 2.** Profiling glucose-binding proteins in *E. Coli* cell lysate. (A) *E. coli* lysate spiked with different amounts of β-glucosidase, was incubated with 40 μM of **4**. A fluorescent picture (lanes 1 and 2) and silver stain (lanes 3 and 4) of the same gel are shown. (B) Comparison between the fluorescent patterns of **4** (lane 5) and **2** (lane 6), which showed only minor differences. The boxes indicate the position of β-glucosidase.

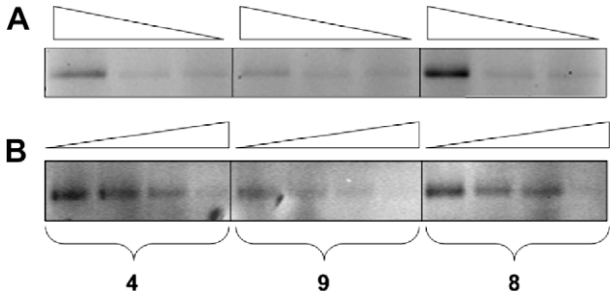



**Figure 3.** Labeling Ceredase® with different probe concentrations (A), or with increasing concentrations of deoxynojirimycin (DNM, 5) (B). (A) Each lane contains 100 ng of enzyme. Concentrations are from left to right for each probe: 10, 1 and 0.1 μM. (B) The same amount of Ceredase® (200 ng) is present in all samples. Probe concentrations are 5 μM in all samples. From DNM **5** a concentration range was prepared: from left to right for all probes 0, 10, 100 and 500 μM.

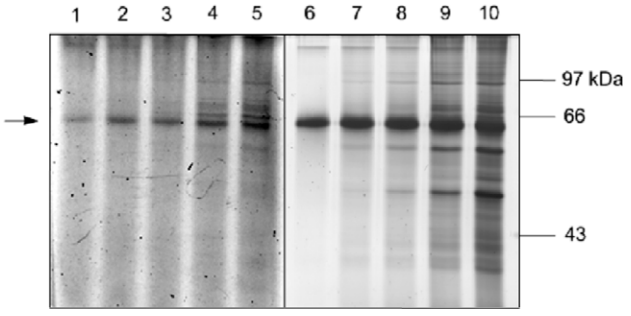



**Figure 4.** Labeling of Ceredase by probe 8 (3 μM) in the presence of a human cell lysate. In all of the lanes, 300 ng of Ceredase® is present. The amount of total protein per lane increases from left to right: 0, 0.3, 0.7, 1.3 and 2.7 per lane. Lanes 1–5: fluorescent picture; lanes 6–10: silver stain of lanes 1–5.

label galectin-3.[12] It is likely that **8** binds the enzyme in a deep pocket in accordance with a recent crystal structure.[27] In contrast the galectin-3 probe binds in a surface groove benefiting from electrostatic interactions with arginines.[28] Therefore, the galectin-3 probe is probably more likely to capture additional proteins than probe **8**.

## 3. Conclusions

We started our project with the easy to synthesize glucose-probe **4**. A disadvantage of this probe is that it is a substrate of the enzyme as well, which causes cleavage of the benzophenone moiety and subsequent non-specific labeling. Furthermore, it turned out that the relatively small sugar had only small influence on binding specificity, when it was coupled to a large, bulky, hydrophobic photolabel. We could have changed to a less hydrophobic photolabel, such as a diazirine moiety, however this label is reported to possess lower labeling efficiency, and a higher reactivity towards nucleophiles and the solvent, than aryl ketones.[29,30] We instead opted to switch to the high affinity iminosugar DNM, and we were able to couple our benzophenone photolabel, as well as the less bulky and hydrophobic acetophenone photo affinity label. The prepared DNM-based labels were tested for their affinity towards three different glycosidases, and were compared with the glucose-probe **4**. Especially probe **8** showed a very high inhibitory potency with an $IC_{50}$ of 20 nM for the non-lysosomal glucoceramidase (GBA2) assay. The probes were tested for their ability to label different β-glucosidases, and their binding specificity through competition experiments with DNM. From those experiments, it was concluded that all the probes bound the enzyme specifically in its binding pocket. Although in general, labeling efficiencies were low, lysosomal glucocerebrosidase could be labeled specifically in the presence of a complex protein mixture. The labeling efficiency can possibly be improved by coupling the photolabel directly, or with a shorter spacer, to the iminosugar. However, a possible negative influence of this bulky substituent on binding specificity cannot be excluded. Nevertheless, the chemical probes presented here do label three different β-glucosidases specifically, and two of them bind with exceptionally high affinity. Further-

more, the experiments clearly show that creating a successful probe is a multidimensional challenge involving issues of lipophilicity, affinity and photoreactivity.

## 4. Experimental

### 4.1. General methods

All reagents were purchased from commercial sources and were used without further purification. The compound 1-deoxynojirimycin **5** was prepared according to the reported protocol.[13] Dowex 50X8 (H+ form, 20–50 mesh) was purchased from Sigma. Analytical thin layer chromatography (TLC) was performed on Merck pre-coated Silica Gel 60 F254 (0.25 mm) plates. Spots were visualized with UV light, $H_2SO_4$, or ninhydrin. Column chromatography was carried out using Merck Kieselgel 60 (40–63 mm). $^1$H NMR and $^{13}$C NMR were obtained on a Varian 300 MHz spectrometer. Chemical shifts are given in ppm with respect to internal TMS for $^1$H NMR. Two-dimensional $^1$H–$^1$H correlation and total correlation spectroscopy (COSY and TOCSY) and $^1$H–$^{13}$C correlated heteronuclear single quantum coherence (HSQC) NMR spectra (500 MHz) were recorded at 300 K with a Varian Unity INOVA 500 spectrometer. Low resolution ESI-MS experiments were performed on a Shimadzu LC–MS QP8000 system. Exact masses were measured by nanoelectrospray time-of-flight mass spectrometry on a Micromass LCToF mass spectrometer at a resolution of 5000 fwhm. Gold-coated capillaries were loaded with 1 μL of sample (concentration 20 μM) dissolved in a 1:1 (v/v) mixture of $CH_3CN$–$H_2O$. NaI or poly(ethylene glycol) (PEG) was added as internal standard. The capillary voltage was set between 1100 and 1350 V, and the cone voltage was set at 30 V.

### 4.2. General procedures

#### 4.2.1. 4-Hydroxy-4′-(prop-2-ynyloxy)benzophenone (2)

4,4′-Dihydroxybenzophenone (1.00 g, 4.67 mmol), was dissolved in acetone (30 mL) and $K_2CO_3$ (600 mg, 4.34 mmol) was added. Propargylbromide (80% w/v solution in toluene, 500 μL, 3.61 mmol) was slowly added to the solution and the mixture was stirred for 16 h. The mixture was purified by silica gel column chromatography (hexane/EtOAc, 5:1, v/v). The product was obtained as a white solid (362 mg, 31% yield). $^1$H NMR ($CD_3OD$): $\delta$ = 3.02 (s, 1H), 4.84 (s, 2H), 4.92 (br s, 1H), 6.89 (d, 2H, $J$ = 8.3 Hz), 7.11 (d, 2H, $J$ = 8.8 Hz), 7.69 (d, 2H, $J$ = 8.3 Hz), 7.75 (d, 2H, $J$ = 8.3 Hz); $^{13}$C NMR ($CDCl_3$): $\delta$ = 55.6, 76.2, 78.0, 114.9, 129.1, 131.6, 132.0, 132.6, 161.3, 162.2, 194.2.

#### 4.2.2. Acetylated glucose-probe (3)

The two compounds **1** (258 mg, 0.60 mmol) and **2** (75 mg, 0.30 mmol) were dissolved in dry $CH_2Cl_2$ (10 mL) and stirred at -20 °C. Trimethylsilyl triflate (13 μL, 0.06 mmol) was added and the mixture was stirred for 16 h allowing the mixture to warm to rt The mixture was neutralized with $Et_3N$, concentrated in vacuo and purified by silica gel column chromatography (hexane/EtOAc, 5:1, v/v) to give **3** as a white solid (41 mg, 0.070 mmol). $^1$H NMR ($CD_3OD$): $\delta$ = 2.00, 2.01, 2.05 (3 × s, 12H), 3.03 (s, 1H), 4.18 (d, 2H, $J$ = 11.7 Hz, H-6ab), 4.33 (dd, 1H, $J$ = 5.3 Hz, 8.8 Hz, H-4), 4.86 (s, 2H, $OCH_2$), 5.15 (t, 1H, $J$ = 9.8 Hz, H-5), 5.23 (t, 1H, $J$ = 8.8 Hz, H-2), 5.42 (t, 1H, $J$ = 9.5 Hz, H-3), 5.52 (d, 1H, $J$ = 7.8 Hz, H-1), 7.12 (d, 2H, $J$ = 8.3 Hz), 7.15 (d, 2H, $J$ = 8.3 Hz), 7.76 (d, 2H, $J$ = 7 Hz), 7.77 (d, 2H, $J$ = 7 Hz); $^{13}$C NMR ($CDCl_3$): $\delta$ = 20.6–20.7 (4 × $COCH_3$) 55.8, 61.8, 68.1, 71.0, 72.1, 72.5, 76.2, 77.7, 98.2, 114.4, 116.0, 131.0, 132.0, 132.2, 132.9, 159.6, 160.9, 169.4, 170.2, 170.6, 194.2.

#### 4.2.3. Glucose-probe (4)

To a solution of compound **3** in MeOH (5 mL) was added a few drops of NaOMe (30% w/v in MeOH) and the mixture was stirred for 30 min. The mixture was neutralized with Dowex $H^+$-resin, filtered and concentrated in vacuo to give **4** in 23% yield over two steps as a white solid. $^1$H NMR ($CD_3OD$): $\delta$ = 3.02 (s, 1H, C≡CH), 3.37- 3.56 (m, 3H, H-3, H-2, H-6a), 3.72 (dd, 1H, $J$ = 5.8, 17.3 Hz, H-5, H-6b), 3.92 (∼d, 2H, $J$ = 11.8 Hz, H-4), 4.86 (s, 2H, $OCH_2$), 5.06 (d, 1H, $J$ = 6.9 Hz, H-1), 7.12 (d, 2H, $J$ = 8.8 Hz), 7.22 (d, 2H, $J$ = 8.8 Hz), 7.76 (d, 2H, $J$ = 5.5 Hz), 7.79 (d, 2H, $J$ = 5.2 Hz); $^{13}$C NMR: $\delta$ = 52.8, 58.5, 67.3, 70.8, 73.9, 74.3, 97.7, 111.7, 113.2, 128.2, 129.1, 158.5, 158.7, 192.6; HR-ESI-MS calcd for $C_{22}H_{22}O_8$ 437.1213 $[M+Na]^+$, found 437.1192.

#### 4.2.4. 3-Bromo-propyl-1-NHBoc (6)

The compound 3-bromopropylamine (500 mg, 2.28 mmol) and $Boc_2O$ (548 mg, 2.51 mmol) were dissolved in dioxane/$H_2O$ (10/5 mL). 5 mL 1 M NaOH-solution in $H_2O$ was added and the mixture was stirred for 1½ h. The reaction mixture was diluted with 50 mL of EtOAc and 20 mL of $H_2O$. The organic layer was washed once with 1 N $KHSO_4$, dried with $Na_2SO_4$, filtered and concentrated in vacuo. Compound **6** was obtained as a colorless oil in 91% yield (516 mg, 2.17 mmol). $R_f$ (hexane/EtOAc, 4:1 v/v) = 0.43; $^1$H NMR (300 MHz, $CDCl_3$): $\delta$ = 1.45 (s, 9H, 3 × $CH_3$), 2.05 (quintet, 2H, $J$ = 6.6 Hz, $CH_2CH_2CH_2$), 3.27 (quartet, 2H, $J$ = 6.3 Hz, $CH_2NH$), 3.44 (t, 2H, $J$ = 6.6 Hz, $CH_2Br$), 4.77 (br s, 1H, NH); $^{13}$C NMR (75 MHz, $CDCl_3$): $\delta$ = 28.5 (3 × $CH_3$), 30.9 ($CH_2Br$), 32.9 ($CH_2CH_2CH_2$), 39.1 ($CH_2NH$), 79.5 ($C(CH_3)_3$), 156.1 (C=O).

#### 4.2.5. *N*-(Propylamide-benzophenone)-1-deoxynojirimycin (8)

Compound **5** (17 mg, 0.1 mmol) was reacted with **6** (36 mg, 0.15 mmol) in DMF (0.5 mL) with $K_2CO_3$ (42 mg, 0.3 mmol) at 85 °C for 18 h. Reaction mixture was filtered and concentrated in vacuo. The crude product was dissolved in 1 mL MeOH, cooled to 0 °C, and 1 mL TFA and a droplet $H_2O$ were added. The mixture was stirred for 1 h at 0 °C, and evaporated to dryness. The crude solid was dissolved in dry DMF (2 mL) and benzophenone-succininmidyl ester **10** (63 mg, 0.17 mmol) and DiPEA (0.1 mL, 0.6 mmol) were added. The mixture was stirred for 18 h, concentrated in vacuo and **8** was obtained after preparative HPLC and lyophilization in 13% isolated yield (34 mg) as colorless oil. $^1$H NMR (500 MHz, $CD_3OD$): $\delta$ = 2.02 (m, 2H, $CH_2CH_2NH$), 2.91–3.01 (m, 3H, H-5, H-$1_{ax}$, C≡CH), 3.24 (∼s, 2H, $CH_2N_{imino}$), 3.31 (t, 1H, $J$ = 9.5 Hz, H-3), 3.41–3.56 (m, 4H, H-4, H-$1_{eq}$, $CH_2NHC$=O), 3.63 (m, 1H, H-2), 3.86, 4.05 (2 × d, 2H, $J$ = 12 Hz, H-6ab), 4.78 (s, 2H, $OCH_2$), 7.06 (d, 2H, $J$ = 9.0 Hz, $CH_{ar}$), 7.75 (t, 4H, $J$ = 8 Hz, $CH_{ar}$), 7.90 (d, 2H, $J$ = 8.0 Hz, $CH_{ar}$); $^{13}$C NMR (125 MHz, $CD_3OD$): $\delta$ = 33.4 ($CH_2CH_2NH$), 36.1 ($CH_2NHC$=O), 47.3 ($CH_2N_{imino}$), 52.9 (C-1, C-6), 54.9 ($OCH_2$), 65.7 (C-5, C-2), 66.9 (C-4), 76.2 (C-3), 75.4 (C≡CH), 77.0 (C≡CH), 113.6, 126.4, 128.6 (6 × $C_{ar}$), 129.1 ($C_{ar,\ ether}$C=O), 131.5 (2 × $C_{ar}$), 136.1 ($C_{ar}$C(=O)NH), 140.0 ($C_{ar,\ amide}$C=O), 161.2 ($C_{ar}OCH_2C$≡CH), 167.6 (C(=O)NH), 195.7 (C=O). HRMS (*m/z*): calcd for $C_{26}H_{31}N_2O_7^+$ $[M+H]^+$ 483.2131; found 483.2114.

#### 4.2.6. *N*-(Propylamide-acetophenone)-1-deoxynojirimycin (9)

Compound **5** (33 mg, 0.2 mmol) was reacted with **6** (71 mg, 0.3 mmol) in 1 mL DMF with $K_2CO_3$ (83 mg, 0.6 mmol) at 85 °C for 18 h. Reaction mixture was filtered and concentrated in vacuo. The crude product was dissolved in 2 mL of MeOH, cooled to 0 °C, and 2 mL of TFA and a drop of $H_2O$ were added. The mixture was stirred for 1 h at 0 °C, and evaporated to dryness. The crude solid was dissolved in dry DMF (4 mL) and acetophenone derivative **11** (139 mg, 0.6 mmol), EDC (115 mg, 0.6 mmol), NHS (69 mg, 0.6 mmol) and DiPEA (0.2 mL, 1.2 mmol) were added. The mixture was stirred for 18 h, concentrated in vacuo and **9** was obtained

after preparative HPLC and lyophilization in 39% isolated yield (34 mg) as yellow oil. $^1$H NMR (500 MHz, DMSO): $\delta$ = 1.79 (∼br s, 2H, C$H_2$CH$_2$NH), 2.45 (t, 2H, $J$ = 6.5 Hz, C$H_2$C(=O)NH), 2.50 (s, 2H, C$H_2$N$_{imino}$), 2.90 (quartet, 1H, $J$ = 10.5 Hz, H-1$_{ax}$), 2.97 (t, 1H, H-5), 3.12 (∼d, 2H, $J$ = 5.5 Hz, C$H_2$NHC=O), 3.21 (m, 4H, CH$_2$C(=O)C$_{ar}$, H-1$_{eq}$, H-3), 3.33 (m, 1H, H-4), 3.56 (br s, 1H, H-2), 3.63 (s, 1H, C≡CH), 3.75, 3.90 (2 × d, 2H, $J$ = 11 Hz, H-6ab), 4.91 (s, 2H, HC≡CC$H_2$O), 7.09 (d, 2H, $J$ = 9.0 Hz, CH$_{ar}$), 7.97 (d, 2H, $J$ = 8.5 Hz, CH$_{ar}$), 8.03 (s, 1H, NH), 9.28 (br s, 1H, NH$^+_{imino}$); $^{13}$C NMR (125 MHz, DMSO): 22.9 (*C*H$_2$CH$_2$NH), 29.3 (*C*H$_2$C(=O)NH), 33.0 (*C*H$_2$C(=O)C$_{ar}$), 35.7 (*C*H$_2$NHC=O), 39.8 (CH$_2$N$_{imino}$), 53.3 (C-1, C-6), 55.7 (OCH$_2$C≡ CH), 65.0 (C-5), 66.1 (C-2), 67.1 (C-4), 76.2 (C-3), 78.5 (C≡CH), 114.4, 129.7 (4 × C$_{ar}$), 160.6 (*C*$_{ar}$OCH$_2$C≡CH), 171.4 (C(=O)NH), 198.5 (C=O). HRMS (*m/z*): calcd for $C_{22}H_{31}N_2O_7^+$ [M+H]$^+$ 435.2131; found 435.2129.

### 4.3. General procedures to label β-glucosidase with a chemical probe

#### 4.3.1. Labeling study of β-glucosidase with (4)

The following components were incubated and irradiated for 30 min under a 366 nm UV lamp at 4 °C: 3 μL of a 1 mg/mL stock solution of β-glucosidase (from almonds, Sigma) and 4 μL of a 1 mM stock solution of **4** in 5% DMSO in water, in 100 μL final volume of 50 mM HEPES-buffer, 150 mM NaCl, pH 7.4. The control sample without UV treatment was protected from daylight with aluminium foil. After photoincubation, 10 μL TCEP (freshly prepared 50 mM stock solution in water), 10 μL of $CuSO_4$ (50 mM stock solution in water) and 1 equiv, with respect to probe **4**, of fluorescein-$N_3$[14] (40 μL of a 100 μM stock solution in water) were added and the volume was adjusted to 200 μL (click conditions adapted from[15]). The samples were gently shaken for 1 h at rt and denatured by the addition of 100 μL sample buffer (10% SDS, 40% glycerol and 2% DTT solution) and subsequent incubation at 95 °C for 5 min. From the total volume of 300 μL, 20 μL was loaded onto a 12% Tris–HCl gel for SDS–PAGE. After extensive washing of the running gel (1 h in water) to eliminate excess dye reagent, fluorescence was detected with a Typhoon fluorescence scanner and the gel was subsequently silver stained (BioRad Silver staining plus kit).

#### 4.3.2. Labeling studies with probe (4) (and (2) as control) in bacterial lysate

Bacterial lysate (40 μL, 1 mg/mL as determined by standard BCA protein assay), 6, 1.5 or 0.75 μL of a 0.5 mg/mL stock solution of almond β-glucosidase, and 4 (Fig. 4A) or 10 μL (Fig. 4B) of a 1 mM stock solution of probe **4** (or **2**) in 5% DMSO in water, in 50 mM HEPES-buffer, 150 mM NaCl, pH 7.4 (total volume 100 μL), were photoactivated and submitted to conjugation to fluorescein-$N_3$[14] and loaded onto a gel and visualized as described above.

#### 4.3.3. Labeling almond β-glucosidase with different probe concentrations

β-Glucosidase (1.5 μL of a 1 mg/mL stock solution, Sigma) was incubated and irradiated for 30 min under a 366 nm UV lamp at 4 °C in 50 μL samples containing 10, 5, 1, 0.5, 0.1 μM of probe **4**, **8** or **9** (stock solutions in water) in 50 mM HEPES-buffer, 150 mM NaCl, pH 7.4. After photoincubation, 2 μL TCEP (freshly prepared 50 mM stock solution in water), 2 μL of $CuSO_4$ (50 mM stock solution in water) and 15 μL of fluorescein-$N_3$[16] (100 μM stock solution in water) were added and the volume was adjusted to 100 μL (click conditions adapted from[13]). The samples were gently shaken for 16 h at 4 °C and denatured by the addition of 50 μL sample buffer (10% SDS, 40% glycerol and 2% DTT solution) and subsequent incubation at 95 °C for 5 min. From the total volume of 150 μL, 20 μL was loaded onto a 12% Tris–HCl gel for SDS–PAGE. After extensive washing of the running gel (1 h in water) to eliminate excess dye reagent, fluorescence was detected with a Typhoon fluorescence scanner.

#### 4.3.4. Labeling Ceredase® with different probe concentrations, in competition with DNM (5), or in the presence of human cell lysate

Ceredase® (1.5 or 2.3 μL, 1 mg/mL,) was incubated and irradiated for 30 min under a 366 nm UV lamp at 4 °C in 50 μL samples containing 10, 5, 3, 1, 0.5, 0.1 μM of probe **4**, **8** or **9** (stock solutions in water), 0, 0.5, 5 or 25 μL of DNM **5** (1 mM stock solution in water), 0, 5, 10, 20 or 40 μL HeLa cell lysate (0.5 mg/mL as determined by standard BCA protein concentration assay) in 50 mM HEPES-buffer, pH 6.1. Samples were submitted to conjugation to fluorescein-$N_3$[16] and loaded onto a gel and visualized as described for the labeling of almond β-glucosidase with different probe concentrations.

### 4.4. Lysate preparation procedures

#### 4.4.1. Bacterial lysate preparation

Bacterial cells (BL21(DE3) from Novagen, $OD_{600}$ = 2.2, 1.5 mL) were pelleted by centrifugation at 5000 rpm for 10 min. The cells were resuspended in 300 μL of B-Per® Reagent (Pierce) and vortexed for 2 min. the homogeneous mixture was centrifuged at 13,000 rpm for 5 min. The supernatant was taken for the labeling studies. Protein concentrations of the solutions were determined by standard BCA protein concentration assay, using bovine serum albumin as a standard.

#### 4.4.2. HeLa cell lysate preparation

$2.5 \times 10^5$ cells were washed twice with PBS and pelleted by centrifugation at 1400 rpm for 5 min. The cells were resuspended in 100 μL of B-Per® Reagent (Pierce) and vortexed for 1 min. the homogeneous mixture was centrifuged at 12,000 rpm for 5 min. The supernatant was taken for the labeling studies. Protein concentrations of the solutions were determined by standard BCA protein concentration assay, using bovine serum albumin as a standard.

### 4.5. Affinity determination procedures

#### 4.5.1. Determination of $IC_{50}$-values of probes 8 and 9 for inhibition of the enzymes lysomal glucocerebrosidase (GBA) and non-lysomal glucoceramidase (GBA2)

Experiments were performed as described in the literature.[14]

#### 4.5.2. Determination of $K_i$-values of probes 5, 8 and 9 for inhibition of almond β-glucosidase

The activity of almond β-glucosidase was determined with 0.2–5 mM *p*-nitrophenol-β-D-glucopyranoside (PNPG) as a substrate in 0.1 M acetate-buffer pH 5.0. The amount of liberated *p*-nitrophenol was measured at 405 nm after 15 min incubation at 37 °C. $K_i$-values were determined by variation of inhibitor concentrations ($I$) and substrate concentrations (PNPG, 0.2–5 mM). The assay was performed in 96-wells format; samples of 150 μL were prepared in each well, consisting of 50 μL PNPG-solution (dilutions from a freshly prepared 20 mM stock in water) and 100 μL inhibitor in acetate-buffer pH 5.0. The reaction was started by adding 50 μL β-glucosidase (in 0.2% BSA in 0.01 M $Na_2HPO_4$, pH 7.0). The Michaelis constant $K_M$ and $K_i$-values were determined from the obtained curves using the equation:[31,32]

$$\frac{1}{V} = \frac{1}{V_{max}} + \frac{K_M}{V_{max}}\left(1 + \frac{[I]}{K_i}\right) * \frac{1}{[PNPG]}.$$

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## Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.10.060.

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