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Galactose Oxidase Variants for the Oxidation of Amino Alcohols in Enzyme Cascade Synthesis

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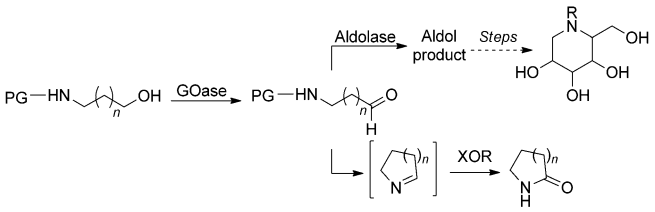
The use of selected engineered galactose oxidase (GOase) variants for the oxidation of amino alcohols to aldehydes under mild conditions in aqueous systems is reported. GOase variant F_2 catalyses the regioselective oxidation of *N*-carbobenzyloxy (Cbz)-protected 3-amino-1,2-propanediol to the corresponding α-hydroxyaldehyde which was then used in an aldolase reaction. Another variant, M_{3-5}, was found to exhibit activity towards free and *N*-Cbz-protected aliphatic and aromatic amino alcohols allowing the synthesis of lactams such as 3,4-dihydronaphthalen-1(2*H*)-one, 2-pyrrolidone and valerolactam in one-pot tandem reactions with xanthine dehydrogenase (XDH) or aldehyde oxidase (PaoABC).

In a move towards designing more sustainable chemistry for the future, the selective oxidation of alcohols to their more activated carbonyl products is a fundamental and central reaction in organic synthesis.[1] Many stoichiometric reagents and catalysts have been developed, although usually with a view to single step reactions rather than compatibility with other reagents/catalysts for multistep cascade sequences.[2] Additional challenges are regiocontrol in the oxidation of polyhydroxylated compounds and also control of the level of oxidation.[3] Thus, selective chemical oxidation of a diol to an α-hydroxyaldehyde may be difficult to achieve in high yield under conditions that would be compatible with subsequent reactions. In addition, the chemoselective oxidation of amino alcohols and diols usually requires prior *N*-carbamoyl/amide protection to prevent the more reactive amine undergoing oxidation.[4] In this paper we report enzymatic cascade oxidations using evolved variants of galactose oxidase (GOase). We show that the aldehyde products obtained can be directly combined with both aldolases, for C–C-bond formation, and in tandem enzyme reactions for oxidative cyclisation of unprotected amino alcohols to give lactams (Scheme 1).



Scheme 1. Galactose oxidase (GOase) catalysed oxidation of *N*-protected (PG) or free amino alcohols to amino aldehydes in combination with aldolase or xanthine oxidoreductase (XOR) for the synthesis of the amino sugars and lactams.

The aldolase mediated synthesis of amino sugars (iminocyclitols) generally *N*-Cbz-protected amine or azide containing acceptor aldehydes for coupling with dihydroxyacetone phosphate (DHAP).[4c,d,5] However, *N*-protected acceptor aldehydes are not commercially available and hence are typically synthesised by chemical oxidation of the *N*-Cbz-protected aminol involving either (i) trichloroisocyanuric acid/TEMPO/CH_2Cl_2,[6a] (ii) IBX/organic solvent/reflux[6b] or (iii) a Dess–Martin periodinane reaction.[5a] The current trend in favour of greener reactions has highlighted biocatalytic methods as a favourable alternative for the synthesis of pharmaceutical building blocks particularly where multiple protection steps or the use of hazardous reagents are involved. Mifsud et al reported a biocatalytic approach towards the oxidation of a *N*-Cbz-protected amino alcohol (*N*-*Z*-ethanolamine, **5a**) using a laccase/TEMPO mediator system.[7] The product of this reaction has been used with DHA-dependent fructose-6-phosphate aldolase to produce an aldol product which was then cyclised by hydrogenation yielding a five-membered iminocyclitol. Notwithstanding the advantages of laccase-mediator systems (LMS), drawbacks have to be taken into account in terms of catalytic efficiency, lack of full mediator regeneration and a possible over-oxidation of aldehydes to carboxylic acids.[8] Moreover, LMS catalyses the oxidation of alcohols with poor regioselectivity so would not be an ideal choice for the oxidation of vicinal diols to for example, an α-hydroxyaldehyde product.

We have previously developed variants of GOase, such as M_{3-5},[9] which is able to oxidise both primary and secondary alcohols, as well as GOase F_2[10] which has an increased activity towards the non-natural substrate glucose. To determine whether these galactose oxidase variants possessed activity towards amino alcohols and diols, we firstly examined the wild-type (wt) enzyme as well as variants M_{3-5} and F_2 towards a set

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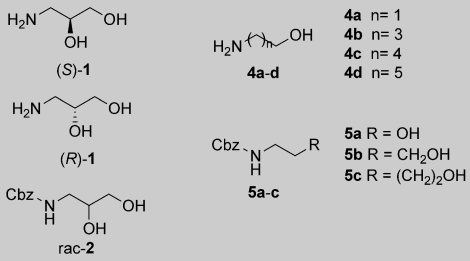
Table 1. Specific activities of wild-type (wt) GOase and variants M_{3-5} and F_2 towards free and *N*-Cbz-protected amino alcohols. Cbz = carbobenzyloxy.



Compound	Specific activity [mU mg^{-1}][a] GOase wt	GOase M_{3-5}	GOase F_2
Galactose	310	6	330
(S)-**1**	0	7	30
(R)-**1**	0	35	41
rac-**2**	0	49	142
4a	0	14	22
4b	0	13	20
4c	0	31	46
4d	0	19	60
5a	0	154	127
5b	0	132	35
5c	0	132	78

[a] Conditions: ABTS/horseradish peroxidase-coupled assay (200 μL) in NaPi (50 mM, pH 7.4) with substrates applied in 5 mM concentration (5% v/v DMSO), λ = 420 nm, 30 °C.

of amino alcohols and diols with or without *N*-Cbz-protection (Table 1). Wild-type GOase showed no activity towards any of the various amino alcohols. However, GOase enzyme variants M_{3-5} and F_2 showed good to moderate activity against all of the compounds investigated. Both variants revealed activity against enantiomeric diols *(S)*- and *(R)*-**1**. GOase F_2 showed higher activity against both enantiomers than M_{3-5}; however the latter enzyme was more enantioselective. Also, aliphatic amino alcohols **4a**–**d** showed slightly higher activity with variant F_2 and this was more pronounced with the longer chain substrate **4d**. In general, initial investigations clearly indicated *N*-Cbz-protection to improve oxidation of aliphatic alcohol moieties (*rac*-**2**, **5a**–**c**) as compared to non-protected derivatives, with variant F_2 having almost 3-times the specific activity towards diol *rac*-**2** compared to M_{3-5}. However, GOase variant M_{3-5} exhibited highest specific activities towards *N*-Cbz-protected alcohols **5a**–**c**. Also of note for this variant was a 26-fold increase in activity relative to the activity towards the natural substrate galactose.

To confirm these preliminary findings, kinetic constants for the GOase variant F_2 towards *(S)*- and *(R)*-**1**, *rac*-**1** as well as *rac*-**2** were measured. In addition, kinetic studies for substrate **5a** with both F_2 and M_{3-5} were carried out (Table 2). Interestingly, GOase F_2 displayed similar affinities (K_m) and catalytic efficiencies (k_{cat}/K_m) for diols *(S)*- and *(R)*-**1** as well as *rac*-**1**, confirming that F_2 does not differentiate between these enantiomers. *N*-Cbz-protection present in substrate *rac*-**2** did not lead to a remarkable change of affinity (K_m) compared to non-protected substrates **1**. However, we identified an almost 8-fold

Table 2. Kinetic constants determined for GOase variant F_2 towards *(S)*- and *(R)*-**1**, *rac*-**1**, *rac*-**2** and of variants F_2 and M_{3-5} towards **5a**.[a]

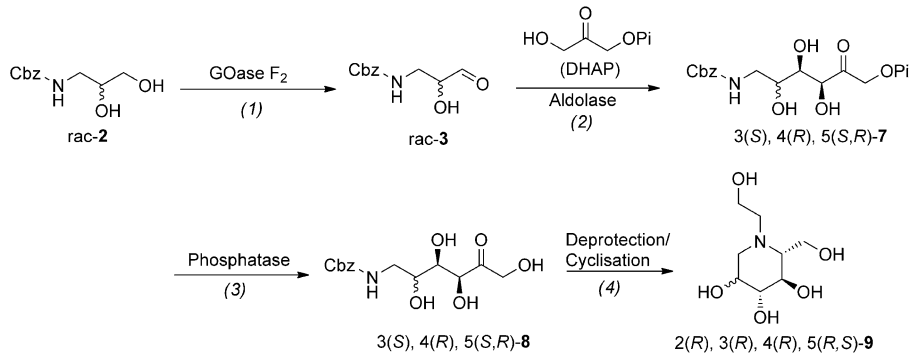
Compound	Variant	K_m [mM]	V_{max} [U mg^{-1}]	k_{cat} [s^{-1}]	k_{cat}/K_m [s^{-1} mM^{-1}]
(S)-**1**	F_2	7.66	0.027	0.031	0.004
(R)-**1**	F_2	8.13	0.019	0.022	0.003
rac-**1**	F_2	6.42	0.019	0.022	0.003
rac-**2**	F_2	7.41	0.149	0.171	0.023
5a	F_2	167.81	1.87	2.13	0.013
5a	M_{3-5}	67.25	2.71	3.09	0.046

[a] Conditions: ABTS/horseradish peroxidase-coupled assay (200 μL) in NaPi (50 mM, pH 7.4) with substrates *(S)*-**1**, *(R)*-**1**, *rac*-**1** and *rac*-**2** dissolved in NaPi and substrate **5a** dissolved in DMSO (co-solvent concentration thoroughly adjusted to 6%, v/v), λ = 420 nm, 30 °C (Figure S8–S13 in the Supporting Information).

higher velocity (V_{max}), turnover (k_{cat}) and catalytic efficiency (k_{cat}/K_m) for *rac*-**2**, highlighting that *N*-Cbz protection facilitates a significant improvement in catalytic turnover. In contrast to diol substrates **1** and *rac*-**2**, both variants revealed a notably lower affinity (K_m) towards *N*-*Z*-ethanolamine **5a**. However, M_{3-5} had a 2.5-fold higher affinity (K_m) together with a 3.5-fold elevated catalytic efficiency (k_{cat}/K_m) in comparison to F_2.

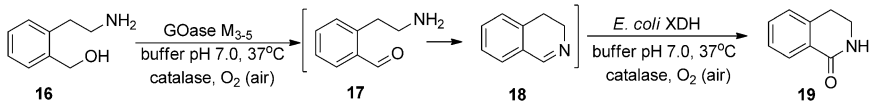
Encouraged by the kinetic studies, variant F_2 was applied to the oxidation of diol *rac*-**2** to give α-hydroxyaldehyde *rac*-**3** and variant M_{3-5} was used for the oxidation of alcohol **5a** to give **6a** on analytical scale. High (78%, [S] = 20 mM) to *quantitative* conversions (100%, [S] = 7.5 mM) of *rac*-**2** were obtained depending on initial substrate concentrations (Table S1, Figure S18 in the Supporting Information). Despite improved kinetic constants of GOase M_{3-5} towards **5a**, compared with variant F_2 (cf. Table 2), this amino alcohol was transformed with lower conversions (75–43%, [S] = 7.5-20 mM) (Table S2, Figure S18 in the Supporting Information). In order to exemplify the potential application of GOase variant F_2 as a biocatalyst for the synthesis of α-hydroxyaldehyde *rac*-**3** from *N*-Cbz-protected amino diol *rac*-**2**, analytical scale reactions ([S] = 7.5 mM, 48 h) were supplemented with 1 equiv. of DHAP and rabbit muscle aldolase (Scheme 2). After 18 h, LC/MS analysis revealed a new peak possessing a *m/z* of 392.0 [M−H$^+$] which corresponds to the phosphorylated aldol product 3(*S*), 4(*R*), 5(*S*,*R*)-**7** as a mixture of diastereoisomers ($m/z_{theoret.}$ 393.28) (Figure S19 in the Supporting Information). An adjustment of the pH value to pH 4.8 and addition of acid phosphatase resulted in complete consumption of the previously observed aldol product peak. Subsequent hydrogenation according to Concia et al.[5a] produced a peak which mass (*m/z* 208.3 [M+H]$^+$, *m/z* 230.2 [M+Na]$^+$) corresponded to an authentic standard of the enantiopure amino sugar *N*-(2-hydroxyethyl)-1-deoxynojirimycin (C5 = *S*) ($m/z_{theoret.}$ 207.11; Figure S20,21 in the Supporting Information) present as one of the diastereoisomers of **9**.

In the present work, the synthesis of other amino sugars was not pursued although we believe that either of the GOase enzymes would be suitable for the generation of aldolase acceptor aldehydes from amino alcohols **1**–**5** based on enzyme activity profiles determined (cf. Table 1).



Scheme 2. Galactose oxidase F_2 catalysed oxidation of *N*-Cbz-3-amino-1,2-propanediol *rac*-**2** to aldolase acceptor aldehyde *rac*-**3** and subsequent steps for the synthesis of the amino sugar **9** (C5 = *S* and *R*). Cbz = carbobenzyloxy.

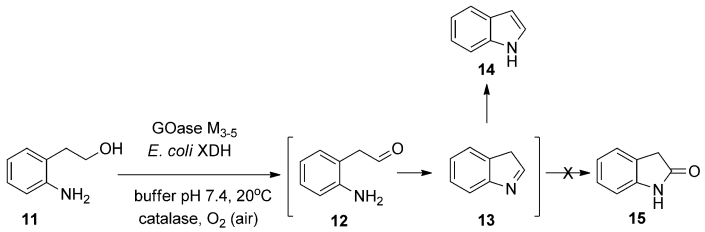
We have previously demonstrated that 3,4-dihydroisoquinoline (DHIQ) **18** is an excellent substrate for *E. coli* XDH when used in a cascade with monoamine oxidase (MAO-N) which catalyses the oxidation of tetrahydroisoquinolines.[9c] Hence, we tested (2-(2-aminoethyl)phenyl)-methanol **16** as a substrate for the GOase M_{3-5}-XDH cascade as a route to 3,4-dihydroisoquinolin-1(2*H*)-one **19** (Scheme 4). We found that the oxidation of **16** by GOase M_{3-5} was remarkably



Scheme 4. GOase M_{3-5}-XDH catalysed synthesis of 3,4-dihydroisoquinolin-1(2*H*)-one **19**.

Oxidative cyclisations of amino alcohols to give benzo-fused or simple aliphatic lactams have previously been reported using Cp*Rh[11] or a Ru complexes respectively.[12] These reactions were carried out in organic solvents and required harsh conditions of temperature and pressure. Having established the oxidation of amino alcohols by engineered variants of GOase we considered reaction sequences in which the initially formed amino aldehyde product could undergo cyclisation to generate an intermediate imine followed by subsequent oxidation to form a lactam. For the second step we targeted molybdenum-dependent enzymes known as xanthine oxidoreductases (XORs), including xanthine dehydrogenase/oxidase (XDH/XO) and aldehyde oxidase (PaoABC). From drug metabolism studies it is known that XORs have the ability to catalyse the oxidation of aldehydes to acids and cyclic imines to lactams.[13]

Initially we investigated the GOase-XDH cascade for oxidation of 2-(2-aminophenyl)ethan-1-ol **11** to give indan-2-one **15**, a core motif in a number of anticancer drugs (Scheme 3). The



Scheme 3. Attempted GOase M_{3-5}-XDH cascade to form indan-2-one **15**.

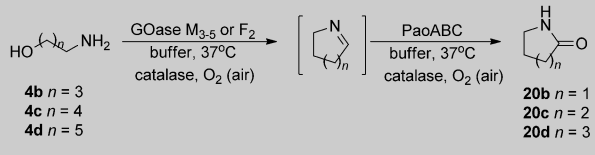
GOase M_{3-5} catalysed step gave good to excellent conversions (84–100%) to amino aldehyde **12** at [S] = 1–5 mM with lower conversions at [S] > 7.5 mM (55% conversion; Table S3). However, the initially formed 3*H*-indole **13** readily tautomerised to 1*H*-indole **14** under the reaction conditions employed.

pH sensitive within the range pH 7.0–8.5, with a pH of 7.0 required to achieve full conversion to DHIQ **18** (Table S4 in the Supporting Information). Formation of **18** is presumed to occur by cyclization of the initially formed amino aldehyde **17**.

GOase M_{3-5} and *E. coli* XDH were then combined in a one-pot reaction at pH 7.0 for the direct synthesis of the lactam **19** with an overall conversion of 69%. HPLC analysis showed that DHIQ **18** was only present in trace amounts under the cascade conditions and was consumed immediately by XDH upon formation (Figure S23 in the Supporting Information). NMR analysis of DHIQ **18** in deuterated potassium phosphate buffer at pH 6.0–8.0 indicated no amino aldehyde or DHIQ hemi-aminol present under the conditions employed (Figure S7 in the Supporting Information). Only DHIQ signals were observed which suggests that DHIQ is the true substrate for XDH rather than the amino aldehyde. Under the cascade conditions, GOase M_{3-5} oxidised amino alcohol **16** more slowly than with GOase M_{3-5} alone in which over the same time period DHIQ **18** was obtained in quantitative conversion (Table S4 and S5 in the Supporting Information). This difference is possibly due to the inhibition of the GOase M_{3-5} by the lactam product **19**.

We then turned our attention to a series of aliphatic amino alcohols **4b–d** for cascade oxidative cyclisation reactions (Table 3). Both GOase M_{3-5} and F_2 and three different xanthine oxidoreductase enzymes were tested: bovine xanthine oxidase (XO), *E. coli* xanthine dehydrogenase (XDH) and *E. coli* periplasmic aldehyde oxidase (PaoABC).[14] Conversions were determined by HPLC (Figure S3 and S4 in the Supporting Information). Neither the bovine XO nor the XDH enzyme gave any lactam products at pH 7.0–8.5 (Table S6). However the combination of GOase M_{3-5} and PaoABC was more promising. Sub-

Table 3. GOase-PaoABC cascade for the oxidative cyclisation of linear aliphatic amino alcohols **4b-d** to give 2-pyrrolidone **20b** and valerolactam **20c**.[a]



4b $n = 3$
4c $n = 4$
4d $n = 5$

20b $n = 1$
20c $n = 2$
20d $n = 3$

Entry	Substrate	GOase	pH	Conversion **20b–d** [%][b]
1	**4b**	M_{3-5}	7.0	10
2	**4b**	M_{3-5}	7.5	41
3	**4b**	M_{3-5}	8.0	56
4	**4b**	M_{3-5}	8.5	85
5	**4b**	F_2	8.5	22
6	**4c**	M_{3-5}	7.0	trace
7	**4c**	M_{3-5}	7.5	3
8	**4c**	M_{3-5}	8.0	20
9	**4c**	M_{3-5}	8.5	26
10	**4c**	F_2	8.5	8
11	**4d**	M_{3-5}	8.5	0
12	**4d**	F_2	8.5	0

[a] Reaction conditions: 7 mM **4b–d**, 103 μL GOase M_{3-5} or F_2 (3.7 mg mL^{-1}), 33 μL catalase (3.3 mg mL^{-1}), 5 μL *E. coli* PaoABC (13.3 mg mL^{-1}), KPi buffer (300 μL final volume). [b] Yields calculated by calibration curves of 2-pyrrolidone **20b** and valerolactam **20c** (Figure S3,S4 in the Supporting Information).

strate **4c** ($n = 4$) yielded a trace of valerolactam **20c** at pH 7.0 (entry 6) but at pH 8.5 the conversion increased to 26% (entry 9). With the shorter chain homologue **4b**, the same pH effect was evident (entries 1–4), however in this case the conversion to 2-pyrrolidone **20b** reached 85% at pH 8.5 (entry 4).

The longer chain substrate **4d** ($n = 5$) gave no conversion to caprolactam **20d**. The higher conversion to 2-pyrrolidone **20b** compared with valerolactam **20c** presumably reflects the more rapid formation of the 5-membered rather than the 6-membered ring imine, despite the fact that substrate **4c** undergoes oxidation by GOase at a 2.5 fold higher rate compared to **4b** (cf. Table 1). For the cascade reaction GOase M_{3-5} is operating at above its pH optimum whereas PaoABC is known to work over a broad pH range. An alternative explanation is that the increase in pH facilitates cyclisation of the assumed amino-aldehyde intermediate giving a higher concentration of the imine for the PaoABC enzyme. We also tried the GOase F_2 mutant with PaoABC which gave lower conversions for **4b** and **c** (entries 5 and 10) and no conversion for **4d** (entry 12). Again the results with **4b,c** are contrary to what would have been expected from the specific activities of the GOase step (cf. Table 1).

In summary the present work has identified two variants of GOase that are useful biocatalysts for the oxidation of amino alcohols and amino diols to produce aldehydes for subsequent enzymatic transformation. GOase can be used with *N*-Cbz protected or free amino groups, affording flexibility in synthesis. Furthermore, we have shown that a previously unexploited aldehyde oxidase, PaoABC from *E. coli*, can be used in a tandem cascade to oxidise cyclic imines, formed by cyclisation of the GOase amino-aldehyde products, to afford valerolactam and 2-pyrrolidone in up to 86% conversion.

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Keywords: aldehyde oxidase · amino alcohols · cascade reactions · enzyme catalysis · lactams

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