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Selective Oxygenation of Ionones and Damascones by Fungal Peroxygenases

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ABSTRACT: Apocarotenoids are among the most highly valued fragrance constituents, being also appreciated as synthetic building blocks. This work shows the ability of unspecific peroxygenases (UPOs, EC1.11.2.1) from several fungi, some of them being described recently, to catalyze the oxyfunctionalization of α- and β-ionones and damascones. Enzymatic reactions yielded oxygenated products such as hydroxy-, oxo-, carboxy- and epoxy-derivatives that are interesting compounds for the flavor and fragrance and pharmaceutical industries. Although a variable regioselectivity was observed depending on the substrate and enzyme, oxygenation was preferentially produced at the allylic position in the ring, being especially evident in the reaction with α-ionone, forming 3-hydroxy-α-ionone and/or 3-oxo-α-ionone. Noteworthy were the reactions with damascones, in the course of which some UPOs oxygenated the terminal position of the side chain, forming oxygenated derivatives (i.e. the corresponding alcohol, aldehyde and carboxylic acid) at C-10, which were predominant in the *Agrocybe aegerita* UPO reactions, and first reported here.

KEYWORDS: aromas, apocarotenoids, ionones, damascones, peroxygenases, biocatalysis, UPO

INTRODUCTION

Apocarotenoids or norisoprenoids, which are usually formed by partial oxidative degradation of carotenoids, include a wide range of compounds with different chemical structures and biological activities. Among these compounds, those having thirteen carbon atoms, such as ionones and damascones, are essential constituents of the aroma of tea, grapes, roses, tobacco and wine, and are also relevant flavors or fragrances that constitute an important economic resource for chemical industries.[1] Moreover, damascones and their derivatives have also been identified as a novel class of potential cancer chemopreventive phytochemicals.[2]

The introduction of hydroxyl or keto functionalities in these compounds reduces their volatility and increases the long lasting odor.[3] Most of these derivatives (3-hydroxy and 3-oxo-α-ionone, 4-hydroxy- and 4-oxo-β-ionone and hydroxy-β-damascone isomers) are present in plants but in very small amounts and extraction is not a viable process for their industrial use. For that reason, they are usually prepared by chemical synthesis and, therefore, alternative methods for the bioproduction of these compounds are of high industrial interest.[1]

Biotransformation of α- and β-ionones, and their respective α- and β-damascones isomers, to a number of hydroxy and keto derivatives has been reported for several fungi.[3-7] However, microbial whole-cell biotransformations require long incubation times, and often suffer from low conversion rates and substrate partial degradation. On the other hand, biotransformations with isolated enzymes (enzymatic *in vitro* conversion), such as some cytochrome P450 monooxygenases (P450s)[8-10] including engineered P450 BM-3 variants,[11,12] have also been reported. Most P450s, however, have the disadvantages of requiring expensive cofactors and auxiliary enzymes, and their stability is usually low due to their intracellular nature.

Unspecific peroxygenases (UPOs, EC.1.11.2.1) represent a relatively new and appealing type of biocatalysts for organic synthesis that, unlike P450s, are extracellular enzymes (therefore more stable) and only require H_2O_2 for activation.[13] However, in spite of all recent progresses in our understanding of UPO catalysis and application,[14] some difficulties in UPO application are still to be solved. They include, in addition to inactivation by hydrogen peroxide that affects enzyme reuse, the present limitations to heterologously express UPOs in bacterial (and even in fungal systems), due to the more recent discovery of these enzymes and their fungal origin. The latter aspects, to be overcome in the future, currently limit enzyme engineering to tailor UPOs for specific substrates and processes, as well as their production scale up. UPOs have been shown to catalyze a diversity of interesting oxygenation reactions with aromatic substrates,[15,16] aliphatic compounds such as fatty acids, alkanes, fatty alcohols,[17-21] steroids and secosteroids,[22-24] and other flavor and fragrance compounds, such as isophorone.[25] The first UPO was described in the basidiomycetous fungus *Agrocybe aegerita* (*Aae*UPO)[26] and since then, several other UPO enzymes have been purified from other Basidiomycota and Ascomycota species such as *Coprinellus radians,*[27] *Marasmius rotula* (*Mro*UPO)[28] and *Chaetomium globosum* (*Cgl*UPO),[29] which is indicative for their widespread occurrence in the fungal kingdom. In addition to these wild-type (i.e. non-recombinant) enzymes, there are other UPOs, e.g. from *Coprinopsis cinerea* (r*Cci*UPO)[18] and *Humicola insolens* (r*Hin*UPO),[29] which are only known as recombinant proteins heterologously expressed by Novozymes A/S (Bagsvaerd, Denmark) in the mold *Aspergillus oryzae.*[30] Very recently, a new UPO from the ascomycetous mold *Daldinia caldariorum* has become available from Novozymes, after gene expression in *A. oryzae*, being also expressible in *Escherichia coli* as a soluble and active enzyme.[31]

In the present work, the oxygenation of α- and β-ionones, and their respective isomers α- and β-damascones by several UPOs is shown for the first time. These are new reactions of interest for the biotechnological synthesis of different natural flavors, pharmaceuticals and synthetic building blocks to be added to the existing portfolio of reactions catalyzed by these exciting enzymes.[14]

MATERIALS AND METHODS

Enzymes. *Aae*UPO (isoform II), a wild UPO from cultures of *A. aegerita* TM-A1 (syn. *Cyclocybe aegerita*, DSM 22459) grown in soybean-peptone medium, was purified as previously described.[26] *Mro*UPO is another wild-type UPO, from cultures of *M. rotula* DSM-25031 (German Collection of Microorganisms and Cell Cultures, Braunschweig), which was purified as described by Gröbe et al.[28] *Cgl*UPO, the third wild-type UPO originates from cultures of *C. globosum* DSM-62110, which was purified as recently described.[29] r*Cci*UPO corresponds to the protein model 7249 from the sequenced *C. cinerea* genome available at the JGI (http://genome.jgi.doe.gov/Copci1). The recombinant enzymes r*Cci*UPO (44 kDa), r*Hin*UPO and r*Dca*UPO were provided by Novozymes A/S after expression in *A. oryzae*.[30] 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 dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and UV-vis spectroscopy following the characteristic heme-maximum around 420 nm (Soret band of resting-state UPOs). Enzyme concentration was estimated according to the characteristic UV-vis band of the reduced UPO complex (Fe^{2+}-heme) with carbon monoxide.[34]

Chemical compounds. Ionones, *rac*-(3*E*)-4-(2,6,6-trimethyl-2-cyclohexen-1-yl)-3-buten-2-one (also known as α-ionone) and (3*E*)-4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-3-buten-2-one (also known as β-ionone), and damascones, *rac*-(2*Z*)-1-(2,6,6-trimethyl-2-cyclohexen-1-yl)-2-buten-1-one (also known as α-damascone) and (2*E*)-1-(2,6,6-trimethyl-1-cyclohexen-1-yl)-2-buten-1-one (also known as β-damascone) were tested as substrates of the above UPOs. All the compounds were purchased from Sigma-Aldrich except α-ionone that was supplied by Fluka.

Enzymatic reactions. Reactions (1 mL volume) with ionones and damascones (0.5 mM) were performed at 30 °C, in 50 mM phosphate buffer, pH 7.0 (pH 5.5 in *Mro*UPO reaction). The enzyme concentration was 0.5 μM and the H_2O_2 was added every 6 min in doses of 0.5 μmol to a final concentration of 2.5 mM.[25] In control experiments, substrates were treated under the same conditions (including H_2O_2) but without enzyme. The blank experiments did not give any oxidation product. Samples, at 30 min reaction, were extracted with ethyl acetate and directly analyzed by GC-MS. On the other hand, a time course experiment with all substrates and enzymes was performed, and the corresponding samples within several reaction times (5 min, 10 min, 15 min and 20 min) were analyzed by GC-MS. In addition, samples were dried under N_2 to prepare trimethylsilyl (TMS) derivatives with *N,O*-bis(trimethylsilyl)trifluoroacetamide (Supelco) that were also analyzed by GC-MS. Reactions with higher substrate concentration were also performed using 5 mM of β-ionone, 1 μM of enzyme and by adding H_2O_2 with a syringe pump over 6 hours at 3 μmol h^{-1}. Total turnover number (TTN) (mol product x number of conversions/mol enzyme) as well as total turnover frequency (TOF) (TTN/time) were calculated in these reactions.

GC-MS analyses. The analyses of samples (with and without silylation) 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 mass fragmentography and comparing their mass spectra with those of the Wiley and NIST libraries, and those previously reported, [3,4,7,9,10,35-37] and relative quantification was obtained from total-ion peak area, using response factors of the same (in the case of substrates) or similar compounds. The mass spectra and chemical structures of substrates and their reaction products (underivatized and as TMS derivatives) are included in Supporting Information (**Table S1**).

RESULTS AND DISCUSSION

In the present work, several fungal UPOs –namely *Aae*UPO, *Mro*UPO, *Cgl*UPO, r*Cci*UPO, r*Hin*UPO and r*Dca*UPO– were tested for their ability to oxygenate apocarotenoids such as α-ionone, β-ionone, α-damascone and β-damascone (**Figures 1-4**), using H_2O_2 as co-substrate and O-donor. The performance of enzymatic reactions were evaluated by GC-MS, and the different activities and selectivities attained by the UPOs are described and discussed in the following sections.

Reactions with α-ionone. All UPOs were capable of completely transforming α-ionone within 30 min reaction time, except r*Cci*UPO that only reached 21% conversion under these conditions (**Table 1**). The time course of the reactions showed that r*Hin*UPO and r*Dca*UPO completely converted the substrate within 5 min, while *Aae*UPO, *Mro*UPO and *Cgl*UPO needed 15-30 min (**Figure 5A**).

GC-MS analyses of enzymatic reactions revealed that all enzymes selectively (86-98%) oxygenated α-ionone at C-3 (allylic position) producing 3-hydroxy-α-ionone (3-

OH-α-I) and 3-oxo-α-ionone (3-CO-α-I) (**Table 1**, **Figures 1, S1)**. In addition to these derivatives, the tautomer of the keto-derivative, 3-hydroxy-2,3-dedihydro-α-ionone (3-OH-2,3-DH-α-I) (7%), was observed in r*Hin*UPO reactions. Small amounts (1-14%) of 4-epoxy-α-ionone (4-epoxy-α-I) were also detected in all reactions. The mass spectra of these compounds (**Table S1**) were in agreement with those published in the NIST library and in the literature.[7,9,10]

Different proportions of both *cis*-3-OH-α-I and *trans*-3-OH-α-I isomers were observed in the reactions with the different UPOs, the *trans*-diastereoisomer being generally the most abundant, except in *Aae*UPO reactions where the proportion of both isomers was similar (**Figures 6A, S1**) after 30 min. The higher proportion of the *trans*-diastereoisomer may be attributed to the faster further oxygenation of the *cis*-diastereoisomer by these enzymes via a *gem*-diol intermediate that is in equilibrium with the corresponding keto derivative (**Figure 1**),[17] although an enzymatically preference for hydroxylation of α-ionone to the corresponding *cis* or *trans* diastereomers may not be discarded.

Interestingly, all UPOs were able to over-oxygenate the 3-OH-α-I to form 3-CO-α-I (via the *gem*-diol), except *Aae*UPO that only formed 5% of the latter compound (**Table 1**). Low over-oxygenating activity of *Aae*UPO for hydroxy-derivatives was also observed in the hydroxylation of the related α-isophorone.[25] The contrary was observed in r*Hin*UPO reactions, in which 3-CO-α-I was the predominant product and the OH-tautomer was also observed. The reason why the enol form was only found in r*Hin*UPO reactions may be due to the high amount of the keto-form in these reactions. With other UPOs, the lower amount of the 3-CO-α-I formed may cause that the OH-tautomer is below the detection limit.

Similar oxygenated derivatives were reported for fungi-mediated biotransformation of α-ionone, although generally with lower substrate conversion rates[3,5,7]. On the other hand, the biotransformation of α-ionone catalyzed by P450 CYP109D1 showed the regioselective formation of 3-OH-α-I.[9] The selectivity found here for most UPOs is similar to that reported for cytochrome CYP101B1, where the *trans* diastereoisomer was preferentially obtained (66%).[10]

Reactions with β-ionone. All UPOs were able to completely convert β-ionone under the same conditions within 30 min of reaction, except r*Cci*UPO that only reached 41% conversion (**Table 2**). Time courses of the reactions show similar conversion degrees as those observed with α-ionone for the different UPOs, with the exception of *Aae*UPO, which achieved almost complete substrate conversion within just 5 min (**Figure 5B**). On the other hand, different regioselectivities were noticed for the different UPOs tested, and oxygenation occurred at different positions (**Table 2**, **Figures 2, S2**). *Cgl*UPO and r*Hin*UPO were most selective (around 80% regioselectivity) towards the C-4 position, resulting in the formation of 4-hydroxy-β-ionone (4-OH-β-I) and its over-oxygenated (via a *gem*-diol intermediate) derivative 4-oxo-β-ionone (4-CO-β-I). All UPOs oxygenated, in addition to C-4, other ring positions (C-2 and C-3) although to lesser extent. These compounds were tentatively identified by MS, and their mass spectra (**Table S1**) matched with those published in the NIST library and literature.[9,10,12,37,38] Moreover, other hydroxylated derivatives resulting from a second and third oxygenation step (di-hydroxy and/or hydroxy-keto derivatives, **Figures S2-S3**) were also detected in almost all reactions.

Only in the case of r*Cci*UPO, the oxygenation of the terminal C-10 position in the side chain (10-hydroxy-β-ionone, 10-OH-β-I) was observed (**Figure S2C**). The position of the hydroxyl group was determined by the mass spectrum of the TMS derivative that

showed a major peak at *m/z* 177 corresponding to the loss of the terminal hydroxyl group (**Figure S4A**). This compound was only evidenced in the silylated sample. To our best knowledge, the occurrence of this oxygenated derivative has not been reported so far. The terminal oxygenation of side chains of other aliphatic cyclic compounds by UPOs has been described for steroids, secosteroids and *trans*-β-methylstyrene.[22,23,39]

On the other hand, the presence of 7,11-epoxymegastigma-5(6)-en-9-one (EME) and its hydroxylated derivative 4-hydroxy-7,11-epoxymegastigma-5(6)-en-9-one (4-OH-EME) was only observed in the reaction with r*Dca*UPO (**Figures 2, S2F**), and to our best knowledge , not reported so far for P450s or microbial cultures. This unusual bicyclic ionone derivative would be formed by the oxygenation of the C-13 followed by ring closure between position 7 and 13 of the ionone framework as reported for the chemical synthesis of EME (in several steps) starting from racemic γ-ionone epoxidation.[35] The presence of the hydroxylated derivative of β-ionone at C-13 (**Figures S2F, S4B**) supports this mechanism. Moreover, the absence of the dihydroxylated derivative of β-ionone at C-4 and C-13 suggests that 4-OH-EME is not formed from 4-hydroxy-β-ionone although this could not be absolutely discarded.

Finally, reactions with higher substrate (5 mM) and enzyme (1 μM) loading were also performed, in which the co-substrate H_2O_2 was continuously supplied over 6 hours with a syringe pump to preserve the enzyme stability (that is decreased by high local concentrations of peroxide). Under these conditions, nearly complete substrate conversion (except with r*Dca*UPO) and slightly increased selectivity were achieved, taking into account that five times less enzyme/substrate ratio was used. That way, total turnover numbers of 1,700-6,200 were reached (**Table 3**).

Reactions with α-damascone. All UPOs tested transformed the substrate to high extents (88-100%) within 30 min, except r*Cci*UPO that only accomplished 11%

conversion under the same conditions (**Table 4**). The time course of the reaction showed that r*Hin*UPO and r*Dca*UPO were more efficient than the other UPOs, since they completely transformed the substrate within 5 min of the reaction (**Figure 5C**).

All UPOs (except r*Cci*UPO) oxygenated the C-3 position to produce 3-hydroxy-α-damascone (3-OH-α-D) (**Table 4, Figures 3, S5**). In a similar way as in the reactions with α-ionone, the hydroxylation of α-damascone by several UPOs yielded both diastereoisomers, *cis*-3-OH-α-D and *trans*-3-OH-α-D, whose mass spectra were in accordance with those previously reported.[4] *Mro*UPO, *Cgl*UPO, r*Dca*UPO and r*Hin*UPO were more selective giving principally the *trans*-diastereoisomer, while *Aae*UPO mainly produced the *cis*-diastereoisomer (**Figure 6B**). In addition, *Cgl*UPO, r*Hin*UPO and r*Dca*UPO over-oxygenated 3-OH-α-D to the 3-oxo derivative, and most UPOs (*Mro*UPO, *Cgl*UPO, r*Dca*UPO and r*Hin*UPO) formed 4-epoxy-α-damascone (4-epoxy-α-D) as well, although in minor amounts. Again, similar products were obtained in fungi-mediated biotransformation of α-damascone but with lower conversion rates, maybe due to the toxicity of these compounds to fungal cells/hyphae.[3]

Interestingly, in addition to ring oxygenation, *Aae*UPO, *Mro*UPO and *rCci*UPO oxygenated the terminal position of the side chain (**Figure 3**) being the predominant reaction (80% of total products) for the former UPO (**Table 4, Figure S5**). In these reactions, the formation of the terminal alcohol (10-hydroxy-α-damascone, 10-OH-α-D) was followed by its over-oxygenation producing the corresponding aldehyde (4-oxo-4-(2,6,6-trimethylcyclohex-2-en-1-yl)but-2-enal, 10-CHO-α-D) and the carboxylic acid (4-oxo-4-(2,6,6-trimethylcyclohex-2-en-1-yl)but-2-enoic acid, 10-COOH-α-D). These compounds were tentatively identified by the mass spectra of their TMS derivatives (**Figure S6**). The spectra show the molecular ions at *m/z* 280 (10-OH-α-D), *m/z* 206 (10-CHO-α-D) and *m/z* 294 (10-COOH-α-D) as well as the fragments corresponding to

the loss of a methyl group $[M–CH_3]^+$ at *m/z* 265, 191 and 279, respectively, and the fragment at *m/z* 123 from the intact ring moiety. Additionally, diagnostic fragments at *m/z* 157 corresponding to the side chain butenoyl group in the 10-OH-α-D (**Figure S6A**), and at *m/z* 177 due to the loss of the aldehyde group $[M\text{-}CHO]^+$ (**Figure S6B**) are also observed. To our best knowledge, such damascone derivatives at C-10 have not been reported so far. This may be due to the fact that most authors analyzed these compounds without derivatization, and we found that they can only be detected after derivatization (silylation).

Reactions with β-damascone. Among all UPOs tested, only *Cgl*UPO and r*Hin*UPO completely converted β-damascone within 30 min, while *Aae*UPO, *Mro*UPO and r*Dca*UPO) attained conversions of 81%, 72% and 34%, respectively, under the same conditions; r*Cci*UPO was practically incapable of oxidizing it (**Table 5, Figures 4, S7**). Noteworthy is the low conversion of this compound by r*Dca*UPO compared to the other substrates tested. The time course of the reaction showed that r*Hin*UPO was more efficient than the other UPOs, since it completely transformed the substrate within 5 min (**Figure 5D**).

In most cases, the hydroxylation at C-4 position was dominant over that at C-3 position with the exception of *Aae*UPO. On the other hand, r*Hin*UPO, and to minor extent *Cgl*UPO, were the only enzymes that over-oxygenated the 4-OH-β-D to 4-oxo-β-damascone (4-CO-β-D) (**Figures S7D-E**). The mass spectra of these compounds (**Table S1**) are in agreement with those previously reported.[3,36,38] On the other hand, as in α-damascone reactions, oxygenated derivatives (alcohol, aldehyde and carboxylic acid) at C-10 were also formed (**Figure 4**), preferentially by *Aae*UPO and *Mro*UPO (**Figure S7**). These compounds were identified by the mass spectra of their TMS derivatives (**Figure S8**), which showed similar characteristic fragments to those of α-damascone

287 derivatives, as the molecular ions at *m/z* 280 (10-OH-β-D), *m/z* 206 (10-CHO-β-D) and
288 *m/z* 294 (10-COOH-β-D) and the fragments corresponding to the loss of a methyl group
289 $[M–CH_3]^+$ at *m/z* 265, 191 and 279, respectively. Likewise, the fragment at *m/z* 177
290 originated by the loss of the aldehyde group $[M\text{-}CHO]^+$ (**Figure S8B**) is also observed.

291 **Comparison of oxygenation patterns by different UPOs.** Generally, all UPOs
292 accomplished high conversion rates for all substrates tested, except r*Cci*UPO. r*Hin*UPO
293 and r*Dca*UPO were the most efficient ones (that converted more substrate in less time
294 under the same conditions) in oxidizing all substrates tested (with the exception of β-
295 damascone that was only moderately converted by r*Dca*UPO) (**Figure 5**). On the other
296 hand, *Aae*UPO showed higher efficiency with β-ionone than with any other substrate.

297 Regarding selectivity of the reactions with ionones, all UPOs showed higher
298 regioselectivities (up to 99%) with α-ionone than with β-ionone (**Tables 1-2**). Although
299 α- and β-ionones differ only in the position of the cyclohexene double bond, UPOs
300 realized different reactivities towards them. Analyses of the different oxygenation
301 products formed by the UPOs revealed that the position of the double bond in the
302 cyclohexenyl ring had seemingly an effect on the regioselectivity of hydroxylation,
303 directing it towards the allylic position. In this sense, α- and β-ionone were mainly
304 hydroxylated by most UPOs at C-3 and C-4 to form 3-hydroxy-α-ionone and 4-
305 hydroxy-β-ionone, respectively. However, this effect was more pronounced with α-
306 ionone, since several oxygenated derivatives at other positions (C-2 and C-3) of the ring
307 (or in the side-chain) were formed with β-ionone. The opposite was reported for P450
308 BM-3 mutants that selectively produced 4-OH-β-I, while they contrarily oxidized α-
309 ionone to a mixture of four products.[12] Interestingly, r*Hin*UPO was the UPO that most
310 pronouncedly oxygenated mono-hydroxyl derivatives further into the corresponding
311 keto derivatives (that are in equilibrium with the *gem*-diol counterparts formed first). On

312 the other hand, most of the tested UPOs left unaffected the C-13 methyl group of both
313 α- and β-ionone. Only in the reaction with r*Dca*UPO the hydroxylation of the C-13
314 carbon atom of β-ionone was observed, producing the interesting EME and 4-OH-EME
315 products.

316 The preference for the hydroxylation of the allylic position in the cyclohexene ring
317 by UPOs was also observed in the reactions with α- and β-damascones. A strict
318 regioselectivity for this position was especially evident in the reaction of α-damascone
319 with r*Hin*UPO and r*Dca*UPO, followed by *Cgl*UPO, and in the reaction of β-damascone
320 with r*Hin*UPO and *Cgl*UPO. Interestingly, in damascones reactions, a different
321 regioselectivity with respect to that observed with ionones was ascertained. Therefore,
322 in addition to the cyclohexenyl ring, hydroxylation was produced at the terminal
323 position of the butenoyl side chain, being predominant in *Aae*UPO reactions but
324 completely absent in r*Hin*UPO, r*Dca*UPO and *Cgl*UPO reactions. Thus, the change in
325 the position of the carbonyl and alkene moieties in the butenoyl side chain caused a
326 drastic change in regioselectivity of *Aae*UPO towards damascones compared to ionones.

327 It can be concluded that the oxyfunctionalization of the ionone and damascone
328 isomers catalyzed by UPOs reveal clear advantages over P450 catalysis (e.g. engineered
329 P450 BM-3 variants) due to the higher conversion rates, enzyme stabilities and little
330 requirements concerning cofactors. These enzymes can therefore be of high interest for
331 the production of valuable compounds, of interest for the flavor and fragrance, cosmetic
332 and pharmaceutical industries. However, it is necessary to mention that some current
333 limitations of UPOs, related to their large-scale production and application, need to be
334 solved for their efficient industrial implementation.

335

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Supporting Information

Chemical structures and mass fragmentations of ionones and damascones and their oxygenated derivatives from enzymatic reactions with several UPOs (Table S1), GC-MS analyses of ionones and damascones reactions with several UPOs (Figures S1, S2, S5 and S7), Chemical structures of the dihydroxy and keto-hydroxy-derivatives from UPO reactions with β-ionone (Figure S3), and mass spectra and formulae/fragmentations of several oxygenated derivatives of ionones and damascones from reactions with several UPOs (Figures S4, S6 and S8).

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FIGURE LEGENDS

Figure 1. α-Ionone (**α-I**) oxygenation by UPOs, showing the 3-hydroxy-α-ionone (**3-OH-α-I**), 3-oxo-α-ionone (**3-CO-α-I**) formed via a *gem*-diol intermediate, its tautomer 3-hydroxy-2,3-didehydro-α-ionone (**3-OH-2,3-DH-α-I**) and 4-epoxy-α-ionone (**4-epoxy-α-I**).

Figure 2. β-ionone (**β-I**) oxygenation by UPOs showing the different oxygenated derivatives: 4-hydroxy-β-ionone (**4-OH-β-I**), 3-hydroxy-β-ionone (**3-OH-β-I**), 2-hydroxy-β-ionone (**2-OH-β-I**), 10-hydroxy-β-ionone (**10-OH-β-I**), 13-hydroxy-β-ionone (**13-OH-β-I**), 4-oxo-β-ionone (**4-CO-β-I**), 2-oxo-β-ionone (**2-CO-β-I**), 7,11-epoxymegastigma-5(6)-en-9-one (**EME**), and 4-hydroxy-7,11-epoxymegastigma-5(6)-en-9-one (**4-OH-EME**).

Figure 3. α-damascone (**α-D**) oxygenation by UPOs, showing the, 3-hydroxy-α-damascone (**3-OH-α-D**), 10-hydroxy-α-damascone (**10-OH-α-D**), 4-epoxy-α-damascone (**4-epoxy-α-D**), 3-oxo-α-damascone (**3-CO-α-D**), 4-oxo-4-(2,6,6-trimethylcyclohex-2-en-1-yl)but-2-enal (**10-CHO-α-D**), 4-oxo-4-(2,6,6-trimethylcyclohex-2-en-1-yl)but-2-enoic acid (**10-COOH-α-D**) derivatives.

Figure 4. β-damascone (**β-D**) oxygenation by UPOs showing the 3-hydroxy-β-damascone (**3-OH-β-D**), 4-hydroxy-β-damascone (**4-OH-β-D**), 10-hydroxy-β-damascone (**10-OH-β-D**) and the over-oxygenated compounds 4-oxo-β-damascone (**4-**

507 **CO-β-D**), 4-oxo-4-(2,6,6-trimethylcyclohex-1-en-1-yl)but-2-enal (**10-CHO-β-D**) and
508 4-oxo-4-(2,6,6-trimethylcyclohex-1-en-1-yl)but-2-enoic acid (**10-COOH-β-D**).
509
510 **Figure 5.** Time course of the transformation of 0.5 mM α-ionone (**A**), β-ionone (**B**), α-
511 damascone (**C**) and β-damascone (**D**) by 0.5 μM *Aae*UPO (purple), *Mro*UPO (yellow),
512 r*Cci*UPO (light blue), *Cgl*UPO (green), r*Hin*UPO (red) and r*Dca*UPO (blue). The
513 standard deviations (SD) calculated for duplicates (at 30 min) are indicated.
514
515 **Figure 6.** Abundance (relative percentage) and chemical structures (relative
516 configuration) of the different diastereoisomers formed in the reactions at 5 min and 30
517 min of α-ionone (**A**) and α-damascone (**B**) with several UPOs. The standard deviations
518 (SD) calculated for duplicates (at 30 min) are indicated.

Table 1. Conversion (percentage of substrate transformed) of α-ionone (α-I) by several UPOs (30 min) and abundance (relative percentage) of the reaction products, including hydroxy (OH), keto (CO) and epoxy derivatives

	*Aae*UPO	*Mro*UPO	r*Cci*UPO	*Cgl*UPO	r*Hin*UPO	r*Dca*UPO
Conversion (%)	>99	>99	21	>99	>99	>99
Products (%)						
3-OH-α-I	93	55	63	72	1	45
3-CO-α-I	5	31	35	22	98*	53
4-epoxy-α-I	2	14	2	6	1	2

* including the keto derivative (91%) and its tautomer 3-hydroxy-2,3-didehydro-α-ionone (7%)

Table 2. Conversion (percentage of substrate transformed) of β-ionone (β-I) by several UPOs (30 min) and abundance (relative percentage) of the reaction products, including hydroxy (OH) and keto (CO) derivatives, and/or 7,11-epoxymegastigma-5(6)-en-9-one (EME), and dihydroxy or hydroxy keto derivatives of β-I (others)

	*Aae*UPO	*Mro*UPO	r*Cci*UPO	*Cgl*UPO	r*Hin*UPO	r*Dca*UPO
Conversion (%)	>99	>99	41	>99	>99	>99
Products (%)						
4-OH-β-I	55	55	61	64	21	53
3-OH-β-I	22	-	19	-	-	19
2-OH-β-I	13	7	2	1	-	3
10-OH-β-I	-	-	14	-	-	-
13-OH-β-I	-	-	-	-	-	7
EME	-	-	-	-	-	11
2-CO-β-I	-	18	-	-	-	-
4-CO-β-I	3	1	2	20	58	-
4-OH-EME	-	-	-	-	-	5
Others	7	19	2	15	21	2

Table 3. Conversion (percentage of substrate transformed), abundance (relative percentage) of reaction products - including hydroxy (OH), keto (CO) and dihydroxy or hydroxy keto derivatives (others) - total turnover number (TTN) and total turnover frequency (TOF) in the enzymatic transformation of 5 mM β-ionone (β-I)

	*Aae*UPO	*Mro*UPO	r*Cci*UPO	*Cgl*UPO	r*Hin*UPO	r*Dca*UPO
Conversion (%)	95	93	22	98	91	52
Products (%)						
4-OH-β-I	87	67	83	70	57	54
4-CO-β-I	-	13	-	24	24	-
Others	13	20	17	6	19	46
TTN	5200	5900	1700	6200	6000	2800
TOF (min^{-1})	14	16	5	17	17	8

Table 4. Conversion (percentage of substrate transformed) of α-damascone (α-D) by several UPOs (30 min) and abundance (relative percentage) of the reaction products, including hydroxy (OH), aldehyde (CHO), keto (CO), carboxy (COOH) and epoxy derivatives

	*Aae*UPO	*Mro*UPO	r*Cci*UPO	*Cgl*UPO	r*Hin*UPO	r*Dca*UPO
Conversion (%)	>99	88	11	>99	>99	>99
Products (%)						
3-OH-α-D	18	55	-	68	27	83
4-epoxy-α-D	-	6	-	16	3	4
10-OH-α-D	-	6	41	-	-	-
3-CO-α-D	-	-	-	16	70	13
10-CHO-α-D	2	14	52	-	-	-
10-COOH-α-D	80	19	7	-	-	-

Table 5. Conversion (percentage of substrate transformed) of β-damascone (β-D) by several UPOs (30 min) and abundance (relative percentage) of the reaction products, including hydroxy (OH), aldehyde (CHO), keto (CO), carboxy (COOH) and epoxy derivatives

	*Aae*UPO	*Mro*UPO	r*Cci*UPO	*Cgl*UPO	r*Hin*UPO	r*Dca*UPO
Conversion (%)	81	72	1	>99	>99	34
Products (%)						
3-OH-β-D	32	8	-	6	5	24
4-OH-β-D	9	46	-	88	53	71
10-OH-β-D	3	6	>99	-	-	5
4-CO-β-D	-	-	-	6	42	-
10-CHO-β-D	8	17	-	-	-	-
10-COOH-β-D	48	23	-	-	-	-

Figure 1

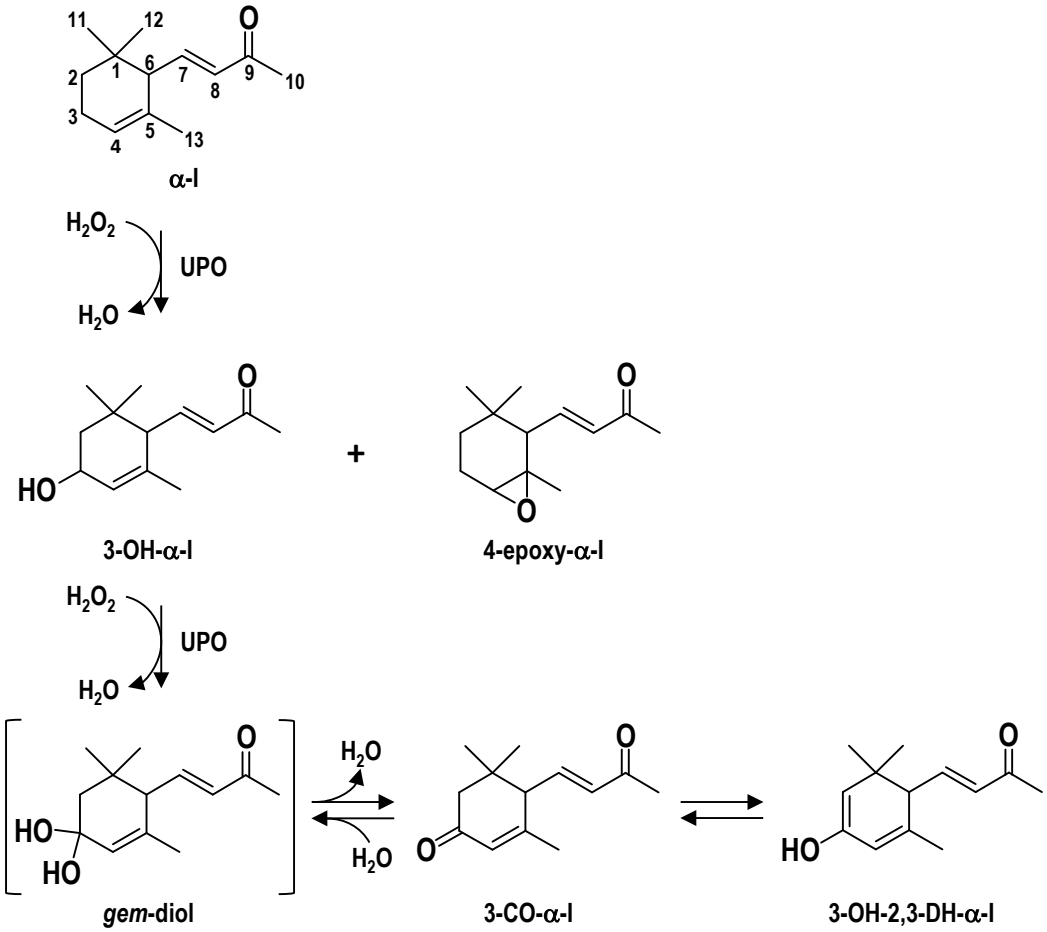

Figure 2

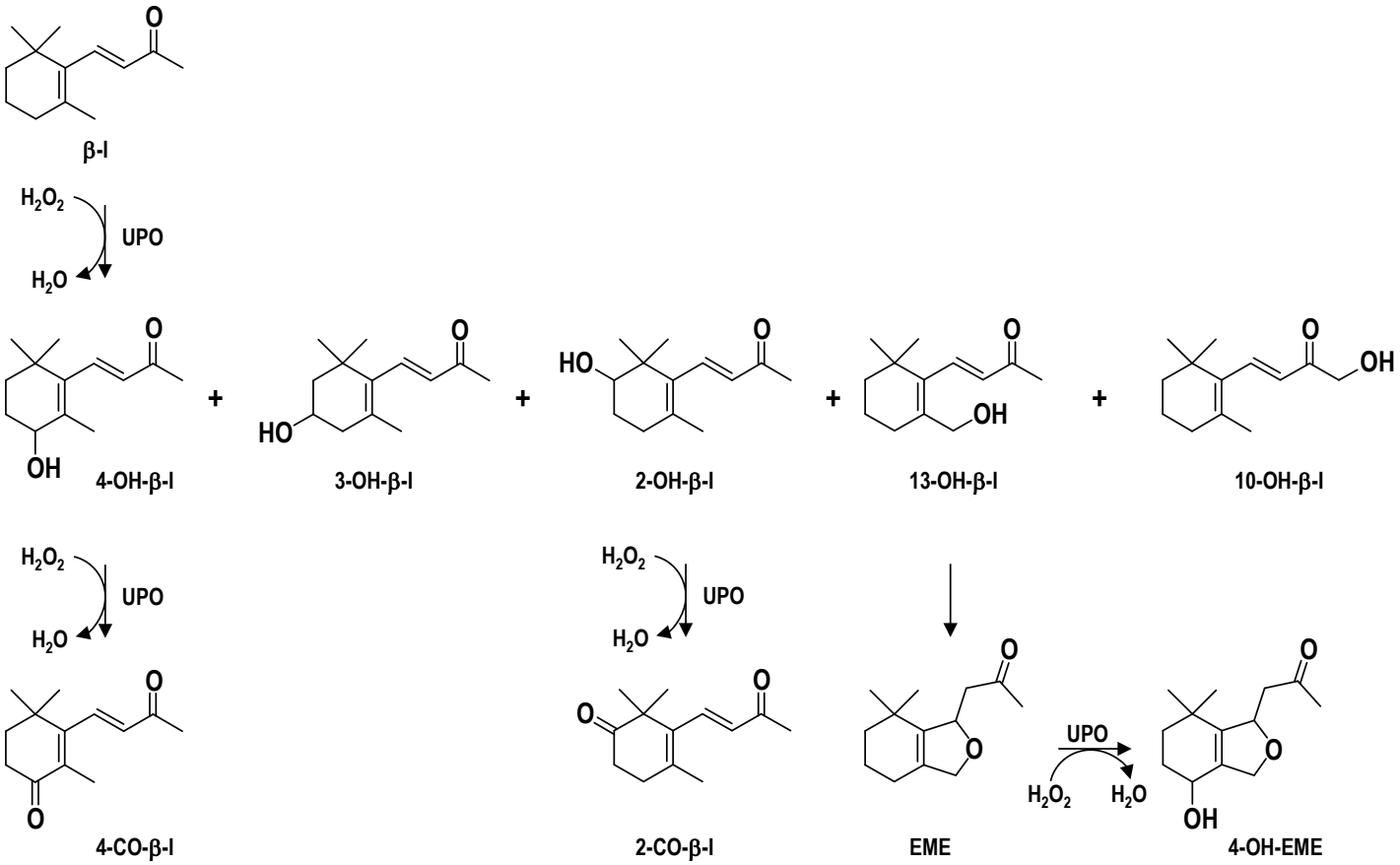
β-I
H2O2
UPO
H2O
4-OH-β-I
3-OH-β-I
2-OH-β-I
13-OH-β-I
10-OH-β-I
4-CO-β-I
2-CO-β-I
EME
4-OH-EME

Figure 3

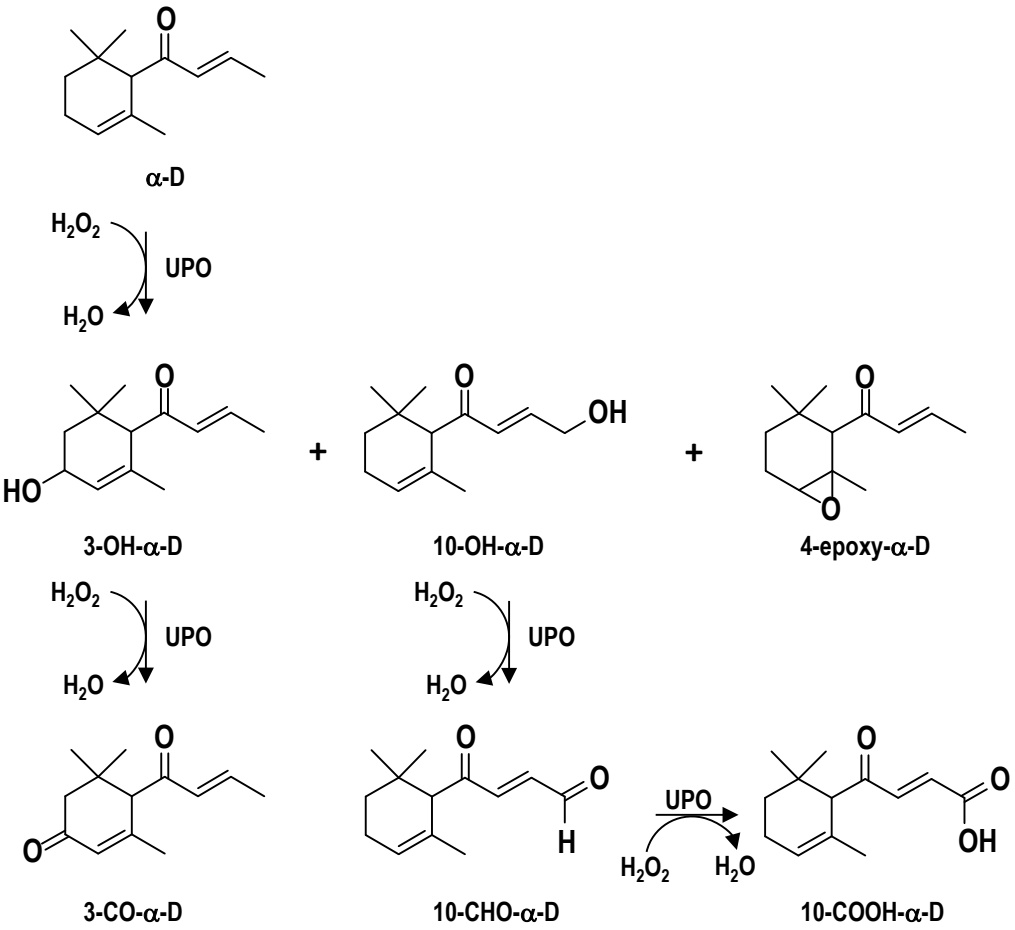
α-D
H2O2
UPO
H2O
3-OH-α-D
10-OH-α-D
4-epoxy-α-D
3-CO-α-D
10-CHO-α-D
10-COOH-α-D

Figure 4

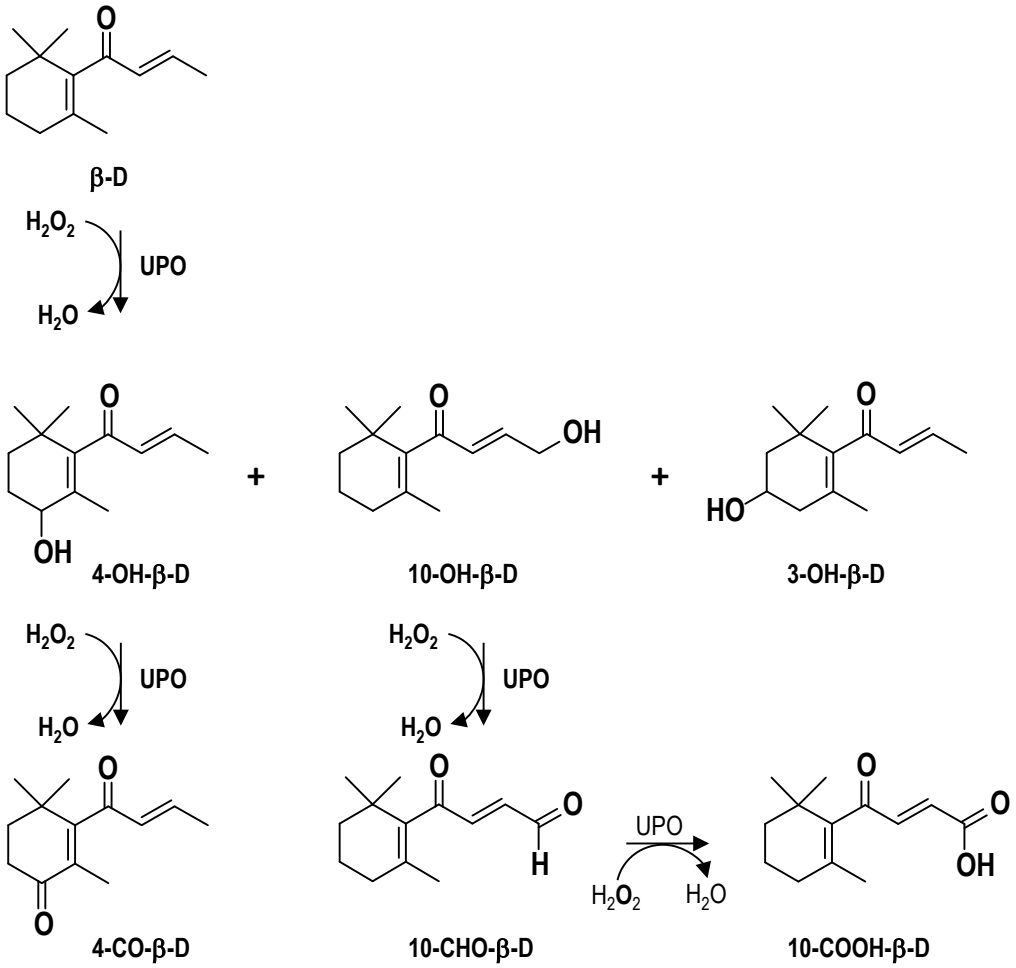

Figure 5

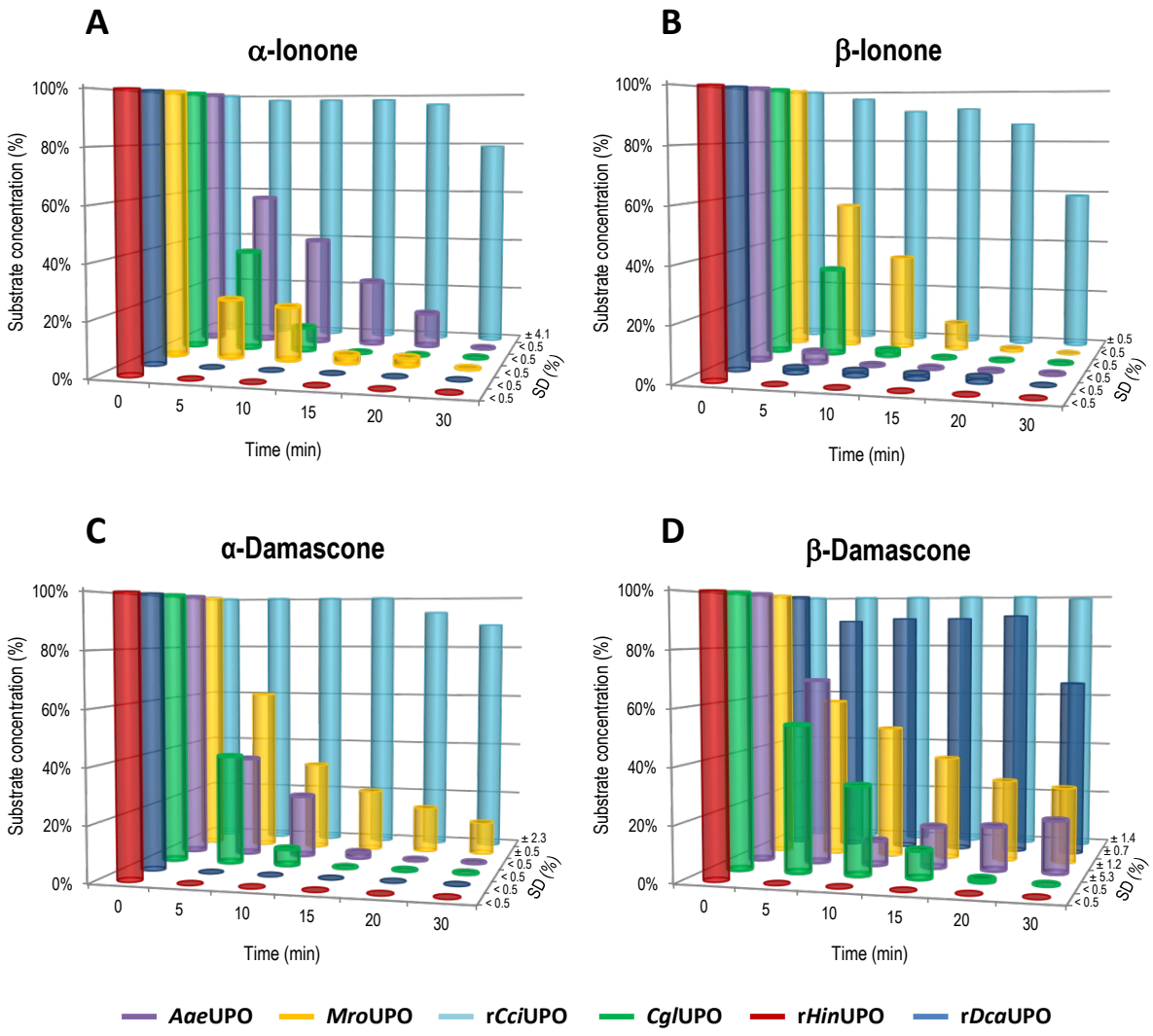
A
α-Ionone
B
β-Ionone
C
α-Damascone
D
β-Damascone
Substrate concentration (%)
100%
80%
60%
40%
20%
0%
0
5
10
15
20
30
Time (min)
SD (%)
AaeUPO
MroUPO
rCciUPO
CglUPO
rHinUPO
rDcaUPO

Figure 6

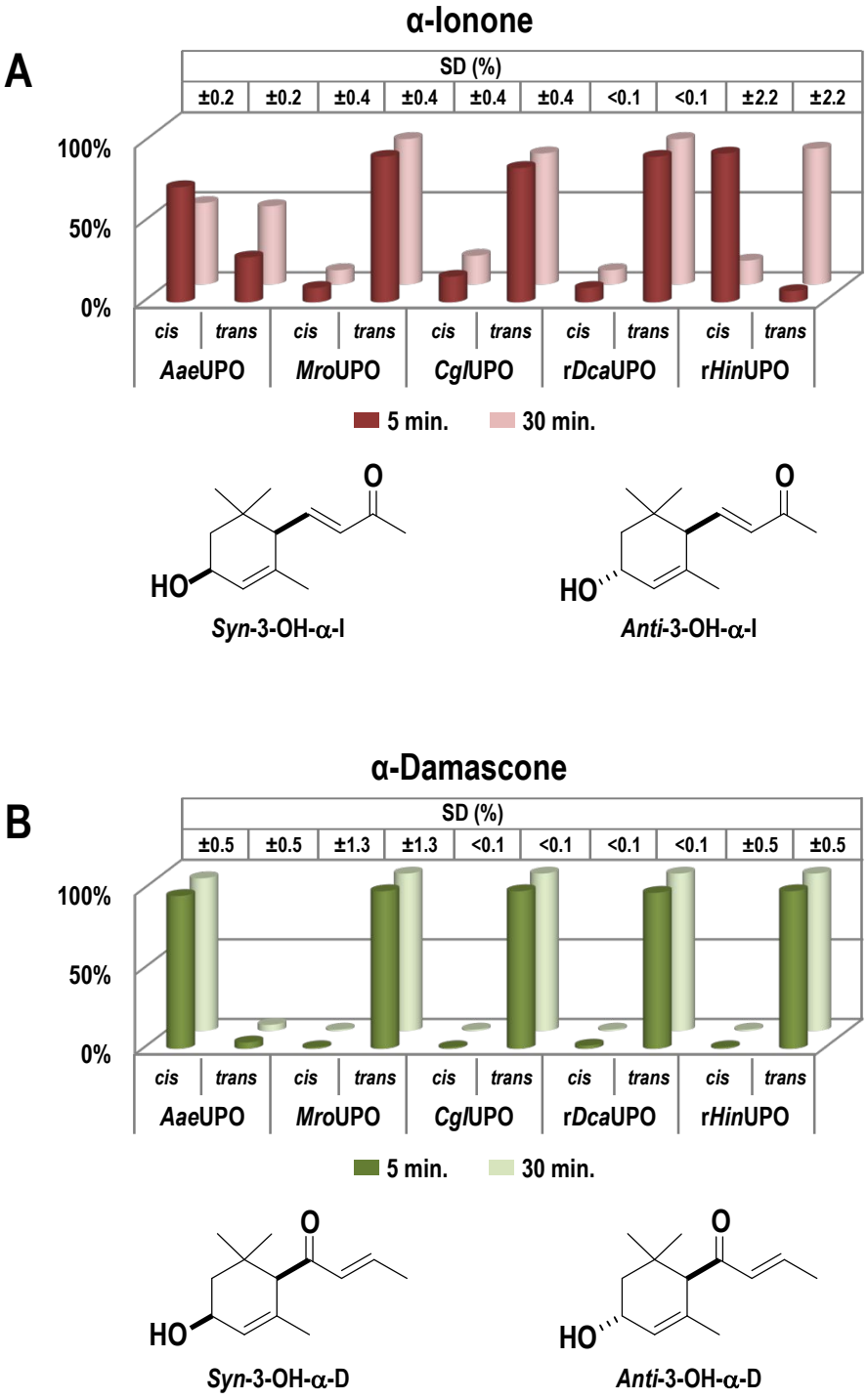
A
α-Ionone
SD (%)
±0.2 ±0.2 ±0.4 ±0.4 ±0.4 ±0.4 <0.1 <0.1 ±2.2 ±2.2
100%
50%
0%
cis trans cis trans cis trans cis trans cis trans
AaeUPO MroUPO CglUPO rDcaUPO rHinUPO
5 min. 30 min.
HO O
Syn-3-OH-α-I
Anti-3-OH-α-I
B
α-Damascone
SD (%)
±0.5 ±0.5 ±1.3 ±1.3 <0.1 <0.1 <0.1 <0.1 ±0.5 ±0.5
100%
50%
0%
cis trans cis trans cis trans cis trans cis trans
AaeUPO MroUPO CglUPO rDcaUPO rHinUPO
5 min. 30 min.
HO O
Syn-3-OH-α-D
Anti-3-OH-α-D

Table of Contents graphic