COMMUNICATION

An *in vitro-in vivo* sequential cascade for the synthesis of iminosugars from aldoses

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Here, we report a chemoenzymatic approach for the preparation of a small panel of biologically important iminosugars from readily available aldoses. Our approach involves an *in vitro* transaminase-mediated amination of aldoses in combination with an *in vivo* selective oxidation of the chemically protected transaminase products, using *Gluconobacter oxydans* DSM 2003 whole cells. Chemically catalysed deprotection and reduction steps afford a selection of valuable iminocyclitols.

Iminosugars or iminocyclitols are naturally occurring carbohydrate mimics, in which the endocyclic oxygen has been replaced by a nitrogen.[1-3] These small molecules are known inhibitors of several carbohydrate processing enzymes, but many have failed to reach the clinic due to insufficient potency or lack of selectivity.[4] Although synthetic routes have been established to access naturally occurring iminosugars and derivatives, the synthesis of these molecules remains complicated.[5-8] The absence of efficient synthetic routes for the preparation of structurally diverse derivatives has been highlighted as one of the key contributing factors to the limited development of second-generation iminosugars as therapeutics.[4] A robust biocatalytic route for the preparation of libraries of natural iminosugars and their derivatives has not yet been developed and has the potential to (i) greatly simplify and streamline their synthesis, (ii) allow access to derivatives that are currently inaccessible and (iii) provide a sustainable route from readily available carbohydrates.

The direct amination and subsequent selective oxidation of readily available aldoses, such as glucose, mannose, and fructose represents an ideal approach for the synthesis of chiral aminopolyols that can be readily converted to iminosugars and circumvents the need for petrochemical-derived feedstocks. We recently reported that transaminases (TAs) could be used to introduce amine functionality into aldoses to access a variety of aminopolyols.[9] A study showing activity on ketoses was reported shortly after.[10] Such activity had not been previously observed towards monosaccharides which exist predominantly in their cyclic form at equilibrium, where the carbonyl functionalities are predominantly masked. This opened a range of new synthetic possibilities for the preparation of high-value targets from chiral pool building blocks by using combinations of enzymes.

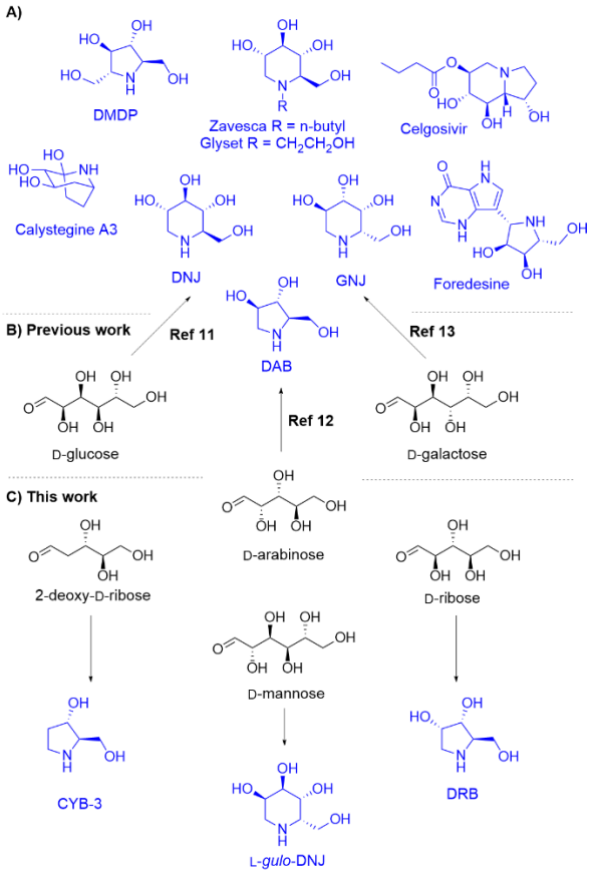

Figure 1. The chemo-enzymatic synthesis of piperidine and pyrrolidine iminosugars from aldoses. A) The structural diversity of natural and synthetic iminosugars is shown. Iminosugars with known biological activity are highlighted in blue. **B)** Previously reported syntheses of DNJ[11], DAB[12] and GNJ[13] from aldoses feature a key microbial oxidation by *Gluconobacter oxydans*. **C)** This work expands the methodology and substrate scope *via* a two-enzyme, four-step sequence. Aldoses are shown in open chain conformation.

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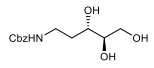
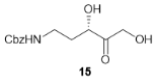
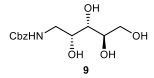
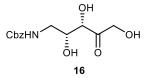
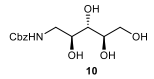
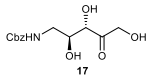
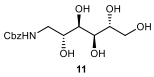
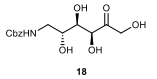
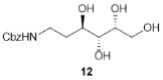
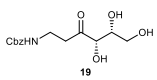
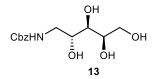
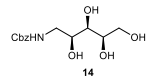
G. oxydans DSM 2003 has been previously reported to display activity towards aminopolyol substrates; analogous to those accessible from aldoses using TAs.[12] Similar to galactose oxidases and aldolases, *G. oxydans* has been shown to only accept aminopolyols where the amine functionality is protected.[14,15] The single enzyme chemo-enzymatic synthesis of iminosugars 1-deoxynojirimycin (DNJ), 1-galactonojirimycin (GNJ), and 1,4-dideoxy-1,4-imino-D-arabinitol (DAB), from D-glucose, D-galactose and D-arabinose respectively has been previously reported (Figure 1B), proceeding *via* a *G. oxydans*-mediated C4/C5 oxidation of the *N*-protected aldose-derived aminopolyols.[11-13] We sought to explore the substrate scope of *G. oxydans* DSM 2003 towards additional aminopolyols and develop methodology to enable a chemoenzymatic approach for the preparation of iminosugars, combining a TA-mediated amination with the oxidising capabilities of *G. oxydans* (Figure 1C).

To explore the substrate scope of *G. oxydans* DSM 2003, the aldoses 2-deoxy-D-ribose (**1**), D-arabinose (**2**), D-ribose (**3**), D-mannose (**4**), 2-deoxy-D-galactose (**5**), D-lyxose (**6**) and D-xylose (**7**) were selected, and the corresponding aminopolyols were chemically synthesized and *N*-Cbz-protected, to provide potential substrates **8-14** (Table 1). Whole-cell biotransformations were performed on a 13-15 mM scale and analysed by NMR to determine conversion. 1D and 2D NMR experiments were carried out to ascertain the site of oxidation. The *N*-Cbz-aminopolyols **8-12** were quantitatively converted to the oxidised products **15-19**. Selective oxidation to the corresponding ketone proceeds regioselectively, at position C4 for the pentose derivatives **8-10**, and at position C5 for hexose-derived **11** (Table 1). The oxidised products were isolated in good to excellent yields (purification was not optimised), with different ratios of linear and cyclic anomers observed. In general, the linear structures were characterised by a distinctive carbonyl peak around 212 ppm in the ^{13}C NMR spectrum, while the cyclic anomers contained hemiaminal peaks at approximately 90 ppm, where a mixture of anomers is most evident for **15**. The oxidised aminopolyols **16** and **17** exist predominantly in linear form, whereas **18** is only detectable as the cyclic acetal (see SI for more details). The cyclisation of 5-membered Cbz-protected aminopolyols has previously been reported.[15] While **12** was quantitatively converted by *G. oxydans*, oxidation took place at position C3 and product **19** was in equilibrium with its cyclic anomer. However, **19** proved to be unstable, resulting in a poor isolated yield of 15%, and was therefore not carried forward to the next step. The corresponding unprotected aminopolyols of **1**-**7** were also tested (data not shown). As expected, these unprotected derivatives were either not oxidised by the organism, or where oxidation occurred, it is thought that the product suffered from instability.[16]

Having established activity on a small panel of substrates, our focus turned to optimising the reaction conditions of the preparative scale biotransformations, focusing on substrates **8-11**. It is possible that more than one *G. oxydans* enzyme is responsible for catalysing these transformations and therefore

Table 1 Exploring the substrate scope of *G. oxydans* towards *N*-Cbz-protected aminopolyols **8-14**

Sugar[a]	Substrate	Product	Conv.[b]	Isolated yield [%]
1	 **8**	 **15**	Quantitative	68
2	 **9**	 **16**	Quantitative	54
3	 **10**	 **17**	Quantitative	63
4	 **11**	 **18**	Quantitative	95[d]
5	 **12**	 **19**	Quantitative	15
6	 **13**	n.d.[c]	n.d.	N/A
7	 **14**	n.d.	n.d.	N/A

Reaction conditions: 20 mL reaction mixture containing substrate (13-15 mM), resting *G. oxydans* DSM 2003 cells (100 mg mL^{-1} wet weight), H_2O (20 mL), pH 6.8, 16 h, 280 rpm, 30 °C. The reaction was performed exclusively in H_2O (20 mL) with initial pH 6.8. [a]*N*-Cbz-protected aminopolyols are derived from; 2-deoxy-D-ribose (**1**), D-arabinose (**2**), D-ribose (**3**), D-mannose (**4**), 2-deoxy-D-galactose (**5**), D-lyxose (**6**) and D-xylose (**7**). [b]Conversion determined by crude NMR analysis, monitoring consumption of starting material and appearance of product peaks. [c]n.d. = not detectable. [d]Compound exists predominantly in its cyclic form at equilibrium – the linear form is presented here to show the site of oxidation clearly.

the effect of varying the temperature and substrate concentration for each substrate was evaluated (Figure 2). In general, the organism maintained high activity over a wide range of temperatures. Next, the effect of substrate concentration on conversion was evaluated by running the transformations at concentrations between 5–50 mM, while keeping the concentration of wet cells constant (100 mg mL^{-1}). The cells converted all four substrates quantitatively to the corresponding oxidation products **15-18** at 20 mM (**8** and **9**) or 25 mM (**10** and **11**). Conversions of up to 90% and 77% were also achieved at 30 mM and 50 mM, respectively.

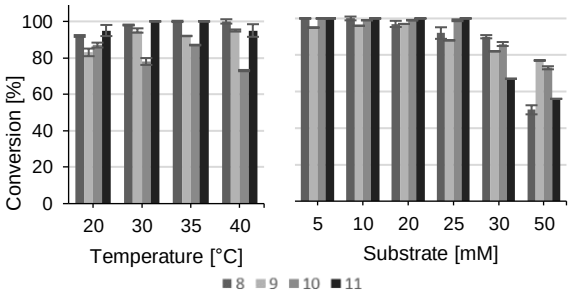


Figure 2. Analytical scale biotransformations to optimise temperature and substrate concentration for substrates 8-11. **Reaction conditions for temperature optimisation (left)**: 1 mL reaction mixture containing 10 mM substrate, resting cells of *G. oxydans* DSM

2003 (100 mg mL^{-1} wet weight), H_2O (1 mL), pH 6.8, 16 h, 280 rpm, temperature 20-40 °C**. Reaction conditions for substrate concentration optimisation:** 1 mL reaction mixture containing 5 – 50 mM substrate, resting cells of *G. oxydans* DSM 2003 (100 mg mL^{-1} wet weight), H_2O (1 mL), pH 6.8, 16 h, 280 rpm, temperature 30 °C (for 11) or 35 °C (for 8, 9 and 10). Conversion determined by HPLC-UV analysis, based on substrate consumption. All data are presented as a mean ± standard deviation. Three independent replicas of the analytical scale biotransformation experiments were performed in this study.

While it is clear that reaction temperatures between 20-40 °C have little impact on the conversion of **8-11** by *G. oxydans* (Figure 2), we observed that the organism was metabolising the oxidised products at 20 °C, as complete conversion was achieved, but isolated yields were often low (Table 2, entries 1-3). Additionally, the prolonged reaction time (>48 h) led to an almost complete disappearance of the product. Interestingly, the optimum growth temperature for *G. oxydans* is between 25–30 °C, while temperatures above 37 °C are known to inhibit growth. Bacterial growth and metabolism can be controlled by adjusting the temperature or pH or by applying osmotic pressure. We investigated the effect that performing biotransformations at 35 °C had on the isolated yields (Table 2, entries 5-8). Quantitative conversions were achieved for substrate **11**, while **8-10** were converted in 90–95% based on HPLC analysis. The oxidised products were purified using silica gel column chromatography in very good yields (65–89%) and the products isolated in multi-milligram quantities (70–138 mg). This minor temperature modification had a very positive effect on the biotransformations and may be a strategy that could be explored for other whole-cell systems, where substrates and/or products are being metabolised.

Table 2 Preparative-scale biotransformations with substrates **8-11**[a]

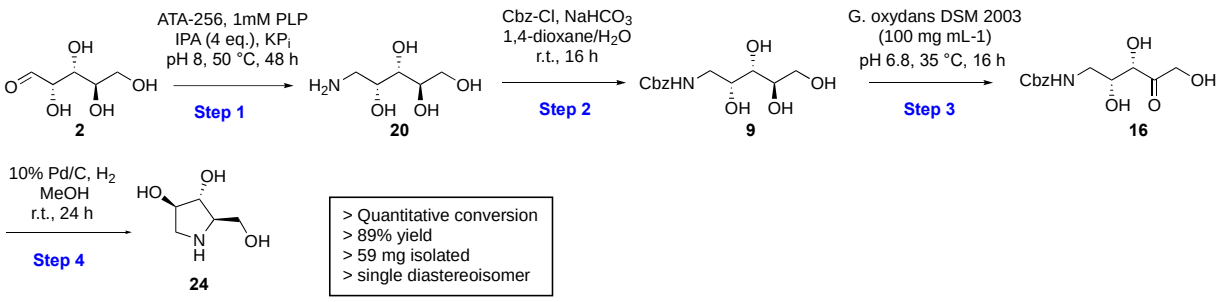
Entry	Substrate	Conc. [mM]	Temp [°C]	Conv.[b] [%]	Yield [%]	Isolated [mg]
1	**8**	20	20	97	46	49
2	**9**	20	20	95	42	47
3	**10**	25	20	93	53	74
4	**11**	25	20	>99	86	135
5	**8**	20	35	90	65	70
6	**9**	20	35	95	89	101
7	**10**	25	35	93	72	102
8	**11**	25	35	>99	88	138

[a]Reaction conditions: 20 mL reaction mixture containing substrate (20 or 25 mM), resting cells of *G. oxydans* DSM 2003 (2 g wet weight), H_2O (20 mL), pH 6.8, 16 h, 280 rpm, 20 °C or 35 °C. The reaction was exclusively performed in water (20 mL) with initial pH 6.8. [b]Conversion determined by HPLC-UV analysis, based on substrate consumption.

The next step involved concurrent deprotection, cyclisation and reduction using Pd/C as the hydrogenation catalyst. Upon deprotection, the amine reacts at the newly oxidised carbonyl site (C4 or C5) to form a polyhydroxylated cyclic imine with the elimination of water. The imine is reduced affording the target iminocyclitols **24**-**29** in 87–93% yield (Scheme 1/Table 3). The cyclic imines of **11** and **16** undergo a stereoselective reduction, yielding the single diastereoisomers, **29** and **24**, respectively. In the case of the oxidised aminopolyols **8** and **10**, the reduction is non-stereoselective and a mixture of diastereoisomers (**25 – 28**) is isolated (Table 3). The purification of diastereomeric mixtures was not undertaken in this work and is likely to be a challenge, due to the highly polar nature of these compounds. 2D NMR experiments (HSQC, HMBC, COSY, and NOESY) were used to structurally and stereochemically characterise each of the iminosugar isomers (see ESI).

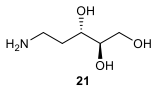
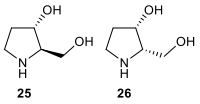
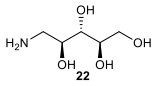
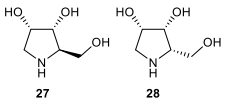
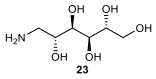
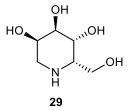
While the need to protect the aminopolyols currently prevents the design of a one-pot cascade, we were still keen to demonstrate that biologically important iminosugars could be readily prepared from monosaccharides using a chemoenzymatic approach. D-aldoses **1-4** were selected, which are precursors of the natural iminosugar products 2-hydroxymethyl-3-hydroxypyrrolidine **25** (CYB-3), DAB **24**, 1,4-dideoxy-1,4-imino-D-ribitol **27** (DRB), and L-1-deoxygulonojirimycin **29** (L-*gulo*-DNJ). The commercially available transaminase ATA-256 was used to convert these aldoses to the corresponding aminopolyols (Table 3). Biotransformations were carried out using 50–200 mM of substrate and 4 eq. of IPA as the amine donor.

Conversions were analysed by ^{1}H NMR spectroscopy using our previously reported method. Conversions ranged from 62–86%, and purification was carried out using ion-exchange column chromatography (Dowex 50WX8 resin, H^+ form). The transaminase-derived aminopolyols were then *N*-Cbz-protected before being subjected to *G. oxydans*-mediated oxidation. Excellent conversions and isolated yields were achieved with this microbial oxidation and the oxidised products were treated with Pd/C under an atmosphere of hydrogen, to afford the target iminosugars in excellent yield.



Scheme 1. The four-step, chemo-enzymatic cascade for the synthesis of DAB. Step 1 - Transamination of D-arabinose **2**. Step 2 - *N*-Cbz protection of aminopolyol **20**. Step 3 - *G. oxydans*-mediated selective oxidation at C4. Step 4 - concomitant deprotection, cyclization and reduction of a cyclic imine to yield **24**.

Table 3. The cascade applied to **1, 3** and **4** to yield iminosugars CYB-3 and DRB as a mixture of diastereomers, and L-*gulo*-DNJ **29** as a single diastereomer.

[a]	**Step 1** Transamination	Conv.: Yield: Isolated:	**Step 2** *N*-Cbz protection	**Products** after **steps** **3** & **4**	d.r.	Yield [%]	Isolated [mg]
1	 **21**	86% 61% 330 mg	**8**	 **25** **26**	1:1	87	35
3	 **22**	62% 34% 77 mg	**10**	 **27** **28**	1:2	87	52
4	 **23**	74% 65% 146 mg	**11**	 **29**	Single	93	74

Conclusions

Our work has highlighted *G. oxydans* DSM 2003 as a useful whole-cell catalyst for the selective oxidation of *N*-Cbz protected aminopolyols derived from aldoses, with an expanded substrate range. We demonstrated that *via* a four-step sequential cascade, featuring a biocatalytic transamination and oxidation, a range of valuable iminosugars could be accessed in excellent yields. While efforts are ongoing to find biocatalysts that can oxidise aminopolyols without the need for protecting group manipulations, this work takes an important step towards developing a robust approach for the preparation of valuable iminosugars.

Author Contributions

JK and FT carried our experiments and assisted with analysis. JR carried out experiments and also contributed to the directing of the research. KY contributed to the writing of the paper and supporting information. EOR was the PI and research director on the project and also contributed to the writing of the paper.

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Conflicts of interest

There are no conflicts to declare.

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