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Glycosidase profiling with immobilised glycosidase-inhibiting iminoalditols—A proof-of-concept study

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Abstract—Three typical glycosidase-inhibiting iminoalditols were attached to a polyamine surface displayed on a silicon chip. Exposure to a representative β-glucosidase revealed selective binding events reflecting the different structural features of the inhibitors probed in this study. This provides a proof-of-concept for the successful exploitation of microarrays of typical reversible glycosidase inhibitors of the iminosugar family.

Iminosugars including iminoalditols and related bicyclic alkaloids are the most prominent family of low molecular weight competitive glycosidase inhibitors.[1] Paradigmatic examples such as compounds **1**, **2** and **3** (Fig. 1) and quite a few of their derivatives have found important roles as diagnostic compounds in the investigation of glycoprotein trimming glycosidases[2] or as pharmaceuticals in the treatment of, for example, diabetes type II symptoms[3] and hereditary enzyme deficiency diseases.[4] Other significant biological activities associated with their glycosidase inhibitory properties are anti-viral, anti-cancer and anti-metastatic, anti-infective as well as insect anti-feedant and plant growth regulatory effects.[5] Recently, we found that various fluorescently labelled derivatives of compounds **1** and **2** are strikingly powerful glycosidase inhibitors exceeding the parent compounds' activities by two orders of magnitude. This, we believe, is due to the interaction of the dansyl moiety with the putatively lipophilic aglycon binding site. Consequently compounds **4**–**6** were deemed to be highly useful diagnostic tools for activity-based high-throughput-analyses as well as gel-staining.[6] Carbohydrate microarrays are becoming increasingly important tools in the postgenomic era for profiling of, for example, lectin binding affinities.[7] However, they are of limited use for profiling glycosidase specificities, since they are necessarily degraded upon interaction. A more appropriate alternative would be a specific glycosidase inhibitor chip. The concept of an inhibitor microarray has recently been shown for activity-based recognition of lipophilic enzymes.[8] Here we report the design of an iminoalditol chip and a proof-of-concept study by exposure of the microarray to β-glucosidase from *Agrobacterium* sp. (Abg).

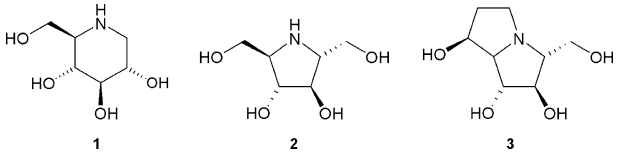

Figure 1. Typical structures of iminosugars.

Soluble D-glucosidase inhibitors **4a**[9] and **5a**[10] as well as D-mannosidase inhibitor **6a**[10] were conveniently transformed into the corresponding hydroxysuccinimide esters employing a two-step procedure outlined in Scheme 1: saponification of the methyl ester followed by reaction with TSTU (*O*-(*N*-succinimidyl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate) provided activated iminosugar moieties **4b**,[11] **5b**[12] and **6b**[13] ready for immobilisation by simple coupling to a starburst PAMAM polyamine surface chip.[14]

Keywords: Iminoalditol; Iminosugar chip; Immobilisation; Glycosidase inhibitor.
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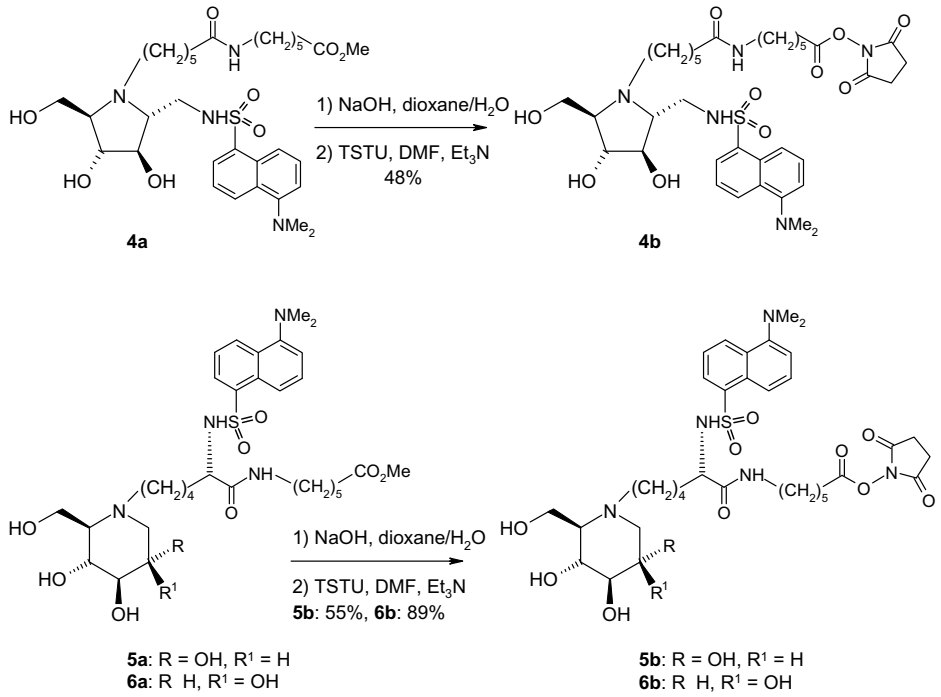
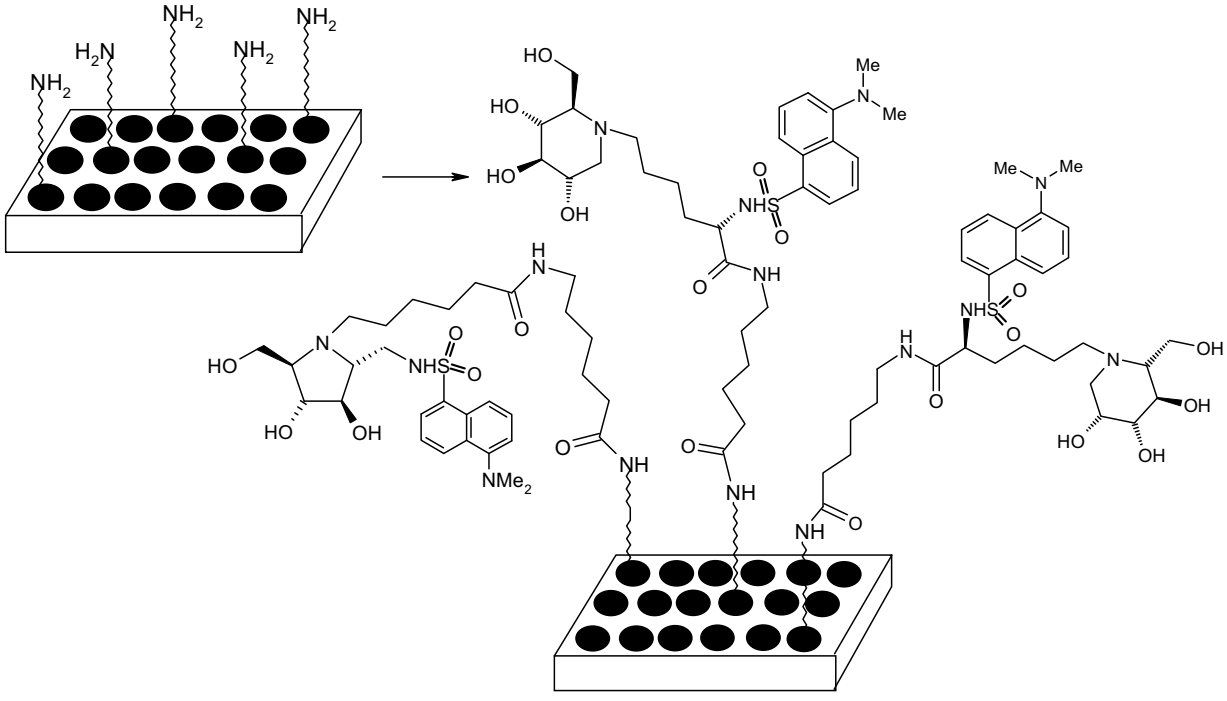


Scheme 1. Preparation of iminosugars **4b**, **5b** and **6b** for immobilisation.

In the example presented here, spots in lines 1–6 of the array contain compound **4b** spotted on, in duplicate lines at concentrations of 1000, 500 and 250 μg/mL. Compound **5b** was spotted in the same fashion in lines 7–12 and compound **6b** in lines 13–18 (Scheme 2).

In order to probe the relevant affinities of the different immobilised glycosidase inhibitors the chip was incubated with the β-glucosidase Abg from *Agrobacterium* sp. This well-characterised glucosidase was expected to strongly interact with immobilised inhibitors **4b** and **5b** but not with compound **6b** which is a highly selective D-mannosidase inhibitor exhibiting the wrong configuration at C-2 of the sugar ring.

Figure 2 shows the interactions with compounds **4b**–**6b** by the bacterial β-glucosidase, which was labelled with Alexa 647 for sensitive and convenient detection. Paralleling the enzyme inhibitory activities of the soluble par-



Scheme 2. Spotting of iminosugars to the chip.

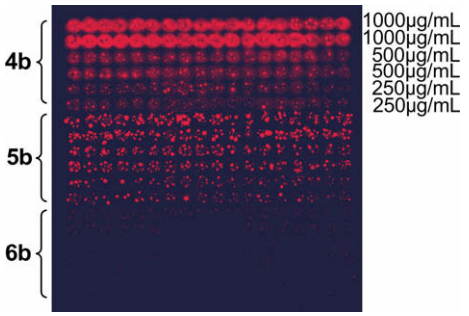


Figure 2. Iminosugar chip carrying compounds **4b**, **5b** as well as **6b** incubated with β-glucosidase from *Agrobacterium* sp. The top six rows show **4b** attached, in duplicate rows, at concentrations of 1000, 500 and 250 μg/mL. The next six rows are **5b** spotted identically and the bottom six rows are **6b** likewise.

Table 1. K_i values in (μM) of iminosugars with β-glucosidase from Abg for compounds **4a**–**6a** in solution[9,10]

Compound	K_i (μM)
4a	0.9
5a	6.2
6a	1000

ent compounds (**4a**–**6a**, Table 1), five membered ring **4b** displays strong interaction with this glucosidase. The same is true, albeit to a slightly lesser extent for iminoglucitol **5b**. As expected, D-mannosidase inhibitor **6b** did not give any appreciable binding with the glucosidase.

In conclusion we have immobilised three potent glycosidase inhibitors thereby constructing a first generation iminoalditol chip designed as a glycosidase detecting device. This proof-of-concept study employing a representative glucosidase nicely reflects the inhibitory properties of the ligands in solution thereby confirming the viability of the concept for the detection and characterisation of glycosidases. Likewise, inhibitor profiling can conveniently be achieved. Inhibitors of other configuration such as D-galactosidases, L-fucosidases and D-hexosaminidases should be amenable to the same approach. This will extend the scope of iminoalditol glycosidase inhibitor arrays to a wide range of diagnostic applications.

Acknowledgments

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11. NMR spectra were recorded on a Varian INOVA 500 operating at 500.619 MHz (^{1}H), and at 125.894 MHz (^{13}C) in methanol-d_4. Chemical shifts are listed in delta employing residual, non-deuterated solvent as the internal standard. MALDI mass spectra were recorded on a MALDI Micro MX (Waters) time-of-flight instrument used in reflectron mode with 2.3 m effective flight path. Analytical TLC was performed on precoated aluminium plates silica gel 60 F254 (E. Merck 5554), detected with UV light (254 nm), 10% vanillin/sulfuric acid as well as ceric ammonium molybdate (100 g ammonium molybdate/8 g ceric sulfate in 1 L 10% H_2SO_4) and heated on a hotplate. For column chromatography silica gel 60 (230–400 mesh, E. Merck 9385) was used. To a solution of **4a** (164 mg, 0.26 mmol) in dioxane/H_2O (10 mL, 1/1 (v/v)), 0.5 M aq NaOH (0.5 mL) was added at 0 °C and the resulting reaction mixture stirred at ambient temperature for 10 h. After neutralisation with ion exchange resin Amberlite IR-120 [H^+] (washed with water) and filtration, the solvents were removed under reduced pressure. The residue was dissolved in dry DMF (5 mL), triethylamine (45 μL, 0.32 mmol) and TSTU (85 mg, 0.28 mmol) were added and the reaction mixture was stirred at room temperature for 20 min. Removal of the solvent under reduced pressure and purification of the residue by column chromatography on silica gel ($CHCl_3$/MeOH v:v 8:1) yielded **4b** (90 mg, 0.13 mmol, 48%) as green syrup. ^{1}H NMR (500 MHz, CD_3OD) δ 8.56 (d, 1H, dansyl), 8.33 (d, 1H, dansyl), 8.20 (d, 1H, dansyl), 7.58 (m, 2H, dansyl), 7.27 (d, 1H, dansyl), 3.91 (m, 1H, $J_{3,4}$ 2.0 Hz, H-4), 3.86 (m, 1H, H-3), 3.64 (dd, 1H, $J_{6a,5}$ 4.9 Hz, H-6a), 3.58 (dd, 1H, $J_{5,6b}$ 3.4 Hz, $J_{6a,6b}$ 11.2 Hz, H-6b), 3.17 (t, 2H, H-6″), 3.02 (dd, 1H, $J_{1a,2}$ 2.9 Hz, $J_{1a,1b}$ 12.2 Hz, H-1a), 2.97–2.93 (m, 2H, H-1b, H-5), 2.87 (s, 6H, 2× NCH_3-dansyl), 2.89–2.85 (m, 1H, H-2), 2.82 (s, 4H, 2× CH_2succinimidyl), 2.62 (t, 2H, H-2″), 2.46 (m, 1H, H-6′), 2.27 (m, 1H, H-6′), 2.12 (t, 2H, H-5′), 1.72 (m, 2H, H-3′), 1.48 (m, 4H, H-3″, H-5″), 1.33 (m, 3H, H-5′, H-4″), 1.19 (m, 1H, H-5′), 1.06 (m, 2H, m, H-4′); ^{13}C

NMR (125 MHz, CD_3OD) δ 174.91 (C-1′), 170.2 (2C, succinimidyl), 152.1, 135.0, 130.2, 130.0, 129.8, 129.4, 128.1, 123.1, 119.3, 115.3 (dansyl), 79.5 (2C, C-3, C-4), 69.2, 68.2 (C-2, C-5), 59.3 (C-6), 46.4 (C-1), 44.7 (2C, NCH_3-dansyl), 41.2 (C-6′), 39.0 (C-6″), 35.9 (C-2′), 30.3 (C-2″), 28.7 (C-5″), 27.4 (C-5′), 26.7 (C-4′), 25.9 (C-4″), 25.6 (C-3′), 25.3(2C, succinimidyl), 25.1 (C-3″). MALDI TOF calcd. for $C_{34}H_{49}N_5O_{10}S$ 719.94, found 720.415.

12. To a solution of **5a** (121 mg, 0.185 mmol) in dioxane/H_2O (10 mL, 1/1 (v/v)), 0.5 M aq NaOH (1.0 mL) was added at 0 °C and the resulting reaction mixture was stirred at ambient temperature for 4 h. After neutralisation with ion exchange resin Amberlite IR-120 [H^+] (washed with water) and filtration, the solvents were removed under reduced pressure. The residue was dissolved in dry DMF (10 mL), triethylamine (90 μL, 65 mg, 0.64 mmol) and TSTU (61 mg, 0.20 mmol) were added and the reaction mixture stirred at room temperature for 30 min. Removal of the solvent under reduced pressure and purification of the residue by column chromatography on silica gel ($CHCl_3$/MeOH v:v 8:1) gave **5b** (75 mg, 0.10 mmol, 55%) as green syrup. ^{1}H NMR (500 MHz, CD_3OD) δ 8.57 (d, 1H, dansyl), 8.36 (d, 1H, dansyl), 8.21 (d, 1H, dansyl), 7.62 (t, 1H, dansyl), 7.58 (t, 1H, dansyl), 7.28 (d, 1H, dansyl), 3.87 (dd, 1H, $J_{5,6a}$ 2.4 Hz, $J_{6a,6b}$ 12.2 Hz, H-6a), 3.78 (dd, 1H, $J_{5,6b}$ 2.9 Hz, H-6b), 3.63 (m, 1H, H-2′), 3.52 (ddd, 1H, $J_{1eq,2}$ 4.9 Hz, $J_{1ax,2}$ 10.3 Hz, $J_{2,3}$ 8.8 Hz, H-2), 3.40 (dd, 1H, $J_{3,4}$ 9.3 Hz, $J_{4,5}$ 9.3 Hz, H-4), 3.34 (dd, 1H, H-3), 3.00 (dd, 1H, $J_{1eq,1ax}$ 11.2 Hz, H-1eq), 2.88 (s, 6H, 2× NCH_3-dansyl), 2.84 (s, 4H, 2× CH_2succinimidyl), 2.82–2.74 (m, 1H, H-6″a), 2.65 (m, 2H, H-6Cb, H-6′a), 2.57 (m, 1H, H-6′b), 2.55 (t, 2H, 2 × H-2″), 2.42 (dd, 1H, H-1ax), 2.35 (ddd, 1H, H-5), 1.64–0.98 (m, 12H, 2× H-3″, 2× H-4″, 2× H-5″, 2× H-3′, 2× H-4′, 2× H-5′). ^{13}C NMR (125 MHz, CD_3OD) δ 172.4 (C-1′), 170.8, 170.8 (succinimidyl), 169.1 (C-1″), 152.1, 135.5, 130.5, 129.9, 129.8, 129.6, 128.3, 123.3, 119.3, 115.4 (dansyl), 78.5 (C-3), 69.9 (C-4), 68.6 (C-2), 66.0 (C-5), 58.9, 56.9, 55.6, 52.2 (C-1, C-6, C-2′, C-6′), 44.7, 44.7 (dansyl), 38.7 (C-6″), 32.5 (C-3′), 30.3 (C-2″), 28.5 (C-5″), 25.4, 25.4 (succinimidyl), 25.7, 24.1, 23.0, 22.7 (C-3″, C-4″, C-4′, C-5′). MALDI TOF calcd. for $C_{34}H_{49}N_5O_{11}S$ 735.85, found 736.402.

13. To a solution of **6a** (182 mg, 0.279 mmol) in dioxane/H_2O (10 mL, 1/1 (v/v)), 0.5 M aq NaOH (1.0 mL) was added at 0 °C and the resulting reaction mixture stirred at ambient temperature for 16 h. After neutralisation with ion exchange resin Amberlite IR-120 [H^+] (washed with water) and filtration, the solvents were removed under reduced pressure. The residue was dissolved in dry DMF (14 mL), triethylamine (40 μL, 29 mg, 0.29 mmol) and TSTU (84 mg, 0.28 mmol) were added and the reaction mixture was stirred at room temperature for 30 min. Removal of the solvent under reduced pressure and purification of the residue by column chromatography on silica gel ($CHCl_3$/MeOH v:v 8:1) gave **6b** (182 mg, 0.247 mmol, 89%) as green syrup. ^{1}H NMR (500 MHz, CD_3OD) δ 8.56 (d, 1H, dansyl), 8.35 (d, 1H, dansyl), 8.21 (d, 1H, dansyl), 7.60 (t, 1H, dansyl), 7.57 (t, 1H, dansyl), 7.28 (d, 1H, dansyl), 4.00 (dd, 1H, $J_{5,6a}$ 2.4 Hz, $J_{6a,6b}$ 12.7 Hz, H-6a), 3.85 (m, 2H, H-2, H-6b), 3.66 (dd, 1H, $J_{3,4}$ 9.3 Hz, $J_{4,5}$ 9.3 Hz, H-4), 3.64 (m, 1H, H-2′), 3.50 (dd, 1H, $J_{2,3}$ 3.0 Hz, H-3), 2.88 (s, 6H, dansyl), 2.84 (s, 4H, 2× CH_2 succinimidyl), 2.80–2.70 (m, 3H, H-1eq, 2× H-6′), 2.67 (m, 2H, 2× H-6″), 2.56 (m, 1H, H-1ax), 2.15 (m, 3H, H-5, 2× H-2″), 1.64-1.08 (m, 12H, H-3″, H-4″, H-5″, H-3′, H-4′, H-5′). ^{13}C NMR (125 MHz, CD_3OD) δ 172.1 (C-1′), 170.8, (2C, succinimidyl), 169.0 (C-1″), 152.1, 135.5, 130.5, 129.9, 129.8, 129.6, 128.3, 123.2, 119.2, 115.3 (dansyl), 73.3, 66.4, 66.3 (C-2, C-3, C-4), 66.1 (C-5), 56.6, 56.6, 55.5, 52.7 (C-1, C-6, C-2′, C-6′), 44.7, (2C, NCH_3-dansyl), 38.7 (C-6″), 32.2 (C-3′), 30.3 (C-2″), 28.4 (C-5″), 25.3 (2C, succinimidyl), 25.6, 25.1, 24.0, 22.6 (C-3″, C-4″, C-4′, C-5′). MALDI TOF calcd. for $C_{34}H_{49}N_5O_{11}S$ 735.85, found 736.402.

14. Compounds **4b**, **5b** as well as **6b** were spotted in 30% DMSO saline sodium citrate buffer at pH 7.50 at 1000, 500 as well as 250 μg/mL using a Qu-pins (150 μm split pins, Genetix UK) at 21–24 °C and 25–37% humidity on dendrimer coated (starburst PAMAM dendrimer generation 5, that is, 128 primary surface amino groups) Slide E barcoded microarray slides (Schott Nexterion) in replicates of 36 (2 rows and 18 columns). β-Glucosidase from *Agrobacterium* sp. was labelled with ALEXA 647-NHS (Molecular Probes, Netherlands). Protein labelling and purification of the conjugate was performed as recommended by *Molecular Probes* (46 μL 1 M $NaHCO_3$ solution was added to 455 μL β-glucosdiase Abg. Therefore, 4.4 mg/mL in 50 mM P_i-buffer, equivalent to 2 mg, 39 nmol, protein in PBS and 400 nmol ALEXA-647-NHS reactive dye (10-fold excess) were dissolved in 15 μL DMSO and added to the ABG solution. After shaking at room temperature in the dark for 2 h, 50 μL 1.5 M hydroxylamine (pH 8.5) was added and the resulting solution was shaken in the dark for 1 h. Unbound dye was removed by passing the reaction solution through a Sephadex G-50 fine column equilibrated with PBS containing 0.9 NaN_3 as a preservative. Three fractions of purified, labelled Abg were collected containing different protein concentrations. Protocols can be downloaded from http://probes.invitrogen.com). Slides were incubated with 100 mU/mL of the labelled enzyme at 37 °C and the pH optimum of the respective enzyme for 2 h. After incubation the microarray was extensively washed with TBST (0.1% Tween-20) and fluorescence scanning was performed employing an ArrayWorx^e automicroarray scanner (Applied Precision, Issaquah, WA, USA). Scanner parameters: for Alexa 647: λ_{ex}: 635 nm; λ_{em}: 685 nm; exposure time 3 s, sensitivity: high dynamic range.