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Efficient terpene hydroxylation catalysts based upon P450 enzymes derived from Actinomycetes

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The hydroxylation of α-ionone **1** and β-ionone **2** to their corresponding mono-hydroxylated derivatives has been examined using a recombinant *E. coli* whole cell system, in which cytochromes P450 SU1 and SU2, and P450 SOY were over-expressed with their cognate ferrodoxins. Both substrates are hydroxylated with a high degree of regioselectivity and for α-ionone **1** the reaction is highly diastereoselective yielding the anti-isomer.

Introduction

One of the most challenging reactions in contemporary organic synthesis is the selective hydroxylation of unactivated C–H bonds. This transformation has considerable potential for the efficient synthesis of fine chemicals, as well as pharmaceutical and agrochemical intermediates. Unfortunately, there are few chemical methods available for the selective hydroxylation of a target compound. Biological systems, on the other hand, are well suited to such transformations. Cytochrome P450 monooxygenases, in particular, have been found to mediate these reactions, including the associated hetereoatom oxidation of organic substrates.[1–3]

Among the P450 monooxygenases, the most extensively characterised is $P450_{CAM}$, isolated from the soil bacterium *Psedomonas putida*. $P450_{CAM}$ catalyses the stereospecific hydroxylation of (1*R*)-(+) camphor to 5-*exo*-hydroxycamphor, which constitutes the initial step in the degradation pathway in this organism.[4,5] One limitation of this system is its relatively tight substrate specificity, even though the widening of its substrate range by protein engineering has been reported.[6] In recent years, the Actinomycetes, and in particular those of the genus *Streptomyces*, have been found to be a rich source of cytochrome P450s.[7] To date, there are at least ten *Streptomycete* P450s which have been cloned and biochemically characterised. The sulfonylurea herbicide-inducible P450 SU1 and P450 SU2 from the soil bacterium *Streptomyces griseolus*, and P450 SOY from *Streptomyces griseus*, are three such examples. Recently, an improved *E. coli* expression system for these P450s has been developed.[8,9]

Unusually for microbial P450s, the P450s from *Streptomyces* sp. possess relatively broad substrate specificity, and are able to catalyse a wider range of hydroxylation reactions with various classes of substrate.[3] Earlier investigations have focused on the transformation of terpenes into hydroxylated derivatives by various wild-type *Streptomyces* species.[10] Compounds containing a trimethylcyclohexane fragment (*e.g.*, the ionones) constitute essential aroma elements in many plant oils. Because of their organoleptic properties, ionones and their more valued oxygenated derivatives have attracted the attention of manufacturers of perfumes, soaps, cosmetics and fine chemicals.[11,12] For instance, hydroxy-β-ionone is an important intermediate for the synthesis of the carotenoids[13–17], and also of deoxyabcisic acid, a synthetic analogue of the phytohormone abscisic acid.[18,19]

In this work, we report the biotransformation of α-ionone **1** and β-ionone **2** into hydroxylated derivatives, using a recombinant *E. coli* whole cell system in which cytochromes P450 SU1 and SU2, and P450 SOY and their cognate ferrodoxins were overexpressed. A comparison is also made between the recombinant P450 SU2 and the wild type cell system for the transformation of β-ionone, both in terms of efficiency and product profile.

Results

Protein expression and spectral studies

Total soluble cell extracts of recombinant *E. coli* were analysed by carbon monoxide reduced difference spectra to determine the amount of P450. A typical spectrum of *E. coli* BL21 (DE3) expressing P450 SU2 is shown in Fig. 1. Similar spectra were obtained for P450 SU1 and P450 SOY. The P450 content of the cell extract was calculated using an extinction coefficient of 91 mM^{-1} cm^{-1} at 450 nm.[20] The absence of a peak at 420 nm in the CO-reduced difference spectrum indicates that all the P450 is correctly folded. Typically, we obtained concentrations of 250 nmol l^{-1} (11.5 mg l^{-1}) for P450 SU2, 100 nmol l^{-1} for P450 SU1 (4.5 mg l^{-1}) and 150 nmol l^{-1} (7.0 mg l^{-1}) for P450 SOY. We also repeated the experiment using the wild type *S. griseolus*, but unfortunately could not obtain a CO-reduced difference spectrum and so were unable to calculate the P450 content. Therefore, biocatalyst efficiencies (ratio of product to protein) between the recombinant system (*e.g.*, P450 SU2) and the wild type *S. griseolus* were based on total protein concentrations. Using the standard Bradford assay,[23] total protein contents were found to be 2.27 mg ml^{-1} for the recombinant system and 1.39 mg ml^{-1} for the wild-type organism.

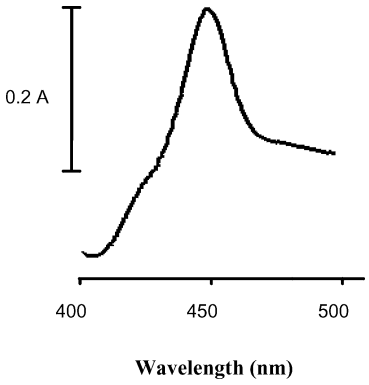

Fig. 1 Reduced carbon monoxide difference spectrum of cytochrome P450 SU2 from *Streptomyces griseolus* expressed in *E. coli* strain BL21 (DE3) in cell-free extract.

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Table 1 Biotransformation of α-and β-ionone by various recombinant systems. Conversion rate were estimated by GLC analysis of reaction mixture after 48 h of incubation. Individual percentages are given with respect to sum of metabolites extracted from reaction mixture. Molecular weights of unidentified metabolites are given in parenthesis

	α-Ionone (**1**)		β-Ionone (**2**)	
Recombinant systems	Conversion rate to **3**	Conversion rate to other products, (MW)	Conversion rate to **4**	Conversion rate to other products, (MW)
BL21 (DE3) (control)	—	—	—	—
P450 SOY C	6.7%	7.1% (233)	11.5%	4.2% (235)
P450 SU1	<1%	—	3.4%	1.7% (244)
P450 SU2	57.1%	3.4% (208), 8.5% (208), 5.0% (208), 5.5% (208)	39.3%	1.54% (208), 1.26% (208)

Biotransformation of α-ionone

α-Ionone biotransformations were carried out using the recombinant expression system for P450 SOY, SU1 and SU2 *in vivo*. A gas-chromatograph of the product distribution for such a bioconversion, using an *E. coli* strain containing the expression plasmid encoding P450 SU2, is shown in Fig. 2. Similar chromatogrphs and product profiles, but with lower conversion rates, were also observed for *E. coli* strains harbouring P450 SU1, and P450 SOY. The overall results are summarised in Table 1. The major product (peak 3 in Fig. 2) from these biotransformation was isolated and, by direct comparison of MS and NMR data,[10] unequivocally found to be 3-hydroxy-α-ionone **3** (Scheme 1). Along with this allylic hydroxylation product, we also detected four additional metabolites with the same mass as 3-hydroxy-α-ionone, (*ca.* 208, peaks a, b, c and d in Fig. 2). These products were not characterised fully since there was insufficient material produced. However, since the GC-MS analyses indicated that these metabolites having a molecular weight of 208, they are likely to be the regioisomers of 3-hydroxy-α-ionone. The best yield obtained was *ca.*57% for 3-hydroxy-α-ionone using the recombinant P450 SU2 system, in which at about 20% of the starting material was recovered after 48 h incubation.

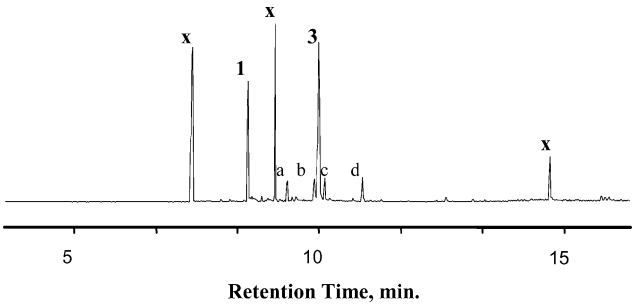
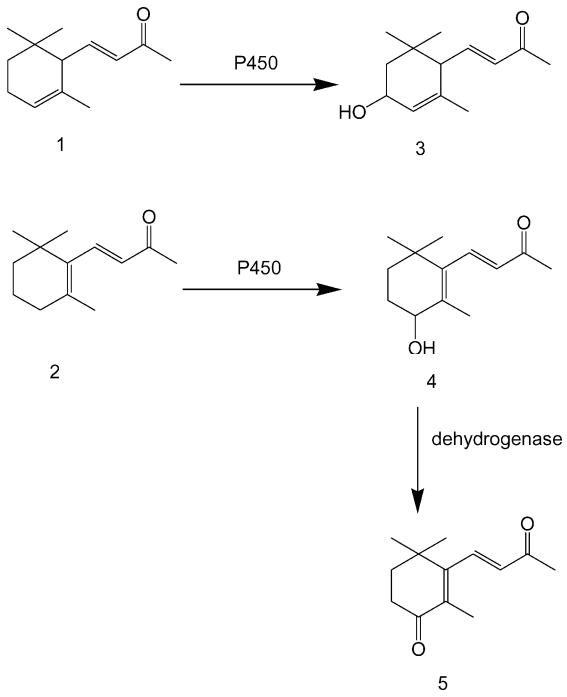
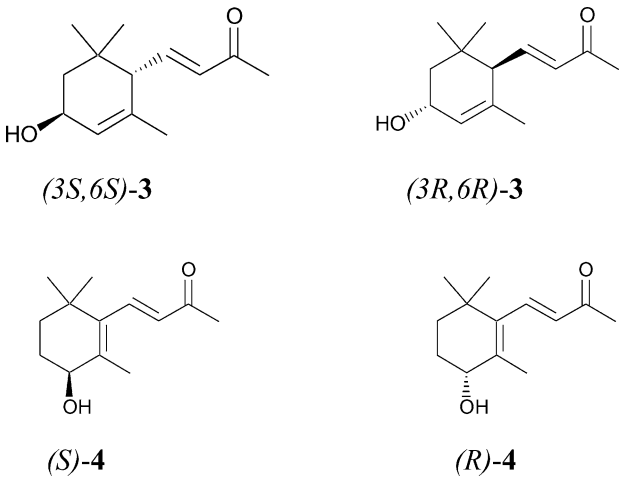


Fig. 2 GC spectra of metabolites from α-ionone biotransformation. The peaks are identified as: (**1**) α-ionone; (**3**) 3-hydroxy-α-ionone; (a),(b),(c), and (d) possible regioisomers of 3; (x) compounds extracted from reaction medium which are also present in the control experiments.



Scheme 1

Hydroxylation of a racemic mixture of α-ionone results in a mixture of diastereoisomers. If the bioconversion was an unselective process, four isomers in an equimolar ratio would be expected to be detected by chiral analysis, and two by achiral analysis. The latter analysis, by GC-MS, revealed one major product from the bioconversion, indicating that the reaction does indeed proceed in a selective manner. Furthermore, only one major peak was observed on the gas-chromatograph. From ^{1}H and ^{1}H–^{1}H 2D COSY NMR analysis, the possible isomers were assigned as (3*S*,6*S*)- and (3*R*,6*R*)-3-hydroxy-α-ionone. No further work was carried out to determine the absolute configuration (Scheme 2).



Scheme 2 Enantiomers of 3-hydroxy-α-ionone (**3**) and 4-hydroxy-β-ionone (**4**).

Biotransformation of β-ionone

The biotransformation of β-ionone **2** was carried out using the same recombinant expression system as the α-ionone conversion, and monitored by GC-MS. A gas-chromatograph showing the product distribution for this bioconversion, using *E. coli* harbouring the P450 SU2 gene, is shown in Fig. 3, and the results are summarised in Table 1. The product (peak 4 in Fig. 3) was found to be 4-hydroxy-β-ionone **4** (Scheme 1).

To allow a direct comparison with the recombinant system, a β-ionone biotransformation by *S. griseolus* cells was also conducted. A time-course profile for the biotransformation

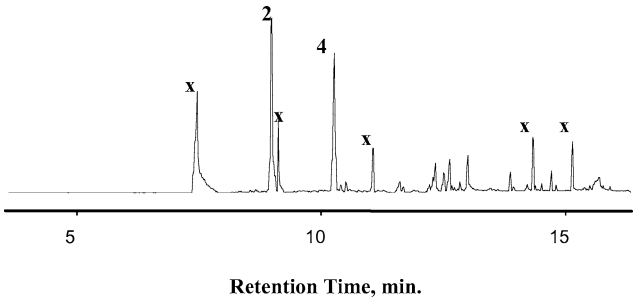
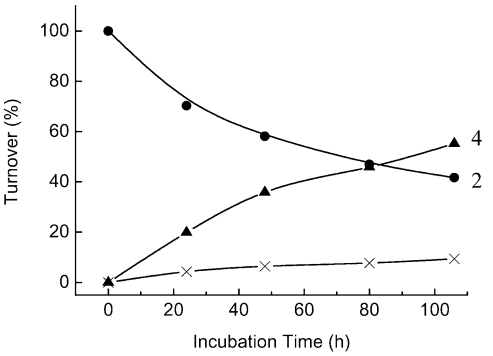
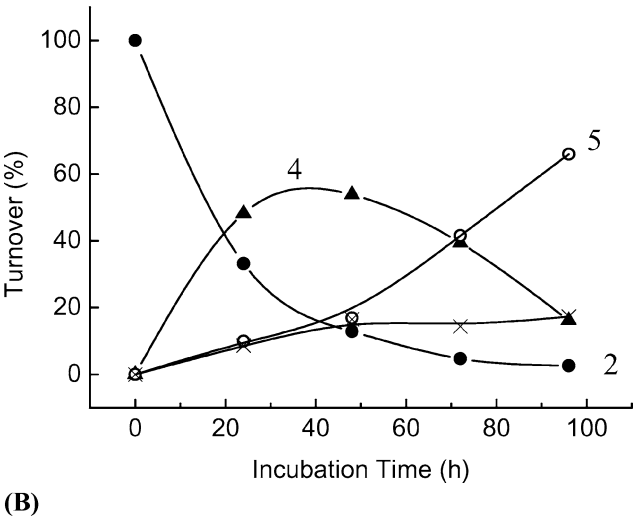


Fig. 3 GC spectra of metabolites from β-ionone biotransformation. The peaks were identified as: (**2**) β-ionone; (**4**) 4-hydroxy-β-ionone; (x) compounds extracted from reaction medium which are also present in the control experiments.

using the recombinant system and wild type cells is given in Fig. 4A and 4B, respectively. In the former biotransformation, the production of **4** slowly increased with time, along with a few other metabolites, while the amount of β-ionone **2** decreased during the incubation period. At the end of the 100 h biotransformation period, approximately 50% of **2** had been converted to **4**, along with 10% of other bioconversion products. In the latter biotransformation, β-ionone was introduced, after 2 d of *S. griseolus* growth into the growth medium, and the consumption of **2** and subsequent formation of products were monitored using GC-MS. After 48 h, the total consumption of β-ionone reached about 85%, by 96 h the bioconversion was almost complete. In addition to **4**, two other metabolites were observed, both of which possess the same molecular weight as that of **4** (208). Interestingly, the formation of **4** peaked (*ca.* 55%) after about 48 h of incubation and then gradually decreased,



(A)



(B)

Fig. 4 (A) Time-courses for the biotransformation of β-ionone by the recombinant P450 SU2 cells. (B) Time-courses for the biotransformation of β-ionone by *S. griseolus* cells.

while the amount of **5**, identified as 4-oxo-β-ionone, continually increased over the same period. The formation of **5** was not observed when **4** was incubated in absence of cells under the same conditions. The oxidation of **4** to form **5** was probably catalysed by dehydrogenase enzymes present in *S. griseolus*.

Enantiomeric excess of 4-hydroxy-β-ionone

Enantiomeric excess (ee)determination of 4-hydroxy-β-ionone **4**, isolated from the two biotransformation systems, was carried out by chiral HPLC using synthetically prepared racemic material as a standard. The absolute configuration of the enantiomers were assigned by a literature method (see Materials and Methods section).[21] The (*S*)- and (*R*)-enantiomers eluted at retention times of 16 and 18 min, respectively. The ee for **4** isolated from the *S. griseolus* biotransformation was calculated to be 3 to 8% (*R*). In contrast, the ee of **4** isolated from the recombinant system was 35% in favour of the (*S*)-enantiomer (Scheme 2).

Discussion

We have described a whole cell biocatalysis system to obtain 3-hydroxy-α-ionone and 4-hydroxy-β-ionone. Biotransformation of a racemic mixture of α-ionone with the *E. coli* recombinant system, which expressed P450 SU1, SU2 or SOY, yielded hydroxy-α-ionone with a high degree of regioselectivity, the major product being 3-hydroxy-α-ionone in all cases. Similarly, β-ionone biotransformation was catalysed by the recombinant system with almost complete regioselectivity, and significant enantioselectivity, to give 4-hydroxy-β-ionone. Wild-type *S. griseolus* was also shown to catalyse β-ionone biotransformation to yield the same product as a major metabolite. However, the product isolated from this biotransformation showed almost no enantioselectivity, as opposed to the recombinant system in which a significant degree of enantioselectivity was observed. This result could indicate the involvement of more that one P450 in the metabolism of β-ionone in *S. griseolus*, since *Streptomyces* are known to be a rich source of P450s. Sequence analysis has identified at least four P450 enzymes present in *S. griseolus*.[7] P450 enzymes that hydroxylate β-ionone to 4-hydroxy-β-ionone, but produce different enantiomers, could cancel out the individual enantioselectivity.

There is no apparent rate increase observed over wild type *S. griseolus* when P450 SU2 is overexpressed in the *E. coli* system. Indeed, when the comparison is based on total soluble protein content, *S. griseolus* is more efficient than the recombinant system. Poor coupling of the endogenous *E. coli* flavoproteins with the recombinant P450, despite the presence of recombinant ferredoxin, seems the most likely explanation for these findings. This kind of interaction was also previously observed for P450 SOY.[22] We therefore conclude that the ferrodoxin reductase activity was limiting in this system and that the endogenous redox proteins coupled poorly to the *S. griseolus* ferrodoxin, leading to insufficient electron transfer to the P450 enzyme.

In the context of applications to biocatalysis, P450 SU2 showed moderate enantioselectivity towards β-ionone. This enzyme therefore provides an ideal target for biocatalyst improvement, either by rational protein design or directed evolution.

Materials and methods

Materials

Substrates for the biotransformation reactions, α- and β-ionones, were purchased from Sigma. Yeast extract and tryptophan are purchased from Difco. Lipase from *Chromobacterium viscosum* was obtained from Fluka.

Bacterial strains and growth procedures

Wild-type *Streptomyces griseolus* was purchased from the American Type Culture Collection. For protein analysis, the following procedure was used to obtain a cell free extract. A starter culture (10 ml Luria-Bertani Medium (LB) in 100 ml flask), inoculated from slant agar stocks, was incubated for 2 d in an orbital shaker (shaking speed 250 rpm at 30 °C). Glucose medium (100 ml in a 500 ml baffled flask fitted with a stainless steel metal spring) was inoculated with 2 ml of starter culture, and incubated at 30 °C with shaking at 250 rpm for 2 d. Cells were harvested by centrifugation, then resuspended in 100 mM potassium phosphate buffer at pH 7.6 containing 2 mM ethylenediaminetetraacetic acid (EDTA), 20% (*v*/*v*) glycerol, 1.5 mM dithiothreitol (DTT) and 1 mM phenylmethylsulfonyl fluoride (PMSF). Cell breakage was achieved using a French Press, and the cell debris was removed by centrifugation at 25,000 rpm. The soluble fraction was analysed spectrophotometrically to determine the P450 content. In addition, the total protein content was determined by Bradford assay.[23]

Dr H. Hussain and Dr J. Ward (University College London) kindly provided the recombinant expression systems described in this paper. *E. coli* strains containing the expression plasmids were picked from a single colony and grown overnight at 37 °C. The overnight culture (1 ml) was used to inoculate 100 ml of LB medium. The culture was incubated at 37 °C until absorbance at 600 nm reached 0.6–0.8. α-Aminolevulinic acid (ALA) and $FeCl_3$ were added to a final concentration of 1 mM and 0.5 mM, respectively. Following further incubation (30 min at 37 °C), expression was induced by the addition of 1 mM of isopropyl-β-D-thiogalactopyranoside (IPTG). The cells were then incubated at 25 °C for a further 16 h in a shaking incubator at the speed of 200 rpm. Cells were harvested by centrifugation, then resuspended in 100 mM potassium phosphate buffer pH 7.6 containing 2 mM EDTA, 20% glycerol, 1.5 mM DTT and 0.5 mg ml^{-1} lysozyme. The suspension was incubated on ice at a 45° angle and shaken for 30 min. After addition of PMSF (1 mM), the mixture was sonicated. Cell debris was removed by centrifugation (14,000 rpm), and the soluble fraction was isolated by high-speed centrifugation at 25,000 rpm. The soluble fraction was then analysed for P450 and total protein.

Determination of cytochrome P450 content

Total P450 content was measured according to the protocol described by Omura and Sato.[20] The cell-free extract (0.5 ml) was added to 2.5 ml of 0.1 M potassium phosphate buffer, pH 7.6. A few crystals of sodium dithionite was added, and the mixture divided into two cuvettes. After auto zeroing, the baseline was run between 400 to 500 nm using a spectrophotometer (Shimadzu). The sample was saturated with about 30 to 40 bubbles of CO at a rate of about 1 bubble per second. The spectrum of the sample was recorded.

Determination of total protein content

Total protein content was determined, for both *S. griseolus* and *E. coli*, using the Bradford procedure.[23] Protein solutions (cell-free extracts from *S. griseolus* and *E. coli*), containing about 50 μg protein in 100 μl volume, were dispensed into test tubes, followed by 5 ml of protein reagent (0.01% Coomassie Brilliant Blue G-250, 4.7% (*w*/*v*) ethanol, and 8.5% *w*/*v* phosphoric acid), and the contents were mixed by brief vortexing. The absorbance at 595 nm was measured after 5 min in 3 ml cuvettes against a reagent blank prepared from appropriate buffer and protein reagent. Protein concentrations were calculated according to the standard curve produced using BSA standards in the same manner.

Bioconversion of α- and β-ionones by the recombinant system

Unless specified otherwise, the following procedure was used for the biotransformation. *E. coli* cells (BL21 DE3) containing the relevant plasmids were grown overnight in LB medium containing 100 μg ml^{-1} ampicillin and 34 μg ml^{-1} chloramphenicol. An aliquot (100 μl) of overnight culture was used to inoculate 20 ml of super broth medium containing the same concentration of antibiotics. When absorbance at 600 nm reached 0.6–0.8, δ-aminolevulinic acid and $FeCl_3$ were added to a final concentration of 1 mM and 0.5 mM, respectively. The cultures were incubated for a further 30 min at 37 °C, 250 rpm. IPTG (1 mM) was then added to induce expression, and the substrate (α- or β-ionone) (*ca.* 2 mM) was added to the incubation medium. The biotransformations were then incubated at 25 °C, 180 rpm and samples taken for analysis at various time points. A 2 ml aliquot was withdrawn every 2 d from the each flask, the cells were harvested by centrifugation (4000 rpm, 2 h) and the supernatants were extracted with diethyl ether (3 × 2 ml). The extract was washed with a saturated aqueous NaCl solution, dried with $MgSO_4$, and then evaporated down to the desired volume. The extracts were then subjected to chromatographic analyses. After 4 d of cultivation, the remaining cultures were centrifuged, and the supernatant was extracted accordingly with diethyl ether and then subjected to the final product analysis.

Bioconversion of β-ionone by wild-type *Streptomyces griseolus*

Starter cultures (10 ml LB medium in 100 ml flask) were inoculated from slant agar stocks, and incubated for 2 d in an orbital shaker (250 rpm) at 30 °C. A glucose medium (100 ml in 500 ml flask) was inoculated with 2 ml of starter culture, and then incubated at 30 °C with shaking (250 rpm). β-Ionone (0.070%) (v/v) was added to each of the cultures after 1d of cultivation, and the biotransformation was continued for a further 4 d. Samples were taken for analysis at various time points. Extractions and analysis of biotransformation products were carried out in the same manner as described above.

Synthesis of standards: 4-hydroxy β-ionone

Regioselective epoxidation of α-ionone, using *m*-chloroperbenzoic acid (MCPBA), gave the corresponding epoxide, which was isomerised to 4-hydroxy-β-ionone by treatment with potassium carbonate in hot methanol:[24] MCPBA (2.0 g, 11.6 mmol) and sodium bicarbonate (1.0 g) were placed in a flask containing dichloromethane (35 ml) cooled to 0 °C. The cold stirred suspension was treated dropwise with a solution of α-ionone (90% pure; 1.4 g; 6.6 mmol) in dichloromethane (3.5 ml). The reaction mixture was stirred for about 8 h with gradual warming to room temperature. The solid was filtered off, and the filtrate was successively washed with 10 ml of saturated sodium carbonate solution, sodium metabisulfite solution, and brine. The organic phase was dried over Na_2SO_4, and concentrated to give the crude epoxide as pale yellow oil (1.70 g; *ca.* 100%). The epoxide (1 g) was then dissolved in methanol (3 ml) and the solution was treated with anhydrous potassium carbonate (4-fold excess). The resulting orange mixture was refluxed for 4–5 h. Most of the methanol was then evaporated, and the residue was dissolved in ethyl acetate (15 ml). The organic phase was washed successively with water and brine, and then dried over Na_2SO_4. The solution was evaporated and the residue was purified by silica gel column chromatography (ethyl acetate–petroleum ether 3 : 1) to give the final compound as a pale yellow oil (650 mg).

Enantiomers of 4-hydroxy-β-ionone standard were obtained using a lipase-catalysed acylation based upon a published procedure.[21] To a stirred solution of racemic 4-hydroxy-β-ionone (200 mg) in vinyl acetate (2 ml) was added lipase from *C. viscosum* (25 mg). The mixture was heated at 40 °C for 2 d. After

cooling, the mixture was filtered, and the filtrate concentrated under vacuum. The residue was separated using reversed-phase HPLC (Phenomenex RP column; 4.6 mm × 250 mm) at 25 °C, using acetonitrile–water gradient at a flow rate of 1 ml min^{-1} with detection at 254 nm. The (*S*)-enantiomer enriched 4-hydroxy-β-ionone (30% *e.e.*) was then separated and used as a standard for further chiral HPLC analysis. Assignment of enantiomers of 4-hydroxy-β-ionone from cytochrome P450-catalysed reactions was made by direct comparison. Chiral HPLC analysis was performed using chiralcel OH–H column (Diacel), hexane–isopropanol (95 : 5), with flow rate of 0.5 ml min^{-1} at 5 °C; the two enantiomers of 4-hydroxy-β-ionone were baseline-separated, and the retention times were 17.4 min for (*S*)-4-hydroxy-β-ionone **4** and 19.4 min for (*R*)-4-hydroxy-β-ionone **4**.

Thin layer chromatography (TLC)

For immediate results, TLC analysis was utilised. Samples were spotted onto TLC plates (silica gel layer thickness, 0.2 mm; Silica Gel 60 F254; Merck), and the plates were developed with hexane–ethyl acetate (3 : 2). Product spots were visualized by spraying with a 2.5% (*w*/*v*) vanillin solution in 95% ethanol–H_2SO_4, and subsequent heating.

Gas chromatography-mass spectrometry (GC-MS)

GC-MS analysis was performed with a crosslinked 5% siloxane column (30 m × 0.25 mm, HP–5MS, Hewlett Packard), with a Hewlett Packard 6890 GC and 5973 mass spectrum analyser with electron impact (EI) ionisation. The temperature programming used was: 100 °C for 5 min, ramp at 50 °C min^{-1} to 200 °C, hold at 200 °C for 5 min, ramp at 75 °C min^{-1} to 300 °C, hold at 300 °C for 3 min. Samples were applied to the gas chromatograph in ethyl acetate solution.

3-Hydroxy-α-ionone 3.

GC-MS (EI). (t_R = 10.0 min.), (208, M^+, 5%), (175, 28%), (152, 24%), (147, 29%), (137, 29%), (131, 13%), (124, 54%), (109, 100%), (105, 22%), (92, 30%), (81, 22%), (43, 26%).

NMR. δ_H ($CDCl_3$; 200 MHz) 6.47 (1 H, dd, J = 15.7 and 10 Hz); 6.03 (1 H, d, J = 15.8 Hz); 5.56 (1 H, br); 4.20 (1 H, br); 2.43 (1 H, d, J = 10 Hz); 2.19 (3 H, s); 1.77 (1 H, dd, J = 13.6 and 6.0 Hz); 1.51 (3 H, s); 1.33 (1 H, dd, J = 13.6 and 6.6 Hz); 0.96 (3 H, s); 0.82 (3 H, s).

4-Hydroxy-β-ionone 4.

TLC. R_f = 0.15, ethyl acetate–hexane (1 : 4)

GC-MS. (t_R = 10.53 min), (208, M^+, 50%), (193, 16%), (175, 17%), (165, 31%), (151, 20%), (137, 36%), (123, 43%), (109, 100%), (91, 38%), (77, 25%), (65, 15%), (55, 17%), (43, 90%).

NMR. δ_H ($CDCl_3$; 200 MHz) 7.26 (1 H, d, J = 16.4 Hz); 6.18 (1 H, d, J = 16.4 Hz); 4.08 (1 H, bm); 2.38 (3 H, s), 2.00–1.20 (4 H, m); 1.94 (3 H, s), 1.21 (3H, s); 1.17 (3 H, s).

IR. ν_{max}/ cm^{-1} (FT) (thin film) 3449, 3055, 2967, 2935, 1664, 1266, 739.

4-Oxo-β-ionone 5.

TLC. R_f = 0.41, ethyl acetate–hexane (1 : 4)

GC-MS. (t_R = 10.48 min), (206, M^+, 59%), (191, 13%), (177, 5%), (163, 100%), (149, 26%), (135, 32%), (121, 50%), (105, 18%), (91, 29%), (77, 16%), (65, 12%), (55, 12%), (43, 42%).

NMR. δ_H ($CDCl_3$; 200 MHz) 7.29 (1 H, d, J = 16.4 Hz); 6.24 (1 H, d, J = 16.4 Hz); 2.60 (2 H, t, J = 7.2 Hz); 2.41 (3 H, s); 1.95 (2 H, t, J = 7.2 Hz); 1.86 (3 H, s); 1.25 (6 H, s).

IR. ν_{max}/ cm^{-1} (FT) (thin film) 3055, 2967, 2931, 1671, 1353, 1266, 738.

Acknowledgements

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