A Journal of the Gesellschaft Deutscher Chemiker

Angewandte International Edition

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To be cited as: *Angew. Chem. Int. Ed.* 10.1002/anie.201708668
Angew. Chem. 10.1002/ange.201708668

Link to VoR: http://dx.doi.org/10.1002/anie.201708668
http://dx.doi.org/10.1002/ange.201708668

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10.1002/anie.201708668

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Selective activation of C-H bonds by cascading photochemistry with biocatalysis

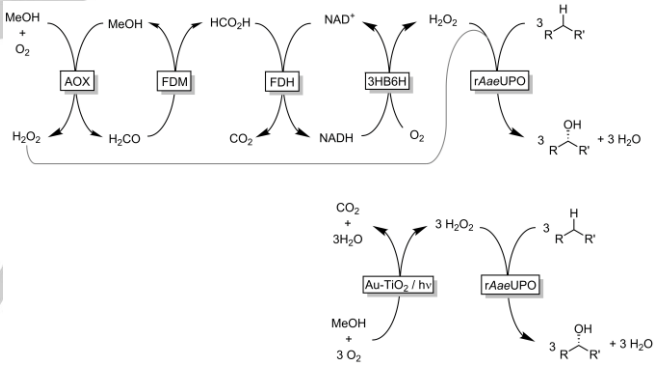
Wuyuan Zhang[†], Bastien O. Burek[†], Elena Fernández-Fueyo, Miguel Alcalde, Jonathan Z. Bloh[*], and Frank Hollmann[*]

Abstract: Selective oxyfunctionalisation of inert C-H bonds under mild conditions can be achieved using peroxygenases. This approach, however, is impaired by the poor robustness of these enzymes in the presence of hydrogen peroxide as the stoichiometric oxidant. Here, we demonstrate that inorganic photocatalysts such as gold-titanium dioxide efficiently provide H_2O_2 from methanol-driven reductive activation of ambient oxygen in suitable amounts to ensure high reactivity and robustness of the enzyme. Using this approach stereoselective hydroxylation of ethyl benzene to (*R*)-1-phenyl ethanol in high enantioselectivity (>98% ee) and excellent turnover numbers of the biocatalyst (>71.000) was achieved.

Selective oxyfunctionalisation of (non-)activated C-H bonds still represents one of the major challenges in organic synthesis. Heme-dependendent oxygenases are valuable catalysts for this task as they confine highly reactive Fe(IV)O species in the sterically well-defined active site of an enzyme.[1] Today, mostly P450 monooxygenases are considered as biocatalysts but peroxygenases (E.C.1.11.2.1) represent a practical alternative especially due to their ease of application. Instead of relying on complex electron supply chains providing the enzymes with reducing equivalents as in case of P450 monooxygenases, peroxygenases use hydrogen peroxide (H_2O_2) directly to form the catalytically active oxyferryl species (Compound I).[2]

H_2O_2, however, also is a potent inactivator of heme-enzymes via oxidative decomposition of the prosthetic group. Therefore, *in situ* generation of H_2O_2 in low concentrations is the preferred approach to alleviate this challenge.[1b] Generally, this is achieved through *in situ* reduction of O_2 to H_2O_2, posing the question about the nature of the electron donor used for this reaction. Next to electrochemical methods, oxidation of stoichiometric cosubstrates such as EDTA, amino acids, alcohols and other reductants[1b] have been investigated. Today, the most common system for *in situ* generation of H_2O_2 certainly is glucose/glucose oxidase. The poor atom efficiency of this system (glucose is oxidised only once to the corresponding lactone generating one equivalent of H_2O_2) together the pH shift originating from gluconic acid accumulation pose significant technological challenges to this approach (especially if used at preparative scale, Table S5 for further details). Therefore, we recently reported an enzymatic cascade to fully oxidise methanol to CO_2 and utilise the reduction equivalents liberated for H_2O_2 generation to promote peroxygenase reactions (Scheme 1).[3] For this, a rather complicated cascade comprising four enzymes and one cofactor was established. Despite the success of this reaction system, we asked ourselves whether a simpler and more elegant *in situ* H_2O_2 generation method is possible.

Inspired by recent work by Choi and Tada,[4] we set out to evaluate gold-loaded TiO_2 (Au-TiO_2) as plasmonic photocatalyst for the oxidation of methanol coupled to reductive activation of molecular oxygen to promote peroxygenase-catalysed oxyfunctionalisation reactions (Scheme 1).



Scheme 1. Comparison of the previously reported *in situ* H_2O_2 generation to promote peroxygenase-catalysed hydroxylation of alkanes using the recombinant peroxygenase from *Agrocybe aegerita* (r*Aae*UPO). Upper: the previously reported multi-enzyme cascade comprising alcohol oxidase (AOx), formaldehyde dismutase (FDM), formate dehydrogenase (FDH), 3-hydroxy benzoate-6-hydroxylase (3HB6H) as well as the nicotinamide cofactor ($NADH/NAD^+$);[3] lower: photochemical oxidation of methanol using Au-loaded TiO_2 (Au-TiO_2).

To test our hypothesis, we synthesised Au-loaded TiO_2 (rutile phase)[5] as methanol oxidation catalyst (SI for details) and used it for the selective hydroxylation of ethyl benzene to (*R*)-1-phenyl ethanol catalysed by the recombinant evolved peroxygenase from *Agrocybe aegerita* (r*Aae*UPO) as model reaction.[6]

Pleasingly, the proof-of-concept reaction proceeded smoothly to full conversion (Figure 1). Overall 10.7 mM of (*R*)-1-phenylethanol (98.2 % ee) was obtained within 72 h corresponding to a turnover number ($TN=mol_{product} \times mol_{catalyst}^{-1}$) of more than 71.000 for the biocatalyst. The sole by-product detectable was traces of acetophenone originating from the over-oxidation of the product by r*Aae*UPO (commencing upon

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depletion of the starting material). Omitting the biocatalyst resulted in small amounts (<0.15 mM) of racemic 1-phenyl ethanol. In the absence of the photocatalyst or performing the reactions in the darkness resulted in no detectable product formation. In the absence of methanol, some product formation was observed, which we attribute to Au-TiO_2-catalysed water oxidation (Figures S 29, 30).

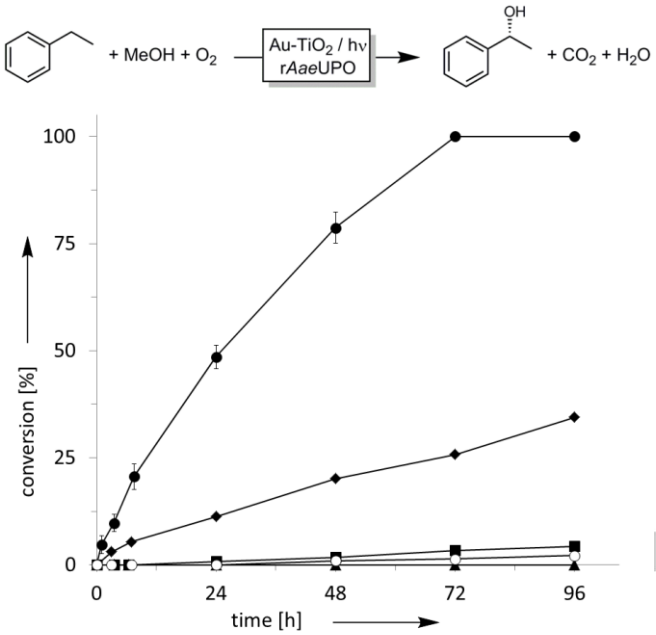


Figure 1. Photochemoenzymatic hydroxylation of ethyl benzene to (*R*)-1-phenyl ethanol combining Au-TiO_2 as photocatalyst for *in situ* H_2O_2 generation and r*Aae*UPO for the stereospecific hydroxylation reaction (●). Negative controls excluding enzyme (■), light (▲), methanol (◆) or rutile Au-TiO_2 (O). Reaction conditions: [methanol] = 250 mM, [rutile Au-TiO_2] = 5 mg mL^{-1}, [r*Aae*UPO] = 150 nM and [ethylbenzene] = 15 mM in 60 mM phosphate buffer (pH 7.0) under illumination.

It should be mentioned that evaporation of the reagents can be a challenge for the current reaction setup. Especially reactions with volatile reagents suffered from poor mass balances if exposed to the ambient atmosphere. Optimised setups, particularly closed vessels, circumvent this apparent limitation (Table S 2).

Next, we systematically investigated the influence of the single reagents on the rate of the photoenzymatic hydroxylation reaction (Table 1, and Figures S17-25). The concentration of MeOH had a significant effect on the initial rate steadily increasing with [MeOH] (Table 1, entries 1-6) and correlating well with the increasing formation rate and steady-state concentrations of H_2O_2. Au-TiO_2 is known to also oxidise H_2O_2 to O_2 thereby preventing its continuous accumulation in the reaction mixture.[4a, 7] Hence, both H_2O_2 and MeOH compete for oxidation at the catalyst surface which explains the higher steady state concentration of H_2O_2 in the presence of methanol. Above approx. 250 mM MeOH the photocatalyst surface appeared to be fully saturated as no further increase of the product formation rate was observed. It is also worth mentioning here that MeOH not only increased the overall reaction rate but also positively influenced the robustness of the process (Figure S 31 and Table S3).

In terms of photocatalyst concentration there seemed to be an optimal value around approx. 10 g L^{-1} with respect to the rate of the photoenzymatic hydroxylation reaction (Table 1, entries 5, 9 and 10). This observation falls into place considering the decreasing optical transparency of the reaction mixture with increasing photocatalyst loading (Figure S 26). Hence, the increasing H_2O_2 generation activity with increasing photocatalyst concentration was counteracted by the decreasing transparency of the reaction mixtures. Again, there was a good correlation between the overall rate with the steady state H_2O_2 concentration.

Increasing the enzyme concentration above 150 nM resulted in no further increase of the overall reaction rate (Table 1, entries 5, 7 and 8). A plausible explanation is that above this value the system was entirely H_2O_2-limited, *i.e.* almost every H_2O_2 molecule generated was consumed productively by the enzyme. Since the H_2O_2 formation rate under these conditions was 0.52 mM h^{-1} and the initial enzymatic product formation rate was 0.45 mM h^{-1}, the efficiency for the enzymatic H_2O_2 utilisation was approximately 87%. On the contrary, when the enzyme concentration was decreased to a third, the reaction rate was approximately halved, indicating that H_2O_2 was no longer the (sole) limiting factor. Under these conditions, the H_2O_2 utilisation efficiency dropped to 52%, as not all of the peroxide was consumed by the enzyme anymore and the excess was degraded by the photocatalyst and other unproductive processes.

The photon flux inside the reaction vessel, determined using ferrioxalate actinometry[8] was 2851 mE L^{-1} h^{-1}. Consequently, under standard conditions (150 nM UPO, 250 mM methanol) the photonic efficiencies of hydrogen peroxide and *(R)*-1-phenyl ethanol formation were 0.036% and 0.032%, respectively. Assuming that only fraction of light corresponding to the band gap of the rutile photocatalyst (≥3 eV / ≤413 nm, 0.7% of the lamp intensity, Figure S 7) was responsible for the activity, a photonic efficiency of 5.2% for hydrogen peroxide and 4.5% for the enzymatic conversion product can be estimated, respectively. In view of previously reported photonic efficiencies of only 1% for TiO_2[9] this may suggest that the photocatalyst used here could also harvest some of the visible fraction as well, presumably via the gold plasmonic resonance at approximately 550-600 nm (Figure S6).

^{1}H NMR analysis revealed that the Au-TiO2-catalysed oxidation of methanol did not stop at the formaldehyde level but also produced formic acid and, presumably, CO2 (Figures S27, 28).

Table 1. Photochemical *in situ* H_2O_2 generation to promote peroxygenase catalysed oxyfunctionalisation reaction.[a]

Entry	Electron donor	[r*Aae*UPO] [nM]	[electron donor] [mM]	[Au-TiO_2] [g L^{-1}]	Initial rate [mM h^{-1}]		Steady-State [H_2O_2] [μM] [b]	[(*R*)-1-phenyl ethanol] [mM][c]	GC-yield [%][d]	TON (r*Aae*UPO) $\times 10^{-3}$ [e]
					Product	H_2O_2				
1	MeOH	150	0	5	0.17	0.37	42	2.9	26	19
2	MeOH	150	5	5	0.20	0.56	55	3.3	24	22
3	MeOH	150	50	5	0.26	0.28	128	5.9	71	39
4	MeOH	150	100	5	0.24	0.56	231	6.4	76	42
5	MeOH	150	250	5	0.45	0.52	156	10.7	>99	71
6	MeOH	150	500	5	0.46	n.d.	n.d.	10.4	97	69
7	MeOH	50	250	5	0.27	0.52	156	2.8	36	55
8	MeOH	350	250	5	0.47	0.52	156	10.7	97	31
9	MeOH	150	250	10	0.46	1.05	160	11.9	>99	79
10	MeOH	150	250	20	0.29	0.44	97	10.1	>99	67
11	HCHO	150	250	5	0.73	1.01[g]	1050[g]	13.7	>99	91
12	$NaHCO_2$	150	250	5	0.58	0.98[g]	193[g]	12.6	99	84
13	EtOH	150	250	5	0.20	0.32[h]	154	3.8	33	25
14	^{i}PrOH	150	250	5	0.26	0.36[h]	122	5.3	46	35

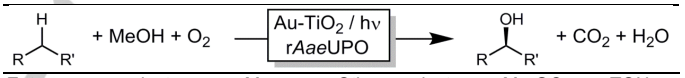
[a] reaction conditions: [ethylbenzene] = 15 mM in 60 mM phosphate buffer (pH 7.0) at 30 °C for 72 hours under illumination; [b] as determined in comparative experiments illuminating Au-TiO_2 in the reaction buffer (Figures S11, S14, S18 and S21); n.d. = not determined. [c] Product with 98% ee was obtained unless indicated otherwise; [d] GC-yield = $[(R)\text{-1-phenyl ethanol}]_{final} \times ([(R)\text{-1-phenyl ethanol}]_{final} + [\text{ethyl benzene}]_{final})^{-1}$; [e] TON = $[(R)\text{-1-phenyl ethanol}]_{final} \times [\text{r}Aae\text{UPO}]^{-1}$; [g] determined at 100 mM of the sacrificial reductant.

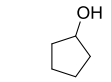
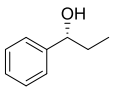
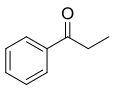
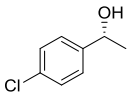
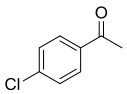
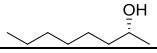
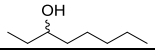
To further investigate this (desired) overoxidation of methanol, a set of experiments was conducted substituting methanol with formaldehyde and formate, respectively, under otherwise identical conditions (Table 1, entries 11, 12). Formaldehyde and formate gave approximately 32% and 18% faster reaction rates than methanol, respectively. This can be readily explained by the higher hydrogen peroxide formation rates observed for these compounds. Formaldehyde also suppressed H_2O_2 degradation, resulting in a higher steady state concentration of H_2O_2. The fact that the increase in peroxide formation was somewhat diminished in the enzymatic reaction rate might be explained by two effects. On the one hand, the response of the enzyme to a higher H_2O_2 formation rate is non-linear, as at some point the enzyme approaches its maximum turnover rate. On the other hand, the experiments with methanol are automatically superimposed by the reaction rate of formaldehyde and formate as they are formed during the reaction. This would be more pronounced in the photoenzymatic experiments than in the photocatalytic H_2O_2 formation due to the longer timescale of the experiments which allow for a higher fraction of the methanol to be converted. Nevertheless, especially formate may represent an attractive alternative to methanol as sacrificial electron donor (Figures S24, 25).

Also other alcohols such as ethanol or isopropanol could be used as sacrificial electron donors to promote the overall reaction, albeit at lower rates as compared to methanol (Table 1, entries 13, 14). The relative rates found with ethanol and isopropanol are in good correlation with the steady-state concentration and formation rate of H_2O_2 and roughly correlate with the oxidation potentials of the alcohols.[10]

Finally, we also evaluated the substrate scope of the proposed photochemobiocatalytic reaction sequence (Table 2). In line with the reported substrate scope of r*Aae*UPO[11] a range of (cyclo)alkanes and alkylaromatic compounds were converted into the corresponding alcohols.

Table 2. Preliminary substrate scope of the photochemobiocatalytic hydroxylation reaction.[a]



Entry	product	mM	ee [%]	Other products	mM	GC-yield [%][b]	TON (r*Aae*UPO) $\times 10^{-3}$
1		6.6	-		0.5	92.4	43.9
2		9.2	-		0.3	>99	61.5
3		4.3	-		0.4	55.7	28.6
4		6.9	>99		1.6	72	45.8
5		8.9	95.0		1.6	91.2	59.6
6		8.0	93.3		1.3	83.5	53.5
7		1.0	89		1.6	67.8	17.5

[a] Conditions: [substrate] = 10.0 mM, [rutile Au-TiO_2] = 10 gL^{-1}, [r*Aae*UPO] = 150 nM, [MeOH] = 250 mM in phosphate buffer (pH 7.0, 60 mM), T = 30 °C,

70 h, under illumination; [b] = $[alcohol]_{final} \times ([ketone]_{final} + [starting\ material]_{final})^{-1}$;

The regio- and enantioselectivity was essentially the same as in previous studies. The only side reaction observed was a minor overoxidation to the corresponding ketone as described above.

Very pleasingly, high turnover numbers could be achieved throughout these experiments that compare well with the numbers reported so far with more complicated *in situ* H_2O_2 generation systems.[1b] Hence, we are optimistic that further optimisation of the reaction setup may well lead to an economically attractive oxyfunctionalisation reaction. Indeed, a preparative scale hydroxylation reaction of ethyl benzene yielded more than 100 mg of essentially enantiopure product (75% conversion, 51% isolated yield). Further optimisation is currently underway.

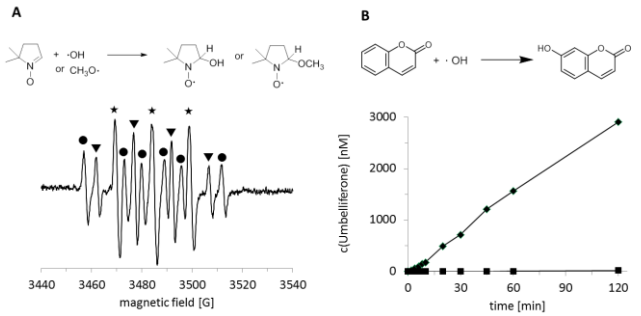


Figure 2. Qualitative and quantitative determination of radicals occurring during the photocatalytic process. (A) EPR spectra recorded during the illumination of and rutile Au-TiO_2 in water with methanol for 20 min. Signals marked with asterisk (★) belong to the existing oxidation product of DMPO, 5,5-dimethyl-2-oxopyrroline-1-oxyl (DMPOX).[12] Signals marked with triangles (▼) belong to the spin-adduct •DMPO-OH. Signals marked with circles (●) belong to the spin-adduct •DMPO-CH_2OH from methanol.[13] Reaction condition: [Au-TiO_2] = 5 g L^{-1}, [DMPO] = 30 mM, [methanol]= 100 mM, RT, under illumination; (B) Time course of the photocatalytic umbelliferone generation from coumarin as a specific detection method for •OH radicals. Reaction conditions: 60 mM phosphate buffer (pH 7), [Au-TiO_2] = 5 g L^{-1}, [coumarin] = 0.1 mM, [methanol]= 0 (◆) or 250 mM (■), T = 30 °C, under illumination.

As mentioned above, methanol not only accelerated the overall reaction but also contributed to its robustness (Figures S 29, 31). In the absence of methanol rAaeUPO lost its catalytic activity almost instantaneously under illumination whereas in the presence of methanol the enzyme activity was retained for several hours (Figure S 31). We suspected reactive oxygen species formed by the photocatalysts to account for this, which was qualitatively confirmed with EPR spectroscopy (Figure 2A).[13] More quantitatively, the coumarin method[14] showed that hydroxyl radicals were formed in significant amounts only in the absence of methanol (Figure 2B). Upon addition of methanol (250 mM) the hydroxyl radical formation rate dropped to only 0.6% of the original value.

Apparently, methanol oxidation occurs significantly faster than water oxidation, which is comprehensible considering the redox potentials of water to hydroxyl radicals, +2.8 V,[15] and methanol to methanol radicals, +1.2 V,[16] respectively. Moreover, due to the strongly reducing nature of the methanol radical (-1.3 V), it can readily inject an electron into TiO_2, forming formaldehyde and resulting in up to two conduction band electrons per reactive photon, an effect also known as current doubling (Figure S32).[17] Hence, methanol oxidation not only accelerated the H_2O_2 generation rate but also prevented the formation of ROS from water oxidation (Figure S32 and Table S3 for further details).[18]

Overall, this study demonstrates the application of methanol as sacrificial reductant for *in situ* H_2O_2 generation from O_2 to promote selective, peroxygenase-catalysed oxyfunctionalisation reactions. Admittedly, the productivities reported here do not reach preparatively useful values yet. Also the very high turnover numbers for r*Aae*UPO reported previously have not been reached yet. Future efforts will therefore focus on optimizing the light penetration into the reaction medium and increasing the H_2O_2 generation rate, e.g. by using photochemical flow-chemistry setups[19] or wirelessly powered internal illumination.[20]

Acknowledgements

F.H and W.Z. gratefully acknowledge financial support by the European Research Council (ERC Consolidator Grant No. 648026). B.O.B. and J.Z.B are grateful for financial support from the German Research Foundation (DFG, grant no. BL 1425/1-1). The authors thank Ben Norder (Delft University of Technology) for XRD, Dr. Wiel H. Evers (Delft University of Technology) for TEM and Prof. Fred Hagen (Delft University of Technology) for EPR measurements.

Keywords: Biocatalysis • Photocatalysis • Oxyfunctionalisation • TiO_2 • Peroxygenase

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10.1002/anie.201708668

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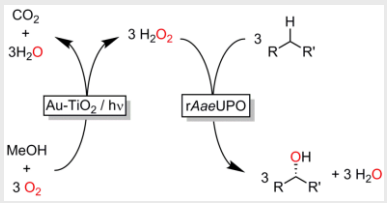
Layout 1:

COMMUNICATION

Selective oxyfunctionalisation reactions are achieved by combining inorganic photocatalysis with selective enzymatic oxyfunctionalisation catalysis.



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Selective activation of C-H bonds by cascading photochemistry with biocatalysis