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Published in:
ChemSusChem

DOI:
10.1002/cssc.201802580

Published: 01/01/2019

Please cite the original version:

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Biocatalytic Production of Amino Carbohydrates through Oxidoreductase and Transaminase Cascades

Ville Aumala, Filip Mollerup, Edita Jurak, Fabian Blume, Johanna Karppi, Antti E. Koistinen, Eva Schuiten, Moritz Voß, Uwe Bornscheuer, Jan Deska, and Emma R. Master

Plant-derived carbohydrates are an abundant renewable resource. Transformation of carbohydrates into new products, including amine-functionalized building blocks for biomaterials applications, can lower reliance on fossil resources. Herein, biocatalytic production routes to amino carbohydrates, including oligosaccharides, are demonstrated. In each case, two-step biocatalysis was performed to functionalize α-galactose-containing carbohydrates by employing 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-α-galactose, 2-amino-2-deoxy-β-galactose, and 2-amino-2-deoxy-6-aldo-α-galactose was confirmed by mass spectrometry. The activity of Cvi-ω-TA was highest towards 6-aldo-β-galactose, for which the highest yield of 6-amino-6-deoxy-α-galactose (67%) was achieved in reactions permitting simultaneous oxidation of β-galactose and transamination of the resulting 6-aldo-β-galactose.

Introduction

Given their wide availability and structural versatility, carbohydrates from plant cell walls are an important raw material for the production of new biobased products that reduce reliance on petroleum. To date, most applications of plant carbohydrates begin by deconstructing polysaccharides to monomers for fermentation to fuels and platform chemicals. For example, diacids, diamines, and AB monomers (e.g., molecules containing both carboxylic acid and amine groups) are required for polyamide synthesis, and diacids and diols for polyester synthesis.

An emerging area of research aims to utilize the versatility and ensuing useful properties of native structures present in plant carbohydrates instead of degrading the structures to monomers. Biocatalyst pathways would create a new class of telechelic, amino-functionalized building blocks or polymers with complementary functionalities (e.g., carboxyl groups).

Existing chemical pathways for carbohydrate amination include applications of 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) to oxidize primary hydroxyl groups and sodium periodate to oxidize secondary hydroxyl groups, followed by reductive amination to assemble the corresponding amines. These routes, however, often require toxic transition metal catalysts, produce volatile organic compounds, and result in decreased polymer chain length. As a gentle alternative to chemical oxidation procedures, the galactose oxidase from F. graminearum was employed to oxidize α-galactose to the corresponding aldehyde. Subsequently, the ω-transaminase from Chromobacterium violaceum (Cvi-ω-TA) was employed for transamination to the corresponding α-amino carbohydrate. In addition, ω-transamination can be performed directly from biomass-derived carbohydrates.

Supporting Information and the ORCID identification number(s) for the author(s) of this article can be found under: https://doi.org/10.1002/cssc.201802580.

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**Results and Discussion**

**Activity of Cvi-ω-TA towards oxidized 6-galactose and 5-galactosamine**

The yields of FgrGaOx and AbiPDH1 produced in *Pichia pastoris* were 108 and 3.9 mg L\(^{-1}\), respectively, and the yields of Cvi-ω-TA and Vff-ω-TA M1 produced in *E. coli* were 115 and 98 mg L\(^{-1}\), respectively (Figure S1 in Supporting Information). These values are in the same range as previous reports describing the recombiant production of these enzymes.\(^{[58, 59]}\)

Activity of Cvi-ω-TA towards the oxidized carbohydrates produced by FgrGaOx or AbiPDH1, and towards pyruvate and 5-erythrose (1), was measured by using the acetophenone assay.\(^{[46]}\) Here, Cvi-ω-TA exhibited significant activity toward aldehyde 2b (160 ± 1 U g\(^{-1}\)), which, albeit lower than the Cvi-ω-TA activity measured toward pyruvate (2995 ± 90 U g\(^{-1}\)), was in

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*Amarium graminearum* (FgrGaOx, UniProt: A0A2H3HJK8) has been used to introduce an aldehyde functionality at the C-6 position of 6-galactose and in 6-galactose-containing oligo- and polysaccharides.\(^{[15, 17, 28]}\) Specificity of FgrGaOx towards the C-6 position of 6-galactose has been well documented,\(^{[29]}\) and the resulting oxidized positions have served as sites for further derivatization, including reductive amination, cross-linking through acetal formation, and phosphorylation.\(^{[15, 30, 31]}\) Furthermore, we recently described the application of the oligosaccharide oxidase from *Saccharomyces cerevisiae* 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, chemo-enzymatic routes to aminated carbohydrates through copper-catalyzed azide–alkyne cycloaddition have been demonstrated; however, fully biocatalytic cascades for amination of nonactivated carbohydrates are unprecedented yet highly desirable to simplify reaction pathways, increase sustainability, and to achieve greater control over reaction products.

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 carbonyl compounds acting as amine acceptor.\(^{[42]}\) Briefly, transaminases operate through a ping-pong, bi-bi reaction in which the first half-reaction involves the transfer of the amino group from the amine donor to the PLP cofactor. The deaminated amino donor (i.e., the respective ketone/aldheyde 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), on the basis of amine donor and acceptor specificity. Whereas α-TAs transfer amino groups from the α-carbon atom of amino acids to α-keto acids, ω-TAs are more versatile as they do not require a carbonyl group in the amine acceptor and can tolerate 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 is a distinguishing advantage 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 direct amination of biomass-derived carbohydrates.

Amines in general are greatly underrepresented in renewable biomass compared with the frequent need for amines in chemicals 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 reviews.\(^{[41–43–45]}\) In this context, enzymatic pathways from primary and secondary alcohols to the corresponding amines, by utilizing oxidases or alcohol dehydrogenases coupled with an ω-TA, have been demonstrated.\(^{[46–48]}\) By contrast, application of ω-TAs for bioproduction development from renewable biomass has only been investigated in a few studies.\(^{[49, 50]}\) For example, Lerchner et al. showed the two-step conversion of isosorbide to the corresponding diene using an alcohol dehydrogenase and an ω-TA.\(^{[49, 51]}\) More recently, Dunbabin et al. demonstrated transaminase-catalyzed production of furfurylamines from furfurals.\(^{[52]}\) On the other hand, the ability of FgrGaOx to oxidize C-6 hydroxyl groups 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.\(^{[35–37]}\)

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 6-erythrose (1), glycolaldehyde, and glyceraldehyde.\(^{[57, 56]}\) In the present study, Cvi-ω-TA was investigated for its potential to aminate aldol- and keto-carbohydrates initially formed through oxidation by FgrGaOx or the pyranose dehydrogenase from *Agaricus bisporus* (AbiPDH1, UniProt: Q3LD11),\(^{[3, 33]}\) respectively. Cvi-ω-TA activity on oxidized carbohydrates was also compared against the M1 variant of the ω-TA from *Vibrio fluvialis* (Vff-ω-TA, UniProt: F2XBU9) engineered by the group of Bornscheuer 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 biobased polymers.
Vfl, pulsed amperometric detection (HPAEC-PAD) or mass spectrometry. Activity of Vfl-α-TA M1 towards FgrGaOx and AbiPDH1 products was tested, but was found to be less than 10% of that of Cvi-α-TA, which is why experiments with Vfl-α-TA M1 were not continued. 

The purified FgrGaOx and Cvi-α-TA, or AbiPDH1 and Cvi-α-TA, were then tested in combination to establish a two-step pathway to amino carbohydrates. Specifically, 2a was oxidized to aldehyde 2b by FgrGaOx and then treated with Cvi-α-TA in an attempt to produce amine 2c. Alternatively, α-galactose was oxidized to ketone 2d by AbiPDH1 and then treated with Cvi-α-TA in an attempt to produce 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 for corresponding reaction mixture and confirmed their production through the oxidoreductase-transaminase cascade reactions.

Having confirmed the production of 2e, we ventured to produce diamine 2g, which is expected to permit carbohydrate coupling through imine bond formation. The activity of FgrGaOx toward 50 mM 2e was determined with the ABTS assay to be 50.1 ± 4.9 U mg⁻¹ enzyme, which is about 10% of that of FgrGaOx toward 2a. Formation of the corresponding bifunctional intermediate product 2f was confirmed by ESIC-Q-TOF MS (Figure 1C), and nearly complete conversion in the subsequent transaminase reaction was verified by HPAEC-PAD (Figure S3 in the Supporting Information). Despite this, diamine 2g was not detected by mass spectrometry, possibly Scheme 1. Biocatalytic cascades to aminated carbohydrates. A) Oxidation of a α-galactosyl subunit on a carbohydrate molecule to 6-aldo-α-galactosyl (2b-6b; see Table S1 for structures of 3b-6b) by FgrGaOx and subsequent amination of the aldehyde group to 6-amino-6-deoxy-α-galactosyl (2c-6c) by Cvi-α-TA. B) Oxidation of α-galactose (2a) to 2-keto-α-galactose (2d) by AbiPDH1 and subsequent amination of ketone 2d to amine 2e by Cvi-α-TA. C) Oxidation of 2e by FgrGaOx to bifunctional intermediate 2f and putative amination of the aldehyde group to the diamine 2g by Cvi-α-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 Cvi-α-TA. Abbreviations: AbiPDH1, pyranose dehydrogenase from Agaricus bisporus; Cvi-α-TA, α-TA from Chromobacterium violaceum; FgrGaOx, galactose oxidase from Fusarium graminearum; HRP, horseradish peroxidase from horseradish; ScoSLAC, small laccase from Streptomyces coelicolor.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Structure</th>
<th>Activity ± SD [U g⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>o-erythrose (1)</td>
<td><img src="https://example.com/figure1a.png" alt="Image" /></td>
<td>700 ± 20</td>
</tr>
<tr>
<td>6-aldo-α-galactose (2b)</td>
<td><img src="https://example.com/figure1b.png" alt="Image" /></td>
<td>160 ± 1</td>
</tr>
<tr>
<td>2-keto-α-galactose (2d)</td>
<td><img src="https://example.com/figure1c.png" alt="Image" /></td>
<td>45 ± 1</td>
</tr>
<tr>
<td>2-amino-2-deoxy-6-aldo-α-galactose (2f)</td>
<td><img src="https://example.com/figure1d.png" alt="Image" /></td>
<td>60 ± 3</td>
</tr>
</tbody>
</table>

[a] Reaction conditions: V = 200 μL, 10 mM amine acceptor substrate, 10 mM 1-PEA, 20 μM PLP, 30 μg (2.9 μM) Cvi-α-TA in 50 mM HEPES–NaOH buffer (pH 7.5) at 37 °C and 700 rpm. [b] Activity toward 2f was measured at an amine acceptor concentration of 5 mM owing 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 the conversion of A) d-galactose (2a) to 6-amino-6-deoxy-d-galactose (2c) expected from sequential action of FgrGaOx and Cvi-ω-TA; B) d-galactose (2a) to 2-amino-2-deoxy-d-galactose (2e) expected from sequential action of AbiPDH1 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 FgrGaOx. Similar spectra were collected from each of the three reaction replicates.
owing to the formation of unknown adducts or side reactions. Although depletion of intermediate 2f through formation of imine derivatives cannot be ruled out, Cvi-ω-TA accepted 2f as a substrate, as shown by using the acetophenone assay (Table 1).

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

The comparison of different oxidized forms of 2a showed that the highest Cvi-ω-TA activity was obtained with aldehyde 2b (Table 1). Therefore, the sequential, two-step enzymatic conversion of 2a to amine 2c was monitored by HPAEC-PAD to quantify product formation (Figure 2).

As previously reported,[26,28,62] oxidation of 2a by FgrGaOx 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 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, two-step oxidation and amination reaction, in which the formation of 2c from 2a was 18 ± 2 mol% prior to any optimization. Notably, the formation of 2c on the basis of the consumption of 2a inevitably underestimates the efficiency of the amination step, as side reactions can occur after formation of intermediate 2b,[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. Indeed, performing the oxidation and transamination steps simultaneously increased the formation of 2c nearly 2.5-fold (Table 2). A similar relative increase from sequential to simultaneous reaction was observed when L-alanine was used as the amine donor, but the yields obtained with L-alanine were roughly ten times lower than those with 1-PEA as amine donor. This was consistent with the unfavorable equilibrium for this reaction.[47,48,63]

It is also notable that increasing the concentration of the PLP cofactor 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 owing to its low cost and easy removal of the acetate byproduct,[53] IPA was tested herein as a means to further increase the formation of 2c from 2b. However, replacing 1-PEA by IPA resulted

Table 2. Influence of reaction setup on the formation of 2-amino-2-deoxy-D-galactose (2c) from D-galactose (2a).[10]

<table>
<thead>
<tr>
<th>Amine donor</th>
<th>PLP concentration</th>
<th>Product (2c) formation [mol %] sequential[b]</th>
<th>simultaneous[c]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-PEA</td>
<td>20 μM</td>
<td>18</td>
<td>N/A[40]</td>
</tr>
<tr>
<td>L-Ala</td>
<td>1 mM</td>
<td>27</td>
<td>67</td>
</tr>
<tr>
<td>[a] Reaction conditions: 50 mM HEPES buffer containing 10 mM D-galactose (2a), 10 mM amine donor (1-PEA or L-Ala), and 20 μM or 1 mM PLP.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enzyme concentrations were 0.44 μM FgrGaOx, 0.53 μM catalase, 0.12 μM HRP, and 2.9 μM Cvi-ω-TA.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[b] Sequential reactions proceeded for 4 ± 1.5 h for the oxidation and transamination steps, respectively.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[c] Simultaneous reactions proceeded for 5.5 h.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Product (2c) formation was quantified by HPAEC-PAD.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[d] Data not available.</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 2. Staggered HPAEC-PAD chromatograms tracking the conversion of 2a to 2c. A) 2a and 2c standards in ddH2O. B) FgrGaOx treatment of 20 mM 2a in ddH2O (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 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 was 90 μg/mL. 1 = amine 2c (tR = 4.7 ± 0.1 min); 2 = 2a in A (tR = 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 (tR between 19.5–33.0 min).
in undetectable product formation (data not shown), consist-
tent with the comparatively high sensitivity of \(Cvi-\omega\)-TA to IPA.

**Activity of \(Cvi-\omega\)-TA toward selected oxidized 6-aldo-\(\alpha\)-galac-
tosyl-containing carbohydrates**

Besides monosaccharide substrates, \(Cvi-\omega\)-TA was tested on a
series of \(\beta\)-galactose-containing oligosaccharides, each of
which was first oxidized with \(FgrGaOx\). As done for \(2a\), near-
quantitative oxidation of each oligosaccharide was confirmed
by TLC (data not shown), and subsequent \(Cvi-\omega\)-TA activity
toward each oxidized carbohydrate was measured by using
the acetophenone assay with 1-PEA as amine donor.

\(Cvi-\omega\)-TA activity was detected for all tested oxidized oligo-
saccharides generated by using \(FgrGaO\), including aldo-meli-
biose (20 ± 4 U mg \(^{-1}\)), aldo-lactose (50 ± 6 U g \(^{-1}\)), aldo-raffinose
(50 ± 3 U g \(^{-1}\)), and aldo-xyl glucan oligosaccharides (32 ±
4 U g \(^{-1}\)). Whereas mass-spectrometric options must be opti-
mized to unequivocally confirm the identity of resulting pro-
ducts, substrate docking studies showed that all investigated
saccharides dock similarly, with the catalytically relevant alde-
hyde group oriented towards the catalytically active exocy-
clic amino group of PMP (Figure S4 in the Supporting Informa-
tion). Notably, comparison with the docked aldo-xyl glucan oli-
gosaccharide and the crystal structure of \(Vfl-\omega\)-TA (PDB ID:
4E3Q) shows the beneficial architecture of the active site of
\(Cvi-\omega\)-TA towards oligosaccharides, since the active site of \(Cvi-
\omega\)-TA is more exposed (Figure S5).

**Conclusions**

We have demonstrated the application of two different fully
biocatalytic cascades that employ carbohydrate oxidoreductas-
es to transform specific hydroxyl groups into carbonyl groups,
and \(Cvi-\omega\)-TA to introduce amine functionalization at the oxidized
positions of the substrates. The pathways produced amino gal-
cactoses with two different regioselectivities: 1) the combina-
tion of \(FgrGaOx\) and \(Cvi-\omega\)-TA yielded galactose derivatives
aminated at the C-6 position, and 2) the combination of
\(AbiPDH1\) with \(Cvi-\omega\)-TA yielded galactose derivatives aminated
at the C-2 position. Production of 6-amino-6-deoxy-\(\alpha\)-galacto-
syl-containing oligosaccharides through pathway 1 was also
detected by acetophenone activity assay. Notably, a multistep
synthetic route was required to synthesize the aminogalactose
derivative used as an analytical standard in the current study,
and this further highlights the benefits of the biocatalytic ap-
proach. Steps taken to maximize product formation included
1) establishing a simultaneous oxidation plus transamination re-
action to the aminated carbohydrate, 2) increasing PLP concen-
tration, and 3) testing of different amine donors. The greatest
gains in the formation of product \(2c\) were achieved by perfor-
mating the oxidation and transamination steps simultaneously
rather than sequentially, consistent with reduced formation of
undesired side products from aldehyde intermediate \(2b\). \(^{[28]}\)
This work takes the first step in unlocking the potential of \(\omega-
TAs for carbohydrate functionalization and thus expands the
pool of building blocks available for new biobased materials.

**Experimental Section**

**Materials**

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

**Production and purification of \(F. graminearum\) galac-
tose oxidase (\(FgrGaOx\)) and \(A. bisporus\) pyranose de-
hydrogenase (\(AbiPDH1\))**

\(F. graminearum\) (\(FgrGaOx\); \(\beta\)-galactose:oxygen 6-oxidoreductase, EC
1.1.13.9, CAZy family AAS 2) and \(A. bisporus\) pyranose dehydrogen-
ase (\(AbiPDH1\); pyranose:acceptor oxidoreductase, EC 1.1.99.29
CAZy family AA3 2) were heterologously expressed in \(P. pastor-
is\) KM71H. Genes (GenBank accession number: AH005781.2 coding
\(FgrGaOx\); KMB51045 \(AbiPDH1\)) with C-terminal 6-His tags were
tested in shake-flask cultivations, as previously described. \(^{[49]}\)
Briefly, precultures were grown in up to 750 mL of buffered glycero-
complex medium (BMGY; 100 \(mM\) potassium phosphate buffer,
\(pH\) 6.0, 2% (w/v) peptone, 1% (w/v) yeast extract, 4 \(\times 10^{-2}\)% (w/v) biotin, 1% (v/v) glycerol) at 30°C, 220 rpm.
Methanol induction was performed over 4 d at 25°C, 220 rpm, in buffered methanol-complex medium (BBMY with 0.5% (v/v) methanol), whereby 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 \(\mu\)m PES filter unit (Millipore, Germany). The filtrate was loaded
directly onto 6 mL of Ni-NTA resin (Qiapgen, 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 with 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) by using a 10 or 30 kDa Vivaspin 20 spin column (Sartorius
AG, Germany).
\(FgrGaOx\) and \(AbiPDH1\) were concentrated to 13.5 mg mL \(^{-1}\) and
1.8 mg mL \(^{-1}\), respectively, and stored at –80°C until further use.
Protein concentration was measured by the Bradford method (Bio-
Rad Laboratories, US), and protein purity was assessed by SDS-
PAGE (Figure S1).

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

A \(pET29a\) plasmid containing the \(Cvi-\omega\)-TA gene (GenBank:
AAQ59697.1), obtained from GenScript, and the \(pET24b\) plasmid
encoding \(Vfl-\omega\)-TA M1 \(^{[49]}\) were transformed into chemically compe-
tent 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 (5000 g and 4 °C for 45 min). For each production, the cell pellet was suspended in 50 mL of 50 mM potassium phosphate buffer (pH 7.5) containing 0.1 mM PLP and lysed by using an Emul-siflex-C3 French press (Avestin Inc., Canada) at 10000 PSI for 20 min.

Immediately after cell lysis, the lysates were clarified by centrifugation at 15 000 g and 4 °C for 45 min. The supernatants each containing the soluble protein were filtered through Stierivex-GP 0.22 μm PES filter units (Millipore). Cvi-ω-TA and Vf-ω-TA M1, each containing a C-terminal His tag, were purified to homogeneity by adding 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 sodium phosphate buffer (pH 7.5) containing 0.1 mM PLP by using a 10 kDa Vivaspins 20 spin column (Sartorius AG, Germany). Cvi-ω-TA was concentrated to 9.5 mg/mL and Vf-ω-TA M1 to 3.3 mg/mL and then stored at −80 °C until further use. Protein concentration was measured by the Bradford method (Bio-Rad Laboratories, USA), and protein purity was assessed by SDS-PAGE (Figure S1 in Supporting Information).

Galactose oxidase assay
The activity of FgrGaOx was measured by 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 NaH2PO4–NaOH (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 on the basis of the extinction coefficient (36 000 L mol⁻¹ cm⁻¹) at 420 nm.[27] Each reaction was performed in triplicate, at minimum.

Enzymatic production of oxidized carbohydrates
Enzymatic oxidation of 4-galactosyl-containing substrates with FgrGaOx (reaction volume: 3 or 5 mL) was performed in 15 mL Cellstar tubes (Greiner BioOne) containing 20 mM 4-galactose equivalents of substrates (i.e., 4-galactose, melibiose, lactose, raffinose, or xylohexulcan oligosaccharides), 150 μg (0.44 μM) FgrGaOx, 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 NaH2PO4–NaOH (pH 7.5). Catalase was used as the primary means of removing hydrogen peroxide in the cascade, and HRP was included owing to its known ability to activate FgrGaOx.[72] The effect is based on the ability of HRP to maintain the copper radical in the active site of FgrGaOx in the correct oxidation state (CuII).[73] The enzyme concentrations were chosen on the basis of 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 precoated silica gel plates (TLC Silica gel 60 F254; AhlPBDH1 oxidation of 4-galactose was performed as described by Sygmond and co-workers,[74] with minor modification. Specifically, reaction mixtures contained 58 μg (3.61 μM) of AhlPBDH1, 50 mM α-galactose, and 5 mM benzoinone in double-distilled water (ddH2O). The small laccase (ScoSALC; 260 μg, 32.5 μM) from Streptomyces coelicolor was used to recycle the electron acceptor (benzoinone), 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 chromogenic detection of acetophenone from 1-phenylethylamine (1-PEA),[60] Activity of Vf-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-α-d-galactosyl groups in oligosaccharide substrates, 20 μM PLP, and 30 μg (2.9 μM) of purified Cvi-ω-TA or Vf-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 transamination reaction. Reactions (200 μL) in which the oxidized carbohydrate was substituted with 10 mM pyruvate and 30 ng ω-TA was added served as positive controls, whereas reactions in which ω-TA was substituted by ddh2O served as negative controls. Initial rates were determined by colorimetric detection of acetophenone over 30 min at 245 nm.[60] Reaction mixtures were then transferred to a Thermomixer (Eppendorf, Germany) to continue incubation at 37 °C and 700 rpm for a further 1 h prior to product measurement by HPAEC-PAD 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 the reaction mixtures contained 10 mM 2a, 1 mM PLP, and 10 mM 1-PEA or 10 mM l-ala-nine. Enzyme concentrations were 0.44 μM FgrGaOx, 0.53 μM catalase, 0.12 μM HRP, and 2.9 μg Cvi-ω-TA. For each simultaneous oxidation–transamination reaction, a sequential oxidation–transamination reaction was performed under 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-PAD.

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 FgrGaOx oxidation of 2a containing up to 100 ppm 2b, 3) product of the amination reaction containing up to 1000 ppm of the corresponding 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 with a Sartorius Vivaspins 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 3500 V, and nebulizer pressure was 103.4 kPa. Samples were injected directly into 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 ¹³C NMR spectra were recorded with a Bruker AV-400 (Germany) instrument at 20 °C (see Supporting Information).

1,2,3,4-Di-O-isopropylidene-α-L-galactopyranose (1.5 g, 5.7 mmol) was dissolved in EtOAc (38 mL) and iodoxbenzoic acid (4.8 g, 17 mmol) was added. After complete oxidation, monitored by TLC, yielding 1,2,3,4-di-O-isopropylidene-α-L-galactohexodialdo-1,5-pyranose, the precipitate was removed by filtration and the crude reaction mixture was concentrated under reduced pressure. The crude product was purified by column chromatography (SiO₂, cyclohexane/EtOAc 85:15 to 70:30), which afforded the product as a clear, viscous oil (1.00 g, 3.87 mmol, 67%).

1,2,3,4-Di-O-isopropylidene-α-L-galactopyranose trifluoroacetate (800 mg, 10.4 mmol) in methanol (5 mL). After 15 min, NaCNBH₃ (100 mg, 1.6 mmol) was added. The reaction mixture was fixed in place. Plausible docking results were selected by evaluating the active site of the receptor. The flexible R416 residue 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. The docking ligands 2b (6-aldo-d-galactose) and the corresponding 6-aldo-d-galactosyl-containing aldo-lactose and aldo-raffinose were constructed with the built-in oligosaccharide building tool of YASARA, by 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 by 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 with PyMOL (The PyMOL Molecular Graphics System, Version 2.2.0 Schrödinger, LLC).

### Quantification of reaction yields by HPAEC-PAD

Amination reactions were performed as described above. Prior to analysis, the reaction samples for HPAEC-PAD were diluted with ddH₂O to 50–100 ppm of carbohydrate. Eluents used were 100 mm NaOH (A) and 100 mm NaOH with 1 mm NaOAc (B). The chromatographic runs were performed with a Dionex CarboPac PA1 IC column and with a flow rate of 0.6 mL min⁻¹, whereby 100% eluent A was used for the first 5 min followed by 0–100% eluent B over the next 50 min. Thermo Scientific Dionex Chromelone 7 Chromatography Data System (version 7.2 SR4, Thermo Fisher Scientific) was used for data analysis. The conversion of α-galactose in the oxidation reaction was calculated from decrease in area of the α-galactose peak, and the yield of 6-amino-6-deoxy-α-galactose was calculated on the basis of the peak area of the 6-amino-6-deoxy-α-galactose standard 2c-TFA (Figure S2).

### Enzyme–substrate docking

For visualization of the substrates in the active site of the Cvi-α-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 by using the YASARA 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. The docking ligands 2b (6-aldo-d-galactose) and the corresponding 6-aldo-d-galactosyl-containing aldo-lactose and aldo-raffinose were constructed with the built-in oligosaccharide building tool of YASARA, by 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 by 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 with PyMOL (The PyMOL Molecular Graphics System, Version 2.2.0 Schrödinger, LLC).

### Acknowledgements

This study was financially supported by the Academy of Finland (decision numbers 308996, 252183, and 298250) and the European Research Council (ERC) Consolidator Grant to E.R.M. (BHIVE-722610) for funding. U.B. thanks the German Research Foundation (DFG, Grant No. Bo1862/16-1) and the EU (Horizon2020, Grant No. 722610) for funding.

### Conflict of interest

The authors declare no conflict of interest.

### Keywords

amination · biocatalysis · carbohydrates · domino reactions · enzymes

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[1] T. Werpy, G. Petersen, Top Value Added Chemicals from Biomass: Volume I—Results of Screening for Potential Candidates from Sugars and Synthe-
Two-step biocatalysis: Fully biocatalytic cascades involving a carbohydrate oxidoreductase (galactose oxidase or pyranose dehydrogenase) and an ω-transaminase are developed for the functionalization of d-galactose-containing carbohydrates to amino carbohydrates, and thus the production of amine-functionalized building blocks for biomaterials applications is facilitated.