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FULL PAPER

Biocatalytic production of amino-carbohydrates through oxidoreductase and transaminase cascades

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Abstract: Plant-derived carbohydrates constitute an abundant renewable resource. Transformation of carbohydrates into new products, including amine-functionalized building blocks for biomaterial applications, can lower reliance on fossil resources. Herein, we demonstrate biocatalytic production routes to amino-carbohydrates, including oligosaccharides. In each case, we performed a two-step biocatalysis to functionalized D-galactose-containing carbohydrates, which employed either the galactose oxidase from *Fusarium graminearum* or a pyranose dehydrogenase from *Agaricus bisporus* followed by the ω-transaminase from *Chromobacterium violaceum* (*Cvi*-ω-TA). Formation of 6-amino-6-deoxy-D-galactose, 2-amino-2-deoxy-D-galactose and 2-amino-2-deoxy-6-aldo-D-galactose was confirmed by mass spectrometry. *Cvi*-ω-TA activity was highest towards 6-aldo-D-galactose, where highest yield of 6-amino-6-deoxy-D-galactose (67%) was achieved in reactions permitting simultaneous oxidation of D-galactose and transamination of the resulting 6-aldo-D-galactose.

Introduction

Given their wide availability and structural versatility, plant-derived carbohydrates represent an important raw material for the production of new bio-based products that reduce reliance on petroleum. To date, most applications of plant carbohydrates begin by deconstructing corresponding polysaccharides to monomers for fermentation to fuels and platform chemicals[1–5]. Bifunctional molecules containing a terminal acid and an amine functionality are among the desired products, because they are key building blocks in the synthesis of different types of polymers[6,7]. For example, diacids, diamines and AB monomers (e.g. molecules containing both the carboxylic acid and amino groups) are required for polyamide synthesis[8], and diacids and diols for polyester synthesis[9–13].

An emerging area of research aims to utilize the versatility and the ensuing useful properties of the native structures of plant based carbohydrates instead of degrading the structures to monomers[14–17]. Bifunctional carbohydrates (e.g., diacids, diamines or AB monomers) from native carbohydrate structures are among the desired products. Besides enzymatic synthesis of amino-sugars from activated sugar nucleotides and sugar phosphates[18–20], biocatalytic cascades to amino-carbohydrates from biomass-derived, native carbohydrates are still missing. If established, these pathways would create a new class of telechelic, amino-functionalized building blocks that retain inherent attributes of native carbohydrate structures (e.g., biocompatibility, hydrophilicity), while being primed for assembly (e.g., through stable amide linkages) with other building blocks or polymers with complementary functionalities (e.g. carboxyl groups)[15,21,22].

Existing chemical pathways for carbohydrate amination include applications of 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) to oxidize *primary* hydroxyl groups[23], and sodium periodate to oxidize *secondary* hydroxyl groups[24], followed by subsequent reductive amination to assemble the corresponding amines[25]. These routes, however, often require toxic transition metal catalysts, produce volatile organic compounds, and result in decreased polymer chain length[26]. As a gentle alternative to chemical oxidation procedures, the galactose oxidase from *Fusarium graminearum* (*Fgr*GaOx, UniProt: A0A2H3HJK8) has been used to introduce an aldehyde functionality at the C6 position of D-galactose in D-galactose-containing oligo- and polysaccharides [15,27,28]. Specificity of *Fgr*GaOx towards the C6 position of D-galactose has been well documented[29], and the resulting oxidized positions have served as sites for further derivatization, including reductive amination, crosslinking through acetal formation, and phosphorylation[15,30,31]. Our group recently described the application of the oligosaccharide oxidase from *Sarocladium strictum* to permit amide bond formation with clickable monomers, leading to telechelic molecules from xylo-oligosaccharides that are primed for reassembly through copper-catalyzed azide-alkyne cycloaddition[22]. Thus, while chemo-enzymatic routes to aminated carbohydrates have been demonstrated, fully biocatalytic cascades for amination of non-activated carbohydrates are unprecedented yet highly desirable in order to simplify reaction pathways, increase sustainability, and to achieve higher control over reaction products.

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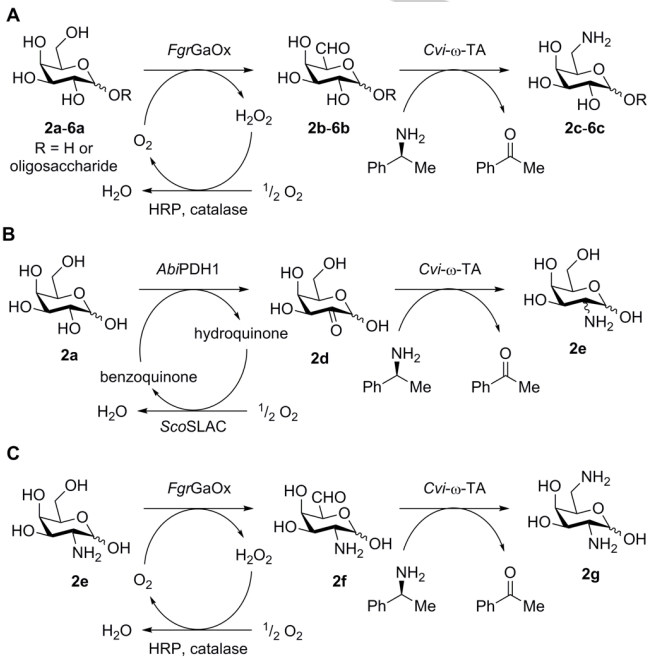
Transaminases are pyridoxal 5'-phosphate (PLP) dependent enzymes that catalyze the transfer of an amino group from *primary* amines acting as the amine donor to carbonyls acting as amine acceptor[32]. Briefly, transaminases operate via a ping-pong, bi-bi reaction, where the first half-reaction comprises the transfer of the amino group from the amine donor to the PLP cofactor. The deaminated amine donor (i.e. the respective ketone/aldehyde product) is then released, leaving the cofactor as pyridoxamine 5'-phosphate (PMP). In the second half reaction, the amino group is transferred from enzyme-bound PMP to the acceptor, regenerating the PLP cofactor and completing the transamination cycle. Transaminases have been functionally classified as α-transaminases (α-TAs) and ω-transaminases (ω-TAs), based on amine donor and acceptor specificity. Whereas α-TAs transfer amino groups from the α-carbon of amino acids to α-keto acids, ω-TAs are more versatile as they do not require a carboxylate group in the amine acceptor and can donate the amino group to α-keto acids as well as other ketones and aldehydes[32–34]. In addition to substrate versatility, no requirement for cofactor regeneration or nucleotide sugars as substrates are distinguishing advantages of ω-TAs relative to other types of enzymes potentially capable of producing carbohydrate amines (e.g. reductive aminases)[35–37]. So-called sugar transaminases have been shown to accept nucleotide sugars as amine donors and acceptors[19,20]; however, these are not suitable for amination of plant-based carbohydrates.

Amines in general are greatly underrepresented in renewable biomass, when compared to the frequent need of amines in chemical and polymer synthesis[38,39]. This makes biocatalytic production of amines from alcohols highly desirable, yet challenging, because no known single enzyme can catalyze such a transformation, and the chemical or chemo-enzymatic methods involving oxidation and reductive amination often involve toxic chemicals and require complicated synthetic procedures[40–42]. To date, ω-TAs have been studied extensively for asymmetric synthesis of pharmaceuticals, which has been summarized in several excellent reviews[41,43–45]. In that context, enzymatic pathways from *primary* and *secondary* alcohols to the corresponding amines, utilizing oxidases or alcohol dehydrogenases coupled with an ω-TA have been demonstrated[46–48]. By contrast, application of ω-TAs for bioproduct development from renewable biomass has only been investigated in a few studies.[49,50]. For example, Lerchner *et al.* (2013) showed the two-step conversion of isosorbide to the corresponding diamine using an alcohol dehydrogenase and an ω-TA[49,51]. In another, more recent study, Dunbabin *et al.* (2017) demonstrated transaminase-catalyzed production of furfurylamines from furfurals[52]. On the other hand, the ability of *Fgr*GaOx to oxidize C-6 hydroxyls on galactose-containing mono- oligo- and polysaccharide substrates has been shown to be an efficient way to produce aldehyde-functionalized carbohydrates[27,28], which might be accepted by ω-TAs. Moreover, carbohydrate oxidoreductases with different regio- and substrate specificities beyond the oxidation of the *primary* C-6 hydroxyl group (e.g., pyranose dehydrogenases) can help extend the range of available carbonyl-containing carbohydrates to ketone-functionalized carbohydrates, which are also potential substrates for ω-TAs[53–55].



Scheme 1. Biocatalytic cascades to aminated carbohydrates. (A) Oxidation of a D-galactosyl subunit on a carbohydrate molecule to 6-aldo-D-galactosyl (**2b-6b**; Table S1 for 3b-6b structures) by *Fgr*GaOx and the subsequent amination of the aldehyde group to 6-amino-6-deoxy-D-galactosyl (**2c-6c**) by the *Cvi*-ω-TA. (B) Oxidation of D-galactose (**2a**) to 2-keto-D-galactose (**2d**) by *Abi*PDH1[53], and the subsequent amination of the ketone **2d** to the amine **2e** by the *Cvi*-ω-TA. (C) Oxidation of **2e** by *Fgr*GaOx to the bifunctional intermediate **2f**, and the putative amination of the aldehyde group to the diamine **2g** by the *Cvi*-ω-TA. R = remaining oligosaccharide. Note: whereas the α-configuration of galactose is drawn, both α- and β-isomers occur. The conformation of the C-2 amino group in reaction product **2e** is unknown. Chiral (*S*)-(−)-PEA was used instead of a racemic mixture due to the strict stereoselectivity of the *Cvi*-ω-TA[56]. Abbreviations: *Abi*PDH1, pyranose dehydrogenase from *Agaricus bisporus*; *Cvi*-ω-TA, ω-TA from *Chromobacterium violaceum*; *Fgr*GaOx, galactose oxidase from *Fusarium graminearum*; HRP, horseradish peroxidase from horseradish; *Sco*SLAC, small laccase from *Streptomyces coelicolor*.

The ω-TA from *Chromobacterium violaceum* (*Cvi*-ω-TA, UniProt: Q7NWG4) is recognized as having a broad substrate range, and has activity towards hydroxylated aldehydes such as D-erythrose (**1**), glycolaldehyde and glyceraldehyde[47,48,56]. In the present study, *Cvi*-ω-TA was investigated for its potential to aminate aldol- and keto-carbohydrates initially formed through oxidation by *Fgr*GaOx or the pyranose dehydrogenase from *Agaricus bisporus* (AbiPDH1, UniProt: Q3L1D1)[53], respectively. *Cvi*-ω-TA activity on oxidized carbohydrates was also compared against the M1 variant of the ω-TA from *Vibrio fluvialis* (*Vfl*-ω-TA) (Uniprot: F2XBU9) engineered by the Bornscheuer group for improved preference towards substrates other than pyruvate, and generally improved activity in the neutral pH range[57]. Our analysis demonstrates biocatalytic cascades to aminated cyclic carbohydrates, including oligosaccharides (Scheme 1). Corresponding pathways generate a new class of renewable

telechelic molecules that were missing from the arsenal of building blocks to new bio-based polymers.

Results and Discussion

Activity of *Cvi*-ω-TA on oxidized D-galactose and D-galactosamine

The yields of *Fgr*GaOx and *Abi*PDH1 produced in *Pichia pastoris* were 108 mg/L and 3.9 mg/L, respectively, whereas the yields of *Cvi*-ω-TA and *Vfl*-ω-TA M1 produced in *E. coli* were 115 mg/L and 98 mg/L, respectively (Figure S1 in Supporting Information). These values are in the same range as previous reports describing the recombinant production of these enzymes[58,59].

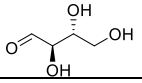
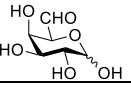
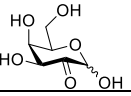
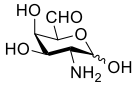
Activity of *Cvi*-ω-TA on the oxidized carbohydrates produced by *Fgr*GaOx or *Abi*PDH1, and on pyruvate and D-erythrose (**1**), was measured using the acetophenone assay[60]. To our delight, *Cvi*-ω-TA exhibited significant activity on the aldehyde **2b** (160 ± 1 U/g), which, while lower than *Cvi*-ω-TA activity measured on pyruvate (2995 ± 90 U/g), was in the same order of magnitude as that on **1** (a preferred substrate of *Cvi*-ω-TA) (Table 1; Scheme 1)[56]. Importantly, hydrate and oxidized derivatives of **2b** will form in the reaction mixture, effectively lowering the concentration of the intermediate aldehyde **2b** in reactions containing D-galactose (**2a**), compared to pyruvate and **1**[28]. *Abi*PDH1 was previously shown to primarily target the C2 position of **2a**[53], suggesting that 2-ketogalactose (**2d**) served as the main substrate for subsequent transamination by *Cvi*-ω-TA. Although the activity of *Cvi*-ω-TA on **2d** was about 30% of that measured using **2b** (Table 1), *Cvi*-ω-TA also showed activity on this sugar substrate yielding the amine **2e** in the multi-enzyme cascade (Figure 1B). It is worth mentioning that, despite a minor fraction of **2a** expected to be in the open chain conformation, transamination of the C1 aldehyde was not observed through the acetophenone assay, nor were the corresponding products detected by HPAEC-PAD or mass spectrometry. Activity of *Vfl*-ω-TA M1 towards the *Fgr*GaOx and *Abi*PDH1 products was tested, but was found to be less than 10% of that of *Cvi*-ω-TA, which is why experiments with *Vfl*-ω-TA M1 were not continued.

The purified *Fgr*GaOx and *Cvi*-ω-TA, or *Abi*PDH1 and *Cvi*-ω-TA, were then tested in combination to establish a two-step pathway to amino-carbohydrates. Specifically, **2a** was oxidized to the aldehyde **2b** by *Fgr*GaOx and then treated with *Cvi*-ω-TA in an attempt to produce the amine **2c**. Alternatively, D-galactose was oxidized to ketone **2d** by *Abi*PDH1 and then treated with *Cvi*-ω-TA in an attempt to produce the amine **2e**. In the case of each sequential reaction, a near quantitative yield for the oxidation of **2a** was confirmed by TLC before initiating the transaminase reaction (data not shown).

Mass spectrometric ESI-Q-TOF analysis verified the enzymatic production of both amines **2c** and **2e**. The masses of protonated and sodiated ion adducts of amines **2c** (Figure 1A) and **2e** (Figure 1B), as well as the expected isotopes, were all found in corresponding reactions, confirming their production through the oxidoreductase-transaminase cascade reactions.

Having confirmed the production of **2e**, we ventured to produce the diamine **2g**, which is expected to permit carbohydrate coupling through imine bond formation[61]. The activity of *Fgr*GaOx on 50 mM **2e** was determined with the ABTS assay to be 50.1 ± 4.9 U/mg enzyme, which is about 10% of *Fgr*GaOx activity on **2a**. Formation of the corresponding bifunctional intermediate product **2f** was confirmed by ESI-Q-TOF (Figure 1C), and near complete conversion in the subsequent transaminase reaction was verified by HPAEC (Figure S3 in Supporting Information). Despite this, diamine **2g** was not detected by mass spectrometry, possibly due to the formation of unknown adducts or side reactions. While depletion of intermediate **2f** through formation of imine derivatives can not be ruled out, *Cvi*-ω-TA accepted **2f** as a substrate as measured using the acetophenone assay (Table 1).

Table 1. Colorimetric activity assay of *Cvi*-ω-TA on selected substrates.

Substrate	Structure	Activity ± SD (U/g)[a]
D-Erythrose (**1**)		700 ± 20
6-Aldo-D-galactose (**2b**)		160 ± 1
2-Keto-D-galactose (**2d**)		45 ± 1
2-Amino-2-deoxy-6-aldo-D-galactose[b] (**2f**)		60 ± 3

[a] Reaction conditions: V = 200 μL, 10 mM amine acceptor substrate, 10 mM 1-PEA, 20 μM PLP, 30 μg (2.9 μM) *Cvi*-ω-TA in 50 mM HEPES-NaOH buffer (pH 7.5) at 37 °C and 700 rpm. [b] Activity on **2f** was measured at an amine acceptor concentration of 5 mM due to the high background absorbance of the substrate at 245 nm. All measurements were conducted in triplicate at minimum.

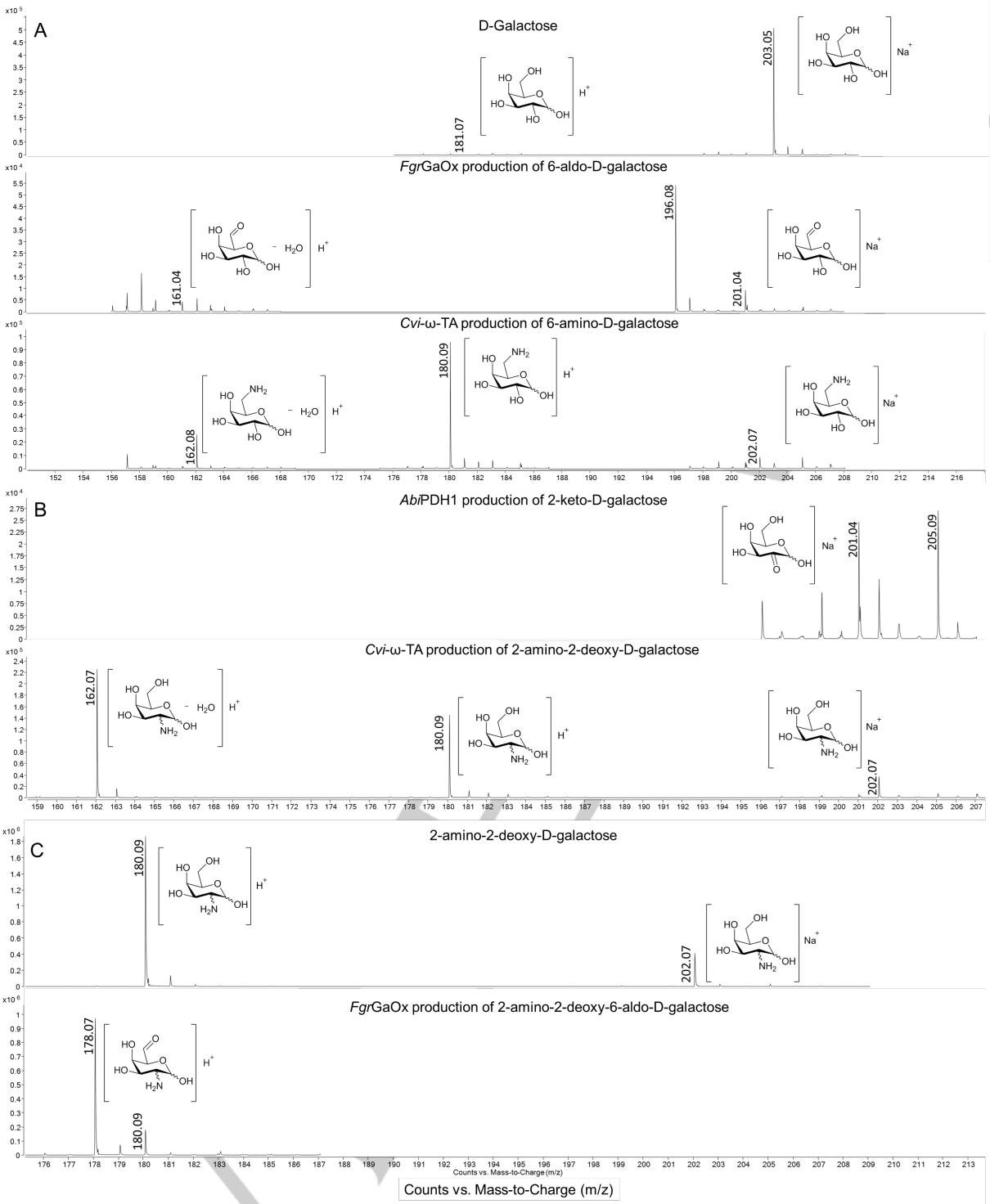


Figure 1. ESI-Q-TOF mass spectra of conversion of A) D-galactose (**2a**) to 6-amino-6-deoxy-D-galactose (**2c**) expected from sequential action of *Fgr*GaOx and *Cvi*-ω-TA; B) D-galactose (**2a**) to 2-amino-2-deoxy-D-galactose (**2e**) expected from sequential action of *Abi*PDH1 and *Cvi*-ω-TA; C) the expected 2-amino-2-deoxy-D-galactose (**2e**) from (B) to 2-amino-2-deoxy-6-aldo-D-galactose (**2f**) through action of *Fgr*GaOx. Similar spectra were collected from each of the three reaction replicates.

Quantification of 6-amino-6-deoxy-D-galactose from D-galactose

The comparison of different oxidized forms of **2a** showed that highest *Cvi*-ω-TA activity was obtained using the aldehyde **2b**

(Table 1). Therefore, the sequential, two-step enzymatic conversion of **2a** to the amine **2c** was followed by HPAEC to quantify product formation (Figure 2).

As previously reported[28,29,62],oxidation of **2a** by *Fgr*GaOx generated several different derivatives of the aldehyde group, including the hydrate (geminal diol) and the corresponding uronic acid due to further oxidation (data not shown). Accordingly, **2a** and the chemically synthesized **2c** were used to generate standard curves to calculate substrate depletion in the oxidation reaction and product formation in the amination reaction (Figure S2 in Supporting Information). Nearly all (95 ± 2 mol%) of **2a** was depleted in the sequential oxidation and amination reaction, where the formation of **2c** from **2a** was 18 ± 2 mol% prior to any optimization. Notably, calculating the formation of **2c** based on the consumption of **2a** inevitably underestimates the efficiency of the amination step as side reactions can occur after formation of the **2b** intermediate[28], and so not all of this intermediate is available for the desired transamination step. In an attempt to reduce the formation of side products from **2b** by reducing the time **2b** remains in aqueous solution, the sequential, two-step enzymatic reactions were instead performed simultaneously. Remarkably, performing the oxidization and transamination steps simultaneously increased the formation of **2c** by nearly 2.5 times (Table 2). A similar relative increase from sequential to simultaneous reaction was observed when using L-alanine as the amine donor, but the yields obtained using L-alanine were roughly 10 times lower than when using 1-PEA as amine donor. This was consistent with the unfavorable equilibrium for this reaction [47,48, 63]. Also notable, increasing PLP cofactor concentration from 20 μM to 1 mM only moderately increased product yields in sequential reactions (Table 2) Because isopropylamine (IPA) is the preferred amine donor used by industry to push reaction equilibria towards the aminated product due to its cheap cost and facilitated acetone by-product removal [63], IPA was tested herein as a means to further increase the formation of **2c** from **2b**. However, substituting 1-PEA for IPA resulted in undetectable product formation (data not shown), consistent with the comparatively high sensitivity of *Cvi*-ω-TA to IPA[64].

Table 2. Influence of reaction set-up on the formation of 2-amino-2-deoxy-D-galactose (2c) from D-galactose (2a)

Amine Donor	PLP Concentration	% mol of product (**2c**) formation	
		Sequential Reaction[a]	Simultaneous Reaction[b]
1-PEA	20 μM	18 %[c]	N/A[d]
1-PEA	1 mM	27 %	67 %
L-Ala	1 mM	2.5 %	6.5 %

Reaction conditions: 50 mM HEPES buffer containing 10 mM D-galactose (**2a**), 10 mM amine donor (1-PEA or L-Ala), 20 μM or 1 mM PLP. Enzyme concentrations were 0.44 μM *Fgr*GaOx, 0.53 μM catalase, 0.12 μM HRP, and 2.9 μM *Cvi*-ω-TA. [a] Sequential reactions proceeded for 4 h + 1.5 h for the oxidation and transamination steps, respectively. [b] Simultaneous reactions proceeded for 5.5 h. Product (**2c**) formation was quantified by HPAEC-PAD. [d] Data not available.

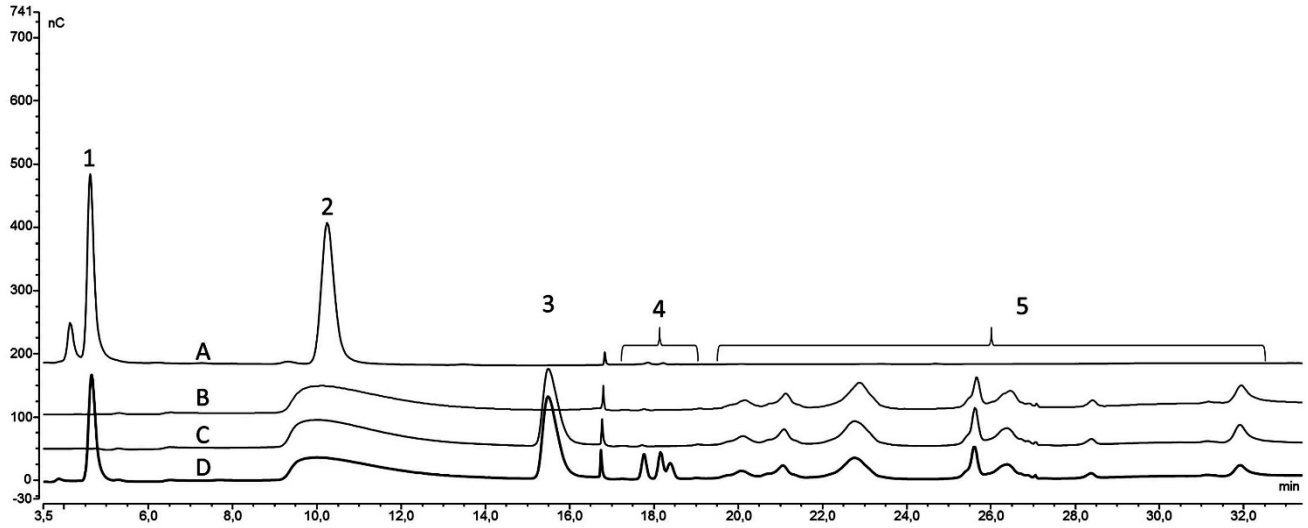


Figure 2. Staggered HPAEC-PAD chromatograms tracking the conversion of **2a** to **2c**. A) **2a** and **2c** standards in ddH_2O; B) *Fgr*GaOx treatment of 20 mM **2a** in ddH_2O (4 h at 30 °C, 700 rpm); C) Control experiment: incubation of the oxidation products (i.e. B) under the conditions of the transaminase reaction but without the addition of transaminase, D) *Cvi*-ω-TA treatment of 10 mM oxidation products containing the aldehyde **2b** (i.e. B) (1.5 h at 37 °C, 700 rpm in 10 mM 1-PEA, 20 μM PLP, 50 mM HEPES-NaOH (pH 7.5)). Prior to analysis, samples were diluted so that the total of the concentrations of **2a**, along with oxidation and amination products, were 90 μg/mL. 1 = amine **2c** (t_R = 4.7 ± 0.1 min); 2 = **2a** in A (t_R =10.3 ± 0.1 min) and overlapping peaks of D-galactose oxidation products in B, C and D; 3 = HEPES; 4 = side products formed in the transaminase reaction; 5 = derivatives formed during the oxidation reaction (t_R between 19.5-33.0 min).

Activity of *Cvi*-ω-TA on selected oxidized 6-aldo-D-galactosyl-containing carbohydrates

In addition to monosaccharide substrates, *Cvi*-ω-TA was tested on a series of D-galactose containing oligosaccharides, each first oxidized using *Fgr*GaOx. As done for **2a**, near quantitative oxidation of each oligosaccharide was confirmed by TLC (data not shown) and subsequent *Cvi*-ω-TA activity on each oxidized carbohydrate was measured with the acetophenone assay using 1-PEA as the amine donor.

Cvi-ω-TA activity was detected using all tested oxidized oligosaccharides generated using *Fgr*GaO, including aldo-melibiose (20 ± 4 U/mg), aldo-lactose (50 ± 6 U/g), aldo-raffinose (50 ± 3 U/g) and aldo-xyloglucan oligosaccharides (32 ± 4 U/g). While mass spectrometry options must be optimized to unequivocally confirm the identity of resulting products, substrate docking studies showed that all investigated saccharides dock similarly, with the catalytically relevant aldehyde group orientated towards the catalytically active exocyclic amino group of PMP (Figure S4 in Supporting Information). Notably, comparison with the docked aldo-xyloglucan oligosaccharide and the crystal structure of the Vfl-ω-TA (PDB ID: 4E3Q) shows the beneficial architecture of the active site of *Cvi*-ω-TA towards oligosaccharides, since the access to the active site of *Cvi*-ω-TA is more exposed (Figure S5 in Supporting Information).

Conclusions

We demonstrate the application of two different, fully biocatalytic, cascades that employ carbohydrate oxidoreductases to transform specific hydroxyl groups to carbonyls, and *Cvi*-ω-TA to introduce amine functionalities at the oxidized positions of the substrates. The pathways produced amino-galactoses with two different regioselectivities: (1) the combination of *Fgr*GaOx and *Cvi*-ω-TA yielded galactose derivatives aminated at the C-6 position, while (2) the combination of *Ab*PDH1 with *Cvi*-ω-TA yielded galactose derivatives aminated at the C-2 position. Production of 6-amino-6-deoxy-D-galactosyl-containing oligosaccharides through pathway 1 was also detected using the acetophenone activity assay. Notably, a multi-step synthetic route was required to synthesise the aminogalactose derivative used as an analytical standard in the current study, further highlighting the benefits of the biocatalytic approach. Steps taken to maximize product formation included (1) establishing a simultaneous oxidation plus transaminase reaction to the aminated carbohydrate, (2) increasing PLP concentration, and (3) testing of different amine donors. Greatest gains in product (**2c**) formation were achieved when performing the oxidation and transamination steps simultaneously rather than sequentially, consistent with reduced formation of undesired side-products from the aldehyde intermediate (**2b**)[28]. This work takes the first step in unlocking the potential of ω-TAs for carbohydrate functionalization, and therefore expands the pool of building blocks available for new bio-based materials.

Experimental Section

Materials

Yeast extract, yeast nitrogen base and peptone were purchased from Lab M Ltd. (UK). D-galactose, lactose, melibiose and raffinose were of analytical grade and purchased from Sigma Aldrich. Xyloglucan oligosaccharides (hepta+octa+nona saccharides) were purchased from Megazyme (O-XGHON; Lot number 20509). 1,2,3,4-Di-*O*-isopropylidene-α-D-galactose, used for preparing the synthetic 6-amino-6-deoxy-D-galactose used as a standard for product quantification, was purchased from Alfa Aesar. All other chemicals were reagent grade and obtained from Sigma Aldrich (Germany) and were used without further purification unless otherwise specified.

Production and purification of *Fusarium graminearum* galactose oxidase (*Fgr*GaOx) and *Agaricus bisporus* pyranose dehydrogenase (*Abi*PDH1)

F. graminearum (*Fgr*GaOx; D-galactose: oxygen 6-oxidoreductase, EC 1.1.13.9, CAZy family AA5_2) and *A. bisporus* pyranose dehydrogenase (*Abi*PDH1; pyranose:acceptor oxidoreductase, EC 1.1.99.29 CAZy family AA3_2) were heterologously expressed in *Pichia pastoris* KM71H. Genes (GenBank accession number: AH005781.2 coding *Fgr*GaOx; KM851045 *Abi*PDH1) with C-terminal 6xHis-tags were obtained from GenScript (New Jersey, US) subcloned into pPICZαA or pPICZB vectors, respectively. Both enzymes were produced in shake flask cultivations, as previously described[69]. Briefly, precultures were grown in up to 750 mL of buffered glycerol-complex medium (BMGY; 100 mM potassium phosphate buffer, pH 6.0, 2% (w/v) peptone, 1% (w/v) yeast extract, 4 × 10-5% (w/v) biotin, 1% (v/v) glycerol) at 30 °C, 220 rpm. Methanol induction was performed over 4 days at 25 °C, 220 rpm, in buffered methanol-complex medium (BMMY with 0.5% (v/v) methanol), where 0.5% (v/v) methanol was added every 24 h to replenish the inducer.

After induction and spinning down the cells, the supernatant was recovered, adjusted to pH 7.4 and filtered through a Sterivex-GP 0.45 μm PES filter unit (Millipore, Germany). The filtrate was loaded directly to 6 mL Ni-NTA resin (Qiagen, Germany) equilibrated in binding buffer (50 mM sodium phosphate buffer, pH 7.4, 500 mM NaCl, 20 mM imidazole) and packed in a XK-16/10 column (GE Life Sciences, Germany). Bound protein was eluted using a linear gradient of 0-100% Ni-NTA elution buffer (50 mM sodium phosphate, pH 7.4 with 500 mM imidazole, 500 mM NaCl). Purified fractions were then exchanged to 50 mM phosphate buffer (pH 7.5) using a 10 or 30 kDa Vivaspin 20 spin column (Sartorius AG, Germany).

*Fgr*GaOx and *Abi*PDH1 were concentrated to 13.5 mg/mL and 1.8 mg/mL, respectively, and stored at −80 °C until further use. Protein concentration was measured using the Bradford method (Bio-Rad Laboratories, US), and protein purity was assessed by SDS-PAGE (Figure S1 in Supporting Information).

Production and purification of the ω-TAs (*Cvi*-ω-TA and *Vfl*-ω-TA M1)

A pET29a+ plasmid containing the *Cvi*-ω-TA gene (GenBank: AAQ59697.1) obtained from GenScript, and the pET24b plasmid encoding *Vfl*-ω-TA M1[57], were transformed into chemically competent *E. coli* BL21. Selected *E. coli* transformants containing each plasmid were grown at 37 °C, 220 rpm in shake flasks containing 250 mL LB medium supplemented with 50 μg/ml kanamycin and 30 μg/ml chloramphenicol. When the OD600 reached 0.8–1, the *E. coli* transformant was induced to express the protein of interest by addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After 15 h of induction at 30 °C, the cells were harvested by centrifugation (5,000 g and 4 °C for 45 min). For each production, the cell pellet was suspended in 50 mL 50 mM potassium phosphate buffer (pH 7.5) containing 0.1 mM PLP and lysed using an Emulsiflex-C3 French press (Avestin Inc., Canada) at 10,000 PSI for 20 min.

Immediately after cell lysis, the lysates were clarified by centrifugation at 15,000 g, 4 °C for 45 min. The supernatants each containing the soluble protein were filtered through Sterivex-GP 0.22 μm PES filter units (Millipore). *Cvi*-ω-TA and *Vfl*-ω-TA M1, each containing a C-terminal His tag, were purified to homogeneity using Ni-NTA resin as described above, except this time 0.1 mM PLP was added to the binding buffer and elution buffer. Purified fractions were then exchanged to 50 mM phosphate buffer (pH 7.5) with 0.1 mM PLP using a 10 kDa Vivaspin 20 spin column (Sartorius AG, Germany). *Cvi*-ω-TA was concentrated to 9.5 mg/mL and *Vfl*-ω-TA M1 to 3.3 mg/mL, and then stored at −80 °C until further use. Protein concentration was measured using the Bradford method (Bio-Rad Laboratories, US), and protein purity was assessed by SDS-PAGE (Figure S1 in Supporting Information).

Galactose oxidase assay

The activity of *Fgr*GaOx was measured using the chromogenic ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) assay, originally described by Baron et al.[70], with modifications. The standard reaction mixture (final volume: 200 μL) contained 270 μg (31 μM, assuming purity) horseradish peroxidase (HRP, from horseradish, Sigma-Aldrich, Germany), 2 mM ABTS, and 50 mM **2a** in 50 mM HEPES (4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid) buffer (pH 7.5); reactions were initiated by adding 5 μL of appropriately diluted enzyme sample to obtain initial rates of reaction. Absorbance was measured at 420 nm at 30 °C for 20 min in an Eon microplate reader (BioTek™, US). Activity values were calculated based on the extinction coefficient (36,000 $M^{-1}cm^{-1}$ at 420 nm)[71]. Each reaction was performed in triplicate, at minimum.

Enzymatic production of oxidized carbohydrates

Enzymatic oxidation of D-galactosyl-containing substrates using *Fgr*GaOx (reaction volume 3 or 5 mL) was performed in 15 mL Cellstar tubes (Greiner BioOne) containing 20 mM D-galactose-equivalent of substrates (i.e., D-galactose, melibiose, lactose, raffinose, or xyloglucan oligosaccharides), 150 μg (0.44 μM)

*Fgr*GaOx, 160 μg (0.53 μM, assuming purity and based on molecular weight of the catalase monomer) catalase (from bovine liver, Sigma-Aldrich, Germany) and 27 μg (0.12 μM) HRP in 50 mM HEPES-NaOH (pH 7.5). Catalase was used as the primary means of removing hydrogen peroxide in the cascade, while HRP was included due to its known ability to activate *Fgr*GaOx[72]. The effect is based on the ability of the horseradish peroxidase to maintain the copper radical in the active site of *Fgr*GaOx in the correct oxidation state (II)[73]. The enzyme concentrations were chosen based on previous experience to achieve maximum oxidation of the galactosyl residues in each substrate[27,28]. Reaction mixtures were incubated for 4 h at 30 °C, with shaking (700 rpm). Conversion of each oxidation reaction was evaluated by TLC (data not shown) on Macherey-Nagel pre-coated silica gel plates (TLC Silica gel 60 F254). *Abi*PDH1 oxidation of D-galactose was performed as described by Sygmund and coworkers[74], with minor modification. Specifically, reaction mixtures contained 58 μg (3.61 μM) of *Abi*PDH1, 50 mM D-galactose, 5 mM benzoquinone in ddH_2O. The small laccase (*Sco*SLAC; 260 μg, 32.5 μM) from *Streptomyces coelicolor* was used to recycle the electron acceptor (benzoquinone) as shown in Scheme 1[75,76].

ω-TA reactions and activity assays

After verifying that the oxidation reaction had reached maximum conversion (evaluated by TLC, data not shown), the activity of the purified *Cvi*-ω-TA towards oxidized carbohydrates was measured using chromogenic detection of acetophenone from 1-phenylethylamine (1-PEA)[60]. Activity of *Vfl*-ω-TA M1 towards aldehyde **2b** was also measured. Briefly, transaminase reactions (200 μL final volume) were carried out at 37 °C in 96-well microtiter plates (96-Well UV Microplate, Thermo Scientific, US) incubated in a plate reader (Biotek Eon, US). The reaction mixture contained 10 mM 1-PEA (i.e. amine donor substrate), up to 10 mM **2b** or 6-aldo-D-galactosyl groups in oligosaccharide substrates, 20 μM PLP, and 30 μg (2.9 μM) of purified *Cvi*-ω-TA or *Vfl*-ω-TA M1. The reaction was buffered with 50 mM HEPES-NaOH (pH 7.5). In sequential reactions, products of the oxidation reactions were formed over 4 h as described above, and then directly used as substrates in the transaminase reaction. Reactions (200 μL) substituting the oxidized carbohydrate with 10 mM pyruvate and containing 30 ng ω-TA served as positive controls, whereas reactions substituting ω-TA with ddH_2O served as negative controls. Initial rates were determined by colorimetric detection of acetophenone over 30 min at 245 nm[60]. Reactions were then transferred to a Thermomixer (Eppendorf, Germany) to continue incubation at 37 °C, 700 rpm for another 1 h prior to product measurement by HPAEC and ESI-Q-TOF mass spectrometry as described below. All reactions were performed in triplicate at minimum.

Reactions (500 μL) permitting simultaneous oxidation of galactose (**2a**) to the aldehyde (**2b**) and transamination of **2b** to the corresponding amine (**2c**) were performed for 5.5 h at 30 °C and 700 rpm in 50 mM HEPES-NaOH (pH 7.0), and contained 10 mM **2a**, 1 mM PLP, and 10 mM 1-PEA or 10 mM L-alanine. Enzyme concentrations were 0.44 μM *Fgr*GaOx, 0.53 μM catalase, 0.12 μM HRP, and 2.9 μM *Cvi*-ω-TA. For each simultaneous oxidation-transamination reaction, a sequential oxidation-transamination reaction was performed in otherwise identical conditions, but by first running the oxidation reaction for 4 h, and then initiating the transaminase reaction by addition of PLP, 1-PEA and *Cvi*-ω-TA, and allowing the transamination reaction proceed for 1.5 h. Product formation was quantified by HPAEC.

Confirmation of the oxidation and amination products by ESI-Q-TOF mass spectrometry

The following samples were analyzed by direct injection ESI-Q-TOF (Agilent 6530 Q-TOF, Singapore): 1) 100 ppm **2a**, 2) product from *Fgr*GaOx oxidation of **2a** containing up to 100 ppm **2b**, 3) product of the amination reaction containing up to 100 ppm of the prospected amine **2c** formed in the reaction (reaction conditions specified above). The product compounds were not isolated prior to analysis. Prior to dilution, samples 2) and 3) were desalted with AG 2-X8 anion exchange resin (BioRad, US), and proteins removed by filtration using a Sartorius Vivaspin 500 spin column (10,000 kDa cutoff). Electrospray ionization was performed in positive mode, and nitrogen gas was used as both the nebulizing and drying gas. The following ionization parameters were used: the drying gas temperature was 250 °C, the drying gas flow was 3 L min^{-1}, the capillary voltage was 3,500 V, nebulizer pressure was 15 PSIG. Samples were injected directly to the ion source by the infusion pump at a flow rate of 250 μL min^{-1} by elution with 0.1% formic acid in 50% acetonitrile. Agilent Masshunter Qualitative Analysis (version B.07.00.Ink) was used for the data analysis. All samples were prepared and analyzed in triplicate.

Synthesis of 6-amino-6-deoxy-D-galactopyranose trifluoroacetate salt (2c*TFA)

Commercially available reagents were used without further purification. Column chromatography over silica gel was performed with Merck Millipore 60, 40-60 μm, 240-400 mesh silica gel. Reactions were monitored by TLC. Visualization of the TLC plates was achieved by UV light or staining with a basic potassium permanganate solution. ^1H and ^{13}C NMR spectra were recorded on a Bruker AV-400 (Germany) instrument at 20 °C (Supporting Information, section 1).

1,2,3,4-Di-*O*-isopropylidene-α-D-galactopyranose (1.5 g, 5.7 mmol) was dissolved in EtOAc (38 mL) and IBX (4.8 g, 17 mmol) was added. After complete oxidation, monitored by TLC, yielding 1,2,3,4-Di-*O*-isopropylidene-α-D-galactohexodialdo-1,5-pyranose[77], the precipitate was removed by filtration and the crude reaction mixture was concentrated under reduced pressure. The crude product was purified by column chromatography (SiO_2, cyclohexane/EtOAc; 85/15 to 70:30) affording the product as clear viscous oil (1.00 g, 3.87 mmol, 67%). R_f = 0.51 (*n*-hexane/EtOAc 2/1).

1,2,3,4-Di-*O*-isopropylidene-α-D-galactohexodialdo-1,5-pyranose (250 mg, 0.97 mmol) was dissolved in a solution of methanol (5 mL) containing ammonium acetate (800 mg, 10.4 mmol). After 15 min, $NaCNBH_3$ (100 mg, 1.6 mmol) was added. The reaction mixture was stirred for 24 h at room temperature, after which all volatiles were removed under reduced pressure. The residue was redissolved in water (15 mL) and extracted with EtOAc (10 mL). This organic layer was discarded. The aqueous phase was brought to a basic level (pH > 12) by addition of solid sodium hydroxide. The aqueous layer was extracted with EtOAc (3 x 10 mL), the combined organic layers were dried over anhydrous $MgSO_4$ and the volatiles were removed under reduced pressure. The product, 6-amino-6-deoxy-1,2,3,4-di-*O*-isopropylidene-α-D-galactopyranose, was obtained as clear viscous oil (180 mg, 0.69 mmol, 71%).

The protected amino sugar (35 mg, 0.14 mmol) was dissolved in deuterium oxide (0.7 mL) and trifluoroacetic acid (11 μL, 0.14 mmol) was added. After stirring for 24 h at room temperature, ^1H NMR analysis confirmed completion of the deprotection and quantitative formation of the trifluoroacetate salt of 6-amino-6-deoxy-D-galactopyranose. The thus obtained solution of the synthetic reference of **2c** (0.2 M, 0.14 mmol, 99%) was used without further manipulation as stock solution. (Supporting Information, section 1).

Quantification of reaction yields with high performance anion exchange chromatography coupled with a pulsed amperometric detector (HPAEC-PAD)

Amination reactions were performed as described above. Prior to analysis, the reaction samples for HPAEC-PAD were diluted with ddH_2O to 50-100 ppm of carbohydrate. Eluents used were 100 mM NaOH (A) and 100 mM NaOH with 1 M NaOAc (B). The chromatographic runs were performed using a Dionex™ CarboPac™ PA1 IC column and with a flowrate of 0.6 mL min^{-1}, where 100% eluent A was used for the first 5 min followed by 0-100% eluent B over the next 50 min. Thermo Scientific™ Dionex™ Chromeleon™ 7 Chromatography Data System (version 7.2 SR4, Thermo Fisher Scientific) was used for data analysis. The conversion of D-galactose in the oxidation reaction was calculated from reduction of the D-galactose peak area, and the yield of 6-amino-6-deoxy-D-galactose was calculated based on the peak area of the 6-amino-6-deoxy-D-galactose standard **2c***TFA (Figure S2 in Supporting Information).

Enzyme-Substrate Docking

For visualization of the substrates in the active site of the *Cvi*-ω-TA, a receptor was constructed by using the crystallized transaminase structure (PDB ID: 4A6T). The crystal structure was slightly altered by modifying the PLP-lysine complex structure to obtain PMP and the unbound K288 residue. Subsequently, the modified receptor was energy minimized using the YASARA[78,79] built-in energy minimization function. The R416 (flipping arginine) side chain was turned slightly upwards away from the PMP to gain space in the large binding pocket, since the sugar substrates did not contain charged residues[80,81]. The docking ligands **2b** (6-aldo-D-galactose), and the corresponding 6-aldo-D-galactosyl-containing aldo-lactose and aldo-raffinose were constructed using the built-in oligosaccharide building tool of YASARA, using the β-D-conformation of each sugar. The docking ligand aldo-melibiose was constructed by oxidizing the corresponding melibiose structure (PubChem Identifier: CID 11458). The XLLG molecule (Figure S6) was extracted as a ligand from the crystal structure PDB ID: 2VH9 and subsequently oxidized to obtain aldo-XLLG. All ligands were energy minimized prior to the docking experiments. The docking was performed with YASARA using the dock_runensemble.mcr macro utilizing the VINA docking method with appropriate simulation cells covering the active site of the receptor. The flexible R416 residue was fixed in place. Plausible docking results were selected by evaluating orientation of the substrate to the PMP cofactor in the active site. In addition to the location and orientation of the substrate, binding energies and dissociation constants reported by YASARA were also considered (Table S2 in Supporting Information). Figures of the dockings were created using PyMOL (The PyMOL Molecular Graphics System, Version 2.2.0 Schrödinger, LLC).

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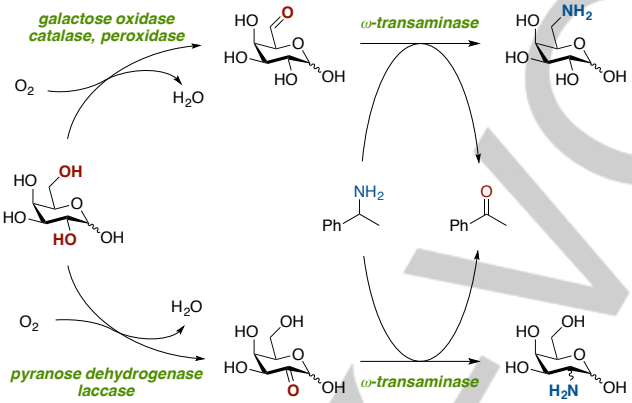

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Biocatalytic production of amino-carbohydrates

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