One-pot Biocatalytic Double Oxidation Process for the Synthesis of Ketoisophorone from α-Isophorone

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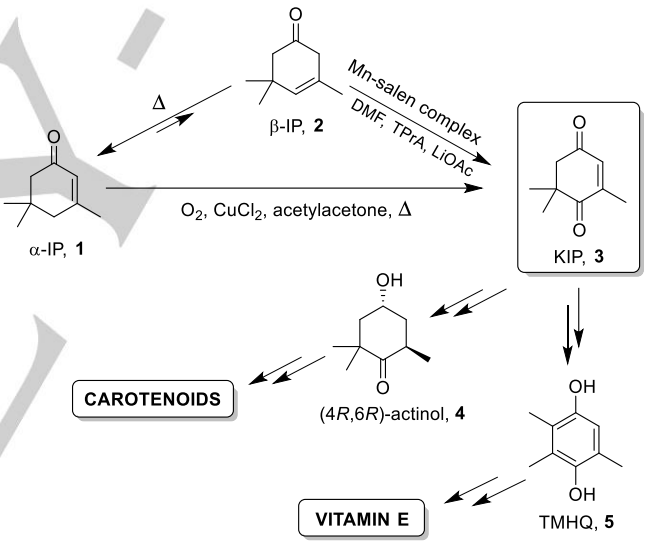
Abstract: The chemical synthesis of ketoisophorone, a valuable building block of vitamins and pharmaceuticals, suffers from a number of drawbacks in terms of reaction conditions and selectivity. Herein, the first biocatalytic one-pot double oxidation of the readily available α-isophorone to ketoisophorone is described. Variants of the self-sufficient P450cam-RhFRed with improved activity have been identified to carry out the first step of the designed cascade (regio- and enantioselective allylic oxidation of α-isophorone to 4-hydroxy-α-isophorone). For the second step, the screening of a broad panel of alcohol dehydrogenases (ADHs) led to the identification of Cm-ADH10 from *Candida magnoliae*. The crystal structure of Cm-ADH10 was solved and docking experiments confirmed the preferred position and geometry of the substrate for catalysis. The synthesis of ketoisophorone was demonstrated both as a one-pot two-step process and as a cascade process employing designer cells co-expressing the two biocatalysts, with a productivity of up to 1.4 g L^{-1} d^{-1}.

Introduction

Monooxygenated terpenoids are relevant target compounds for the synthesis of active pharmaceutical ingredients (APIs), fragrances and nutritional supplements.[1] For instance, ketoisophorone (2,6,6-trimethylcyclohex-2-ene-1,4-dione, KIP, **3**), is one of the key intermediates to access useful building blocks for the synthesis of carotenoids and α-tocopherol (vitamin E),[2] as shown in Scheme 1. For instance, the biocatalytic conversion of KIP to the chiral intermediate (4*R*,6*R*)-4-hydroxy-2,2,6-trimethylcyclohexanone ((4*R*,6*R*)-actinol, **4**), which is an important precursor of several carotenoids, has been recently demonstrated in a one-pot system employing an engineered *Candida macedoniensis* old yellow enzyme (CmOYE) and *Corynebacterium aquaticum* (6*R*)-levodione reductase.[3] Moreover, KIP can be used as a precursor of trimethylhydroquinone (TMHQ, **5**), the key building block in the synthesis of vitamin E.[4] Industrially, KIP is produced from the readily available 3,5,5-trimethyl-2-cyclohexen-1-one (α-isophorone, α-IP, **1**) by isomerization to 3,5,5-trimethyl-3-cyclohexen-1-one (β-isophorone, β-IP, **2**), followed by homogeneous liquid oxidation to KIP.[5a-e] However, the isomerization requires high temperatures and the equilibrium is still heavily shifted towards α-IP (no more than 2% conversion to β-isophorone)[6]. Therefore, the direct oxidation of α-IP has become an attractive alternative route to KIP. However, this selective allylic oxidation proved to be a challenging task and currently such transformation requires toxic heavy metal catalysts,[7] generates undesired by-products[8] and/or requires high temperatures and organic solvents.[9]



Scheme 1. Current syntheses of KIP from α-IP by chemical oxidation, a gateway to the production of high-value building blocks for the industry of carotenoids and vitamins.

In general, the regioselective oxidation of non-activated hydrocarbons under mild reaction conditions remains a "dream reaction" in organic chemistry.[10] With this regard, a biotechnological approach offers a green alternative to traditional chemistry, since biocatalytic reactions generally feature exquisite selectivity and are carried out under mild conditions.[11] Cytochrome P450s (CYPs or P450s) are a diverse superfamily of heme oxidoreductases capable of performing many oxidative reactions, most notably the insertion of oxygen into a chemically inert C-H bond, using oxygen as a benign oxidant and releasing water as by-product.[12a,b,c] During this catalytic cycle, oxygen activation is enabled by two electrons supplied by NAD(P)H and shuttled by redox partners.[13a,b,c,d] Moreover, the outstanding diversity of P450s and their wide distribution in all kingdoms of life imply that their substrate scope

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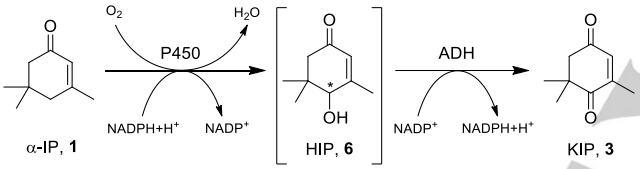
and catalytic versatility could be much superior to other enzymes.[14] Overall, P450s should be considered as valuable catalysts in the enzymatic toolbox of the organic chemist, even if major limitations have to be tackled to implement them in biocatalytic processes.[15a,b,c,d] Kaluzna *et al.* have recently reported the production of 4-hydroxy-α-isophorone (HIP, **6**, Scheme 2) from α-IP to a pilot-scale using the well-known P450-BM3 from *Bacillus megaterium* (CYP102A1),[16] paving the way for a sustainable P450-based KIP production. On the other hand, several reports deal with the application of P450s to catalyse the initial C-H activation in one-pot multi-enzymatic transformations: examples are the oxyfunctionalization of linear hydrocarbons (both *in vitro* and *in vivo*)[17a,b,c], the production of cycloalkanones or lactones from the corresponding cycloalkanes[18a,b,c], the synthesis the grapefruit flavour (+)-nootkatone[19], the conversion of cyclohexane to cyclohexanediols,[20] and the stereoselective benzylic amination of ethylbenzene derivatives.[21] Therefore, considering these biocatalytic strategies, we designed a cascade system to access KIP from α-IP *via* a double allylic oxidation (Scheme 2), employing a P450 in the first step to afford HIP, followed by further oxidation by an alcohol dehydrogenase (ADH).



Scheme 2. Proposed one-pot biocatalytic system from α-IP to KIP.

Herein we report the development and optimization of the envisioned cascade using an improved variant of the chimeric self-sufficient P450cam-RhFRed,[22] consisting of the heme domain of the well-known CYP101A1 (P450cam, camphor 5-monooxygenase, UniProt P00183) from *Pseudomonas putida* fused to the NADPH-dependent reductase domain (RhFRed) of CYP116B2 from *Rhodococcus* sp. (P450RhF, UniProt Q8KU27),[23] in combination with Cm-ADH10 (GenBank AGA42262.1) from *Candida magnoliae*.[24] Along with P450-BM3, P450cam is one of the most studied P450s,[25a,b,c,d,e] and the plethora of information available has been exploited to engineer this enzyme mainly targeting active site hot spots Y96, F87, L244, and V247 in order to generate biocatalysts for terpene oxidation,[26a,b,c] to accept small alkanes[27a,b,c] or industrial pollutants such as large polycyclic aromatic hydrocarbons[28] and polychlorinated benzenes.[29] Our group has previously reported directed evolution methods to redesign P450cam active site[30a,b,c] and recently Kelly *et al.* have developed a colony-based screening based on indigo production from indole which was exploited as a first visual screen to identify self-sufficient P450cam-RhFRed variants with altered promiscuous activities[31]. It was shown that this activity correlates well with that towards structurally different substrates such as ethylbenzenes,[32] corroborating the role of neutral mutations in protein evolution.[33a,b,c,d] Following the same line of thought, we have screened the developed libraries to find variants for the first allylic oxidation of α-IP to HIP. For the second step, a broad panel of commercially available ADHs were evaluated, leading to the identification of Cm-ADH10 as a suitable candidate.

Results and Discussion

Screening and improvement of P450cam-RhFRed variants

The 96 in-house P450cam-RhFRed variants were pooled according to the mutated residues generating five pooled libraries (A, B, C, D, EFG, see Experimental section) of roughly equal size. In general, clustering strategies allow to reduce the screening effort, avoiding the need for screen on a single clone level.[34] In the following step, positive clusters may be broken down to the single clone level in order to identify mutations improving the desired activity. Figure 1 shows the measured biocatalytic performance expressed in terms of the total turnover numbers (TTN), that is the ratio between total product concentration and catalyst concentration, calculated over 24 h. In fact, the variability of P450 expression in *E. coli* may lead to misjudging the observed yields, [35] thus, a fast, whole-cell assay was implemented to determine P450 concentration which was then used to normalize enzyme activity[31].

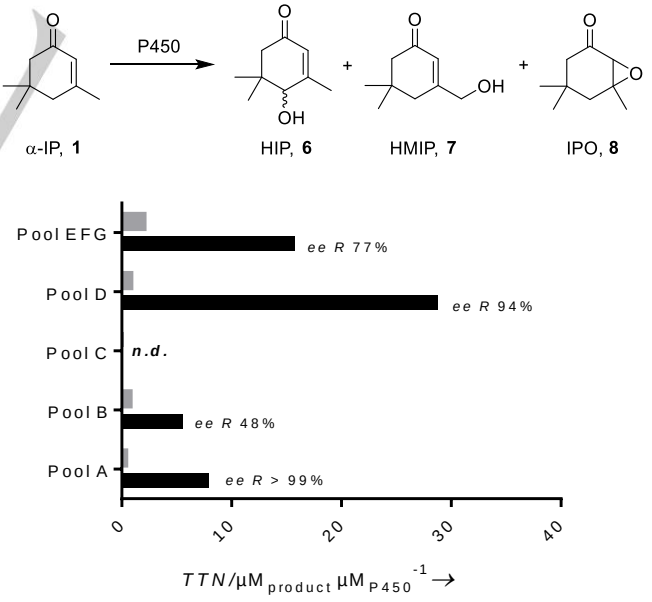


Figure 1. Possible products of the P450 oxidation of α-IP and comparison of TTNs for HIP (black bars) or HMIP (grey bars) obtained for P450cam-RhFRed pooled variants. HIP *ee* values are also given.

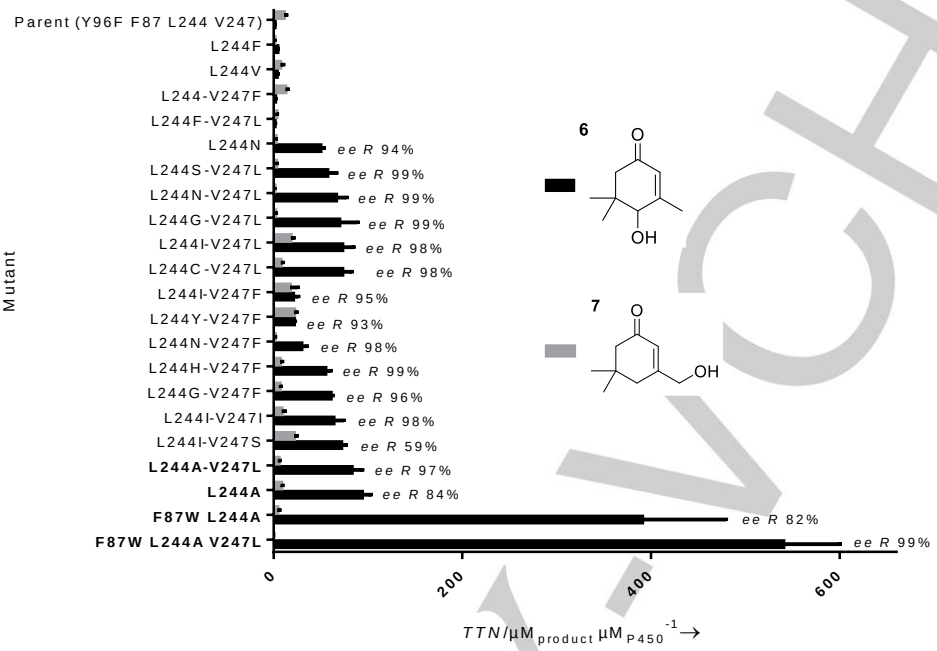


Figure 2. Comparison of TTNs of α-IP to HIP (black bars) or α-IP to HMIP (grey bars) obtained for library D. HIP *ee* values are given, except where the conversions were too low. Variants generated by site-directed mutagenesis are indicated in bold.

Finally, the enantiomeric excess values (*ee*) were also determined by comparison with synthesized standards. The P450-catalysed single oxidation of α-IP may lead to (at least) three major products (Figure 1): HIP (**6**), the regioisomeric allylic oxidation product (3-(hydroxymethyl)-5,5-dimethylcyclohex-2-en-1-one, HMIP, **7**) and isophorone oxide (2,3-epoxy-3,5,5-trimethyl-1-cyclohexanone, IPO, **8**). Product **7** was identified after scale-up, purification and NMR characterization (see Supporting Information). The formation of isophorone oxide was not observed and (*R*)-HIP was the preferred product (Figure 3b), with library D (mutations at residues L244-V247) showing the highest TTNs in the desired α-IP allylic oxidation. Notably, no activity was detected for mutants in pool C (mutations at residues M184-T185).

These initial results drove the next step of the investigation towards the single-clone level analysis of mutants at positions L244-V247. Residues L244 and V247 are positioned over the heme, with L244 located very close to the heme prosthetic group, and V247 in the upper section of the active site.[36] These positions have been often targeted in conjunction with F87-Y96 in order to affect active site volume and manipulate the regio- and enantioselectivity of the oxidation of C-H bonds of unnatural substrates, as discussed in the introduction. The indigo screening adopted previously using P450cam-RhFRed-Y96F as template (indicated as parent)[31] led to the selection of mutants which carried mainly apolar sidechains at position 247 (the only exceptions being V247N and V247S), while more variability was introduced at position 244, with polar residues (asparagine, serine, cysteine) and a positive charged residue such as histidine. Analysis of biotransformation products (Figure 2) demonstrated that positions 244 and 247 greatly affect TTNs of α-IP. Interestingly, the majority of mutants bearing the bulky aromatic phenylalanine residue show not only reduced TTNs but also a decrease in the regioselectivity of the reaction, with the formation of HIP and HMIP in approximately equal amounts by mutants L244Y-V247F and L244I-V247F. Given the observed positive effect of the substitution of L244 with small or apolar residues, the mutation L244A was also introduced, considering the effect of L244A mutation on substrate acceptance, regio- and enantioselectivity.[37] This mutation was studied either in presence of the wild-type V247 or the substitution V247L, which occurred in many positive hits. The two mutants L244A and L244A-V247L showed the best TTN values (94±9 and 83±11, respectively) and were selected for further engineering. Similarly to Bell *et al.*,[26b] we reasoned that the performance of the biocatalyst could be enhanced by improving the enzyme-substrate fit, i.e., by pushing the substrate closer to the heme. With this in mind, the bulky tryptophan was introduced in place of phenylalanine at position 87, known to be involved in substrate access and recognition.[27b,29] As shown in Figure 2, the introduction of F87W in the L244A-V247L background led to more than a 6-fold improvement of TTNs values with respect to L244A-V247L mutant (as full conversion was obtained with this variant, the value might be underestimated), with no HMIP detected and an *ee* of 99% (*R*)-HIP, suggesting a better orienting effect of this biocatalyst towards the highly reactive

iron-oxo species. Finally, the best improved variant (Y96F-F87W-L244A-V247L, now termed P450-WAL) was selected for subsequent optimization experiments for the designed cascade reaction.

Selection of HIP-oxidizing Cm-ADH10 and structural characterization

A screening kit of 116 ADHs from very diverse organisms and with a broad range of accepted substrates was provided by c-LEcta (c-LEcta GmbH, Leipzig, Germany). In order to identify suitable biocatalysts for the oxidation of (*R*)-HIP, the panel was screened against this substrate by HPLC. Several enzymes (>80% of the panel) showed almost no activity, likely because of the steric hindrance of the substrate, however, among the positive hits, two were selected for having the highest activity: Cm-ADH10 from *Candida magnoliae* (GenBank AGA42262.1) and the NADPH-dependent carbonyl reductase (SSCR) from *Sporobolomyces salmonicolor* (UniProt Q9UUN9).[38a,b,c] Notably, both ADHs accept NADP(H) as cofactor, which is desirable to create a self-sufficient cascade with respect to the cofactor.[39] The corresponding genes were cloned into a pET28a vector to carry out expression trials, which revealed good expression levels for Cm-ADH10, as opposed to SSCR (Figure S1, Supporting Information). For this reason, we selected the former for subsequent characterization and combination with the P450. To understand better the catalytic properties of Cm-ADH10, we solved the crystal structure of its complex with $NADP^+$ to a resolution of 1.6 Å (PDB 5MLN, Table S1; Figure S2 and Figure S3). After soaking the crystal with a solution of (*R*)-HIP (10 mM), no substrate was bound to the crystal. However, after docking (performed with AutoDock Vina),[40] the most favoured calculation for the bound ligand (*R*)-HIP confirms that the cavity can accommodate the substrate at a distance and geometry that would favour the transfer of a hydride from the chiral carbon on the 4-(*R*)-hydroxy moiety to C4N of $NADP^+$ (Figure 3). In this position, the 4-hydroxy group would be in the right geometry to interact with the catalytically-important residues S144 and Y157, while the rest of the molecule of 4(*R*)-HIP is possibly stabilized by interactions with residues H149 and Y189 in the entrance of the active site. Coincidentally, this area in the crystal structure is occupied by a tetrad of water molecules that have been reported to be bound to the Tyr-OH and Lys side chain, thus mimicking substrate and ribose hydroxyl group positions.[41] Previous studies support this concept, where Y157 would function as the catalytic base, whereas S144 would stabilize the substrate and K161 would form hydrogen bonds with the nicotinamide ribose moiety and lower the pK_a of the Tyr-OH to promote proton transfer.[42] Since the docking does not reveal any direct interaction with this (or other) residues in the active site, it is very likely that the Cm-ADH10 activity arises from its spacious active site that would allow the binding of (*R*)-HIP in an induced-fit mechanism.

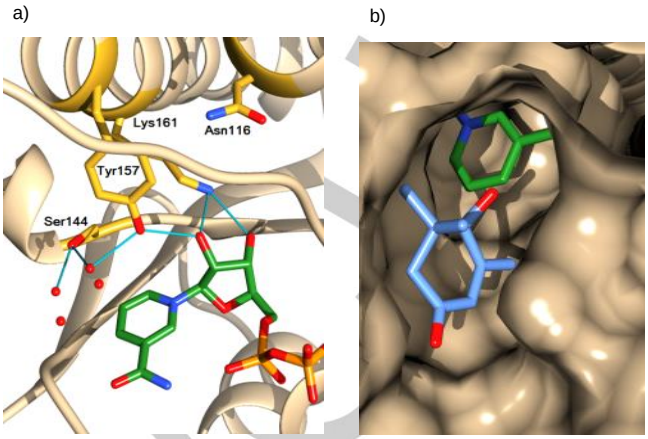


Figure 3. The crystal structure of Cm-ADH10. (a) In the catalytic site four water molecules are within close distance (3.5 Å) of C4N of $NADP^+$ (green), where a substrate would be located for hydride transfer; Tyr157 and Lys161 (yellow, along with other important residues) interact with nicotinamide ribose to orient the C4N carbon towards substrate, and Ser144 would stabilize the substrate. (b) *(R)*-HIP (light blue) docked in the active site cavity with the chiral carbon pointing towards C4N of $NADP^+$.

Preliminary biotransformations were carried out with varying (*R*)-HIP concentrations (10-100 mM) and a constant $NADP^+$ concentration (0.25 mM), using 1 mg mL^{-1} purified Cm-ADH10 and 0.5% v/v ethyl acetoacetate as co-substrate for cofactor regeneration by the same ADH acting as a dual-functional enzyme (see below for further details about co-substrate screening). With this setup, conversions ranged from 92% with 10 mM substrate, down to 48% with 100 mM substrate (see Figure S4, Supporting Information). Thus, Cm-ADH10 lends itself to being applied in the designed bi-enzymatic cascade.

Assembling the system: initial unsuccessful attempts

Initially, we tried to combine the two selected biocatalysts in a one-pot, two-step format, adding 1 mg mL^{-1} of purified Cm-ADH10, cofactor and co-substrate (see above) directly to the supernatant of the whole-cell P450 reaction carried out at 20°C with 200 mg mL^{-1} wet cell load in 50 mM sodium phosphate buffer pH 7.2, 100 mM KCl (indicated as standard biotransformation buffer) and 10 mg mL^{-1} glucose for cofactor regeneration by *E. coli* metabolism. Unexpectedly, we obtained very low conversion values for the ADH catalysed reaction, starting from just 10 mM α-IP in the first step. We reasoned that potential inhibitors for the second step could originate from the growth of the whole-cell biocatalysts in the M9 minimal medium supplemented with glucose. Considering that growth media can influence the performance of biocatalysts,[43] and that glucose is one of the most acidogenic carbon sources for enterobacteria,[44] the pH of the supernatant resulting from the first oxidation step carried out in phosphate buffer with different concentration was measured (Figure 4). Surprisingly, the pH dropped by 1.5 units when the standard biotransformation buffer was used, and even with a 300 mM potassium phosphate (KP_i) buffer the pH decrease was significant. Nevertheless, by increasing the

concentration of the buffer employed in the first step, higher conversions where observed in the second one. Eventually, more than a 2-fold increase in HIP conversion was observed when the P450 catalysed reaction carried out in standard biotransformation buffer was titrated to pH 8.0 before the addition of Cm-ADH10. A similar trend was observed when cells grown in M9 medium where simply resuspended in buffer without any addition of substrate or glucose, suggesting that the observed pH decrease is linked to the metabolism of cells grown to OD_{600} ~ 5.0-6.5. Even if no attempt was made to gain a better understanding of the phenomenon and investigating the effect of washing the biocatalyst, the latter experiment highlighted the need for a careful optimization of each individual step before combination.

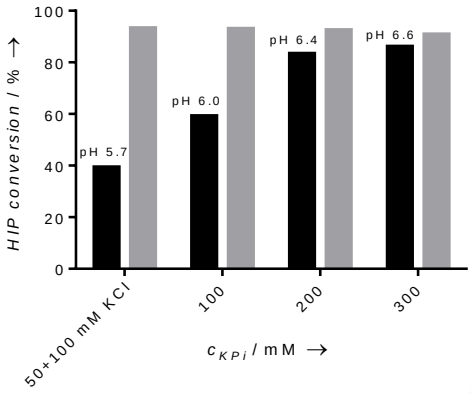


Figure 4. Effect of the pH reached after the P450-catalysed reaction on Cm-ADH10 performance. KP_i buffer concentration used in the first step is given on the x-axis (see text for details). Black bars: HIP conversion after direct addition of Cm-ADH10 (the pH measured after the first step is indicated above). Grey bars: HIP conversion when the supernatant of the first reaction was titrated to pH 8.0 before Cm-ADH10 addition.

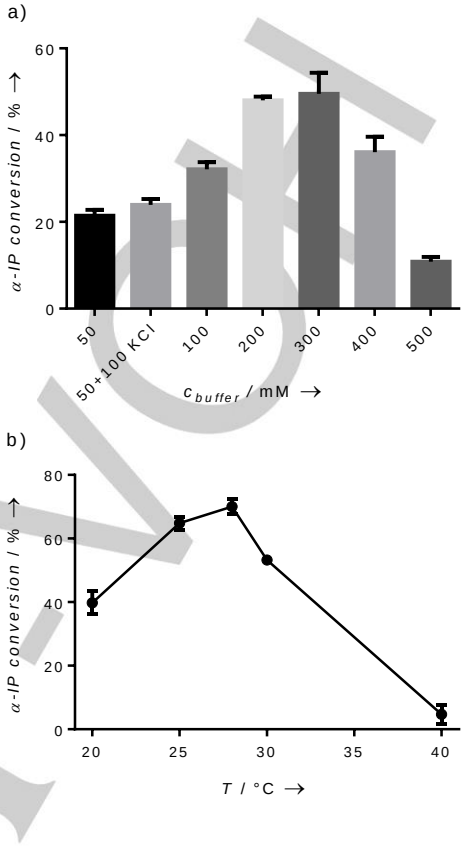


Figure 5. Optimization of the allylic oxidation of α-IP catalysed by P450-WAL. (a) Effect of buffer concentration. Reaction set-up: 200 mg mL^{-1} wet cells resuspended in KP_i buffer pH 8.0 (concentration given on the x-axis), 10 mg mL^{-1} glucose, 20 mM α-IP, 2% DMSO, 20°C, 1 mL final volume in deep-well plate, 24 h reaction time. (b) Effect of temperature. Reaction set-up: same as above, exception being the buffer adopted (200 mM KP_i, pH 8.0) and substrate concentration (15 mM).

Parallel reaction optimization

Next, we proceeded with the parallel optimization of the two oxidative steps with respect to buffer concentration, pH and temperature. For the P450 allylic oxidation step, KP_i buffer (pH 8.0) was chosen for the unique K^+ binding site of P450cam, which displays higher stability and superior camphor binding in presence of K^+ ions.[45] The effect of buffer concentration was examined, along with the temperature optimum. As shown in Figure 5a, conversion improved with increasing buffer concentration up to 200-300 mM, and by comparing biotransformations carried out in 50 mM KP_i without KCl, with KCl or in 100 mM KP_i, it can be concluded that buffer capacity seems to have a greater effect on conversion than K^+ concentration. Eventually, 200 mM KP_i buffer was chosen to investigate the effect of temperature on conversion, finding the optimum at 28°C (Figure 5b).

With respect to the Cm-ADH10 alcohol oxidation, different co-substrates were selected and tested in order to exploit the biocatalyst as a dual-functional enzyme capable of performing the whole substrate oxidation-cofactor regeneration cycle (Figure 6a).[46] As potential effective co-substrates we tested acetone (**9a**), as well as activated ketones such as chloroacetone (**9b**), ethyl acetoacetate (**9c**) and ethyl levulinate (**9d**), since their reduction (and hence cofactor regeneration) is favoured by an additional intramolecular hydrogen bond between the newly formed alcohol functional group and the electronegative moiety.[47] Chloroacetone and ethyl acetoacetate proved to be superior and, even if the latter performed better during the first six hours of reaction, the former pushed the conversion to 84% with 40 mM substrate after 24 h (*vs.* 67% with ethyl acetoacetate). Therefore, chloroacetone was employed as hydrogen acceptor in all the subsequent optimization steps. The pH effect on the Figure 6b) partially explains our initial unsuccessful attempts to combine the two enzymes: in fact, enzyme activity drops below pH 6.0, with an optimum pH range between 7.0 and 9.0. Moreover, the buffer

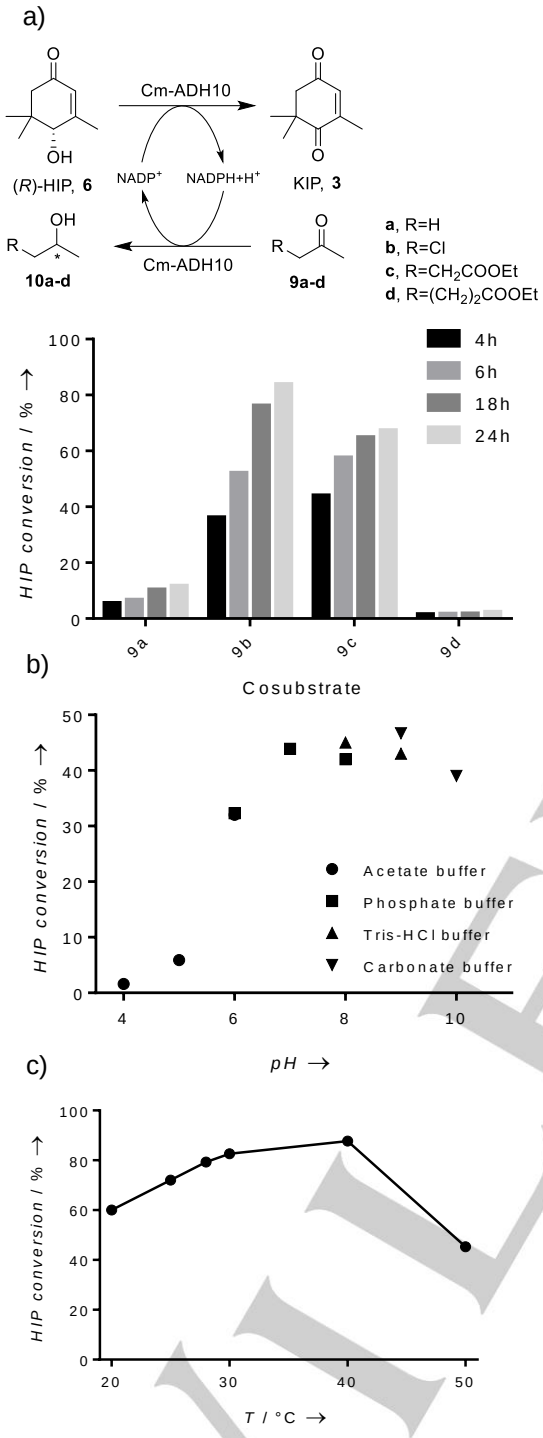


Figure 6. Optimization of the oxidation of (*R*)-HIP catalysed by Cm-ADH10. (a) Effect of different co-substrates. Reaction set up: 100 mM KP_i buffer pH 7.5, 1 mg mL^{-1} Cm-ADH10, 0.25 mM $NADP^+$, 0.5% v/v cosubstrate, 40 mM (*R*)-HIP, 30°C. (b) Effect of pH. Reaction set up: 100 mM indicated buffer, 1 mg mL^{-1} Cm-ADH10, 0.25 mM $NADP^+$, 0.5% v/v chloroacetone, 40 mM (*R*)-HIP, 30°C, 4 h. (c) Effect of temperature. Reaction set up: 200 mM KP_i buffer pH 8.0, 1 mg mL^{-1} Cm-ADH10, 0.25 mM $NADP^+$, 0.5% v/v cosubstrate, 40 mM (*R*)-HIP, 24 h.

system employed in the P450 catalysed reaction (200 mM KP_i) displayed perfect compatibility with the second step (see Figure S5, Supporting Information). Finally, we turned our attention to temperature optimization, finding the highest conversion at 40°C (Figure 6c).

With these optimized conditions in hand, we moved on to the envisaged bi-enzymatic cascade concept to carry out scale-up experiments for product isolation and characterization.

Preparative-scale double oxidation of α-isophorone to ketoisophorone

In order to simplify the entire process further, the oxidation of (*R*)-HIP was accomplished using a concentrated cell-free extract (CFE, corresponding to 1 mg/mL final protein concentration) of *E. coli* overexpressing Cm-ADH10, thus avoiding the expensive protein purification step. The course of the reaction under the optimized conditions was followed over a total reaction time of 50 h, showing that the P450 catalysed allylic oxidation of 10 mM α-IP reached 94±2% conversion in 18 h, whereas the second step was much faster, reaching complete conversion over 6 h (Figure 7). Remarkably, the addition of $NADP^+$ was found to be unnecessary to achieve the highest conversion in the second step, further reducing process costs. Similarly to Kaluzna *et al.*[16], some degree of overoxidation of HIP to KIP was observed during the first step, probably resulting in the subsequent reduction of the C=C bond of KIP by endogenous ene-reductases (<5% by-product).[18c]

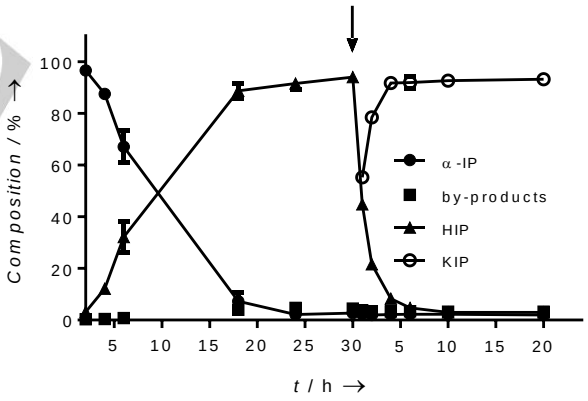
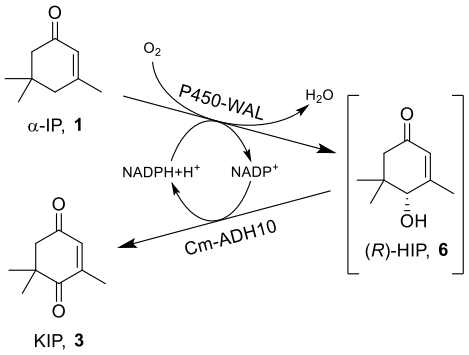


Figure 7. Time-course experiment for the one-pot two-step double allylic oxidation of α-IP. Reaction set up: 200 mg mL^{-1} wet cells resuspended in 200 mM KP_i buffer pH 8.0, 10 mg mL^{-1} glucose, 10 mM α-IP, 2% DMSO (first step, 28°C), then addition of 20% v/v Cm-ADH10 cell-free extract and 1.6 eq. of chloroacetone to the supernatant of the first reaction (second step, 40°C). The arrow indicates the time of addition.

Given these results, we have also attempted to co-express the P450-WAL variant with Cm-ADH10 in the same host with a two vector system to carry out the whole-cell double oxidation of α-IP (Scheme 3). After 24 h, the conversion reached an average value of 65±2%, approximately 30% lower than for the one-pot two-step process carried out at screening scale (Figure S6,

Supporting Information). However, the co-expression of the two enzymes and cofactor preferences make this reaction redox-balanced, avoiding addition of a CFE and co-substrate. Lower conversion can likely be linked to the metabolic burden associated with the co-expression of multiple biocatalysts:[48] in fact, P450 expression level was reduced 3-fold in the "designer" microorganism (6.0 μM for the two step process *vs.* 1.9 μM for the whole-cell process), which accounted for the residual α-IP (see Figure S7, Supporting Information). Based on the quantified products after 24 h, a productivity of 1.4 g L^{-1} d^{-1} and 1.1 g L^{-1} d^{-1} could be calculated for the two-step and the whole-cell process, respectively. However, the one-step process displayed higher TTNs when compared to the two-step process (3421 *vs.* 1567), possibly indicating a more effective cofactor recycling system and/or a positive effect resulting from the removal of the intermediate HIP.[18c]



Scheme 3. Whole-cell double oxidation cascade of α-IP to KIP using *E. coli* co-expressing P450-WAL and Cm-ADH10.

The scale-up of P450-catalyzed whole-cell reactions is notoriously a challenging task, due to biological and process limitations.[15a] In order to evaluate the scalability of our one-pot two-step double allylic oxidation of α-IP, we carried out a linear scale-up (100 mg α-IP, 65 mL total volume). After 24 h, a good scalability was demonstrated, with 80% substrate converted, an excellent 87% recovery and a total isolated yield of 56% after extraction with ethyl acetate and column chromatography (see Figure S6, Supporting Information).

Conclusions

The direct oxidation of inexpensive α-isophorone to ketoisophorone is a sought-after chemical transformation, in that the latter has found many applications in the synthesis of carotenoids. As each chemical process leading to KIP is plagued by many drawbacks, here we demonstrated the first biocatalytic one pot double oxidation of α-isophorone to ketoisophorone by combining an improved variant of the chimeric self-sufficient P450cam-RhFRed and Cm-ADH10 from *Candida magnoliae*. A panel of previously developed P450 variants have been screened and rationally re-designed to improve the enzyme-substrate fit, generating data that could constitute a valid starting point for modelling experiments. Cm-ADH10 was identified as a suitable catalyst for the second step and its crystal structure is provided. Although no substrate is bound, further docking experiments may shed light on key residues involved in the selectivity towards HIP and confirm the preferred position and geometry of the substrate for catalysis. This will enable further intensification of the process. The need for careful and stepwise reaction optimization when designing cascades has been highlighted once again by our initial unsatisfactory results. Eventually, bottlenecks have been identified and the designed cascade has been demonstrated in two different systems: a one-pot, two-step process with Cm-ADH10 acting as a dual-functional enzyme for direct cofactor regeneration (using just 1.6 eq. of chloroacetone) or a one-pot, whole-cell cascade process employing a "designer cell" to co-express the two biocatalysts for a redox-neutral reaction. Despite a lower productivity, the latter system deserves attention in that it allows an easier and faster preparation of the biocatalyst.

Experimental Section

Materials

Chemicals, solvents, and carbon monoxide for CO difference spectroscopy were obtained from Sigma-Aldrich (Poole, Dorset, UK). Gases for GC/FID analysis were purchased from BOC gases (Guildford, UK). (*R*)-HIP was kindly provided by DSM (DSM Ahead R&D B.V.-Innovative Synthesis, Urmonderbaan 22, NL-6167 RD Geleen, The Netherlands). Competent cells and enzymes for molecular biology were purchased from New England Biolabs (NEB). M9 minimal salts (5X) were purchased from Sigma-Aldrich, reconstituted by stirring the recommended amount of powder in water and sterilized by autoclaving. 40% glucose (w/v), antibiotics 1000X, 1 M $MgSO_4$, 1 M $CaCl_2$, and 25% (w/v) $FeCl_3$ were prepared in dH_2O and filter sterilized through a 0.2 μm syringe filter.

Protein expression and purification

Plasmids (pET-14b, pCDF-1b, pET-28a) carrying genes encoding P450cam-RhFRed variants were stored at 4°C. Chemically competent *E. coli* BL21 (DE3) cells were transformed by heat shock according to manufacturer instructions. Transformants were plated on LB agar with added antibiotic (150 μg/ml ampicillin or 50 μg/ml for spectinomycin and kanamycin) and grown at 37°C for 16 hours. Single colonies were picked from plates to prepare starter cultures in LB medium supplemented with antibiotic. After 16 hours, expression cultures were inoculated 1/100 using LB starter cultures and, to guarantee proper aeration, cultures volume was no more than 25% of the total conical flask volume. P450 expression in minimal medium was carried out according to Kelly *et* al.[31]: 1X M9 salts solution were supplemented with 0.4% glucose, 0.05% of $FeCl_3$, 1 mM $MgSO_4$, 1 mM $CaCl_2$ and cells were grown with vigorous shaking (200 rpm) at 37 °C until an OD_{600} of 0.8 was reached. At this stage, isopropyl β-D-1-thiogalactopyranoside (IPTG, 0.4 mM) was added to induce protein expression, along with ALA (0.5 mM) supplementation. Protein expression was carried out at 20 °C with shaking at 200 rpm for 20 h and used immediately. The use of complex media (e.g., LB, TB and auto-induction media) resulted in the expression of the protein in the insoluble fraction or soluble inactive protein (data not shown).

Similarly, production of Cm-ADH10 and SSCR was carried out following a protocol similar to that for P450cam-RhFRed expression, the exception being the medium used (TB instead of M9) and the need for 5-ALA supplementation (not added). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was employed to confirm protein expression. Recombinant Cm-ADH10 was obtained by inoculating 1 L of Terrific broth containing 50 µg/mL kanamycin with transformed cell *E. coli* BL21 (DE3) and incubated until an OD_{600} of 0.6; protein production was induced with 0.4 mM IPTG at 20 °C and production was kept for 16 h. Cells were harvested by centrifugation (2500 *g*, 20 min, 4 °C) and kept at -80 °C until purification. Cells from 1 L of culture were suspended in 50 mL of 20 mM Tris·HCl, pH 7.5. The crude extract was prepared by sonication 5 min, 70% amplitude, 5 s on/off. Cell debris was removed by ultracentrifugation at 31,000 *g* for 45 min, 4 °C. The supernatant was filtrated using a 0.45 um filter. The cell-free extract was applied to a 5 mL HisTrap FF column using an ÄKTA Pure (GE healthcare) at 1 ml/min at 4 °C and a 20 mM imidazole solution in 20 mM Tris·HCl buffer, pH 7.5. The column was washed with 4 – 6 CV of a 50 mM imidazole solution in 20 mM Tris·HCl buffer, pH 7.5. Pure enzyme was eluted using 3 CV of a 500 mM imidazole solution in 20 mM Tris·HCl buffer, pH 7.5. Enzyme was concentrated and excess of imidazole was removed with a concentrator Amicon® Ultra concentrator (10,000 NMWL; Millipore) at 4000 *g*. Typically, the enzyme was diluted to 10 mg/mL in 50 mM KPi buffer (pH 7.5) to carry out biotransformation trials and kinetic measurements, whereas for crystallographic studies, concentrated aliquots (1 – 1.5 mL) were treated with thrombin (0.5 U, 4 °C, overnight shacking) and cleaved 6x-His Tag was removed by gel filtration in a Superdex 200 10/30 GL. Pure protein, as judged by UV chromatogram and SDS-PAGE was concentrated to 77 mg/mL and stored at -80 °C.

Clustering of indigo-positive variants

Plasmids (pET-14b) encoding P450cam-RhFRed mutants generated in a previous work[31] were purified from bacterial glycerol stocks. Variants were produced targeting 7 pairs of residues by saturation mutagenesis using NDT codon degeneracy. This resulted in the production of seven libraries: A (F87-F96), B (F98-T101), C (M184-T185), D (L244-V247), E (G248-T252), F (V295-D297) and G (I395-V396). In order to cluster these variants, 100 ng of each plasmid purified were added to one of five pools according to the mutations introduced, the exceptions being variants from library G248-T252, V295-D297 and I395-V396 that were pooled together because the reduced size of these libraries (giving five pooled libraries in total). These plasmids preparations were then transformed into *E. coli* NEB-5-alpha competent cells and plasmids purified again for transformation into *E. coli* BL21 (DE3) for small-scale expression (125 mL). During subsequent screenings, transformation efficiencies of each pool member were not determined.

Whole cell biotransformations with library D P450cam-RhFRed variants

Expression cultures were centrifuged (2500 *g*, 20 min, 4 °C) and the pellet resuspended to 230 mg/mL (wet weight) in the appropriate biotransformation buffer (50 mM sodium phosphate buffer, pH= 7.2, 100 mM KCl). Biotransformations were carried out in 48-deep well plates on a 1 mL scale, with 880 µL of re-suspended cells, 100 µL of 100 mg/mL glucose and the appropriate final concentration of substrate typically added from concentrated stocks in DMSO (2% v/v final concentration). Plates were sealed with a gas permeable membrane and reaction carried out with 250 rpm orbital shaking for 24 h. Reaction mixtures were extracted with 1 mL of methyl-tert-butyl ether (MTBE) with 1 mM decane (as internal standard for quantitative analysis) vortexed for 30 s and centrifuged 5 minutes before removing the organic layer, which was then transferred to fresh tubes containing $MgSO_4$. Finally, 250 µL of the MTBE extract was taken for GC analysis and diluted when necessary. In order to determine the *ee*%, the samples were also analysed by chiral normal phase HPLC.

Screening of ADHs

A panel of alcohol dehydrogenases enzymes as freeze-dried cell free extracts in 96-deep well plates were kindly supplied by c-LEcta GmbH (Leipzig, Germany). For a first screening, each freeze-dried extract was resuspended in 50 uL of 50 mM sodium phosphate buffer pH= 7.2, 100 mM KCl, and reaction carried out on a 500 µL scale with both 10 mM (*R*)- and (*S*)-HIP, 0.5 mM of both NAD^+ and $NADP^+$ and acetone 5% v/v (30 °C). Then, best hits were chosen for a further screen on a 500 uL scale with 10 mM (*R*)-HIP, 0.5 mM $NADP^+$ and acetone 5% v/v. Reaction mixtures were extracted with methyl-tert-butyl ether (MTBE), vortexed for 30 s and centrifuged 5 minutes before removing the organic layer, which was then transferred to fresh tubes containing $MgSO_4$. Finally, 250 µL of the MTBE extract was taken for chiral normal phase HPLC analysis.

Scale up of the biotransformation of α-isophorone with P450cam-RhFRed L244I-V247S for the identification of 7

In order to characterize α-IP oxidation products, P450cam-RhFRed L244I-V247S was chosen for its good activity profile and reduced selectivity. To a solution of α-IP (2 mM) and glucose (10 mg/mL) and KCl (100 mM) in NaP_i buffer (50 mM, pH 7.2, final volume 200 mL), wet *E. coli* BL21(DE3) cells producing P450cam-RhFRed L244I-V247S were added (200 mg/mL). The mixture was incubated at 20°C, 250 rpm for 24 h, then centrifuged to remove the cells. The reaction mixture was acidified to pH ~ 3.0 with 3 M HCl and centrifuged (2500 *g*, 20 min, 4°C). The supernatant was extracted with EtOAc (2 x 200 mL), the organic layer was dried over $MgSO_4$ and evaporated, to afford an orange residue. Silica gel chromatography purification (cyclohexane with increasing amounts of EtOAc) afforded **6** and **7** of sufficient purity. Compound **7** was identified by comparison of its ^{1}H NMR spectrum with literature data.[49] ^{1}H NMR (400 MHz, $CDCl_3$): δ = 6.15 (s, 1H), 4.23 (s, 2H), 2.27 (s, 2H), 2.14 (s, 2H), 1.05 (s, 6H).

Analytical scale double oxidation of α-isophorone to ketoisophorone

Method A (one-pot, two-step). Step 1. To a solution of α-IP (10-20 mM) and glucose (10 mg/mL) in the appropriate buffer (50-500 mM, pH 8.0, final volume 1 mL, 48-deep well plates), wet *E. coli* BL21(DE3) harbouring a pCDF-1b vector carrying the gene encoding P450-WAL were added (200 mg/mL). The mixture was incubated at 20-40°C, 250 rpm for 18-24 h, then centrifuged to remove the cells. Step 2. Either the supernatant of step 1 or a solution of **6** (10-100 mM) in the appropriate buffer (100-500 mM, pH 4.0-10.0) was used for the ADH oxidation step. Cm-ADH10 was added either as a 10x concentrated cell lysate of *E. coli* BL21(DE3) (~1 mg/mL final protein conc.) or as purified protein (1 mg/mL final conc.) along with the appropriate cosubstrate (0.5 % v/v) and $NADP^+$ (0.25 mM final conc.). The mixture was incubated at 20-50°C, 180 rpm for 6-24 h. Both steps were followed by taking small samples for GC analysis after extraction.

Method B (whole cell cascade). *E. coli* BL-21 (DE3) competent cells were co-transformed with plasmids bearing the sequences encoding P450-WAL (pCDF-1b) and Cm-ADH10 (pET28a) and plated on LB agar containing 25 µg/mL of kanamycin and spectinomycin. After 16 h incubation at 37°C, single colonies were picked and grown at 37°C in M9 medium supplemented with antibiotics. Protein production was carried out similarly to P450cam-RhFRed variants, including 25 µg/mL of

kanamycin and spectinomycin. The whole-cell double oxidation of α-isophorone was carried out in KPi buffer (200 mM, pH 8.0, final volume 1 mL, 48-deep well plates) with α-IP (10 mM), glucose (10 mg/mL) and whole cells (200 mg/mL cell wet weight). The mixture was incubated at 28°C with shaking (200 rpm) for 24 h. Samples were analysed by GC after extraction.

Preparative-scale double oxidation of α-isophorone to ketoisophorone

To a solution of α-IP (10 mM) and glucose (10 mg/mL) in KP_i buffer (200 mM, pH 8.0, final volume 65 mL), wet *E. coli* BL21(DE3) cells producing P450-WAL were added (200 mg/mL). The mixture was incubated at 28°C, 150 rpm for 18 h, then centrifuged to remove the cells. A 10x concentrated cell lysate of *E. coli* BL21(DE3) producing Cm-ADH10 (~1 mg/mL final protein conc.) and chloroacetone (1.6 equiv.) were added to the supernatant. The mixture was incubated at 40°C, 150 rpm for 6 h. Both steps were followed by taking small samples for GC analysis. After completion of the reaction, the mixture was extracted with EtOAc (3 x 30 mL). The organic phase was dried over $MgSO_4$ and evaporated under reduced pressure to afford a yellowish crude product that was submitted to column chromatography purification (petroleum ether 40-60°C with increasing amounts of EtOAc). KIP was obtained as a colourless oil (46 mg, 56% isolated yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 6.55 (s, 1H), 2.71 (s, 2H), 2.00 (s, 3H), 1.24 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 203.6, 197.9, 149.1, 137.2, 52.0, 45.3, 26.2, 17.0; HRMS (ESI+): m/z for $C_9H_{13}O_2^+$ $[M+H]^+$ calcd. 153.0910, found 153.0897.

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Keywords: alcohol dehydrogenases • biocatalysis • C-H activation • enzyme cascades • P450 monooxygenases

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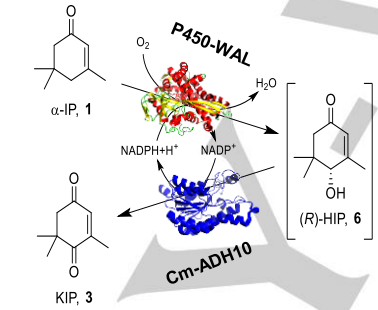
Entry for the Table of Contents (Please choose one layout)

Layout 1:

FULL PAPER

The first biocatalytic one-pot double oxidation of α-isophorone to ketoisophorone is described. To enable it, variants of the self-sufficient P450cam-RhFRed with improved activity have been combined with Cm-ADH10 from *Candida magnoliae*. The synthesis of ketoisophorone was demonstrated with a productivity of up to 1.4 g L^{-1} d^{-1}.



Up to 1.4 g L^{-1} d^{-1}

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One-pot Biocatalytic Double Oxidation Process for the Synthesis of Ketoisophorone from α-Isophorone