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Biocatalytic oxidation of alcohols using galactose oxidase and a manganese(III) activator for the synthesis of islatravir†

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Galactose oxidase (GOase) is a Cu-dependent metalloenzyme that catalyzes the oxidation of alcohols to aldehydes. An evolved GOase variant was recently shown to catalyze a desymmetrizing oxidation as the first enzymatic step in the biocatalytic synthesis of islatravir. Horseradish peroxidase (HRP) is required to activate the GOase, introducing cost and protein burden to the process. Herein we describe that complexes of earth-abundant Mn(III) (*e.g.* $Mn(OAc)_3$) can be used at low loadings (2 mol%) as small molecule alternatives to HRP, providing similar yields and purity profiles. While an induction period is observed when using $Mn(OAc)_3$ as the activator, employment of alternative Mn(III) sources, such as $Mn(acac)_3$ and $K_3[Mn(C_2O_4)_3]$, eliminates the induction period and provides higher conversions to product. We demonstrate that use of the $Mn(OAc)_3$ additive is also compatible with subsequent biocatalytic steps in the islatravir-forming cascade. Finally, to exhibit the wider utility of $Mn(OAc)_3$, we show that $Mn(OAc)_3$ functions as a suitable activator for several commercially available variants of GOase with a series of alcohol substrates.

Introduction

Biocatalysis has been increasingly adopted by the pharmaceutical industry in the synthesis of complex molecules.[1–3] The exquisite chemo-, regio- and stereoselectivity conferred by enzymes render them an attractive option as green and sustainable catalysts for many synthetic transformations. Recently, our laboratories disclosed a fully biocatalytic route to synthesize islatravir, an HIV reverse transcriptase translocation inhibitor (Scheme 1).[4] Islatravir was formed from 2-ethynylglycerol (**1**) in a three-step cascade (single aqueous stream) using nine enzymes, five of which were engineered *via* directed evolution. The first step in the cascade utilized an evolved variant of galactose oxidase (GOase, EC 1.1.3.9) from *Fusarium graminearum*, which catalyzed the desymmetrizing oxidation of prochiral 2-ethynylglycerol to form (*R*)-2-ethynylglyceraldehyde (**2**).[4]

GOase belongs to the copper radical oxidase enzyme family[5] (AA5 in the CAZy enzyme classification[6]) that catalyzes two-electron oxidations of alcohols to aldehydes and uses oxygen as a terminal oxidant. Synthetic applications of GOase require the use of two additional metalloenzymes: catalase serves to decompose *in situ* generated H_2O_2, and horseradish peroxidase (HRP) serves to activate GOase to reach the desired oxidation state of the Cu active site (Scheme 2).

The generally accepted catalytic cycle for GOase[7,8] is shown in Scheme 2. GOase can exist in three unique states, of which two (**II** and **III**) are catalytically active, while species **I** is a catalytically inactive Cu(II) complex. One-electron oxidation of complex **I** generates the Cu(II) radical species **II**, which promotes the oxidation of the alcohol substrate to form the desired aldehyde *via* a sequential two-electron, two-proton transfer process. The concomitant Cu(I) species **III** can then reform complex **II** *via* oxidation with molecular oxygen. It has been suggested that the reactive species **II** can undergo a one electron reduction deactivation pathway and fall off the catalytic cycle,[9] resulting in a slower rate and incomplete substrate conversion. Generally, peroxidase enzymes, such as HRP, have been utilized as enzymatic activators to efficiently (re)generate oxidized species **II** from reduced **I** *via* one electron oxidation,[10,11] although this protein–protein interaction is still not fully understood.[12] At large scale, use of HRP can add significant cost,[11] as well as increase protein burden, adding considerably to the challenges of process development. As our original disclosure utilized 3 wt% of a purified HRP,[4] we recog-

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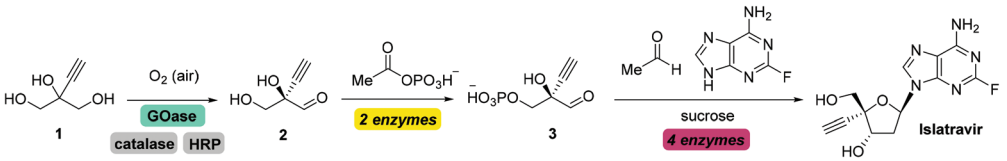
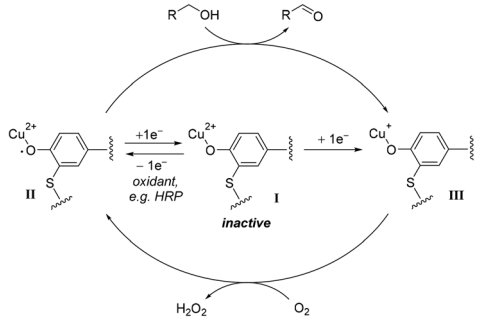


Scheme 1 Overview of the biocatalytic cascade used to synthesize islatravir.



Scheme 2 Simplified catalytic cycle for GOase, as proposed by Whittaker.[7] Adapted from ref. 11.

nized the opportunity for improvement by identifying a small molecule chemical oxidant as a low-cost, non-enzymatic GOase activator. In previous studies, $K_3Fe(CN)_6$ and K_3IrCl_6 have been used as activators for GOase to catalyze the oxidation of galactose[13] and to generate fully activated GOase for spectroscopic studies.[14] Moreover, $K_3Fe(CN)_6$ has been used to activate oxidases in biocatalytic oxidations of benzyl alcohol, although high concentrations relative to substrate were required (40 mol% to equimolar or above, depending on the oxidase).[11,12,15] Despite these advances, there remains a need for a practical and economical small molecule GOase activator for synthetic scale reactions. Herein, we report that catalytic quantities of Mn(III) salts can be used as attractive alternatives to HRP to activate GOase for the biocatalytic oxidation of alcohols, providing significant cost savings while maintaining high levels of catalytic activity.

Results and discussion

To identify suitable one-electron oxidant activators for GOase, we turned to high-throughput experimentation (HTE). HTE screens utilized the best performing GOase variant at the time of initiating screening, GOase-1;[16] using HRP as an activator, this variant yielded *ca.* 60% conversion to the desired aldehyde in 85% ee, with minimal over-oxidation. We sought GOase-compatible oxidants that have suitable oxidation potential to activate species **I**, but are unable to directly oxidize the triol to the racemic aldehyde. Over 100 small molecule oxidants were selected and screened at various loadings (10 to 100 mol% relative to 2-ethynylglycerol, see ESI† for details).

In addition to the oxidants shown in the literature to activate GOase, we evaluated a wide variety of commonly used oxidants such as persulfates, organic peroxides and high-valent transition-metal complexes. HRP (6 wt%) and activator-free conditions were tested as positive and negative controls, respectively. A selection of the screening results is shown in Chart 1. Only marginal conversion (3%) was observed in the reaction in the absence of an activator, presumably due to predominant formation of inactive species **I**; upon addition of $CuSO_4$ to copper-free purified GOase-1, reduced species **I** is observed by UV-vis spectroscopy (see ESI†) indicating this variant primarily forms **I** in the absence of an activator. Conditions using most activators shown in Chart 1 achieved some conversion with similar enantioselectivities to the HRP control, showing the oxidations are primarily GOase-mediated in these cases. Despite this, inclusion of the majority of screened oxidants resulted in low conversions to the desired aldehyde, likely due to one or more of the following reasons: (1) poor solubility of the activator, (2) undesired reactions with enzymes, (3) side product formation (*e.g.* over-oxidation to 2-ethynylglyceric acid, or degradation of 2-ethynylglyceraldehyde to formic acid),[4] or (4) insufficient redox potential. Interestingly, employment of the most commonly used activators in the literature, $K_3Fe(CN)_6$ and K_3IrCl_6, led to poor yields of 2-ethynylglyceraldehyde (17% and 36%, respectively). Promisingly, Mn(III) complexes $Mn(OAc)_3$ and MnF_3 emerged as viable leads, helping to provide conversions (56% and 59%, respectively) comparable to HRP (62% conversion) and with similar purity. To avoid the possibility of *in situ* HF formation on scale, $Mn(OAc)_3$ was selected as our lead for further investigation. In an early literature report, the Mn(III)-EDTA complex was utilized as a GOase activator in the oxidation of galactose, but was observed to activate more slowly than chloroiridate.[17] Further explorations of Mn(III) as a GOase activator have not been reported.[18] $Mn(OAc)_3$[19] has been commonly used as an oxidant in organic reactions, often using acetic acid as the solvent,[20] and has been shown to act as both a Lewis acid and electron transfer reagent for radical initiation.[21] In aqueous solution, the Mn(III) oxidation state is susceptible to disproportionation to Mn(IV) (*i.e.* MnO_2) and Mn(II), although it is stabilized by O-donor ligands,[22] and $Mn(OAc)_3$ was recently shown to operate as an oxidant in aqueous systems for the oxidation of sulfinates.[23]

Following the results from HTE screening, the $Mn(OAc)_3$ conditions were tested on gram-scale to verify the screening result (Fig. 1). For this experiment, a further-evolved GOase variant, GOase-2, was used;[16] during development, process optimization and enzyme evolution were progressing in parallel, and GOase-2 emerged as a catalytically more active variant

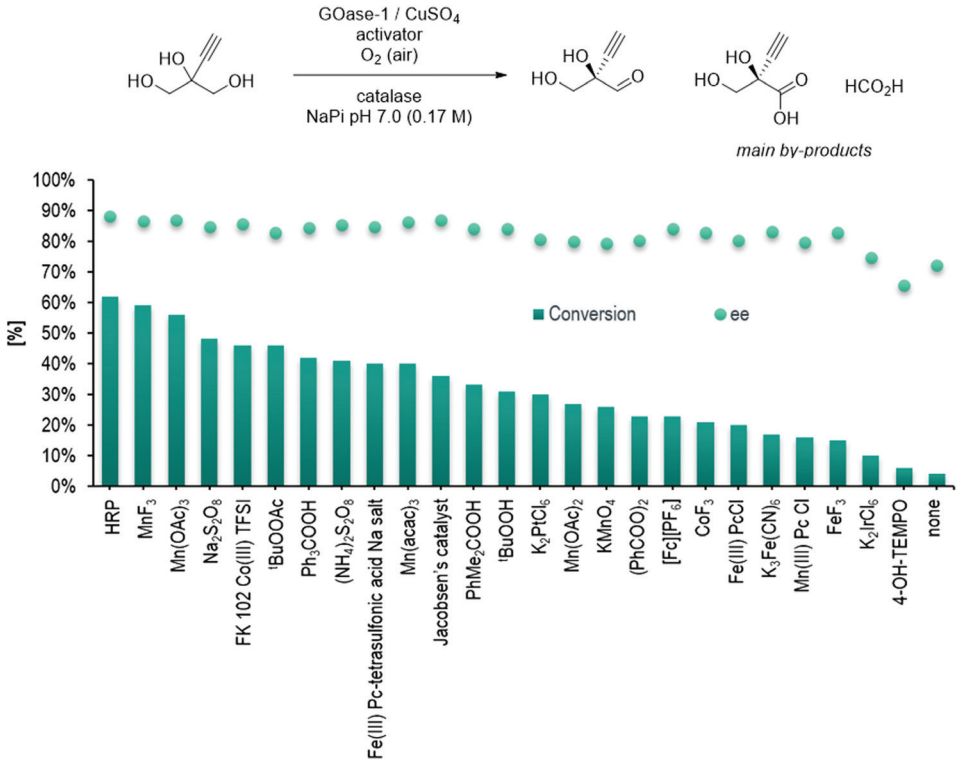


Chart 1 Selected HTE screening results on small molecule activators for GOase-1. Conversion to aldehyde is represented by bars, and the aldehyde ee by circles.

with improved enantioselectivity after two rounds of directed evolution from GOase-1. With 14 wt% of GOase-2 powder and 10 mol% $Mn(OAc)_3$, 2-ethynylglyceraldehyde was obtained in 60% yield, compared with 57% yield using HRP. The enantiomeric excess was comparable between the activators (96% and 95% ee for HRP and $Mn(OAc)_3$, respectively), and purity profiles were similar (see ESI†). The $Mn(OAc)_3$ loading was reduced to only 2 mol% without impacting the 2-ethynylglyceraldehyde yield after *ca.* 20 hours reaction, suggesting that the rate of catalysis is greater than 50 times the rate of enzyme deactivation. Without an activator, 2-ethynylglyeraldehyde was formed in 29% yield after 16 hours (in 82% ee), suggesting that limited activation of this GOase variant occurs in the absence of an activator (Fig. 1).[19] No oxidation was observed in the control reaction of 2-ethynylglycerol with $Mn(OAc)_3$ in the absence of GOase, showing $Mn(OAc)_3$ does not directly promote the oxidation of 2-ethynylglycerol.

Since desymmetrizing oxidation is the first step in the biocatalytic cascade to synthesize islatravir,[4] any chosen alternative to HRP for this process must be compatible with the biocatalytic transformations carried out downstream. Gratifyingly, a representative stream of 2-ethynylglyceraldehyde formed in the presence of $Mn(OAc)_3$ (5 mol% relative to 2-ethynylglycerol) performed well in the subsequent reactions. The stream gave 96% conversion to 2-ethynylglyceraldehyde 3-phosphate (**3**) in the kinase-catalyzed phosphorylation, and the four-enzyme aldol/glycosylation step produced islatravir in 86% isolated yield (relative to 2-fluoroadenine, see ESI† for details).

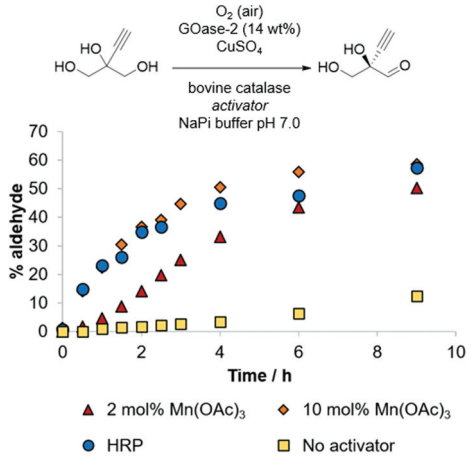


Fig. 1 Desymmetrizing oxidation of 2-ethynylglycerol (0.258 M) using 6 wt% HRP or $Mn(OAc)_3$ (2 or 10 mol%) as activators for GOase-2. Mol% and wt% are relative to 2-ethynylglycerol.

With $Mn(OAc)_3$ identified as a competent HRP alternative, we sought to understand more about the nature of the GOase activation. Closer examination of the reaction profile using 2 mol% $Mn(OAc)_3$ revealed an induction period in the product formation. Profiling of the initial regime was conducted to further probe the activation period using $Mn(OAc)_3$ at 1 to 10 mol% loading. Upon increasing the loading, the induction

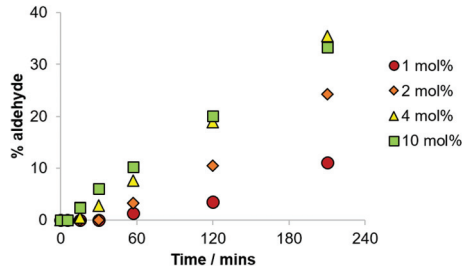


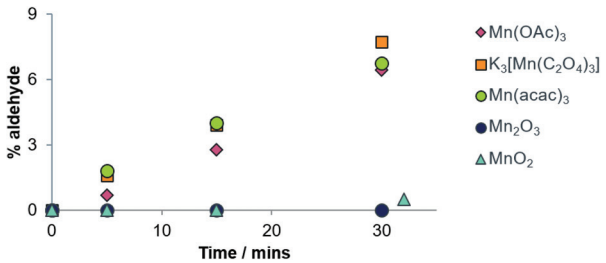
Fig. 2 Initial 2-ethynylglyceraldehyde formation from oxidation of 2-ethynylglycerol (0.258 M) using GOase-2 (14 wt%), catalase (14 wt%), and various $Mn(OAc)_3$ loadings. Mol% and wt% are relative to 2-ethynylglycerol.

period was observed to decrease (Fig. 2). However, following the induction period, the productive regime of the catalysis was shown to proceed at similar rates independent of $Mn(OAc)_3$ loading.

The presence of an induction period suggests that the active state of GOase is not formed immediately on addition of $Mn(OAc)_3$, and also that the activating state of $Mn(OAc)_3$ might not be formed immediately. To further understand the nature of the induction period, several other Mn species were investigated as activators, with the insoluble oxides MnO_2 and Mn_2O_3 proving ineffective activators of GOase-2 (Fig. 3). This observation suggests that MnO_2, potentially formed *in situ* by disproportionation of Mn(III), is unlikely to be the active oxidant derived from $Mn(OAc)_3$. $Mn(acac)_3$ or $K_3[Mn(C_2O_4)_3]$, Mn(III) complexes with distorted octahedral coordination geometry,[22] are competent activators at 2 mol% loading, providing comparable initial catalysis as $Mn(OAc)_3$ (post-induction period). After 16 hours, the aldehyde yield with $Mn(acac)_3$ was 62% (compared with 59% with $Mn(OAc)_3$ under parallel conditions), while $K_3[Mn(C_2O_4)_3]$ enables higher aldehyde yield of 73%. Furthermore, the induction period is not observed with these activators, suggesting that the Mn(III) oxidation state is responsible for the oxidation of **I** to **II**, as has been previously proposed for Mn(III)-EDTA-mediated activation.[17] We speculate that the induction period observed with $Mn(OAc)_3$ may be attributed to modifications of the trimeric structure[19] required to undergo electron transfer with GOase, and/or the limited solubility of $Mn(OAc)_3$.[24]

Varying the loading of water-soluble $K_3[Mn(C_2O_4)_3]$ from 1 to 10 mol% (relative to 2-ethynylglycerol) resulted in almost identical initial reaction rates (see ESI†), as was observed post-induction with $Mn(OAc)_3$, suggesting that the activator loading in this range does not impact the initial catalytic turnover of GOase. While use of 2 mol% and 4 mol% of $K_3[Mn(C_2O_4)_3]$ led to essentially the same yields of 2-ethynylglyceraldehyde (74% and 75%, respectively), decreasing the loading to 1 mol% resulted in a somewhat lower 68% yield. As the initial rates are similar across each loading, the difference in yield is likely due to formation of inactive **I** *via* off-cycle reduction of active **II** during catalysis (Scheme 2). The lower yield observed with 1 mol% $K_3[Mn(C_2O_4)_3]$ may suggest that an off-cycle reduction occurs at *ca.* $1/50^{th}$ to $1/100^{th}$ the rate of catalytic turnover with this variant,[25] although some decomposition of $K_3[Mn(C_2O_4)_3]$, which would reduce the re-activation, and therefore overall yield, cannot be discounted. At 10 mol% loading, only 51% 2-ethynylglyceraldehyde was produced, possibly suggesting additional mechanistic complexity in which higher concentrations of oxalate may irreversibly deactivate GOase or catalase. Moreover, while $K_3[Mn(C_2O_4)_3]$ is a soluble source of Mn(III) and effective activator, its sensitivity to light and heat[26] and lack of commercial availability render it a less attractive candidate for incorporation into a manufacturing process.

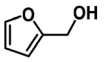
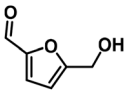
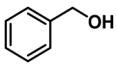
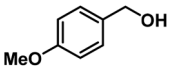
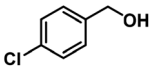
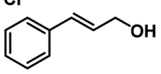
With $Mn(OAc)_3$ established as a convenient alternative to HRP for our substrate and engineered GOase, we next aimed to show that $Mn(OAc)_3$ is an effective HRP replacement for alternative GOase-mediated oxidation reactions. Commercially available GOase variants M_1 and M_{3-5} were examined to demonstrate that the $Mn(OAc)_3$ activation is not specific to enzymes engineered for islatravir synthesis. GOase M_1 was developed by Arnold and co-workers as a variant with higher stability and improved expression in *E. coli* compared with wild-type GOase,[27] while GOase M_{3-5} was evolved by Turner to oxidize secondary alcohols,[28] but has also been shown to operate on a variety of primary alcohol substrates.[29–32] Table 1 shows the comparisons at selected time points between identical conditions proceeding with either no activator, HRP (3 wt%) or $Mn(OAc)_3$ (5 mol%). These examples demonstrate that aldehyde yields with $Mn(OAc)_3$ as the GOase activator are comparable with, or superior to, results obtained using HRP, confirming the viability of using $Mn(OAc)_3$ as a cost-effective and widely available HRP replacement.[33]



Activator	% aldehyde (16h)
$Mn(OAc)_3$	59%
$Mn(acac)_3$	62%
$K_3[Mn(C_2O_4)_3]$	73%
Mn_2O_3	31%
MnO_2	38%

Fig. 3 Initial 2-ethynylglyceraldehyde formation from oxidation of 2-ethynylglycerol (0.258 M) using GOase-2 (14 wt%), catalase (14 wt%), and various activators (2 mol%). Mol% and wt% are relative to 2-ethynylglycerol.

Table 1 Oxidation of various alcohols with GOase comparing no activator, $Mn(OAc)_3$ (5 mol%) or HRP (3 wt%)

Entry	Substrate	GOase variant/loading	Reaction time/h	% aldehyde (no activator)	% aldehyde ($Mn(OAc)_3$)	% aldehyde (HRP)
1		M_{3-5} 10 wt%	24	8	59	54
2		GOase-2 20 wt%	5.5	1	88	92
3		M_1 10 wt%	2.7	20	55	35
4		GOase-2 20 wt%	16	8	73	72
5		GOase-2 20 wt%	8	1	46	54
6		M_{3-5} 20 wt%	2.7	47	89	81

% yields were determined *in situ* by ^{1}H NMR spectroscopy using an internal standard. 10 vol% DMSO was used in all entries except 2. Mol% and wt% are relative to the primary alcohol substrate.

Conclusions

We have demonstrated that Mn(III) complexes are effective activators of GOase for biocatalytic alcohol oxidations. In seeking a convenient and economical replacement for HRP, over 100 potential activators were rapidly screened *via* high throughput experimentation. While incorporation of many activators provided the desired aldehyde in poor yields, presumably due to incompatibility with GOase and/or overoxidation of the product, we showed that loadings as low as 2 mol% $Mn(OAc)_3$ enabled the formation of (*R*)-2-ethynylglyceraldehyde from 2-ethynylglycerol in comparable yield to HRP. As a result, this readily available activator can enable a lower cost process and reduces the protein burden, which simplifies downstream purifications. An induction period was observed when using $Mn(OAc)_3$, but could be eliminated using alternative Mn(III) sources such as $Mn(acac)_3$ or $K_3[Mn(C_2O_4)_3]$. The broad generality of Mn(III) salts as single-electron activators was demonstrated with both the M_1 and M_{3-5} variants of GOase and could be applied to the oxidation of six representative examples of primary alcohol substrates. We expect that this combination of bio- and chemo-catalysis will be a useful method for the synthetic community.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

We thank Margaret Miller for assistance with setup for scaling the oxidation reactions, Gregory Hughes and Rose Mathew for assistance with 2-ethynylglycerol preparation, and Danielle Schultz for useful discussions. We thank Codexis, Inc. for directed evolution and supplying the enzymes.

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