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Divorce in the two-component BVMO family: the single oxygenase for enantioselective chemo-enzymatic Baeyer–Villiger oxidations†

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Two-component flavoprotein monooxygenases consist of a reductase and an oxygenase enzyme. The proof of functionality of the latter without its counterpart as well as the mechanism of flavin transfer remains unanswered beyond doubt. To tackle this question, we utilized a reductase-free reaction system applying purified 2,5-diketocamphane-monooxygenase I (2,5-DKCMO), a FMN-dependent type II Baeyer–Villiger monooxygenase, and synthetic nicotinamide analogues (NCBs) as dihydropyridine derivatives for FMN reduction. This system demonstrated the stand-alone quality of the oxygenase, as well as the mechanism of FMNH₂ transport by free diffusion. The efficiency of this reductase-free system strongly relies on the balance of FMN reduction and enzymatic (re)oxidation, since reduced FMN in solution causes undesired side reactions, such as hydrogen peroxide formation. Design of experiments allowed us to (i) investigate the effect of various reaction parameters, underlining the importance to balance the FMN/FMNH₂ cycle, (ii) optimize the reaction system for the enzymatic Baeyer–Villiger oxidation of rac-bicyclo[3.2.0]hept-2-en-6-one, rac-camphor, and rac-norcarnphor. Finally, this study not only demonstrates the reductase-independence of 2,5-DKCMO, but also revisits the terminology of two-component flavoprotein monooxygenases for this specific case.

Introduction

The Baeyer–Villiger (BV) oxidation converts ketones to esters and lactones by oxidative cleavage of the C–C bond adjacent to the carbonyl group.1,2 The (chemical) usage of peroxide derivatives is, without a doubt, a powerful methodology for this C–C bond-breaking process; however, it also suffers from a lack of enantioselectivity. As often observed with biological processes, the enzyme-catalysed version of BV oxidation overcomes this handicap.3,4

Oxidative enzymes are capable of catalysing a large range of reactions, but often require cofactors, which have to be recycled to enable their catalytic usage. Among these enzymes, the class of oxygenases is immensely diverse5–8 and contains inter alia flavoprotein monooxygenases (FPMOs), a family in which are found enzymes that catalyse BV oxidation. These monooxygenases belong to the groups A, B or C of FPMOs9 and are single- or two-component enzymes (Scheme 1), respectively called type I and type II Baeyer–Villiger monooxygenases (BVMOs).9–11

Despite their structural differences, the one- and two-component oxygenase systems share a dependency on the expensive nicotinamide adenine dinucleotide cofactor (NAD(P)H). Moreover, the stability of NAD(P)H can become a hurdle (under basic conditions), as the adenine dinucleotide moiety is susceptible to hydrolysis.13–15

Synthetic nicotinamide analogues (nicotinamide coenzyme biomimetics, NCBs) address both obstacles, as they can be synthesized at lower costs while remaining stable.16 Moreover, they have been established to replace their natural templates for numerous flavin-dependent enzymes17–19 with a flavin shuttle electron mediation.20–22

Most of the studies on enzymatic BV oxidation focused on one-component BVMOs from group B (also known as type I BVMOs). These enzymes are available in high numbers in protein databanks, easily detectable by consensus sequence research23 and subsequently more frequently investigated and applied in many catalytic processes.3,4,24–26 In contrast, two-
complete reaction. The concept of flavin recycling as the adjusting wheel of two-component system to avoid flavin degradation is discussed and the donors without a loss of activity. Finally, the tunability of the compatibility of the monooxygenase with artificial hydride meters and optimize the final system. The utilization of NCBs was executed to estimate the impact of the seven reaction parameters and co-factors. The mechanism of hydride transfer and the balance component systems referred to as type II BVMOs (from group C of FPMOs) are rare. Only two representatives are known so far, 2,5-diketocamphane 1,2-monoxygenase I (2,5-DKCMO; EC 1.14.14.108) and 3,6-diketocamphane 1,6-monoxygenase (2,6-DKCMO; EC 1.14.14.155), probably because a two-component system is not a trivial biocatalytic arrangement to identify. The mechanism of hydride transfer and the balance of the reductase and oxygenase reactions are still unknown. Consequently, the question of whether the oxygenase can work independently of the reductase component and nicotinamide cofactor remains to be determined.

In this in vitro study, we showed the promising catalytic capacity of the oxygenase component and established its mode of reaction. We report an innovative and simplified reductase-free enzymatic system for BV oxidation, driven by NCB hydride donation for flavin mononucleotide (FMN) reduction and further electron mediation through the flavin. The purified type II BVMO 2,5-DKCMO from *P. putida* ATCC 17453 (NCIMB 10007), which was produced via heterologous expression as an E. coli-codon-optimized version with an N-terminal His-tag for affinity chromatography purification (see ESI section A for further details†). A fast substrate profiling showed that rac-bicyclo[3.2.0]hept-2-en-6-one 1, an established substrate for the evaluation of enantioselective BV oxidation, displayed the highest conversion rate among the assayed compounds (see ESI, Fig. S4†). The biotransformation of rac-1 by 2,5-DKCMO was already investigated by Lau and co-workers, as well as the group of Bornscheuer, but no complete characterization of the reaction had been made. Thus, we selected this compound as the model substrate for our study.

**Biocatalyst formulations**

Interestingly, the biocatalyst was stored at −20 °C for 21 days in a frozen as well as lyophilized form with minimal loss of

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**Model reaction system**

The great interest on type II BMVOs is due to their simple character, as they are separated reductase and oxygenase components. This feature may allow the transfer of the preformed FMNH 2 to the oxygenase component by rapid diffusion without the need for any protein–protein interaction. It has been shown previously that multiple FMN reductases can act as alternative distal donors to the monoxygenase component. Consequently, it is reasonable to assume that it can accept FMNH 2 from an origin other than enzymatically reduced if no protein–protein interaction with a reductase is required. Herein, the possibilities to tune such enzymatic type II BVMO reactions by the substitution of a reductase-mediated reduction of the FMN are very promising, since simplified reaction systems are of great potential for further applications, for instance, for an external electron supply through a variety of electron donors.

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**Results and discussion**

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**Scheme 1** Flavin-dependent single- and two-component monooxygenase systems. (a) One-component FPMO system: the reductase and oxygenase domains are gathered in a single protein. Flavin is tightly bound as a prosthetic group in the active site of the enzyme. (b) Two-component FPMO system: reductase and oxygenase components are in two separated proteins. The flavin reduction catalysed by the reductase is driven by NAD(P)H oxidation, whereas flavin-H 2 oxidation is driven by the oxygenase. The mode of flavin-H 2 transport depends on the two-component monooxygenase, achieved either by free diffusion or via reductase-oxygenase mediation.22

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**Fig. 1** Enantioselective and regioselective enzymatic BV oxidation of rac-bicyclo[3.2.0]hept-2-en-6-one 1. Each enantiomer of the ketone is the precursor of two regioisomeric lactones 2 and 3. The thickness of the arrows represents the preference of 2,5-DKCMO.
activity. Both formulations maintained approx. 70–80% of the conversion rate of the ‘fresh’ biocatalyst, determined directly after purification. In the experiments with ‘fresh’ oxygenase, 84 ± 5% of the model substrate 1 was converted after two hours, whereas in the frozen enzyme system and the lyophilized one, 59 ± 5% and 67 ± 7% of conversion were observed respectively (see ESI, Fig. S6†). This observation indicates that 2,5-DCKMO is particularly resistant against lyophilization and freezing conditions compared to most BVMOs for which low stability is often the greatest hurdle.25,40 From a practical point of view, this quality was highly desirable since it allowed the easy storage of the biocatalyst, and consequently facilitated the comparison of various reaction conditions, avoiding frequent enzyme production and/or tedious normalization of the activity.

**Screening of oxygenase-catalysed reactions with NCBs**

We investigated the potential of two synthetic nicotinamide analogues41 for FMN reduction. The direct FMN reduction requires stoichiometric amounts of hydride donors, which will be oxidized. The inexpensive cost of synthesizing NCBs makes them attractive for this use. Moreover, in terms of electron transfer efficiency and atom economy, the NCBs are superior to the natural system.20 NCB-driven approaches were documented for other flavoenzymes, such as very recently for a regioselective aromatic hydroxylation,41 and other two-component FMPOs.20–22 These systems outperformed the natural enzymatic regeneration system proceeding at four times faster initial reaction rates21 and NCBs showed a very high K_m compared with the natural nicotinamide cofactor.41 However, they have never applied to type II BVMO.

The two partially water-soluble and stable NCBs, 1-benzyl-1,4-dihydronicotinamide (BNAH), and 1-(2-carbamoylmethyl)-1,4-dihydronicotinamide (AmNAH, caricotamide), were selected. To ensure a homogeneous reaction system, both NCBs were supplied from a stock solution in DMSO. The first experiments, with both NCBs and without reductase, showed a slow enantioselective conversion of ketone 1 with an increase of the enantiomeric excess in favour of the (+)-enantiomer and a preferential formation of (+)-(1R,5S)-2-oxabicyclo[3.3.0]oct-6-en-3-one 2, also called (+)-normal lactone. The reaction system is shown in Scheme 2. Both NCBs provided the hydride donation for the reduction of the flavin cofactor FMN before FMNH_2 diffusion into the active site of the oxygenase. However, a difference of performance was observed as shown in Fig. 2 and AmNAH appears as the most suitable hydride donor. Both a higher ee of the substrate and a higher conversion were observed at the two tested concentrations (10 and 50 mM).

The preliminary results did not show the full potential of the approach. The presence of reduced and oxidized flavin, as well as oxygen in the aqueous medium, is a potentially delicate composition.42–46 In absence of the catalase, a change of the colour of the reaction from pale yellow to orange was observed with AmNAH as shown in Fig. 3. This indicates the formation of anionic semiquinone flavin radicals and other flavin decomposition products, due to an uncoupling of the reduced flavins into radicals and hydrogen peroxide.47,48 H_2O_2 is known to be formed with flavoprotein monoxygenases, including two component styrene monoxygenase and halogenases.20,21 On the other hand, it is also known to oxidize substrates as ketone 1 in racemic lactone 2.49 In our case, the addition of catalase improved the biotransformation (Fig. 3) as illustrated by the complete disappearance of the (−)-ketone 1 in six hours along with a better ee of normal lactone 2 (82% and 55% respectively with and without catalase after two hours of reaction). Consequently, it can be concluded that catalase addition alleviates the unselective chemical BV oxidation by H_2O_2. In this regard, the stability of the enzyme

![Scheme 2](image-url)
in the presence of H₂O₂ was also evaluated.† After its storage in up to 50 mM H₂O₂ for four hours, no significant decline of activity was observed (data not shown), confirming the great stability of the oxygenase.

Control experiments for flavin reductase and AmNAH

To transfer electron mediators from one (bio)molecule to another, two basic mechanisms have evolved in nature: (i) either driven in an active fashion e.g. via protein–protein interaction as with cytochrome P450 enzymes (this interaction mediates the electron transfer from the flavin cytochrome P450 reductase to the heme of the oxygenase) or (ii) passively by simple free diffusion as it has been shown for the two-component FPMO bacterial luciferase. In other two-component FPMOs, this transfer has been a pivotal question for the last decades that remains to be clarified, although the concept of free-diffusion of the reduced flavin to the oxygenase was predominant for most of the described systems. On the other hand, in the case of type I BVMO, it has been shown that NADPH contributes to enzymatic catalysis and interferes positively with enantioselectivity, not making its substitution desirable.

†Assay conditions: 50 µL mL⁻¹ oxygenase, 50 µM FMN, 25 mM NADH as the hydride source, 50 mM Tris-HCl buffer at pH 7.5, 20 °C and 160 rpm.

To determine the behaviour of our model type II BVMO system in the presence and absence of a reductase, purified flavin reductase (Fre) from E. coli was selected to act as the reductase counterpart. This enzyme was previously described as a suitable supplier of reduced FMN for 2,5-DKCMO. The Fre enzyme was applied in our experiments in an equimolar amount (compared to the monoxygenase) using NADH as the hydride donor. To evaluate the potential impact of Fre on the catalytic performance of the reaction system, identical setups without Fre served as control experiments (Fig. 4).

Surprisingly, in terms of substrate conversion, the reductase-free system applying AmNAH as the hydride donor outperformed the reductase setup using NADH over time (see Fig. 4a). Similar results were obtained when five times more

 Assay conditions: 50 µL mL⁻¹ oxygenase, 50 µM FMN, 25 mM NADH as the hydride source, 50 mM Tris-HCl buffer at pH 7.5, 20 °C and 160 rpm.

† Fig. 3 Chemo-enzymatic enantioselective BV oxidations of rac-1 with AmNAH in the presence and absence of catalase. Experiments with catalase: plain lines — full marks; without catalase: dashed lines - - - , empty marks. Graph (a): Conversion: 1, 1; Remaining (−) ketone 1, ▲, ▲. Graph (b): Normal lactone 2 ee: ●, Ketone 1 ee: ■, □. The error bars indicate the standard deviation of two independent reactions. The pictures on the right show the reaction mixtures after 8 hours. Experimental conditions: 4 mM rac-1, 50 µM FMN, 50 mM AmNAH in 1 mL of tricine buffer (50 mM, pH 8.5) at 15 °C. 0.5 mg mL⁻¹ lyophilized 2,5-DCKMO and, if mentioned, a great excess (2.5 mg mL⁻¹) of catalase, but no flavin reductase added.

Assay conditions: 50 µL mL⁻¹ oxygenase, 50 µM FMN, 25 mM NADH respectively. 5.0 µM FMN, 1 mL of tricine buffer (50 mM, pH 8.5) at 30 °C. 0.4 mg mL⁻¹ frozen 2,5-DCKMO, 2.5 mg mL⁻¹ catalase, and if required, lyophilized Fre was added in equimolar amounts to the oxygenase. Reactions were performed in duplicate.

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Fre enzyme was applied§ (data not shown). The NADH-driven Fre-system did not exceed a final conversion of 63 ± 0% (reached in less than two hours) in the equimolar Fre system, or 56 ± 2% (reached after three hours) when Fre was applied in five times excess, respectively. The reaction system without the reductase, driven by the direct FMN reduction via AmNAH, reached nearly full conversion (97 ± 1%) after six hours. The evolution of ee of ketone 1 was also in agreement with this observation.

Additionally, we compared the two systems with the reaction only driven by NADH (without Fre). To succeed in comparing the enantioselectivity of the three complex systems, the effect of different FMN reduction rates has to be eliminated. Thus, Fig. 4b shows the substrate ee as a function of the conversion. The plotted curves of the reductase-free reaction setup with AmNAH, as well as with NADH were perfectly supposable, and in agreement with the experiment associating the reductase and NADH.

With these experiments, we demonstrated the independency of 2,5-DKCMO from any flavin reductase enzyme exemplary by its independence from Fre. The oxygenating component of the chosen type II BVMO system maintained its enzymatic capacity in the absence of the reductase. Moreover, the nicotinamide cofactor was substituted by AmNAH, demonstrating that the hydride donor for in situ FMN reduction can be easily replaced. Therefore, we conclude that the mechanism of reduced flavin transfer in this two-component monoxygenase system does not require a protein–protein interaction but is achieved passively by free diffusion.

Characterization of the reductase-free reaction system

Since the stability of the oxygenase is not questioned, we ascribe the early cessation of the reaction observed in some experiments to an imbalance of the reaction system. Indeed, FMN recycling has a particularly delicate balance, as it depends on fine adjustments of the concentrations of the hydride donor, the enzyme, and the flavin itself. To investigate this hypothesis and to further assess the characteristics of the system, we took advantage of the adaptability of 2,5-DKCMO regarding the reductase and hydride donor for FMN reduction.

The impact of different reaction parameters in the reductase-free setup was tested using a DoE (Fig. 5) comprising NCB type and concentration, FMN and oxygenase concentration as well as temperature and buffer type, and pH (for DOE details, see ESI, Table S1†).

The ee of the ketone after three hours and the final conversion were chosen as data for the DoE calculation. They allowed respectively an insight into (i) the reaction rate (activity) of the system in the early stages of the reaction, by monitoring the enantioselectivity of the enzyme, and (ii) the durability of the system by monitoring the capacity of the enzyme to transform the substrate in 22 hours. The latter one is of particular interest because it depends on the process stability of all species present in its environment.

The DoE results are shown in Fig. 5. It is important to note that the length of bars in the figure is not proportional to the quantitative outcomes, but represents the influence of a given parameter on the outcomes. From the DoE results (Fig. 5), we concluded that the buffer (both nature and pH) had a relatively small effect on both outputs, and hence on the whole system. Unsurprisingly, the temperature and oxygenase concentration affected the system during the entire reaction. All other tested parameters affected the response largely but with varying degrees of intensity in the early and later states. The same observation was observed for the FMN concentration, but with a greater effect of lower concentrations at the early stage to a lower impact in the late stage.

The NCBs revealed a clear influence of their nature and concentration on the performance of the system. Interestingly, the impact of the N-substituent type, an amide (AmNAH) or phenyl group (BNAH), was the strongest parameter at the early stage of the reaction (ketone ee at three hours), as shown by the AmNAH bar being considerably longer than its BNAH counterpart. We attribute the early stage-impact to the dissimilar flavin reduction rate (NCB-driven hydride transfer) evoked by different (electro) chemical characteristics e.g. redox potential of the NCBs, but also their chemical stability in aqueous solution. Although

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§ Five times excess compared with the monoxygenase using the same NADH concentration.

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Fig. 5 Screening by DoE of parameter effects on reductase-free 2,5-DKCMO-mediated enantioselective BV oxidations of rac-1. The (i) enantiomeric excess of the ketone 1 at three hours and (ii) total conversion after 22 hours were selected to calculate the DoE output. The parameters are shown to the left, the levels are shown to the right: e.g. FMN is the parameter, and 25–150 µM are the levels. The length of the bars represents the impact of the parameter levels on ketone ee and conversion. Greater effects are shown by larger bars. Blue numbers show some changes in the values of outputs compared to the reference level (black bars and dashed lines).

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BNAH is the stronger reducing agent, its half-life time in aqueous media (approx. 1.5 hours) is more than ten times shorter compared to AmNAH. At the end of the reaction, the impact of both NCBs decreased, as the rate of hydride transfer to FMN became negligible, and therefore insignificant. Hydride donation was not only affected by the type of hydride donor (NCBs) but also by the concentration, as we observed an impact of this parameter at the early stage and the end of the reaction. Interestingly, this shifts over time with an increasing impact of lower concentrations at the later stage of the reaction. Thus, lower NCB concentrations did not result in higher conversion. However, it must be noted that the interpretations gained from the DoE are only valid within its boundaries, e.g. an impact of a pH value below 7.5, or above 8.5 might be considerable.

The analysis of DoE results shows that all parameters with a great impact on the reaction system are associated directly with the FMN/FMNH₂ cycle (see Fig. 6). This applies to the reduction of the flavin, e.g. the type and concentration of the NCBs (see Fig. 6 in blue), but also to its enzymatic FMNH₂ (re) oxidation for which the oxygenase concentration (see Fig. 6 in red) is crucial.

The temperature was also identified as a relevant parameter in the system, which is in accordance with our interpretation, as it affects both flavin reduction and (re)oxidation, albeit to a different extent (reduction = chemically versus oxidation = enzymatically). In some experiments, an increased chemical oxidation of ketone but no bioconversion was observed. Therefore, the impact of the parameter on both the chemical and chemoenzymatic performance of the reaction system was also evaluated.

As a consequence of the identification of the key role of the flavin and its function as the adjusting wheel of the reaction, we assessed the FMN reduction/(re)oxidation cycle to be crucial for a (long-time) catalysis. The reduction and oxidation rates of the flavin need to be balanced to ensure the greater stability of the system’s components, in particular, to minimize the instability of the reduced flavin in the presence of oxygen.

Improvement of the system

A second DoE was performed with AmNAH, identified to give the highest conversions of our model substrate rac-

ketone 1. This DoE aimed to optimise the reaction for maximum conversion and high enantioselectivity. Herein, we also included the other two oxygenase substrates, rac-camphor (rac-4) and rac-norcarnorphor (rac-5) (see ESI, Fig. S4†). Maintaining the reaction parameters affecting FMN reduction (NCB type and concentration), as well as the flavin (re)oxidation (oxygenase concentration) constant, we investigated the optimum FMN concentration and the temperature for the three substrates, respectively within the intervals of 10–25 µM and 12–30 °C. The outputs, conversion and ketone ee were measured after two hours for all the compounds (Fig. 7).

Among the tested substrates, 2,5-DKCMO showed the highest activity towards the model substrate rac-1 with a maximum conversion of approx. 75% (Fig. 7a), followed by rac-4 with >35% (Fig. 7b) with an exclusive conversion of (1R,4R)-camphor. rac-5 is a rather poor substrate achieving conversions less than 20% (Fig. 7c) within the first two hours under the given conditions. For all tested substrates we observed greater conversions (Fig. 5a–c) at higher temperatures. For rac-1, low and medium FMN concentration (up to 20 µM) resulted in better performances. Similar results were observed with rac-

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Fig. 6 Impact on the reaction parameter of the reductase-free NCB driven model reaction. The parameters affecting the (abiotic) reduction of the FMN are the type and concentration of the NCB (in blue). The oxygenase concentration catalyses the (re)oxidation of the fully reduced flavin (in red) and hence is the important counterpart in this cycle. Temperature (not shown) and FMN concentration (in black, in the centre) contribute to both chemical reduction and enzymatic (re)oxidation of the flavin.

Fig. 7 Optimization by DoE of the enzymatic BV oxidation of rac-1 (a and d), rac-camphor (b and e), and rac-norcarnorphor (c and f) in a reductase-free system. The two parameters, [FMN] and temperature, varied from 12 °C to 30 °C and from 10 µM to 25 µM. The outputs are (i) the total conversion (a–c), and (ii) the enantiomeric excess (d–f) after two hours. Note: different scales are displayed for the same colour code regarding the DoE output. Experimental conditions: 5 mM substrate, 0.19 mg mL⁻¹ purified and frozen oxygenase, 25 mM AmNAH, 2.5 mg mL⁻¹ catalase in 50 mM tricine buffer at pH 8.5.
4 or more precisely (1R,4R)(+)-camphor) and rac-5 despite significantly lower rates.

Regarding the ee of the substrates (Fig. 7d–f) we observed (i) an effect of the temperature on the rac-1, resulting in highest ee values at low to moderate temperatures and (ii) an effect of the FMN concentration on rac-4 with a clear trend for higher ee at low FMN concentrations. In this context, we expect the optimal FMN concentration for the reaction to be lower than our tested range. The results of rac-5 however, are difficult to evaluate due to the low conversion in the DOE condition window.

Summarizing the results of the second DoE, we observed the general trend for better enzymatic performance at lower to mediate temperatures and flavin concentrations.

These data support our hypothesis of the flavin/flavin-H₂ cycle being the bottleneck and hence the major adjusting wheel in the reductase-free setup. It is also noticeable that the optima for the three compounds are slightly different, which reflects the influence of different enzymatic oxidation rates. Therefore, in a virtually optimized reaction setup, we expect to accomplish experimental conditions, in which flavin reduction and the (enzymatic) (re)oxidation are appropriately balanced.

**Enantioselective in vitro biotransformation of rac-1 by 2,5-DKCMO**

The biotransformation of rac-1 by 2,5-DKCMO was already tested but no complete description had been made. A typical gas chromatogram of the enzymatic bioconversion is shown in Fig. S5 in the ESI.† The knowledge gained from the DoEs allowed us to apply improved conditions for full conversion and high enantioselectivity as shown in Fig. 8 to characterize the model reaction in detail. This, however, is not trivial, as enzyme reaction rates may slightly vary from batch to batch, which requires the adaption of the flavin reduction rate to ensure the stability of the system. A set of adapted conditions using AmNAH allows examining the evolution of all compounds present in the reaction as reported in Fig. 8. As no alcohol formation was observed, reduction of the ketone by AmNAH was excluded.

The BV oxidation of each enantiomer of rac-1 offers theoretically two possibilities, as shown in Fig. 1. (−)-Ketone 1 can be transformed into either the so-called (+)-normal lactone 2 or (−)-abnormal lactone 3, while (+)-ketone delivers the opposite enantiomers. The temperature of the biotransformation was lowered to 14 °C for a better vision of the evolution of each enantiomer. Indeed, as suggested in Fig. 7d and in accordance with a behaviour already described for various enzymes, enantioselectivity was favourably affected by a drop in temperature. Under our reaction conditions, 2,5-DKCMO converted (−)-(1S,5R)-enantiomer 1 was first obtained as shown in Fig. 8. The beginning of the bioconversion was therefore characterized by the formation of (almost exclusively) (+)-normal lactone 2. This demonstrates the high enantio- and regioselectivity of the reaction. Surprisingly, once the (−)-(1S,5R)-1 was (almost) consumed, the oxygenase converted also the (−)-(1R,5S)-1 at similar rates compared to the enantiomeric counterpart. This conversion gave birth to both (+)-normal lactone 2 as well as the (+)-abnormal lactone 3. To the best of our knowledge, such sequential oxidation of enantiomers of the rac-1 has only been described for the one-component phenylacetone monoxygenase (PAMO) with non-natural FAD derivatives. 60

**Experimental details**

Note: a detailed description of procedures is available in the ESI section A – Material and Methods.†

**Chemicals**

rac-1, rac-4, and rac-5 as well as the other tested substrates were obtained from chemical suppliers. Authentic lactone samples were synthesized by microbiological biotransformation according to described procedures. NCBs were synthesized as described in the literature and used from a stock solution in DMSO (1 M).

**Preparation of the oxygenase**

Cloning and transformation. The gene (camE_{25}; oriB) from the large linear 533 kb CAM plasmid encoding for the 2,5-DKCMO in Pseudomonas putida ATCC 17453 (NCIB 10007) was optimized for *E. coli* and purchased from Thermo Fisher®. The synthetic gene was cloned into a pET15b plasmid via the restriction ligation technique (using Ndel and XhoI restriction sites previously added to the ends of the synthetic gene). For the Fre reductase, the genomic DNA from *E. coli* BL21(DE3)
was isolated. The gene (fre, originating from E. coli K12) was amplified by PCR and cloned into the pET15b vector by the same approach as applied for the oxygenase. Both constructs encode for proteins with an N-terminal Histidine-Tag for downstream protein purification.

**Cell culture.** An *E. coli* BL21(DE3) strain harbouring each construct was incubated in a *Luria-Bertani* medium containing 100 μg mL⁻¹ ampicillin at 37 °C with a shaking speed of 180 rpm. When the OD₆₀₀ value reached 0.5–0.8, IPTG was added to a final concentration of 0.5 mM to induce the expression of proteins for 14 h at 25 °C and 180 rpm.

**Enzyme purification.** Cells were harvested by centrifugation at 4 °C, resuspended in lysis buffer (50 mM Tris-HCl, pH 7.5), and disrupted by sonication. The supernatant was purified by Ni-NTA affinity chromatography and the purified protein was aliquoted after dialysis. The aliquots were used immediately for bioconversions or directly frozen in liquid nitrogen before storage at −80 °C. Protein expression was analysed by SDS-PAGE for each batch.

**Biotransformation and gas chromatography analyses**
Sealed 2 mL glass vials were used for all bioconversions. Typically, the total reaction volume was set to 1 mL or 0.5 mL, respectively. Samples were taken from the same batch over time, directly from the reaction medium (buffer), and extracted in ethyl acetate solution containing 0.5 g L⁻¹ of tridecane as the internal standard, in a volumetric ratio of 1 to 2.

GC analyses of the three ketones and their corresponding alcohols were carried out on a Cyclosil-B column (Agilent J&W GC columns). Product identification was ensured by the comparison of GC retention times with those of authentic samples (see ESI A†).

**Design of experiments (DoE)**
AZURAD software (http://www.azurad.fr) was applied for the DoEs using a polynomial model with linear, quadratic, and interaction effects. Multilinear regression was applied to calculate the coefficients in the modelling step. The details are shown in ESI A in Tables S1, S2 and Fig. S7.†

**Conclusions**
The high stability of the oxygenase part of the two-component BVMO system of 2,5-DCKMO, as well as its independence from the reductase, offers multiple possibilities to apply this biocatalyst in a modular fashion, as long as the availability of fully reduced flavin is provided. In this regard, it is essential to balance (i) FMN reduction, (ii) diffusion-driven transport, and (iii) (re)oxidation of the oxygenase. FMN diffusion is the most difficult to customize. Adjusting the FMN reduction rate, or the (specific) activity of the oxygenase, respectively, offers a more convenient strategy. Notably, tuning should be adopted to the other parameters to ensure the balanced state of the flavin recycling.

The reductase-free setup of the redox system finally demonstrated that the transport mechanism of reduced flavin to the oxygenase in this study proceeded by free diffusion. This reductase-independent enzymatic BV oxidation enables the reduction of the complexity of this two-component enzyme. In addition, it questions the terminology of the system(s), since the reductase might only be required in the natural (*in vivo*) context, but can be removed in artificial systems, still maintaining the oxygenase functionality. 2,5-DKCMO might be characterized as one component with the ability to utilize (hydroquinone) electron mediators, or flavin hydroquinone-dependent monooxygenase. Therefore, for other representatives of two-component FPMOs, each oxygenase component could advantageously be investigated separately from its reductase counterpart.

We gave herein an additional example of the synthetic potential of two-component flavoprotein monooxygenases. 

**Conflicts of interest**
There are no conflicts of interest to declare.

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