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Selective Oxyfunctionalisation Reactions Driven by Sulfite Oxidase-Catalysed *In Situ* Generation of H₂O₂

Morten M. C. H. van Schie,^{*[a]} Alexander T. Kaczmarek,^[b] Florian Tieves,^[a] Patricia Gomez de Santos,^[c] Caroline E. Paul,^[a] Isabel W. C. E. Arends,^[d] Miguel Alcalde,^[c] Günter Schwarz,^[b] and Frank Hollmann^[a]

H₂O₂ can be accepted by several peroxygenases as a clean oxidant, able to supply both the necessary electrons and oxygen atom at the same time. The biocatalysts, in turn, are able to catalyse an array of interesting oxygen insertion reactions at enantio- and regio-selectivities hard to attain with classical chemical methods. The sensitivity of most peroxygenases towards H₂O₂, however, requires this oxidant to be generated *in situ*. Here, we suggest the application of (modified) sulfite oxidases to couple the oxidation of sulfites to the reduction of oxygen. This enables us to use calcium sulfite, an industrial waste product from scrubbing flue gases, as an electron donor to reduce oxygen. This will supply the required peroxide in a controlled manner and enables us to perform these challenging reactions at the expense of simple salts.

Peroxygenases (E.C. 1.11.2.1, UPO for unspecific peroxygenases) are powerful catalysts for the selective oxyfunctionalisation of (inert) C–H and C=C-groups.^[1] These are reactions which are still hard to perform using classic chemical methods. Especially the high catalytic activity, robustness and simplicity of application of these enzymes makes them potentially useful catalysts for organic oxyfunctionalisation chemistry.

Unfortunately, as heme-enzymes, UPOs also suffer from a pronounced instability towards hydrogen peroxide, the stoichiometric oxidant in UPO-reactions.^[2] This issue is met by *in situ* generation of H₂O₂ at rates, which maximize productive UPO-turnover while minimizing the undesired oxidative inactivation of the enzymes.^[3] Today, a broad range of *in situ* H₂O₂ generation systems are available, ranging from biocatalytic,^[4] heterogeneous catalysis,^[5] photochemical^[6] and electrochemical methods.^[7] Now we raise the question about the environmental impact of such systems, especially with large-scale applications in mind, and hypothesize if we can use waste products from other processes for this purpose. In this respect, we drew our attention to sulfite as stoichiometric reductant, as these salts are a by-product from flue gas treatment and are thus produced at large amounts.^[8]

To use sulfite as stoichiometric reductant for the *in situ* generation of H₂O₂, we envisioned the application of sulfite oxidases (SO), which are ubiquitously found in all kingdoms of life. SO play a central role in the sulfur metabolism of living cells.^[9] As the name implies, SO oxidizes sulfite to sulfate using a unique pterin-based molybdenum cofactor (MoCo) in the active site.^[10] In vertebrates, SO localizes into the intermembrane space of mitochondria and consists of three distinguishable domains, the N-terminal heme domain, incorporating a cytochrome *b*₅-type heme cofactor, followed by the MoCo-binding domain, and the C-terminal dimerization domain. Upon oxidation of sulfite, the molybdenum in the active site is reduced from Mo^{VI} to Mo^{IV}. The two electrons are subsequently transferred *via* an intra-molecular electron transfer from the MoCo to the heme domain and finally to cytochrome *c*.^[11] Alternatively, sulfite-derived electrons can be transferred to nitrite, forming the radical nitric oxide.^[12] In contrast, in plants, SO consists only of the MoCo and dimerization domain and localizes in peroxisomes.^[13] Here, the redox balance is closed through reaction with molecular oxygen, forming superoxide, which rapidly dismutates to H₂O₂. When the heme domain in the mammalian SO is deleted, similar behavior of H₂O₂ formation is observed.^[14] So far, SO have been extensively studied from fundamental and therapeutic points of view. Furthermore, these enzymes have also been immobilized on electrodes in biosensors and fuel cells.^[15] In this work, we envision a biocatalytic purpose for this H₂O₂ generation system in the application of peroxygenase reactions (Scheme 1).

To test the viability of the proposed scheme, we chose the evolved, recombinant peroxygenase from *Agrocybe aegerita* (rAaeUPO) as model enzyme for the selective hydroxylation of

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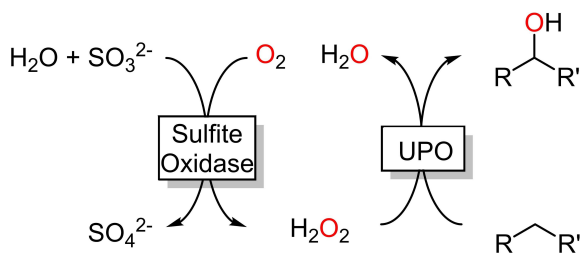
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Scheme 1. The cascade of sulfite oxidase and peroxygenases.

ethylbenzene to (*R*)-1-phenylethanol.^[16] Five SO enzymes from different sources (all recombinantly expressed in *Escherichia coli*) were tested; one plant SO (from *Arabidopsis thaliana*) and two mammalian SO, from mouse and human, either in their natural configuration containing the heme moiety (full) or a

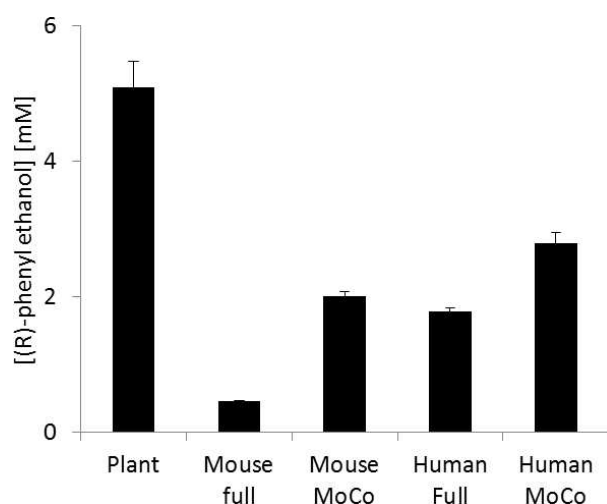


Figure 1. Comparison of different sulfite oxidases to drive the *rAaeUPO*-catalysed hydroxylation of ethylbenzene. General conditions: [SO] = 100 nM, [*rAaeUPO*] = 500 nM, [Na₂SO₃]₀ = 10 mM, [ethylbenzene]₀ = 100 mM in a 50 mM Bis-Tris buffer at pH 7.0. 250 μ l reactions were performed in duplicates in a thermos shaker at 30 °C and 500 rpm for 24 hours.

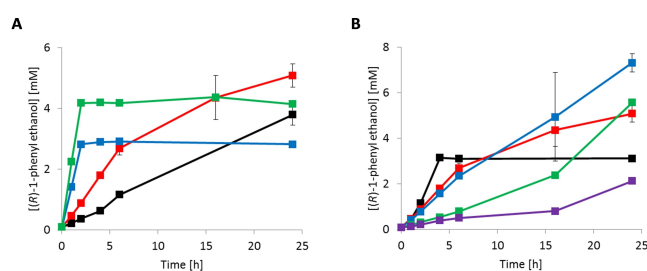


Figure 2. Influence of the reaction pH (A) and sulfite concentration (B) on the plant-SO – *rAaeUPO* cascade performance. General conditions: [PSO] = 100 nM, [*rAaeUPO*] = 500 nM, [Na₂SO₃]₀ = 10 mM, [ethylbenzene]₀ = 100 mM in a 50 mM Bis-Tris buffer at pH 7.0. Reactions were performed in duplicates, with reaction volumes of 250 μ l, in a thermos shaker at 30 °C and 500 rpm. A: performance at pH 6.5 (black), pH 7.0 (red), pH 7.5 (blue) or pH 8.0 (green). B: Sodium sulfite concentration of 5 mM (black), 10 mM (red), 20 mM (blue), 50 mM (green) or 100 mM (purple).

shortened variant, devoid of the heme-subunit (MoCo, Figure 1).

Pleasingly, all SO tested enabled *rAaeUPO*-catalysed hydroxylation of ethylbenzene. The heme-depleted SO gave higher activities (i.e. overall product concentrations) than the heme-containing ones, which is most likely due to the faster direct aerobic reoxidation at the Mo^{IV} centers. For all further experiments we focused on the plant SO (PSO). To get further insights into the parameters influencing the performance of the SO-*rAaeUPO*-oxygenation system we systematically varied some reaction parameters such as pH, enzyme concentrations and sulfite concentrations (Figure 2 and supporting information).

The reaction functioned optimally at neutral pH values, which appears to be a compromise between the preferred conditions for SO (basic) and the peroxygenase (slightly acidic). Changing the ratio between the two enzymes showed careful balance of the two catalysts is required as an excess of sulfite oxidase activity will presumably result in accumulation of the hydrogen peroxide, which deactivates the biocatalysts (Figure S2A). When the PSO concentration was limiting, a turnover frequency of 4.4 (\pm 0.5) s⁻¹ for the oxidase could be determined.

Increased sulfite concentrations corresponded to improved reaction continuity, but also a decreased reaction rate (Figure 2B). Furthermore, we saw less product formation than expected with the amount of sulfite added. When adding 5 mM of Na₂SO₃, for instance (Figure 2B, black time course), only 3.1 mM of product was obtained. Similar observations were made for the human MoCo variant (Figure S1). These effects are most likely related to the spontaneous reaction between sulfite and H₂O₂ yielding sulfate and water.^[13b,17] The direct oxidation of sulfite by molecular oxygen is also possible, which would also decrease oxygen availability.^[18] The reaction with peroxide has been reported to follow first order kinetics; the rate is thus linearly dependent on the *in situ* concentration of both sulfite as peroxide. This is also an explanation for why, during experiments at increased sulfite concentration (Figure 2B, green and purple time courses), the reaction rate increased after 16 hours; at this point the Na₂SO₃ concentration had significantly decreased. We therefore set out to limit the concentration of SO₃²⁻ in the reaction mixture using CaSO₃, which is poorly soluble in water (up to 0.45 mM)^[19] and could form a solid phase in the reaction acting as a sulfite reservoir. Advantageously, CaSO₃ is also the primary product from flue gas desulfurisation with lime stone, and can thus be considered to be a waste product from industry, available in large quantities.^[8] Very pleasingly, this strategy indeed enhanced the product accumulation significantly (Figure 3).

Encouraged by these results, we next decided to explore the scope of the proposed sulfite-driven oxidation reaction. Here, we tested several classical oxygenation reactions applying different peroxygenases in the cascade with PSO (Table 1). The requirement of the CaSO₃ slurry to be homogeneously dispersed in solution did, in some occasions, induce significant standard deviations.

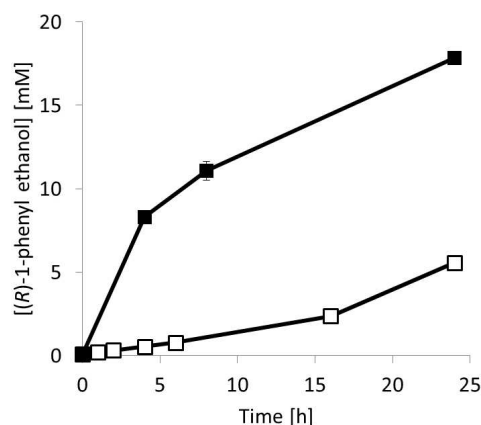


Figure 3. Comparison of Na₂SO₃ (white) and CaSO₃ (black) on the PSO – rAaeUPO cascade. General conditions: [PSO] = 100 nM, [rAaeUPO] = 500 nM, [SO₃²⁻]₀ = 50 mM, [ethylbenzene]₀ = 100 mM in a 50 mM Bis-Tris buffer at pH 7.0. 250 μl reactions were performed in duplicates at 30 °C and 500 rpm. Reactions with Na₂SO₃ were performed in a thermoshaker, while the samples with CaSO₃ were mixed by a magnetic stirrer in a water bath. Mixing (and thus oxygen transfer) by the thermoshaker was actually more efficient than with the magnetic stirring bar (Figure S6).

Table 1. Substrate scope for the reaction of PSO coupled to various peroxygenases.

Product	Catalyst	TTN ^[a] SO/(Peroxyge- nase)	Conversion [mM]	ee [%]
	rAaeUPO	77 000/(30 800)	15.4 (±1.8)	98.2
	rAaeUPO	83 500/(33 400)	16.7 (±4.6)	–
	rAaeUPO	70 000/(28 000)	14.0 (±0.41)	5.8
	rAaeUPO (Solo) ^[b]	26 350/(10 540)	5.72 (±0.52)	–
	CfuCPO	67 000/(n.d.)	13.4 (±0.16)	98.4
	CVCPPO	–	0.0	–

General conditions: [SO] = 200 nM, [rAaeUPO] = 500 nM, [CaSO₃]₀ = 100 mM, [substrate]₀ = 50 mM in a 50 mM Bis-Tris buffer. 500 μl reactions were performed in triplicates at 30 °C with magnetic stirrers mixing at 500 rpm for 24 hours. For dextromethorphan, the substrate concentration was 10 mM. For thioanisole, 100 U ml⁻¹ of CPO from *Caldariomyces fumago* was added as the peroxygenase. ee: enantiomeric excess. [a] TTN: mol product mol⁻¹cat. [b] Via O-dealkylation of dextromethorphan.

As shown in Table 1, a range of classical peroxygenase reactions could be promoted by the proposed SO₃-H₂O₂ generation system ranging from the (stereospecific) hydroxylation of sp³-C–H-bonds *via* oxidative demethylation^[20] and epoxidation^[21] to sulfoxidation reactions.^[22] Conversions and total turnover numbers were in the same order of magnitude,

with the exception for dextromethorphan, where the rAaeUPO catalysed reaction was probably limiting. We also evaluated SO for chemoenzymatic halogenations reaction based on hypohalites. These should be generated *in situ* from halides using the vanadium-dependent haloperoxidase from *Curvularia inaequalis*;^[23] albeit without success. We ascribe this observation to the deactivation of SO by the bleach formed by VCPO. This phenomenon has been observed for other oxidases before and was also confirmed by us in a control reaction where 10 μM NaClO nearly fully inhibited the SO.^[24] Sulfite itself did not seem to affect the activity of VCPO.

In the experiments with styrene as the substrate, we noted a decrease in mass balance and the appearance of a polar compound on TLC. Based on literature,^[25] we deduced this compound be the hydroxyl sulfonate derivative of styrene. This, as sulfite is able to act as a nucleophile to ring-open the epoxides. As for the uncoupling reaction of sulfite with peroxide, this ring opening reaction is reported to follow first order kinetics. Therefore, the epoxide is primarily observed if CaSO₃ is the sulfite source. For Na₂SO₃, only the sulfonated product, mainly the 1-phenyl-2-hydroxyl-ethanesulfonate, was obtained as previously described in literature (Figure 4).^[25b]

In a final set of experiments, we scaled up the reaction to semi-preparative scale in order to show the applicability of the suggested system. As the reactions generally stopped within 24 hours, we added more biocatalyst at regular intervals. Spiking experiments showed only the PSO needed to be added every 24 hours, as the UPO remained active for over 3 days under the applied reaction conditions (Figure S2B). During these initial experiments, over prolonged time and at a larger reaction volume, a decrease in reaction rate was observed as compared to the small scale experiments. We ascribed this to a

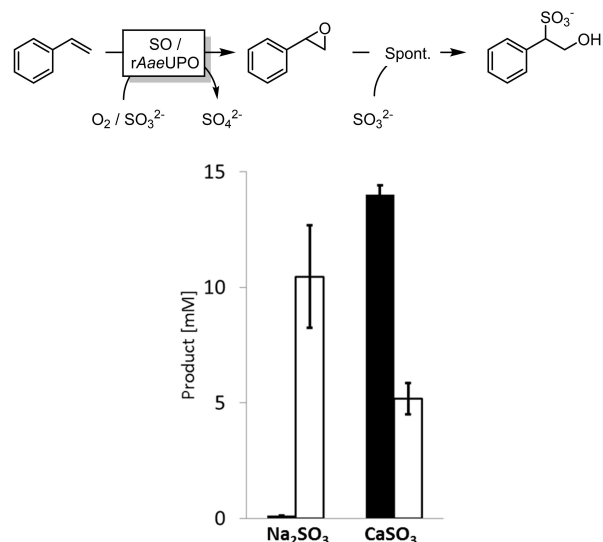


Figure 4. Chemoenzymatic transformation of styrene into styrene oxide (full) or 1-phenyl-2-hydroxyl-ethanesulfonate (open), depending on the sulfite salt used. General conditions: [SO] = 200 nM, [rAaeUPO] = 500 nM, [SO₃²⁻]₀ = 100 mM, [styrene]₀ = 50 mM in a 50 mM Bis-Tris buffer. 500 μl reactions were performed in triplicates at 30 °C with magnetic stirrers mixing at 500 rpm.

decrease in oxygen transfer rates. We therefore supplied pure oxygen to the larger reaction *via* a balloon. Furthermore, we observed no inhibition of activity for the cascade in the presence of the sulfate that would be produced over time (Figure S2C). At the end of the preparative scale reaction, after 96 hours, 47.4 mM of *R*-phenylethanol was produced with an enantiomeric excess of 97.8%. After isolation, 155 mg of product was obtained which contained, next to the phenylethanol, 23% of the overoxidated acetophenone. The overoxidation was approximately ten times higher than the one observed during the reactions at smaller scale, which can stem from the higher phenylethanol concentrations towards the end of the reaction. The acetophenone produced requires two oxidation steps, we can deduce that the rAaeUPO and the PSO made 142000 and 180000 turnovers, respectively. As the reaction rate showed no regression over time (Figure S2D), we assume even higher turnovers can be achieved.

Overall, we provide the proof of principle of using sulfite oxidases coupled to various peroxygenases, thereby enabling oxyfunctionalisation reactions up to preparative scale at the expense of an industrial waste product. The total turnover numbers achieved for the oxidase are already up to 180000 for the arbitrarily chosen sulfite oxidase from *Arabidopsis thaliana*. These turnover numbers are in the same order of magnitude as reported for the best performing other H₂O₂ generating biocatalysts.^[4b] As SO variants can be found in all organisms, other promising catalysts can easily be obtained *via* enzyme database mining.^[26] Finally, in terms of applications, the possibility of sulfite to further participate in chemo-enzymatic cascades opens up new ways to conveniently produce various hydroxysulfonates.^[27]

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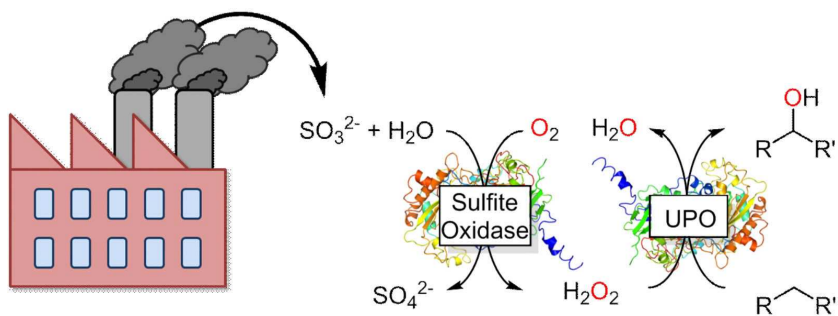
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COMMUNICATIONS



Oxyfunctionalisation: Sulfite oxidases are able to oxidize CaSO_3 , an industrial waste product, into CaSO_4 using a molybdenum cofactor. The redox balance can subsequently be closed by reducing oxygen to H_2O_2 , which is

required by peroxygenases for their selective oxyfunctionalisation reactions. We show this biocatalytic cascade can be applied up to semi-preparative scale.

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1 – 5

Selective Oxyfunctionalisation Reactions Driven by Sulfite Oxidase-Catalysed *In Situ* Generation of H_2O_2

