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Improving the monooxygenase activity and the regio- and stereoselectivity of terpenoid hydroxylation using ester directing groups

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Abstract

The monooxygenase enzyme CYP101B1, from *Novosphingobium aromaticivorans* DSM12444, binds norisoprenoids more tightly than monoterpenoids and oxidized these substrates with high regioselectivity. Ionols bound less tightly to CYP101B1 than ionones but the levels of product formation remained high and the selectivity of oxidation was similar to that observed for the parent norisoprenoid. The structurally related sesquiterpene lactone (+)-sclareolide, **9**, was stereoselectively hydroxylated by CYP101B1 to (*S*)-(+)-3-hydroxysclareolide, **9a**. The turnover of monoterpenoid derivatives showed low levels of product formation and selectivity despite promising binding data. CYP101B1 catalyzed the selective oxidation of (1*R*)-(−)-nopol, **14**, and *cis*-jasmone, **15**, generating >90% (1*R*)-(−)-5-hydroxynopol, **14a**, and 4-hydroxy-*cis*-jasmone, **15a**, respectively. To develop strategies for the efficient and selective oxidation of monoterpenoid based

substrates using CYP101B1 we investigated the binding and catalytic properties of terpenoid acetates. The ester functional group of these substrates mimicked the carbonyl moiety of norisoprenoids and anchored the monoterpenoid acetates in the active site of CYP101B1 with high affinity for the monoterpenoid acetates. The oxidation of these substrates by CYP101B1 occurred with product formation rates in excess of 1000 min^{-1} and total turnover numbers of greater than 5000 being observed in all but one instance. Critically, the oxidations were regioselective with several being stereoselective. (–)-Myrtenyl acetate, **20**, was oxidized regioselectively (> 95%) to yield *cis*-4-hydroxy-myrtenyl acetate, **20a**, which was further oxidized to 4-oxo-myrtenyl acetate, **20b**, using a whole-cell system providing a biocatalytic route to generate intermediates used in the production of cannabinoid derivatives. The ester carbonyl moiety could also be used as a directing group also to enhance the activity and control the selectivity of P450 catalyzed reactions; for example, the turnover of L-(–)-bornyl acetate, **18,** and isobornyl acetate, **19**, by CYP101B1 generated 9-hydroxybornyl acetate, **18a**, and 5-*exo*-hydroxyisobornyl acetate, **19a**, respectively, as the sole products.

Keywords; biocatalysis, norisoprenoids, terpenoids, esters, substrate engineering, directing groups, C–H bond oxidation.

Introduction

Cytochrome P450 (CYP) enzymes constitute a superfamily of heme-containing monooxygenases which have a myriad of physiological roles and have many potential applications in the catalytic synthesis of fine chemicals under mild conditions.[1-5] There is great interest in applying them as biocatalysts for the insertion of an oxygen atom from dioxygen into chemically inert carbon-hydrogen bonds with high regio- and stereoselectivity.[6, 7] Cytochrome P450 activity requires two electrons that are usually derived from NAD(P)H and these are delivered individually to the CYP enzymes by electron transfer proteins.[8] Electron transfer is often the rate determining step in cytochrome P450 catalysis and the identification of complete systems which can catalyze the selective oxidation of hydrocarbons and other organic molecules is a prominent field of research.[9-11]

Many bacterial CYP enzymes have been investigated as potential biocatalysts for the selective oxidation of C–H bonds. Self-sufficient enzymes in which the P450 is fused to the electron transfer partners have been studied.[7, 12] The CYP102 family is highly active for fatty acid substrates but modifications of the enzyme are required to selectively oxidize other desirable target substrates.[7, 10, 13-17] Class I CYP enzymes, whose electron transfer systems consist of a flavin-dependent ferredoxin reductase and a ferredoxin or flavodoxin, are also capable of high monooxygenase activities and selective hydroxylations.[18-22] Biocatalytic oxidations using these systems have been reported on a range of substrates using either the wild-type or mutant forms of the CYP enzyme.[1, 5, 9, 10] The activities of these class I systems or artificial fusion enzymes are often compromised when non-physiological electron transfer partners are used which can hamper the development of efficient biocatalytic procedures.[19, 23-26]

We have reported that the CYP101B1 enzyme from *Novosphingobium aromaticivorans* DSM12444 is a highly active catalyst for the oxidation of norisoprenoids and can oxidize other

structurally diverse classes of substrates.[27-29] A class I electron transfer system, consisting of a flavin-dependent ferredoxin reductase, ArR, and a [2Fe-2S] ferredoxin, Arx, has been identified which supports the high activity of CYP101B1.[19, 27, 28] To date CYP101B1 is one of only two members of this subfamily of P450 enzymes to be identified the other member being found in a different strain of *Novosphingobium*.[29] A whole-cell system capable of product formation on the gram-per-liter scale in shake flasks has also been constructed.[27] As a result, CYP101B1 is a promising monooxygenase system for biocatalytic applications involving C–H bond oxidation reactions.

CYP101B1 is related to CYP101A1 (P450cam), CYP101D1 and CYP101D2 all of which bind and oxidize the stereoselective oxidation of camphor (yielding ≥98% 5-*exo*-hydroxycamphor).[19, 27, 30, 31] It is also related to CYP101C1 which is able to bind and catalyze the hydroxylation of norisoprenoids.[32] CYP101B1 also catalyzes the oxidation of (1*R*)-(+)-camphor but the reaction is not selective, generating five products; 5-*exo*-hydroxycamphor, 5-*endo*-hydroxycamphor, 6-*endo*-hydroxycamphor, 9-hydroxycamphor and an isomer of 3-hydroxycamphor.[28, 33] The CYP101B1 catalyzed oxidation of the related terpenoid 1,8-cineole was more regioselective with >90% of the products arising from oxidation at C3.[33] The oxidation of α-ionone was regioselective, generating isomers of 3-hydroxy-α-ionone. The oxidation of β-ionone and β-damascone was selective for oxidation at C3 (90%).[29] Therefore CYP101B1 has the potential to catalyze routes to high value compounds without the requirement for extensive protein engineering. Here we report an investigation into the substrate range of CYP101B1 and show it is a capable of acting as a highly active and regioselective biocatalyst for the oxidation of norisoprenoids and structurally related terpenoids. We also demonstrate that monoterpenoid acetates can use the ester moiety as a directing group to facilitate and control

substrate binding leading to selective and rapid oxidation of unactivated C–H bonds in terpenoid frameworks. As such we show that the simple addition of an ester group could be used as chemical auxiliary or directing group in substrate engineering experiments, as described by Griengl and others, to improve the activity and selectivity of C–H bond oxidation reactions.[25, 34-36]

Experimental Section

General

General reagents and organics, including all the substrates, were from Sigma-Aldrich, TCI, Acros or VWR. Buffer components, NADH, and isopropyl-β-D-thiogalactopyranoside (IPTG) were from Anachem (Astral Scientific, Australia) or Biovectra, (Scimar, Australia). UV/Vis spectroscopy was performed on Varian Cary 5000 or Agilent Cary 60 spectrophotometers. Gas chromatography (GC) analyses were carried out on a Shimadzu GC-17A instrument coupled to a QP5050A MS detector using a DB-5 MS fused silica column (30 m x 0.25 mm, 0.25 μm) and helium as the carrier gas. Analytical liquid chromatography was performed using an Agilent 1260 Infinity pump equipped with an Agilent Eclipse Plus C18 column (250 mm x 4.6 mm, 5 μm), an autoinjector and UV detector. A gradient, 20 - 95%, of acetonitrile (with trifluoroacetic acid, 0.1%) in water (TFA, 0.1%) was used.

General DNA manipulations and microbiological experiments and the expression, purification and quantitation of CYP101B1 and the electron transfer proteins ArR and Arx from *N. aromaticivorans* were performed as described previously.[27, 28] The CYP101B1 protein concentration was calculated using $\varepsilon_{419} = 113\ mM^{-1}\ cm^{-1}$.[27, 28]

Substrate binding analysis

For substrate binding CYP101B1 was diluted between 1 and 6 μM using 50 mM Tris, pH 7.4. Substrate was added (in 200 μM) aliquots until no shift in the Soret band was observed. The high spin heme content was estimated, to approximately ±5%, by comparison with a set of spectra generated from the sum of the appropriate percentages of the spectra of the substrate-free form (>95% low spin, Soret maximum at 418 nm) and camphor-bound form (>95% high spin, Soret maximum at 392 nm) of wild-type CYP101A1.

To determine the dissociation constant the CYP101B1 enzyme was prepared as above to

between 0.5 and 4.5 μM in a total volume 2.5 mL and used to baseline the spectrophotometer. Aliquots of substrate (0.5–2 μL) were added using a Hamilton syringe from a 1, 10 or 100 mM stock solution in ethanol or DMSO. The solution was mixed and the peak-to-trough difference in absorbance of the Soret band was recorded between 700 nm and 250 nm. Further aliquots of substrate were added until the maximum peak-to-trough difference (A_{386} to A_{419}) did not shift further. The apparent dissociation constants, K_d, were obtained by fitting the peak-to-trough difference of the Soret band against substrate concentration to a hyperbolic function (Eqn. 1):

$$\Delta A = \frac{\Delta A_{max} \times [S]}{K_d + [S]} \qquad \text{Eqn. 1}$$

Where ΔA is the peak-to-trough absorbance difference, ΔA_{max} is the maximum absorbance difference and [S] is the substrate concentration.

Certain norisoprenoid and monoterpenoid acetate substrates exhibited tight binding, with $K_d < 5[E]$, and in these instances the data were fitted to the tight binding quadratic equation (Eqn. 2):[37]

$$\frac{\Delta A}{\Delta A_{max}} = \frac{([E]+[S]+K_d) - \sqrt{\{([E]+[S]+K_d)^2 - 4[E][S]\}}}{2[E]} \qquad \text{Eqn. 2}$$

where ΔA is the peak-to-trough absorbance difference, ΔA_{max} is the maximum absorbance difference, [S] is the substrate concentration and [E] is the enzyme concentration.

Enzyme turnover and product formation analysis

NADH turnover assays were performed with mixtures (1.2 mL) containing 50 mM Tris, pH 7.4, 0.5 μM CYP101B1, 5 μM Arx, 0.5 μM ArR and 100 μg mL^{-1} bovine liver catalase. The mixtures were oxygenated and then equilibrated at 30 °C for 2 min. Substrates were added from a 100 mM stock solutions in ethanol or DMSO to a final concentration of 0.5 - 1 mM. NADH was added to ~320 μM, final A_{340} ~ 2.00, and the absorbance at 340 nm was monitored. The rate of NADH

turnover was calculated using ε_{340} = 6.22 mM^{-1} cm^{-1}. The total turnover number was determined using assays set up like those above with the same ratio of the enzymes but with 0.1 μM CYP enzyme, 2 mM substrate and 4 mM NADH.

The turnovers assays were extracted and product analysis by GC was performed as described previously.[27] The product concentration in incubation mixtures was calculated by calibrating the total ion count response of the GC-MS detector to products when available (or the substrate if the product was not available). Where more than one product was produced the detector response was assumed to be equal, e.g. 4-hydroxy-β-ionol and 3-hydroxy-β-ionol were assumed to have equal detector responses. The coupling efficiency was the percentage of NADH consumed that led to product formation. The product formation rate (PFR) was calculated assuming the NADH was used to form product as the ratio determined by the coupling efficiency. The turnover number (TON) and total turnover numbers (TTN) which are the number of moles of product per mole of P450) were calculated by quantitating the product in the turnovers as above. The analysis methods and retention times are given in the supporting information.

Product isolation and characterization

To isolate and identify products where standards were not available a whole-cell oxidation system utilizing the plasmids pETDuetArx/ArR and pRSFDuetArx/CYP101B1 was used to oxidize substrates as described previously.[27] The plasmids were transformed into competent BL21(DE3) cells and grown on LB plates containing ampicillin, 100 μg mL^{-1}, and kanamycin, 30 μg mL^{-1} ($LB_{amp/kan}$). A single colony was inoculated into 500 mL broth ($2xYT_{amp/kan}$) and grown at 37 ºC overnight with shaking at 110 rpm. Protein expression was induced by the addition of 100 μM IPTG (from a 0.5 M stock in H_2O) and the temperature and the shaker speed were reduced to 20 ºC and 90 rpm. The growths were allowed to continue for another 24 hours before the cell pellet was harvested by centrifugation and washed in *E. coli* minimal media (EMM; K_2HPO_4 7 g,

KH_2PO_4 3 g, Na_3citrate 0.5 g, $(NH_4)_2SO_4$ 1 g, $MgSO_4$ 0.1 g, 20 % glucose (20 mL) and glycerol (1 % v/v) per litre).[38] The cell pellet was resuspended in an equal the volume EMM (~6 g cell wet weight.L^{-1}) and split into 200 mL aliquots in 2L flasks. The substrates were added in 2 and 1 mM aliquots up to 8 mM over 24 hours and the reactions were run for 30 hours. Samples (1mL) were taken for GC-MS analysis and were prepared as described above. To isolate product the supernatant (200 mL) was extracted in ethylacetate (3 x 100 mL), washed with brine (100 mL) and dried with magnesium sulfate and the organic extracts were pooled and the solvent was removed by vacuum distillation and then under a stream of nitrogen. The products were purified using silica gel chromatography using a hexane/ethyl acetate stepwise gradient ranging from 80:20 to 50:50 hexane to ethyl acetate using 2.5% increases every 100 mL. The composition of the fractions was assessed by TLC and GC-MS and those containing single products (≥95%) were combined for characterization. The solvent was removed under reduced pressure.

The purified products (ranging from 2 to 20 mg) after purification were dissolved in $CDCl_3$ or acetone-d^6 and the organics characterized by NMR spectroscopy and GC-MS. NMR spectra were acquired on a Varian Inova-600 spectrometer operating at 600 MHz for ^{1}H and 151 MHz for ^{13}C or an Agilent DD2 spectrometer operating at 500 MHz for 1H and 126 MHz for ^{13}C. A combination of ^{1}H, ^{13}C, COSY, HSQC and HMBC experiments was used to determine the structures of the products. NOESY was used to confirm the stereochemistry were required. Other assignments of minor products produced in low yields were made via co-elution experiments or comparison of MS spectra of standards published by others

Results

The oxidation of norisoprenoids and analogous substrates by CYP101B1

The norisoprenoids, α- and β-ionone, have been shown to induce a large type I spin state shift (≥ 95%) on binding to CYP101B1, the binding is tight and these substrates are efficiently hydroxylated by the enzyme solely or predominantly at C3.[19, 27, 28] The related norisoprenoid β-damascone, which switches the position of the carbonyl and alkene moieties on the butenone side chain, induced a lower spin state shift after addition to the enzyme. The binding was also weaker but the efficiency and regioselectivity of CYP101B1 catalyzed oxidation were maintained (Table 1).[29] In order to investigate the importance of different functional groups within the structural framework of norisoprenoids which result in effective binding and turnover efficiency by CYP101B1 we analyzed a series of substrates related to α- and β-ionone (**1** and **2**, Scheme 1). Substrate binding and enzyme turnover activity were measured using *in vitro* enzyme assays and oxidized metabolites were isolated using a whole-cell oxidation system which produced CYP101B1, Arx and ArR in *E. coli*.

The substrates α- and β-ionol (**3** and **4**) differ from the equivalent ionones by simply replacing the carbonyl with an alcohol functional group (Scheme 1). Both ionols induced a large type I spin state shift when added to CYP101B1 (70% and 90%, Fig. S1) but they bound thirty- to forty-fold less tightly than the ionone equivalents (Table 1, Fig. S2). The turnover and coupling efficiency of CYP101B1 with both were high (product formation rate (PFR); 670 to 764 $nmol.nmol\text{-}CYP)^{-1}.min^{-1}$; henceforth abbreviated to min^{-1}). This is approximately two-thirds that of β-ionone with that of α-ionol being greater than α-ionone (Table 1). α-Ionol oxidation by CYP101B1 generated a mixture of two products arising from monooxygenase activity (Fig. S3(a) and Fig. S4(a)). Using a whole-cell oxidation system generated the ketone, 3-oxo-α-ionol (**3a**), as

the major product which was isolated and identified by NMR (Fig. S3(a) and Fig. S5(a)). The two alcohols (**3b** and **3c**) were isolated as mixtures after silica chromatography but the MS and NMR analysis were consistent with oxidation at C3 and the data of D'Abrosca *et al* (Fig. S4(a)).[39] There was an approximate 2:1 excess of the *cis* isomer in the *in vitro* turnover (Scheme 1) which was consistent with that found for the parent ionone.[29] Two major products were obtained from the CYP101B1 catalyzed oxidation of β-ionol, **4**. Whole-cell oxidation generated three products, two of which were isolated and identified as 3-hydroxy-β-ionol (**4a**) and 4-oxo-β-ionol (**4b**) by matching the NMR spectra and MS data with those previously reported.[39] The other product was assigned as 4-hydroxy-β-ionol (**4c**) based on its NMR and MS data (Fig. S4(b) and S5(b)). The regioselectivity of oxidation of β-ionol is lower for the C3 position (60%) when compared with the oxidation of β-ionone, which was 90% selective for oxidation at the equivalent position (Scheme 1 and Fig. S3 (a)).[29]

Methylionone (≥85% α-isomer, **5**), which introduces an extra methyl group to the butenone side chain (Scheme 1), induced a slightly higher spin state shift in CYP101B1 compared to α-ionone, **1**, but bound three-fold less tightly (Table 1, Fig. S1 and Fig. S2). The rate of NADH oxidation was slower but comparable to that induced by α-ionone. However, both the coupling efficiency and the product formation rate were higher. Three products were identified, one of which was isolated in a pure form and characterized by NMR as 3-oxo-α-methylionone (**5a**, Scheme 1, Fig. S3 (a) and Fig S5 (c)). A second product was purified which had NMR and MS spectra consistent with an isomer of 3-hydroxy-α-methylionone (**5b**, Fig. S4(c) and supporting information).[40] The uncharacterized third product was not isolated in a pure form but the spectroscopic and MS data (Fig. S4 (c)) were consistent with those of another isomer of 3-hydroxy-α-methylionone (**5c**). The main product from the whole-cell oxidation was

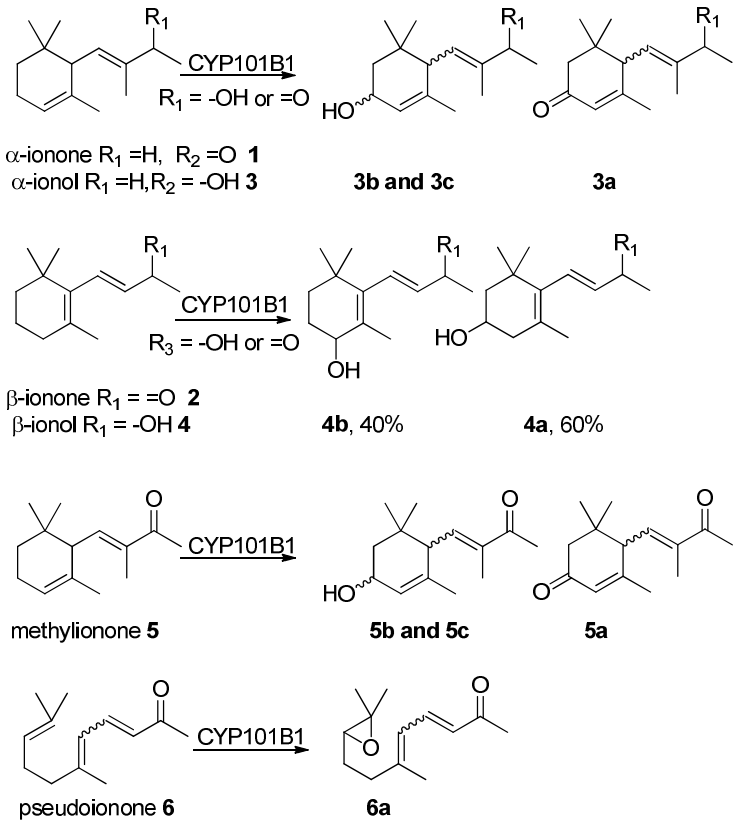
3-oxo-α-methylionone, **5a** while the *cis*- and *trans*-hydroxy products were formed in roughly equal quantities in the *in vitro* turnovers (Fig. S3 (a), Fig. S7).

Table 1 Substrate binding, kinetic and coupling efficiency data for CYP101B1 with norisoprenoids and related substrates.

CYP101B1/ substrate	%HS heme	K_d (μM)	NADH (min^{-1})	PFR (min^{-1})	C %	TON	TTN *vitro*	TTN *vivo*
β-ionone[29] **2**	≥95%	0.23 ± 0.1	1600 ± 100	1010 ± 60	63	418/ *682*	5240	6200[27]
α-ionone[29] **1**	≥95%	0.26 ± 0.04	1380 ± 140	660 ± 60	48	272/ *566*	8660	14750[a]
β-damascone[29]	80%	8.3 ± 0.9	930 ± 13	562 ± 12	60	424/ *700*	7700	6320
β-ionol **4**	90%	7.4 ± 0.2	1030 ± 55	670 ± 84	65	440/ *677*	-	5680
α-ionol **3**	70%	11.4 ± 0.5	1030 ± 50	764 ± 76	75	424/ *565*	-	-
α-methylionone **5**	90%	0.8 ± 0.01	1270 ± 10	980 ± 70	77	500/ *667*	6740	7790[a]
Pseudoionone **6**	90%	5.4 ± 0.1	180 ± 30	103 ± 9.0	58	-	1800	6050
Isophorone **8**	40%	n.d.	73 ± 5	n.d.	n.d.	-	-	-
trans-4-phenyl-3-buten-2-one **7**	55%	n.d.	71 ± 28	n.p.	n.p.	-	-	-

Steady state turnover activities were measured using a ArR:Arx:CYP101B1 concentration ratio of 1:10:1 (0.5 μM CYP enzyme, 50 mM Tris, pH 7.4). Coupling is the percentage efficiency of NADH utilization for the formation of products, NADH is the NADH oxidation rate and PFR the product formation rate. C is the coupling efficiency, TON the average turnover number for the NADH consumption assay (with the maximum TON possible given in italics). TTN *vitro* is the total turnover number observed using *in vitro* assays set up like those above with the same ratio of

enzymes but with 0.1 μM CYP enzyme, 2 mM substrate and 4 mM NADH. TTN *vivo* is the total turnover number obtained from whole-cell turnover assays (concentration of the P450 enzyme in this system was 0.65 μM).[27] The Rates are reported as mean ± S.D. ($n \geq 3$) and given in nmol.(nmol-CYP)$^{-1}$.min^{-1}. n.p no observable product, n.d. not determined. [a] The *in vivo* TTN includes further oxidation to the ketone. Some of the data for the norisoprenoid substrates was reported previously and are included for comparison.



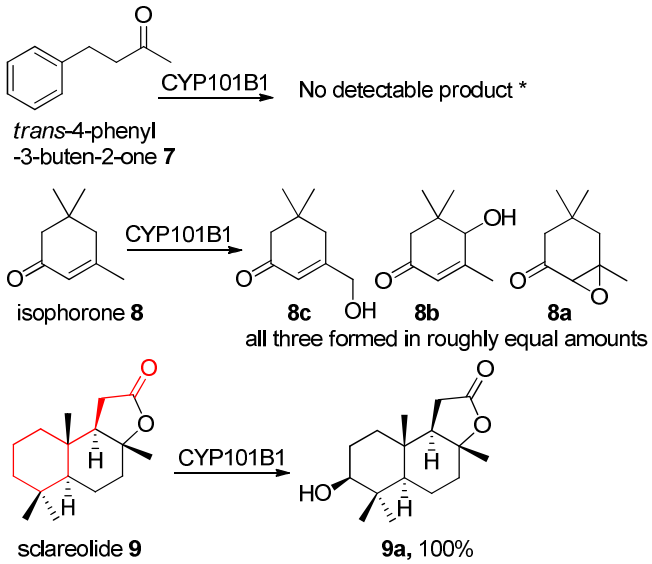
Scheme 1 The products formed from CYP101B1 turnovers with norisoprenoid analogues. The turnovers of α- and β-ionone were as reported previously.[29] Unless otherwise stated the regioselectivities of the turnovers conducted in this manuscript were 100%.

Pseudoionone (**6**), which is a linear analogue of the norisoprenoids, also induced a high-spin state shift when added to CYP101B1 (Scheme 1). The binding was weaker than those of the cyclic ionones but stronger than ionols and β-damascone (Table 1 and Fig. S2). The activity of CYP101B1 with pseudoionone, as measured by the NADH oxidation and product formation rates, was significantly lower when compared to all the cyclic norisoprenoids and ionols though the coupling efficiency was comparable (Table 1). Two monooxygenase products were generated from the *in vitro* turnovers in roughly equal amounts (Fig. S3(a)). These products were generated using the CYP101B1 whole-cell system but were not separated by silica gel chromatography preventing the detailed spectroscopic characterization of the monooxygenase products. The pseudoionone substrate is a mixture of two isomers (*cis* and *trans*) and the MS fragmentation patterns of the two metabolites were similar (Fig. S4 (d)). Both *cis*- and *trans*-pseudoionone are selectively oxidized by perbenzoic acids (e.g. *m*-CPBA) at the alkene furthest from the carbonyl group, to an epoxide (**6a**, 3,5-octadien-2-one, 8-(3,3-dimethyloxiranyl)-6-methyl, (Z,E) and (E,E)).[41-43] The epoxidation of pseudoionone using this method generated two products with identical GC retention times and matching MS spectra and fragmentation patterns to those formed by the CYP101B1 catalyzed oxidation of the same substrate allowing characterization (Scheme 1).

We also tested *trans*-4-phenyl-3-buten-2-one (**7**) with CYP101B1. This substrate has the same butenone side chain as α- and β-ionone but replaces the trimethylcyclohexene ring with an aromatic one (Scheme 2). The proportion of high-spin heme induced by substrate binding to CYP101B1 was lower (55%) than the ionone and ionol substrates (Table 1 and Fig. S1). The lower solubility and weaker binding of this substrate prevented accurate measurement of the dissociation constant but it has a lower affinity than any of the norisoprenoid substrates. The activity of CYP101B1 with this substrate was slower than even pseudoionone, **6** (Table 1) and no product arising from monooxygenase activity could be identified by GC-MS or HPLC analysis of the *in*

vitro turnovers. Whole-cell oxidation turnovers resulted in trace amounts of three potential products (all m/z = 164.1 AMU, data not shown) but their identity could not be determined.

Given the high affinity of CYP101B1 for molecules with the ionone framework we decided to assess how active it would be for larger and smaller substrates with similar structures. Isophorone (**8**, 3,5,5-Trimethyl-2-cyclohexene-1-one) has a similar ring system to α-ionone, **1** ((3*E*)-4-(2,6,6-Trimethylcyclohex-2-en-1-yl)but-3-en-2-one, Scheme 2) but is missing the butenone side chain of the isoprenoids. Isophorone binding to CYP101B1 only induced a 40% shift to the high-spin form and the activity as measured by NADH oxidation was low (Table 1). The level of product formation was meagre and the oxidation was unselective generating three products in similar yields (Fig. S4(e)). Analysis of the MS fragmentation patterns of the three products suggested they were isophorone oxide (**8a**), 4-hydroxy-3,5,5-trimethyl-2-cyclohexen-1-one (**8b**, 4-hydroxyisophorone) and 3-hydroxymethyl-5,5-dimethyl-2-cyclohexen-1-one (**8c,** 7-hydroxyisophorone, Fig. S4(e), Scheme 2).[44, 45] The results from a selection of other smaller monoterpenoid organic molecules are discussed in the next section.



Scheme 2 The products formed from CYP101B1 turnovers with *trans*-4-phenyl-3-buten-2-one, **7**, isophorone, **8**, and (+)-sclareolide, **9**. The portion of the (+)-sclareolide structure which is related to norisoprenoid framework is highlighted in red. * 3 products in very low yield could be detected after whole-cell oxidation.

(+)-Sclareolide (**9**) is a sesquiterpene lactone with structural similarities to the norisoprenoids but is significantly larger (Scheme 2). (+)-Sclareolide, **9**, bound to CYP101B1 with high affinity (K_d, 20 ± 4 μM) and induced a reasonable shift to the high-spin state (50%). Therefore the active site of CYP101B1 can accommodate substrates larger than norisoprenoids (Table 1, Fig. S1 and S2). The oxidation activity was slow but a single product was isolated using the whole-cell oxidation system (Scheme 2 and Fig. S4(f)). The product was identified as (*S*)-(+)-3-hydroxysclareolide (**9a**) by matching the NMR signals with those reported in the literature (Fig. S5d).[46, 47]

The oxidation of terpenes and related substrates by CYP101B1

We have previously shown that both enantiomers of camphor (**10**) are hydroxylated by CYP101B1 but the reactions are unselective generating five products (**10a-e**). 1,8-Cineole (**11**) induced a greater shift to the high-spin form on binding to CYP101B1 when compared to the enantiomers of camphor and the selectivity of oxidation is more favorable with >90% selectivity for the C3 position (**11a-c**). However the activity of product formation for all of these was lower than the norisoprenoid substrates as was the regioselectivity.[28, 29, 33] To further investigate which monoterpenoid frameworks can be regioselectively oxidized by CYP101B1 we measured the substrate binding and turnover activity data of a series of monoterpenes and related substrates.

(+)-Fenchone (**12**), which is an analogue of camphor (1,3,3-Trimethylbicyclo[2.2.1]heptan-2-one *vs*. 1,7,7-Trimethylbicyclo[2.2.1]heptan-2-one,

Scheme 3), bound less tightly to CYP101B1 but had a similar product formation rate and coupling efficiency (Table 2, Fig. S1 and S2). The *in vitro* CYP101B1 turnover of (+)-fenchone, **12**, was more selective than those of camphor with 75% of one major product being formed with two minor products making up the remaining 25% (Scheme 3, Fig. 1(a), Fig. S3(b) and Fig. S4(g)). The major product was isolated after a whole-cell oxidation turnover and silica gel chromatography and identified as 5-*exo*-hydroxyfenchone (**12a**, Fig. S5(e)).[48, 49] The two minor products had MS fragmentation patterns which enabled tentative assignments of 6-*endo*- and 6-*exo*-hydroxyfenchone (**12b** and **12c**, Fig. S4(g)).[50]

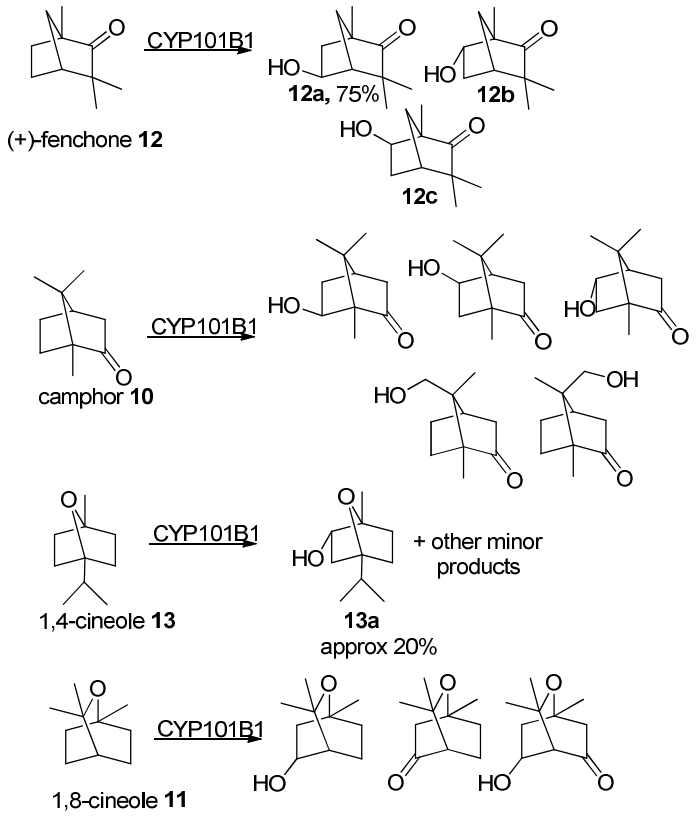
Table 2 Substrate binding, kinetic and coupling efficiency data for CYP101B1 with monoterpenoids and related substrates.

CYP101B1/ substrate	%HS heme	K_d (μM)	NADH	PFR	C %	TON	TTN *vitro*	TTN *vitro*
(1*R*)-(−)-camphor[33] **10r**	55%	334 ± 8	1040 ± 8	150 ± 20	15%	110/ *730*	-	4470
(1*S*)-(−)-camphor[33] **10s**	55%	262 ± 8	1260 ± 90	160 ± 40	13%	100/ *770*	-	-
(+)-fenchone **12**	50%	544 ± 23	1110 ± 80	200 ± 20	18%	122/ *678*	920	1450
1,8-cineole[33] **11**	75%	576 ± 15	940 ± 2	355 ± 12	38%	260/ *684*	420	7490[a]
1,4-cineole **13**	50%	609 ± 25	419 ± 35	42 ± 12	10%	50/ *500*	-	-
(1*R*)-(−)-nopol **14**	45%	64 ± 2	553 ± 87	165 ± 32	30%	206/ *687*	620	2820
cis-jasmone **15**	35%	473 ± 17	133 ± 2	28 ± 11	21%	118/ *562*	-	3390
5-norbornen-2-yl acetate **16**	65%	506 ± 15	140 ± 5	75 ± 18	54%	362/ *670*	-	-

See Table 1 for experimental details. The data for the camphor and 1,8-cineole substrates was reported previously and are included for comparison. [a] The *in vivo* TTN includes further

oxidation to the ketone and hydroxyketone products.

1,4-Cineole (**13**, 1-isopropyl-4-methyl-7-oxabicyclo[2.2.1]heptane) has structural similarities to 1,8-cineole, **11** (1,3,3-trimethyl-2-oxabicyclo[2.2.2]octane) and *p*-cymene (1-isopropyl-4-methylbenzene, Scheme 3). It induced a lower spin state shift upon binding to CYP101B1 than the 1,8-cineole, **11**, but greater than the non-oxygen containing substrate *p*-cymene (Fig. S1).[29, 33] The product formation rate of 1,4-cineole, **13**, was similar to that of *p*-cymene but ten-fold lower than 1,8-cineole, **11** (Table 2). At least seven monooxygenase products were generated from the oxidation of 1,4-cineole with low selectivity (Fig. S3(c)). All seven had masses indicating they were monooxygenase products with one further product which has a mass consistent with that of a ketone further oxidation product (Fig. S4(h)). The low levels of each of the products generated prevented detailed characterization though the major product was isolated and identified as 6-*endo*-hydroxy-1,4-cineole (**13a**, or its enantiomer, 2-*endo*-hydroxy-1,4-cineole, or a mixture of both, Scheme 3, Fig. S5(f)).[51] The MS fragmentation pattern was also in agreement with that reported in the literature (Fig. S4(h)).[51]



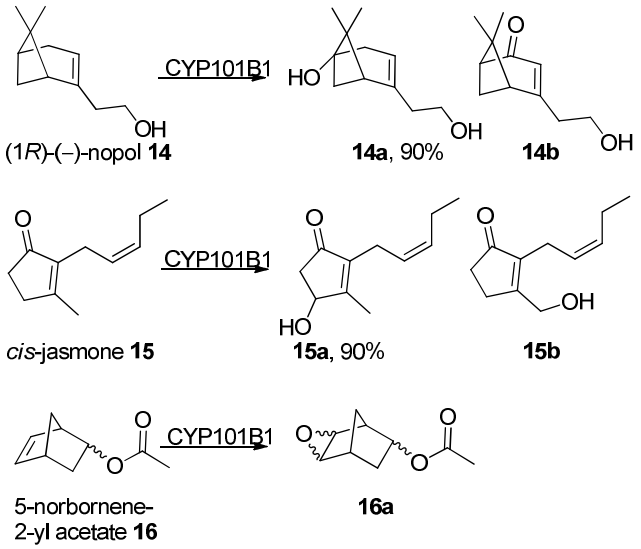
Scheme 3 The products formed from CYP101B1 catalyzed oxidation of (+)-fenchone, 1,4-cineole. The products arising from camphor (R and S enantiomers, **10r** and **10s**) and 1,8-cineole, **11**, are shown for comparison and their selectivity of oxidation were as reported previously.[33]

We tested a range of other monoterpenoid molecules and these resulted in minimal spin state shifts (≤20%) on addition to CYP101B1. These included; (–)-α-pinene (20%), (+)-α-pinene (5%), β-pinene (5%), carvone (20%), α-terpineol (10%), carvacrol (<5%) and thymol (10%). The enzyme catalyzed turnovers with these substrates were very unselective generating multiple products in very low yield or no detectable product. As a result these substrates were not investigated further (data not shown).

We also chose to study other molecules which share structural features with norisoprenoids and terpenes. (1*R*)-(–)-Nopol (**14**) and *cis*-jasmone (**15**) are chemicals used in the flavor and fragrance industry and both contain an alkene containing aliphatic ring system and an alkyl side

chain (Scheme 4). (1*R*)-(−)-Nopol, **14**, is related to α-pinene and the terpenoid myrtenol but has a primary alcohol functionality at the end of an ethyl side-chain. *cis*-Jasmone, **15**, has a pentenyl side chain and contains a carbonyl group on the ring system (Scheme 4). Both induced a type I spin state change upon binding to CYP101B1, with that of (1*R*)-(−)-nopol, **14**, 45%, being greater than that of *cis*-jasmone, **15**, 35% (Fig. S1). (1*R*)-(−)-Nopol, **14**, bound to CYP101B1 with a dissociation constant almost an order of magnitude lower, 64 μM, than any of the monoterpene substrates tested thus far (Table 2, Fig. S1 and Fig. S2). The activity and coupling efficiency of the enzyme turnovers with both substrates was moderate generating observable levels of a single major product *in vitro* (Fig. S3(d) and (e)). The product formation rate of *cis*-jasmone, **15**, 28 min^{-1}, was lower than that of (1*R*)-(−)-nopol, **14** (Table 2). With both substrates additional minor products were formed in the whole-cell oxidation reactions (Fig. S3(d) and (e)).

After purification of the whole-cell oxidation turnover of (1*R*)-(−)-nopol, **14** two out of three products were identified as (1*R*)-(−)-5-hydroxynopol (**14a**, major product) and (1*R*)-(−)-4-oxonopol (**14b**, Fig. S4(i)). (1*R*)-(−)-4-Oxonopol, **14b** was only formed in the whole-cell turnovers and this suggested that (1*R*)-(−)-4-hydroxynopol, (**14c**) must be the other product observed (Scheme 2, GC RT 11.0 min, Fig. S3(d)). The major product (1*R*)-(−)-5-Hydroxynopol, **14a** and (1*R*)-(−)-4-Oxonopol, **14b** were identified by NMR (Fig. S5(g) and (h)) with the data for both closely matching that reported previously.[52] For *cis*-jasmone, **15**, the major product of the enzyme turnover was characterized via NMR as 4-hydroxy-*cis*-jasmone (**15a**) with 11-hydroxy-*cis*-jasmone (**15b**) being the minor product formed *in vivo* (Fig. S4(j), Fig. S5(i) and (j)).[53]

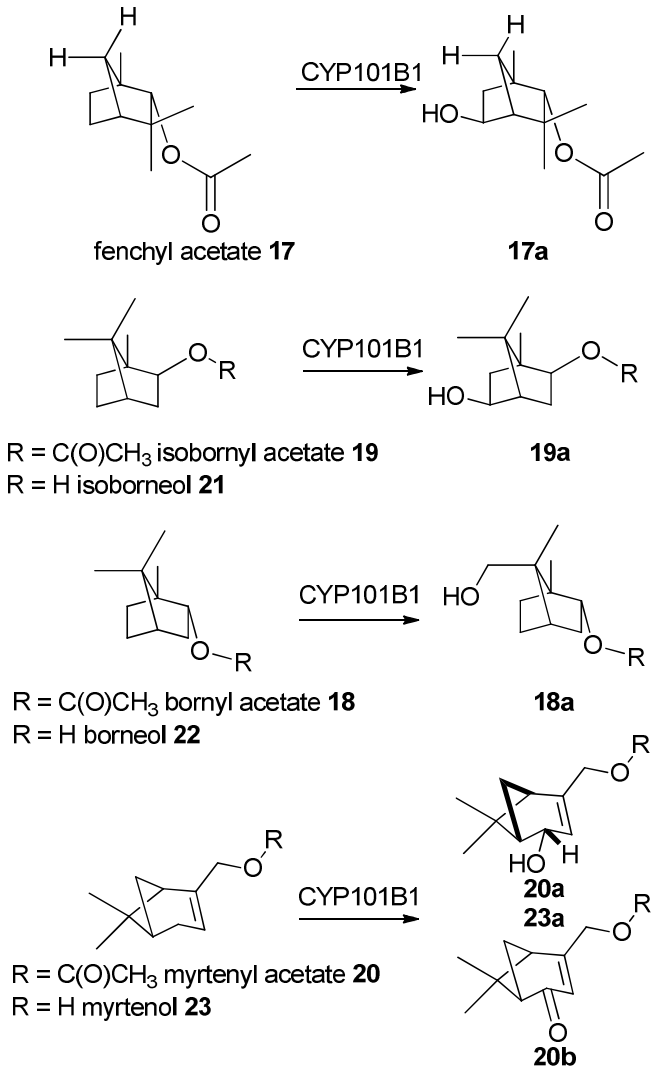


Scheme 4 The products formed from CYP101B1 catalyzed oxidation of (1*R*)-(−)-nopol (**14**), *cis*-jasmone (**15**) and 5-norbornene-2-yl acetate (**16**).

5-Norbornene-2-yl acetate (**16**) was also investigated as it has an acetate side chain attached to a bicyclic ring structure (Scheme 4). The acetate side chain could, in principle, mimic the butenone side chains of norisoprenoids and result in improved binding and turnover by CYP101B1. The spin state shift on binding to CYP101B1 was high (65%, as a comparison that of norcamphor was 30%, Fig. S1). The dissociation constant of CYP101B1 for 5-norbornene-2yl acetate, **16**, was comparable to that of (+)-fenchone, **12**, but the activity as measured by NADH oxidation was low (Table 2 and Fig. S2). However, the coupling of the reducing equivalents to product formation in the enzyme turnovers was 54%. This is greater than any of the monoterpenoid like substrates and was comparable to those of the norisoprenoid substrates (Tables 1 and 2). CYP101B1 catalyzed oxidation of this substrate, which is a mixture of the *endo*- and *exo*-isomers, generated two products (Fig. S3(f)). These were not separated after silica chromatography but the major product could be assigned as an isomer of 2,3-epoxy-5-norbornanyl acetate by MS and NMR (**16a**, Fig. S4(k) and S5(k)).

The oxidation of monoterpene acetates by CYP101B1

Monoterpenoid oxidation with CYP101B1 resulted in lower activity and levels of product formation compared with norisoprenoids. Given the tight binding of (1*R*)-(−)-nopol, **14**, and the increased levels of coupling with 5-norbornene-2-yl acetate, **16**, we decided to investigate if monoterpenoid acetates, which contain the carbonyl containing acetate group, could interact with the amino acid residues of the active site of CYP101B1 in a similar fashion to the butenone side chain of the norisoprenoids. The oxygen atom of the carbonyl group on the acetate ester would therefore act as an anchor and hold the substrate more tightly in the active site of CYP101B1 allowing only specific C–H bonds to be abstracted. If this is case, monoterpenoid acetates would show improved substrate binding to CYP101B1 over the parent terpenes. The acetate ester could function as a directing group and result in enhanced biocatalyst characteristics including turnover activity and product selectivity. Fenchyl acetate (**17**), L-(−)-bornyl acetate (**18**), isobornyl acetate (**19**) and (−)-myrtenyl acetate (**20**, Scheme 5) were chosen and all induced shifts to the high spin state when added to CYP101B1 (80% to ≥ 95%). These are higher than those obtained with the related monoterpenoids (fenchone, **12**, camphor, **10**, isoborneol, **21**, borneol, **22**, (−)-myrtenyl, **23** and (1*R*)-(−)-nopol, **14**; Tables 2 and 3, Fig. S1). The binding affinities of fenchyl, bornyl, isobornyl and myrtenyl acetates (**17-20**) for CYP101B1 were also significantly tighter than those of the corresponding monoterpenoids (Table 3). The tightest binding monoterpenoid acetate to CYP101B1 was L-(−)-bornyl acetate, **18** (K_d = 0.71 μM) which binds more strongly than all of the norisoprenoids bar α- and β-ionone, **1** and **2** (Tables 1 and 3, Fig. S2).



Scheme 5 The products formed from CYP101B1 catalyzed oxidation of monoterpenoid acetates.

Isoborneol, **21**, and borneol, **22**, oxidation yielded at least three products in low yield (Fig. S3(h)).

The activity of the CYP101B1 catalyzed oxidation of the monoterpenoid acetates was also considerably higher than the parent monoterpenoids. (Table 2 and Table 3) The product formation rate of CYP101B1 with L-(−)-bornyl acetate, **18**, was the lowest of the esters at 600 min^{-1} which significantly higher than those of camphor, **10**, borneol, **22**, and isoborneol, **21**. The other three acetates (**17**, **19** and **20**) induced product formation rates greater than 1000 min^{-1}, exceeding those of the norisoprenoids (Table 2). The highest product formation activity was observed for

(−)-myrtenyl acetate, **20**; 1520 min^{-1} which was significantly greater than those of (−)-myrtenol, **23**, and (−)-nopol, **14** (Table 2 and Table 3).

Table 3 Substrate binding, kinetic and coupling efficiency data for CYP101B1 with monoterpenoid acetates. The turnovers were regioselective with the exception of myrtenol acetate which yielded 5% of the epoxide.

CYP101B1/ substrate	%HS heme	K_d (μM)	NADH	PFR	C %	TON	TTN *vitro*	TTN *vivo*
fenchyl acetate **17**	80%	7.3 ± 0.8	1220 ± 5	1110 ± 30	90%	566/ *628*	1790	10810
isobornyl acetate **19**	≥95%	14 ± 0.2	2000 ± 70	1250 ± 80	61%	360/ *590*	6360	5200
L-(−)-bornyl acetate **18**	≥95%	0.71 ± 0.04	3080 ± 70	600 ± 50	19%	130/ *684*	1870	2720
(−)-myrtenyl acetate **20**	≥95%	5.1 ± 0.8	1700 ± 14	1520 ± 30	90%	660/ *733*	3220	8970[a]
borneol **22**	50%	140 ± 24	410 ± 2	11 ± 3	3%	28/ *646*	115	<250
isoborneol **21**	55%	140 ± 8	337 ± 15	32 ± 1	9%	59/ *620*	220	<250
(−)-myrtenol **23**	30%	87 ± 4	336 ± 2	44 ± 2	13%	88/ *638*	75	2020

See Table 1 for experimental details. [a] The *in vivo* TTN includes further oxidation to the ketone.

The coupling efficiency of product formation to the NADH reducing equivalents in the CYP101B1 turnovers was also considerably greater (50-100%) than the monoterpenoids with the exception of L-(−)-bornyl acetate, **18**, which had lower coupling efficiency (19%). GC-MS analysis of the *in vitro* and whole-cell turnovers showed that CYP101B1 oxidized all of the monoterpenoid acetate substrates regioselectively in contrast to the turnovers of camphor, **10** and (+)-fenchone, **12**, which generated multiple products (Fig. 1 and Fig. S3(g)). The turnovers of

(−)-myrtenol, **23**, was selective for a single product while those of borneol, **22** and isoborneol, **21**, generated very little product and were not selective (Table 3 and Fig. S3(h)).

CYP101B1 turnovers with (−)-myrtenyl acetate, **20**, generated one major product (~95%, Fig. S3(g)). Whole-cell oxidation generated two major products which were isolated and identified as, 4-*cis*-hydroxymyrtenyl acetate (**20a**) and the further oxidation product 4-oxomyrtenyl acetate (**20b**, Fig. S4(l), S5(l) and S5(m)). Small levels of a third product (<5%) which has a mass spectrum indicating it arose from monooxygenase activity were also observed (Fig. S4(l)). This metabolite co-eluted (RT 7.5 min, Fig. S3(g)) with the epoxide product obtained from the reaction of (−)-myrtenyl acetate and *m*-CPBA (**20c**). A single major product was obtained in low yield from the turnovers of (−)-myrtenol, **23**, which was characterized as 4-*cis*-hydroxymyrtenol, **23a** (Fig. S4(m) and S5(n)).

Fenchyl acetate, **17**, and isobornyl acetate, **19**, were oxidized stereoselectively by CYP101B1 (Scheme 3, Fig. 1(b), Fig. 1(c) and Fig. S3(f)) and the products were identified using NMR as 5-*exo*-hydroxyfenchyl acetate (**17a**) and 5-*exo*-isobornyl acetate (**19a**), respectively (Scheme 3, Fig. S4(l), Fig. S5(o) and S5(p)). Enzyme catalyzed turnover of L-(−)-bornyl acetate, **18**, also generated a single product but in significantly lower yield (Fig. 1(d)). The product was isolated and identified by NMR as 9-hydroxybornyl acetate (**18a**, Fig. S4(l) and Fig. S5(q)) suggesting that the orientation of the acetate group controls which C–H bond is held closest to the heme iron.

(a) (b)

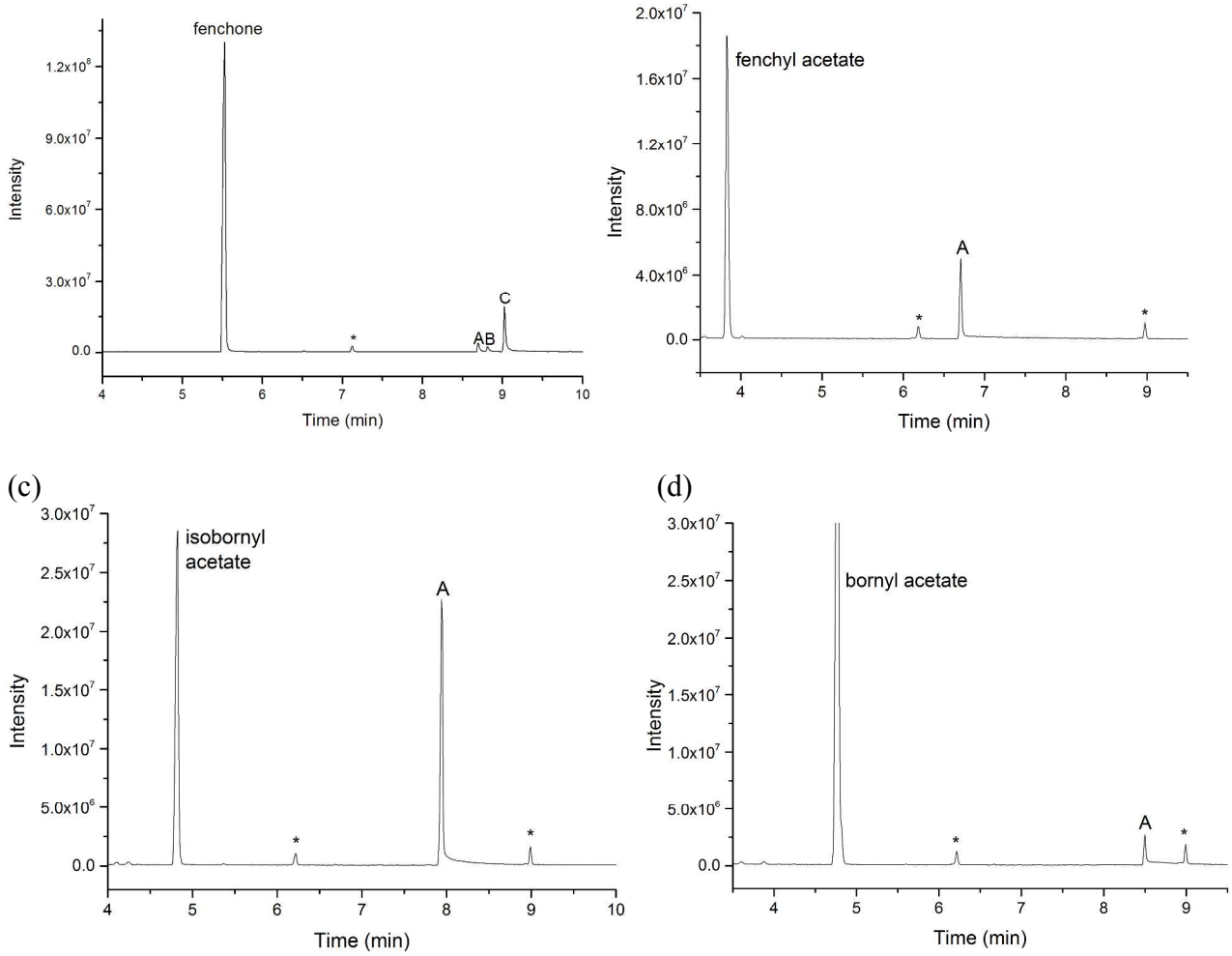


Figure 1 GC-MS analysis of the CYP101B1 turnovers of **(a)** whole-cell oxidation of (+)-fenchone with product A, 6-*endo*-hydroxyfenchone and B 6-*exo*-hydroxyfenchone and C 5-*exo*-hydroxyfenchone. In all traces impurities are labelled (*) **(b)** *In vitro* turnovers of fenchyl acetate with the product marked A, 5-*exo*-hydroxyfenchyl acetate, **(c)** *In vitro* turnovers of isobornyl acetate with the product marked A, 5-*exo*-hydroxyisobornyl acetate and **(d)** *In vitro* turnovers of L-(−)-bornyl acetate with the product marked A, 9-hydroxybornyl acetate.

The turnover data (PFR and coupling efficiency) and the linear NADH oxidation time courses indicate that CYP101B1 is a good biocatalyst for use synthetic applications (Table 1-3 and Fig. S6). The *in vitro* turnover assays have turnover numbers limited by the amount of NADH

added (TON up to a maximum of ~730, Table 1-3). In order to measure the efficiency of the enzyme over a longer period of time we measured the total turnover number (TTN) of the enzyme with selected substrates. *In vitro* the TTN followed a similar trend to the PFR and the TON with the cyclic norisprenoids and terpenoid acetates having high values, 1790 to 8660 compared to the monoterpenoids < 920 (Tables 1-3). The TTN of isobornyl acetate, **19**, 6360, approached than of the best norisoprenoids (5240-8660), and was greater than that of L-(–)-bornyl acetate, **18**, as expected from the PFR (Tables 1 and 3). The TTN of (–)-myrtenyl acetate, **20**, was lower due to competing ester hydrolysis over the longer duration of these *in vitro* turnovers while that of fenchyl acetate was lower than expected and requires further optimization (Table 3).

The products generated using the shake flask whole-cell oxidations were the same as those from the *in vitro* turnovers, though more ketone and further oxidation products were obtained (Tables 1-3, Fig. S7 and Fig. S8). The product yields and total turnovers from these oxidation were also high. In contrast to the *in vitro* turnovers over 7 mM product (~ 87% conversion in a 200 ml reaction) was generated during the oxidation fenchyl acetate, **17** (TTN 10810, ~1.4 g L^{-1}). The TTNs of the other monoterpenoid acetates were also high (>5000), though that of L-(–)-bornyl acetate, **18**, was lower presumably due to its lower PFR (Table 3). These were comparable to those obtained for the norisoprenoids (5680 – 14750, Table 1). The conversions were also high ranging from 50-87% of the 8 mM product added to the 200 mL reaction during the turnover (Fig. S8). The TTN and conversions of isoborneol, **21**, and borneol, **22**, were low but those of (–)-myrtenol, **22**, and (1*R*)-(–)-nopol, **14**, were higher though still significantly lower than that of (–)-myrtenyl acetate (Table 2 and 3). The TTN of (1*R*)-(–)-camphor, **10r**, and 1,8-cineole, **11**, were 4470 and 7790, in line with what was reported previously, though the selectivity was lower than the monoterpenoid acetates.[33] The TTN and conversions of the other ketone compounds, (+)-fenchone,

12, and *cis*-jasmone, **15**, were significantly lower than the norisoprenoids (Table 2).

Overall the data suggests that the monoterpenoid acetates are a good fit for the active site of CYP101B1 and as a consequence are efficiently and regioselectively, and in some instances, stereoselectively hydroxylated. The altered regioselectivity of isobornyl and bornyl acetates, **18** and **19**, shows that the carbonyl group of the acetate plays an important role in anchoring the substrate in the active site and can control the selectivity of oxidation. (–)-Myrtenyl acetate, **20**, turnover resulted in the oxidation of a C–H bond in a different position relative to the other bicyclic terpenoid acetates. This could be due the different location of the acetate group, the presence of a double bond or the modified bicyclic ring structure. The interaction of the carbonyl group with the enzyme appears to regulate the orientation of the substrate in the active site. As a result modification of the location of the carbonyl group in a molecule would allow chemists to govern which C–H bonds are held closest to the heme iron-oxo intermediate and are therefore abstracted.

Discussion

CYP101B1 is able to oxidize a broad range of substrates though optimal catalytic efficiency is achieved for norisoprenoids and monoterpenoid acetates both of which have a carbonyl group on a side chain attached to a substituted cyclohexyl or bicyclic ring systems. These substrates are hydroxylated with high regioselectivity and in certain instances stereoselectivity. Terpene oxidation by natural and modified P450 enzymes is a highly active area of research due to their use as flavor and fragrance compounds and their biological activity.[9, 47, 54] Microbial oxidative biotransformations of norisoprenoids and related substrates have been reported using different bacteria including *Streptomyces*[55] and the fungus *Aspergillus niger*[40] and P450 enzymes from different strains of *Streptomyces*, *Bacillus* and *Myxobacterium*.[56-60] The ability of CYP101B1 to regioselectively epoxidize the linear pseudoionone, **6**, would allow it to be used as a biocatalyst for linear terpenoids and related derivatives.[61]

The importance of the trimethylcyclohexene ring of the norisoprenoids can be clearly seen by comparing their activities with CYP101B1 to those of the linear pseudoionone, **6** and the aromatic *trans*-4-phenyl-3-buten-2-one, **7** with the norisoprenoids. Previously we have shown that the mixed phenyl and cyclohexyl ring substrate, phenylcyclohexane, was solely oxidized on the aliphatic ring by CYP101B1.[29] Cytochrome P450s are capable of efficiently oxidizing aromatic molecules[62-65] and phenylcyclohexane and *trans*-4-phenyl-3-buten-2-one, **7** must be held with the benzene ring held in such a fashion that the initial formation of the arene oxide or heme bound intermediate required for the oxidation of aromatic C–H bonds was strongly disfavored.[66]

The carbonyl moiety in the butenone side chain of the norisoprenoids must also be important for substrate binding as ionols, **3** and **4**, bind less strongly than the equivalent ionones, **1** and **2**. In addition both enantiomers of camphor, **10** and (+)-fenchone, **12**, are better substrates than the isomers of pinene, borneol isomers, **21** and **22**, and α-terpineol. The improved selectivity of

C–H bond activation of (+)-fenchone, **12**, compared to camphor may be related to the more similar position of the alkyl side chains within the bicyclic system to the norisoprenoids (Scheme 3). The high selectivity for the 5-*exo*-hydroxyfenchone product, **12a**, differed from the reaction of microsomal CYP2A6 which converted fenchone to a mixture of 6-*exo*- and 6-*endo*-hydroxyfenchone, **12b** and **12c**. Fenchone, **12**, is also converted to a mixture of metabolites in its biotransformation by several organisms.[48, 67] Overall bicyclic monoterpenoids containing a carbonyl group resulted in better turnover. It is of note that, 1,8-cineole, **11**, shows the best catalytic properties of any monoterpenoids suggesting the ether oxygen may also be able to interact favorably with the CYP101B1 active site.

(+)-Sclareolide, **9**, a plant natural product, is a sesquiterpene lactone which has some structural similarity to norisoprenoids including a carbonyl functional group in a similar position relative to a methyl substituted cyclohexane ring. While the binding of this larger substrate to CYP101B1 was suboptimal it was stereoselectively oxidized to form (*S*)-3-hydroxy-(+)-sclareolide, **9a**, as the sole product. The C–H bonds which are abstracted are six carbons from the carbonyl functionality which is in the same relative position as those predominantly acted upon by the enzyme with the norisoprenoids (C3). Unselective sclareolide, **9**, oxidation by various organisms has been observed,[46, 68-70] but recently variants of the self-sufficient cytochrome P450Bm3 (CYP102A1), have also been reported to selectively hydroxylate (+)-sclareolide to (*S*)-3-hydroxy-(+)-sclareolide, **9a**.[47]

(1*R*)-(−)-Nopol, **14**, and *cis*-jasmone, **15**, were oxidized by CYP101B1 with improved regioselectivity compared to the other monoterpenes tested. The oxyfunctionalized side chain of (1*R*)-(−)-nopol, **14**, allowed comparatively tight substrate binding. The biotransformation of (1*R*)-(−)-nopol, **14**, to 4-hydroxynopol, **14c**, 4-oxonopol **14b**, and 5-hydroxynopol, **14a**, has been reported by the fungus *Glomerella cingulata.*[52] *cis*-Jasmone, **15**, oxidation by the fungal strains

Penicillium, Absidia, Syncephalastrum, Botrytis, Aspergillus, Cunninghamella, Chaetomium, Didymosphaeria also generates 4-hydroxy-*cis*-jasmone as the major product, **15a**.[53, 71] The identification of new products such as 11-hydroxy-*cis*-jamsone, **15b**, may provide compounds with new biological activity.

5-Norbornene-2-yl acetate, **16**, and the monoterpenoid acetates showed the highest efficiency for the conversion of the NADH reducing equivalents into products of any of the monoterpenoid or related substrates tested indicating that an acetate group may be able to mimic the favorable substrate binding properties of the butenone side chain of the norisoprenoids. The binding and turnover data suggests that the carbonyl group is able to interact with amino acids in the active site such that it holds appropriately sized substrates in the optimal position for high activity, total turnover number and selectivity for C–H bond oxidation. The addition of an acetate group to a selection of bicyclic monoterpenoid alcohols improved substrate binding, enzyme activity and selectivity of product formation. For bornyl and isobornyl acetate, **18** and **19**, the C–H bond which is abstracted, by CYP101B1, is from the ring *trans* to the acetate group resulting in significant changes in the selectivity. The lower activity of L-(–)-bornyl acetate, **18**, despite its tighter binding may arise from the less reactive nature of the terminal methyl group, which is oxidized, compared to the methylenes in the isobornyl acetate, **19**. Alternatively the quaternary bridgehead carbon may place the methyl groups in a suboptimal position for C–H bond activation. The absence of the methyl species on the bridgehead of fenchyl acetate, **17**, opposite the acetate group resulted in hydroxylation at C5 in contrast to the oxidation of L-(–)-bornyl acetate, **18**, at C9.

The location of the carbonyl group in (–)-myrtenyl acetate, **20**, is the same number of atoms away from the ring system as that in the norisoprenoid substrates (α- and β-ionone, **1** and **2**).

The activity of the CYP101B1 with myrtenyl acetate, **20**, resulted in the highest product formation rate and coupling among the monoterpene acetates tested and exceeded those of the norisoprenoids (**1** and **2**). The oxidation was also regioselective and hydroxylated the substrate at the allylic C–H bond generating *cis*-4-hydroxymyrtenyl acetate, **20a**, and the further oxidation product 4-oxomyrtenyl acetate, **20b**. Competing epoxidation at the adjacent, and more reactive, alkene double bond was minimal. One explanation for oxidation at this site may be the easier activation of an allylic C–H bond compared to the bridgehead carbon at the equivalent position of C3 of the norisoprenoids and C5 of the terpenoid acetate substrates. The isomers of 4-hydroxy- and 4-oxo-myrtenol and their esters are important intermediates in the synthesis of cannabinoid derivatives such as HU-211 (dexanabinol) and HU-210 both of which have a range of medicinal uses.[72, 73] The isomers of 4-hydroxymyrtenyl esters are synthesized by reacting them with sodium chromate to form the 4-oxo species followed by reduction using sodium borohydride.[74]

The selective oxidation of monoterpenoid acetates, **17-20**, and other substrates, e.g. sclareolide **9**, by CYP101B1 is remarkable given the number of potential sites for oxidation and would be useful for synthetic chemistry. Acetate and other esters could be used as directing groups or chemical auxiliaries to improve selectivity and turnover without protein engineering and this could be beneficial in generating effective biocatalysts.[34-36, 75] Recently the combined approach of utilizing dimethylamine anchoring groups, molecular dynamics and protein engineering of P450 PikC was used to improve the selectivity of oxidation of menthol derivatives.[25, 36] The crystal structure of CYP101B1 has not yet been solved but using ester groups combined with simple P450 substrate binding assays we have been able to improve the activity and selectivity of monoterpenoid oxidation. The *in vitro* activity and total turnovers achieved with CYP101B1 are significantly greater than those reported for the P450 PikC system (TTN ~ 200 in 3 hours for PikC versus > 500 min^{-1} for CYP101B1 and TTN ranging from 2000 to 10000 for the norisoprenoids

and monoterpenoid acetates). CYP101B1 was also a more efficient biocatalyst compared to mammalian enzymes for which similar substrate engineering strategies have been used.[34, 76] This is presumably due to the availability of the natural electron transfer partners for CYP101B1 and the high activity of soluble bacterial enzymes. Large quantities of products could be generated (up to 7 mM conversion) using a non-optimized whole-cell *E. coli* oxidation system with substrate added after 24 hours showing low rates of conversion. These could be significantly improved using higher density cell cultures in a fermentor with more control of the pH, nutrients and a product feeding/substrate removal regime.

The combination of selective oxidation and the high monooxygenase activity and total turnover numbers achieved using a whole-cell oxidation system for the monoterpenoid acetate substrates with CYP101B1 are ideally suited to the biocatalytic generation of hydroxylated products. Acetate and related ester protecting groups are easy to introduce and to remove from alcohol functional groups and as a result could be utilized to improve the activity or the regio- and stereo-selectivity of oxidation.

Conclusion

In summary CYP101B1 is able to efficiently and selectively oxidize a range of norisoprenoids and related substrates such as the sesquiterpenoid (+)-sclareolide. The oxidation of smaller terpenoids and related substrates is less efficient and in some cases less selective. In certain instances novel products such as 11-hydroxy-*cis*-jasmone were isolated. Monoterpenoid acetates, which are useful flavor and fragrance compounds, where efficiently oxidized by CYP101B1. The simple modification of certain monoterpenes by adding an acetate moiety resulted in large improvements in both the activity and selectivity of oxidation. As a result we were able to regioselectively, and in some instances stereoselectively, oxidize monoterpenoid acetates at product formation rates up to 1520 min^{-1} with high turnover numbers. The oxidation of myrtenyl acetate at the allylic 4-position offers a biocatalytic route to important intermediates in cannabinoid derivative synthesis. When incorporated into a whole-cell oxidation system, with the physiological electron transfer partners, ArR and Arx, CYP101B1 was capable of generating high levels of oxidation products of the norisoprenoids and terpenoids. Therefore the combination of efficient and stereoselective turnovers catalyzed by CYP101B1 and the addition of ketone containing and other directing groups will enable the development of new biocatalytic routes to fine chemicals.

Acknowledgements

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Supporting Information

GC analysis methods and retention times, ^{1}H and ^{13}C NMR details of products, substrate spin state shift details, dissociation constant analysis, GC-MS and MS analysis, NMR spectra.

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