Selective synthesis of 4-hydroxyisophorone and 4-ketoisophorone by fungal peroxygenases†

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The recently discovered unspecific peroxygenases (UPOs) from the ascomycetes Chaetomium globosum and Humicola insolens were capable of selectively hydroxylating isophorone to 4-hydroxyisophorone (4HIP) and 4-ketoisophorone (4KIP), which are substrates of interest for the pharmaceutical and flavor-and-fragrance sectors. The model UPO from the basidiomycete Agrocybe aegerita was less regioselective, forming 7-hydroxyisophorone (and 7-formylisophorone) in addition to 4HIP. However, it was the most stereoselective UPO yielding the S-enantiomer of 4HIP with 88% ee. Moreover, using H. insolens UPO full kinetic resolution of racemic HIP was obtained within only 15 min, with >75% recovery of the R-enantiomer. Surprisingly, the UPOs from two other basidiomycetes, Marasmius rotula and Coprinopsis cinerea, failed to transform isophorone. The different UPO selectivities were rationalized by computational simulations, in which isophorone and 4HIP were diffused into the enzymes using the adaptive PELE software, and the distances from heme-bound oxygen in H2O2-activated enzyme to different substrate atoms, and the corresponding binding energies were analyzed. Interestingly, for process upscaling, full conversion of 10 mM isophorone was achieved with H. insolens UPO within nine hours, with total turnover numbers up to 5500. These biocatalysts, which only require H2O2 for activation, may represent a novel, simple and environmentally-friendly route for the production of isophorone derivatives.

Introduction

Isophorone derivatives, such as 4-hydroxyisophorone (4HIP) and 4-ketoisophorone (4KIP), are of interest as flavour-and-fragrance additives,1 and as intermediates in the synthesis of pharmaceuticals, vitamins and natural pigments.2,3 A variety of chemical methods is available for the production of 4HIP and 4KIP. Thus, both derivatives have been synthesized from β-isophorone,1,4 which – on its part – can be obtained by isomerization of isophorone (also known as α-isophorone). The rearrangement of β-isophorone to the α-isomer, however, is a main drawback of this process. The direct oxidation of isophorone to 4KIP with molecular oxygen [O2] appeared to be the solution, using copper(ii) acetylacetonate or molybdenum-based systems as catalysts,5 but required toxic heavy metals and led to the formation of undesired side products. Moreover, a direct chemical oxidation process of isophorone to 4HIP is not available and this compound is usually synthesized by reduction of 4KIP,6,7 which can be a rather expensive starting material.

Alternatively, some biological processes for the synthesis of 4HIP and 4KIP have been described, often using cytochrome P450 monooxygenases (P450s). Among them, the microbial biotransformation of isophorone was described for fungi – like Aspergillus niger, Alternaria alternata and Neurospora crassa – with 4HIP and 7-hydroxyisophorone (7HIP) as main metabolites. More recently, a process using recombinant Escherichia coli transformed with the P450-BM3 gene (together with the gene of NADPH-regenerating glucose dehydrogenase) allowed the scaled-up selective production of 4HIP at kilogram scale.10 On the other hand, 4KIP has been produced either in an one-pot two-step enzymatic process or as a cascade process employing cells co-expressing P450-WAL and Cm-ADH10 dehydrogenase.11 However, isolated P450s generally suffer the disadvantage of rather higher instability and the frequent need of auxiliary enzymes/domains and expensive cofactors.
Unspecific peroxygenases (UPOs, EC.1.11.2.1) are novel and appealing biocatalysts for organic synthesis, since their ‘simplicity’ (only H$_2$O$_2$ is required for activation) and stability (as secreted enzymes) circumvent major disadvantages of P450s while catalyzing the same kind of oxyfunctionalization reactions. 12 The first enzyme of this class was discovered in 2004 in the basidiomycete Agrocybe aegerita 13 and since then, new peroxygenases came out from Coprinellus radians, 14 Marasmius rotula, 15 and more recently from Chaetomium globosum. 16 Their widespread occurrence in the fungal kingdom has been demonstrated by the analysis of basidiomycete, ascomycete and other fungal genomes and revealed over one-thousand putative peroxygenase genes. 17 This allowed the production of recombinant enzymes, like those of Coprinopsis cinerea 18 or Humicola insolens, 16 which are heterologously expressed by Novozymes A/S ( Bagsvaerd, Denmark) in the mold Aspergillus oryzae.

The spectrum of reactions catalyzed by these enzymes is steadily increasing and includes oxygenations of both aromatic 19,20 and aliphatic compounds, 21–24 fatty acids epoxidation 25 and chain-shortening, 26 and also reactions of rather complex and bulky substrates like steroids, 27,28 and sec-osteroids 29,30 that are subject to epoxidation, side-chain hydroxylation or side-chain removal.

In the present work, the hydroxylation of isophorone by several UPOs with different selectivities is presented for the first time, to be included in the portfolio of reactions catalyzed by these novel and exciting enzymes. 12,17

Materials and methods

Enzymes

AaeUPO (isoform II, 46 kDa), the first UPO described in 2004, is a wild-type (i.e. non-recombinant) peroxygenase from cultures of A. aegerita TM-A1, grown in soybean-peptone medium, which was purified as described by Ullrich and Hofrichter. 31 MroUPO is another wild-type peroxygenase (32 kDa) from cultures of M. rotula DSM-25031 (German Collection of Microorganisms and Cell Cultures, Braunschweig), which was purified as described by Gröbe et al. 15 CgUPO (36 kDa) is a third wild-type peroxygenase from cultures of C. globosum DSM-62110, which was purified as recently described by Kiebist et al. 16 The recombinant enzymes rCciUPO (44 kDa) and rHinUPO were provided by Novozymes A/S. rCciUPO corresponds to the protein model 7249 from the sequenced C. cinerea genome available at the JGI (http://genome.jgi.doe.gov/Copci1) used in several studies. 22,27,30 The rHinUPO sequence has been more recently reported 12 and used for oxyfunctionalizations that are not catalyzed by other UPOs. 16 Both UPOs were expressed by Novozymes in Aspergillus oryzae (patent WO/2008/119780). All UPO proteins were purified by fast protein liquid chromatography (FPLC) using a combination of size exclusion chromatography (SEC) and ion exchange chromatography on different anion and cation exchangers. Purification was confirmed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and UV-visible spectroscopy following the characteristic heme-maximum around 420 nm (Soret band of resting-state heme-thiolate proteins). Enzyme concentration was estimated according to the characteristic UV-vis band of the reduced UPO-complex with carbon monoxide. 23

Model compounds

3,5,5-Trimethyl-2-cyclohexen-1-one (isophorone) from Sigma Aldrich (97% purity) was tested as substrate of the above UPOs. 3,5,5-Trimethyl-2-cyclohexen-1,4-dione (4-keto-isophorone, 4KIP) also from Sigma Aldrich, and chemically-synthesized 4-hydroxy-3,5,5-trimethyl-2-cyclohexen-1-one (4-hydroxisophorone, 4HIP) by 4KIP reduction, were used as standards in gas chromatography-mass spectrometry (GC-MS) analyses. 4HIP, obtained as a racemic mixture by chemical reduction of 4KIP, 7 was used as substrate in enzymatic reactions together with isophorone.

Enzyme reactions

Reactions (1 mL volume) with isophorone (0.1 mM) were performed at 30 °C, in 50 mM phosphate buffer, pH 7. The enzyme concentrations ranged from 50 nM to 10 μM, using 2.5 to 5 mM H$_2$O$_2$. In control experiments, substrates were treated under the same conditions (including H$_2$O$_2$) but without enzyme. After 30 min reaction, products were extracted with methyl tert-butyl ether, which was evaporated under N$_2$, and the products dissolved in chloroform to be analyzed by GC-MS. Reactions at higher isophorone concentration (10 mM) were performed with CgUPO and rHinUPO (2–5 μM), H$_2$O$_2$ was added with a syringe pump to give concentrations in the reaction mixture of 1 or 5 mM H$_2$O$_2$. 4KIP reduction, were used as standards in gas chromatography-mass spectrometry (GC-MS) analyses.

Enzyme kinetics

Reactions were carried out with 6.25–6400 μM substrate and 100 nM enzyme. They were initiated adding 0.5 mM H$_2$O$_2$ and stopped by vigorous shaking in 5 mM sodium azide. Reaction times, the reaction velocity of which was linear, were previously selected: 5 min for AaeUPO, 3 min for CgUPO and 1 min for rHinUPO. All reactions were performed in triplicates. Product quantification was carried out by GC-MS using external standard curves, and kinetic parameters — turnover number (k$_{cat}$), Michaelis constant (K$_m$) and catalytic efficiency (k$_{cat}$/K$_m$) — were obtained by fitting the data to the Michaelis–Menten equation, or to the corresponding variation of this equation when substrate inhibition is occurring, using SigmaPlot (Systat Software Inc., San Jose, CA, USA).
GC-MS

The analyses were performed in a Shimadzu GC-MS QP 2010 Ultra system, using a fused-silica DB-5HT capillary column (30 m × 0.25 mm internal diameter, 0.1 μm film thickness) from J&W Scientific. The oven was heated from 50 °C (1.5 min) to 90 °C (2 min) at 30 °C min^-1, and then from 90 °C to 250 °C (15 min) at 8 °C min^-1. The injection was performed at 250 °C and the transfer line was kept at 300 °C. Compounds were identified by comparing their mass spectra and retention times with those of available commercial or synthesized authentic standards, and by search in the NIST library.

Chiral HPLC

Chiral analyses were performed with a Shimadzu LC-2030C 3D system equipped with a photo-diode array detector using a chiral column Chiralpak IG (5 μM particle size, 4.6 mm diameter × 150 mm, Daicel Chemical Industries Ltd.) equipped with a Chiralpak IG guard column (5 μM particle size, 4.0 mm diameter × 10 mm). The column was eluted in isotropic mode with 95% hexane and 5% isopropanol at 0.5 mL min^-1 for 60 min, and the absorbance was monitored at 254 nm. Enantiomers were identified based on the elution order previously reported.

Molecular models

AaeUPO and MrO UPO models were obtained from the chain-A of the 2YP1 and 5FUJ crystal structures, after removing the Cys227 dimerization disulfide bridge from the second structure. Homology models for CglUPO, rHinUPO and rCciUPO were obtained at the Swiss-Model server, with related crystal structures as templates. The heme cofactor and the Mg ion (along with its two coordinated water molecules) were then superimposed using 2YP1 and 5FUJ as templates, followed by an initial minimization to release steric clashes. An oxygen atom (iron-oxo) was finally added to all structures modeling heme compound I. All systems were prepared at pH 7 with the protein preparation wizard from Schrödinger. The Mg ion (along with its two coordinated water molecules) were placed in the active site when exploring diffusion of the ligands. The presence of a water molecule has been highlighted in compound I activity and might be important when diffusing polar substrates such as 4HIP.

Ligand diffusion simulations

The new adaptive-PELE software was used to study ligand diffusion and binding on the different UPO structures. PELE uses a Monte Carlo (MC) procedure to describe the protein-ligand conformational dynamics. At each MC iteration, the algorithm performs: 1) ligand perturbation (translation and rotation); 2) protein perturbation following normal modes; 3) explicit water sampling; 4) side chain prediction; and 5) overall minimization. The final structure is then accepted or rejected based on a Metropolis criterion. The adaptive protocol improves PELE’s sampling by running multiple short simulations (epochs) where initial conditions are selected through a reward function aiming at sampling non-visited areas. We also used a new version of PELE that allows for explicit water sampling, where water molecules are allowed to freely move (with 100 small translations and rotations) after the backbone sampling. Two sets of simulations were performed for each ligand and structure. In the initial one the ligand was placed on the protein surface, next to the entrance to the active site (at ~16 Å from the heme’s iron atom), and allowed to diffuse freely into the heme distal site. The structure with the best (ligand) binding energy from the initial simulation was then selected for a second local refinement run, where the ligand center of mass was constrained to move within 8 Å from the heme’s iron. All simulations used 50 epochs of 16 MC PELE steps each with 128 computing cores. Interaction/binding energies (kcal mol^-1) were derived as Eab = (Ea + Eb), where Eb is the total energy of the complex, Eb the energy of the ligand and Ea the energy of receptor (everything but the ligand), all of them obtained at the OPLS2005 level of theory with a surface GB implicit solvent model.

Results and discussion

Regioselectivity in isophorone transformation by UPOs

In the present work, the ability of several UPOs to oxidize isophorone was analyzed, and different transformation patterns were observed (Fig. 1). The peroxygenases from the ascomycetes C. globosum (CglUPO) and H. insolens (rHinUPO), and the basidiomycete A. aegerita (AaeUPO) were found to transform the substrate, although with different regioselectivities (Table 1). In contrast, the enzymes from the basidiomycetes C. cinerea (rCciUPO) and M. rotula (MrO UPO) were unable to

![Fig. 1 GC-MS analysis of 0.1 mM isophorone reactions (30 min) with 0.1 μM of MrO UPO, rCciUPO, CglUPO, rHinUPO, AaeUPO and control without enzyme, showing the remaining substrate (IP, isophorone) and the hydroxylated (4HIP and 7HIP) and oxo (4KIP and 7FIP) derivatives.](image-url)
Table 1 Comparison of five UPOs in isophorone (0.1 mM) conversion (% of substrate) and relative abundance of the products 4HIP, 7HIP, 4KIP and 7FIP (% of the total products) after 30 min reaction, using different enzyme and peroxide doses

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Conversion</th>
<th>4HIP</th>
<th>7HIP</th>
<th>4KIP</th>
<th>7FIP</th>
</tr>
</thead>
<tbody>
<tr>
<td>AaeUPO&lt;sup&gt;a,d&lt;/sup&gt;</td>
<td>72</td>
<td>46</td>
<td>25</td>
<td>tr</td>
<td>29</td>
</tr>
<tr>
<td>AaeUPO&lt;sup&gt;b,d&lt;/sup&gt;</td>
<td>96</td>
<td>46</td>
<td>19</td>
<td>tr</td>
<td>35</td>
</tr>
<tr>
<td>CglUPO&lt;sup&gt;a,d&lt;/sup&gt;</td>
<td>95</td>
<td>79</td>
<td>—</td>
<td>20</td>
<td>1</td>
</tr>
<tr>
<td>CglUPO&lt;sup&gt;c,e&lt;/sup&gt;</td>
<td>100</td>
<td>—</td>
<td>—</td>
<td>100</td>
<td>—</td>
</tr>
<tr>
<td>rHinUPO&lt;sup&gt;a,d&lt;/sup&gt;</td>
<td>84</td>
<td>53</td>
<td>—</td>
<td>45</td>
<td>2</td>
</tr>
<tr>
<td>rHinUPO&lt;sup&gt;c,e&lt;/sup&gt;</td>
<td>100</td>
<td>3</td>
<td>—</td>
<td>97</td>
<td>—</td>
</tr>
</tbody>
</table>

<sup>a</sup> Reactions using 0.1 μM UPO.  <sup>b</sup> Reactions using 0.25 μM UPO.  <sup>c</sup> Reactions using 0.5 μM UPO.  <sup>d</sup> Reactions using 2.5 mM H<sub>2</sub>O<sub>2</sub>.  <sup>e</sup> Reactions using 5 mM H<sub>2</sub>O<sub>2</sub>. tr denotes traces.

The regioselectivity observed in the hydroxylation of isophorone by some UPOs is similar to that reported for certain P450s. Among them, P450cam-RhFRed variants have been reported to yield 4HIP, 7HIP and isophorone oxide (2,3-epoxy-3,5,5-trimethyl-1-cyclohexanone) as major products, with 4HIP as the only product from one of the variants. 4HIP was also the main product of reactions with CYP102A1 and CYP101A1.

![Scheme 1](image1.png)

Scheme 1 Isophorone (IP) hydroxylation catalyzed by CglUPO and rHinUPO (A) and AaeUPO (B), showing the hydroxylated 4HIP (4-hydroxyisophorone) and 7HIP (7-hydroxyisophorone) and the oxo 4KIP (4-ketoisophorone) and 7FIP (7-formylisophorone) derivatives.

![Fig. 2](image2.png)

Fig. 2 Time course of 10 mM isophorone (IP) reaction with 5 μM CglUPO (A) and rHinUPO (B) and H<sub>2</sub>O<sub>2</sub> (added with a syringe pump to give 5 mM h<sup>−1</sup> concentration), showing substrate and products (4HIP and 4KIP) concentrations.
although minor amounts of the epoxide, 7HIP and further oxidation products were observed.34 P450s, unlike HinUPO or CglUPO, seem to be unable to oxidize 4HIP into 4KIP and, therefore, two enzymes (a P450 and an alcohol dehydrogenase) are necessary to obtain 4KIP from isophorone.11

In view of the higher selectivity to form the products of interest (4HIP and 4KIP), CglUPO and HinUPO were selected to perform reactions with higher (>100) substrate load (Fig. 2). These experiments revealed a faster substrate conversion by HinUPO that completely transformed isophorone within 6 h, while CglUPO needed 12 h for 87% conversion. As expected, a higher proportion of 4KIP was observed in the HinUPO reactions. A higher enzyme dose would be needed to complete conversion into 4KIP, as it was already found when lower substrate concentrations were tested (Table 1).

### Kinetics of isophorone hydroxylation by UPOs

Despite the difficulties to determine initial enzymatic reaction rates by GC-MS, kinetic curves for isophorone hydroxylation by the three UPOs could be obtained (Fig. S4†) and reaction constants \( (k_{\text{cat}}, K_m \text{ and } k_{\text{cat}}/K_m) \) were estimated (Table 2). There were differences in enzyme affinities, since the \( K_m \) values were four- and two-fold higher for AaeUPO than for CglUPO and HinUPO, revealing the higher isophorone affinity of the two latter enzymes. Moreover, HinUPO displayed a ten-fold higher turnover number \( (k_{\text{cat}}) \) compared to CglUPO, which resulted in five-fold higher catalytic efficiency, while the efficiencies of AaeUPO and CglUPO were similar.

The catalytic efficiency of these UPOs hydroxylating isophorone is in the range of previously reported for the hydroxylation of cyclohexane by other UPOs.41 On the other hand, the \( K_m \) of CglUPO and HinUPO for isophorone hydroxylation is similar to values (380–440 \( \mu \)M) reported for CYP102A1 variants when decoy molecules were used, and the turnover numbers of these variants (2.5–5.5 s\(^{-1}\)) were similar as well to that reported herein for CglUPO (4.4 s\(^{-1}\)).34

Higher total turnover numbers \( (\text{TTNs}) \) were attained with HinUPO (2660) than with CglUPO (1820) under the same reaction conditions, although these values could be up to 5600 and 3600 when lower enzyme doses were used (Table 3). The TTNs are in the range of those reported for two-step (1567) or one-step (3421) isophorone oxidation by the combination of P450-WAL and Cm-ADH10.11

### UPO enantioselectivity on isophorone and racemic 4HIP

Enantioselectivity in the synthesis of 4HIP by the three UPOs hydroxylating isophorone was determined by HPLC (Fig. S5†). The results of the chiral analysis showed that only the reaction with AaeUPO can be considered as stereoselective, with an enantiomeric excess (ee) of 88% \( S \)-4HIP (Table 4). The three UPOs preferentially formed the \( S \)-enantiomer, in contrast to P450s that rather formed the \( R \)-enantiomer.11,42

The above values were estimated under reaction conditions where the product concentration was similar for all

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**Table 2** Kinetic parameters of AaeUPO, CglUPO and HinUPO for the hydroxylation of isophorone

<table>
<thead>
<tr>
<th>UPO</th>
<th>( k_{\text{cat}} ) (s(^{-1}))</th>
<th>( K_m ) (( \mu )M)</th>
<th>( k_{\text{cat}}/K_m ) (mM(^{-1}) s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>AaeUPO</td>
<td>20.1 ± 1.1</td>
<td>1380 ± 200</td>
<td>14.6 ± 2.2</td>
</tr>
<tr>
<td>CglUPO</td>
<td>4.4 ± 0.4</td>
<td>309 ± 55</td>
<td>14.2 ± 2.8</td>
</tr>
<tr>
<td>HinUPO</td>
<td>42.0 ± 9.8</td>
<td>633 ± 201</td>
<td>66.4 ± 26.1</td>
</tr>
</tbody>
</table>

a Data represent mean values of three replicates with standard deviations.

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**Table 3** Product concentration and catalytic performance - given by total turnover number (TTN) and turnover frequency (TOF) – of CglUPO and HinUPO after 9 h reaction using a higher isophorone concentration (10 mM)

<table>
<thead>
<tr>
<th>UPO</th>
<th>4HIP (mM)</th>
<th>4KIP (mM)</th>
<th>TTN</th>
<th>TOF (min(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>CglUPO‡</td>
<td>6.7</td>
<td>1.2</td>
<td>1820</td>
<td>3.4</td>
</tr>
<tr>
<td>CglUPO‡</td>
<td>4.8</td>
<td>1.2</td>
<td>3600</td>
<td>6.7</td>
</tr>
<tr>
<td>HinUPO‡</td>
<td>2.3</td>
<td>5.5</td>
<td>2660</td>
<td>4.9</td>
</tr>
<tr>
<td>HinUPO‡</td>
<td>2.4</td>
<td>4.4</td>
<td>5600</td>
<td>10.4</td>
</tr>
</tbody>
</table>

a Reactions using 2 \( \mu \)M enzyme. b Reactions using 5 \( \mu \)M enzyme.

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**Table 4** Results from chiral HPLC analysis of the 10 mM isophorone reaction (60 min) with AaeUPO, CglUPO and HinUPO (5 \( \mu \)M) showing the yields of the \( R \) and \( S \) enantiomers of 4HIP, and the resulting enantiomeric excess (ee), together with the amount of 4KIP formed and the total conversion yield (% of substrate) under the given conditions

<table>
<thead>
<tr>
<th>UPO</th>
<th>Conversion (%)</th>
<th>4HIP (mM)</th>
<th>4KIP (mM)</th>
<th>R-4HIP (%)</th>
<th>S-4HIP (%)</th>
<th>ee (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AaeUPO‡</td>
<td>51</td>
<td>2.5</td>
<td>0.1</td>
<td>6</td>
<td>94</td>
<td>88</td>
</tr>
<tr>
<td>CglUPO‡</td>
<td>24</td>
<td>3.3</td>
<td>0.3</td>
<td>30</td>
<td>70</td>
<td>40</td>
</tr>
<tr>
<td>HinUPO‡</td>
<td>36</td>
<td>2.0</td>
<td>0.4</td>
<td>48</td>
<td>52</td>
<td>4</td>
</tr>
</tbody>
</table>

a Reactions using 20 mM H\(_2\)O\(_2\). b Reactions using 5 mM H\(_2\)O\(_2\).

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**Fig. 3** Example of the initial (black) and refinement (red) simulations on isophorone (IP) diffusion (\( C_{\text{IP}}-\text{oxo} \) distance being monitored with respect to binding/interaction energy) on CglUPO, using two-step adaptive PELE.35
three enzymes (2.0–3.3 mM) and 4HIP over-oxidation was minimal, since it was observed that the ee of the hydroxyl-
ation product changed when 4KIP was formed. This was due to higher velocity in the conversion of the $S$-enantiomer com-
pared to the $R$-enantiomer, as observed in $r$HinUPO reactions with racemic 4HIP as substrate (Fig. S6†). That way, a kinetic 
resolution of the racemate, with ee of 99–100% and 60–75% 
recovery, can be achieved with $r$HinUPO and CglUPO. In con-
trast, AaeUPO just slowly converted the racemic mixture of 
4HIP, as it was also observed in the reaction of the enzyme

Fig. 4 Isophorone (IP, left) and (S)-4-hydroxyisophorone (S-HIP, right) diffusion refinement on five UPOs, with adaptive PELE$^{35}$ monitoring the distance between the substrate and the oxo atom (red sphere) of the H$_2$O$_2$-activated heme with respect to the binding energy ($E_b$). A) $C_4$ distance vs. energy plot for IP diffusion in CglUPO (red), MroUPO (magenta), AaeUPO (green), $r$HinUPO (cyan) and $r$CciUPO (blue) (see Fig. S7† for individual PELE plots of the five UPO systems). B and C) IP at the two lowest binding-energy positions during the CglUPO and AaeUPO simulations (A), re-
spectively. D) $H_4$ distance vs. energy plot for S-HIP diffusion on CglUPO (red) and AaeUPO (green). E and F) S-HIP at the two lowest binding-energy positions during the CglUPO and AaeUPO simulations (D), respectively.
with isophorone, where only traces of 4KIP were formed (Fig. 1), and no enantiomeric enrichment was produced.

Computational analyses: molecular modeling

Isophorone diffusion was done in two simulation steps using PELE software. For the initial simulation, after preparing all systems placing isophorone on the surface next to the entrance channel, the substrate was allowed to move freely within 16 Å of the heme iron. In all systems, the substrate moved easily into the active site. Structures in direct contact with the heme produced the best binding-energy poses, although they presented different substrate orientations. Therefore, after selecting the best binding-energy structure, we run a local refinement, where the ligand was forced to move within ~8 Å of the heme iron. Fig. 3 shows an example of these two runs for CglUPO, where we display the binding-energy profile with respect to the C4-oxo distance for the initial (black) and the refinement (red) simulations.

Fig. 4A shows the binding energy along the C4-oxo distance for isophorone in the refinement runs for the five UPO structures (identified with different colors). Among them, three UPOs show distances lower than 3 Å: CglUPO (red), AaeUPO (green) and rHinUPO (cyan) (see Fig. S7† for the individual PELE plots). Such positioning will largely facilitate the hydrogen-atom abstraction by compound I, in agreement with the experimental results (Table 1). Interestingly, different isophorone reactive poses were detected for the different UPOs, as shown in Fig. 4B and C for CglUPO and AaeUPO, respectively. A closer examination of these structures showed shorter distances to the heme oxo for the pro-S (1.9 Å in both) than for the pro-R (3.5 and 2.6 Å, respectively, not shown) hydrogen atoms, which explained the preferential formation of the 4HIP S-enantiomer, as shown in Table 4.

The resulting S-4HIP was also diffused with PELE, and strong differences in the C4-oxo distances and energies were obtained, as illustrated in Fig. 4D for two of the systems, with CglUPO closely approaching the heme oxo (Fig. 4E) while no catalytic distances were attained by AaeUPO (Fig. 4F). The above results agree with isophorone oxidation to 4KIP by CglUPO, while 4HIP is the main product from AaeUPO (Table 1), as well as with the deracemization results of chiral 4HIP (Fig. S6†). On the other hand, although additional PELE calculations showed a similar C4-oxo distance for the R-enantiomer (data not shown), the slightly worse (5 kcal mol−1) binding energy of the different pose adopted (with respect to S-4HIP) is in agreement with the S preference experimentally observed (Fig. S6†).

Finally, the dual hydroxylation at the isophorone C4 and C2 positions by AaeUPO, compared with the selective oxidation at C3 by CglUPO and rHinUPO (Table 1) was also analyzed in the PELE simulations. The isophorone C2 position is not at a catalytically relevant distance in CglUPO (4.4 Å, Fig. 4B and S8A†), while the C2-oxo and C4-oxo distances for AaeUPO (3.3 and 2.9 Å, respectively, Fig. 4C and S8B†) are within reaction limits (the oxo to hydrogen distances are also shown in Fig. 4B and C). The above results explain the lack of C2 hydroxylation by CglUPO and the similar percentages of C7-derivatives (7HIP + 7FIP) and C8-derivatives (4HIP + 4KIP) by AaeUPO (Table 1).

Conclusions

We report a new enzymatic route for isophorone oxidation to form 4HIP and 4KIP, which are interesting products for the flavour-and-fragrance and pharmaceutical industries. The direct enzymatic oxidation of isophorone to 4KIP (with only one enzyme) is reported here for the first time for two fungal peroxynucleases (CglUPO and rHinUPO). The above represents an advantage over the route with P450s, since the latter needs two enzymes (a P450 and an alcohol dehydrogenase) to obtain 4KIP from isophorone. However, process optimization of isophorone conversion by UPO is needed to attain the high-scale transformations reported for whole-cell P450 systems.33

Conflicts of interest

There are no conflicts to declare.

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