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# Exploring the substrate specificity of Cytochrome $P450_{cin}$

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Dedication: This paper is dedicated to Paul Ortiz de Montellano: inspirational, insightful and interested – what more could one ask?

*Keywords:*
Cytochrome $P450_{cin}$
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ABSTRACT

Cytochromes P450 are enzymes that catalyse the oxidation of a wide variety of compounds that range from small volatile compounds, such as monoterpenes to larger compounds like steroids. These enzymes can be modified to selectively oxidise substrates of interest, thereby making them attractive for applications in the biotechnology industry. In this study, we screened a small library of terpenes and terpenoid compounds against $P450_{cin}$ and two $P450_{cin}$ mutants, N242A and N242T, that have previously been shown to affect selectivity. Initial screening indicated that $P450_{cin}$ could catalyse the oxidation of most of the monoterpenes tested; however, sesquiterpenes were not substrates for this enzyme or the N242A mutant. Additionally, both $P450_{cin}$ mutants were found to be able to oxidise other bicyclic monoterpenes. For example, the oxidation of (*R*)- and (*S*)-camphor by N242T favoured the production of 5-*endo*-hydroxycamphor (65–77% of the total products, dependent on the enantiomer), which was similar to that previously observed for (*R*)-camphor with N242A (73%). Selectivity was also observed for both (*R*)- and (*S*)-limonene where N242A predominantly produced the *cis*-limonene 1,2-epoxide (80% of the products following (*R*)-limonene oxidation) as compared to $P450_{cin}$ (23% of the total products with (*R*)-limonene). Of the three enzymes screened, only $P450_{cin}$ was observed to catalyse the oxidation of the aromatic terpene *p*-cymene. All six possible hydroxylation products were generated from an *in vivo* expression system catalysing the oxidation of *p*-cymene and were assigned based on $^1$H NMR and GC-MS fragmentation patterns. Overall, these results have provided the foundation for pursuing new $P450_{cin}$ mutants that can selectively oxidise various monoterpenes for biocatalytic applications.

## 1. Introduction

Cytochromes P450[1] (P450s) are family of versatile enzymes that catalyse a wide range of oxidative transformations. Typically, P450s employ a high-valent iron-oxo species to insert an oxygen atom into an unactivated carbon hydrogen bond to create an alcohol. Significantly, this is a one-step oxidation process in which the stereo- regio- and enantiospecificity is often highly controlled and is difficult to replicate with traditional organic synthetic methodologies [1]. Hence, P450s can provide a suitable adjunct to chemical synthesis with a wide array of potential biotechnological applications. One of these applications is the production of terpene derivatives, which are essential components in the manufacture of both flavour and fragrance compounds and also find use in the pharmaceutical industry [2,3].

Monoterpenes such as camphor, cineole, limonene and pinene, are the main constituents of many plant oils [4] and are formed from the linking of two isoprene units to create a $C_{10}$ molecule. As monoterpenes are found in nature they are particularly attractive chemical starting materials because they are easily attainable, inexpensive and derived from a renewable resource [3]. A large number of bacterial P450s have been found that naturally catalyse the oxidation of various monoterpenes [3]. One example is $P450_{cam}$ (CYP101A1), which catalyses the oxidation of camphor **1** to 5-*exo*-hydroxycamphor **2a** (Fig. 1; *vide infra*) [5]. Such terpene oxidising P450s are potentially valuable tools that can be employed biotechnologically to produce a variety of modified terpene intermediates [1]. However, to increase the potential utility of bacterial P450s for biocatalytic applications these proteins must be engineered to increase their substrate range while maintaining catalytic efficiency, and stereo- regio- and enantioselectivities. It has been established that enzymes that can only catalyse a transformation at very low levels can be a useful starting point for developing efficient catalysts [6]. Therefore, protein engineering techniques such as site-directed mutagenesis and directed evolution are often used to create P450 mutants that can expand the inherent substrate selectivity of these enzymes and catalyse alternative oxidations [1,7].

Two bacterial P450s most commonly modified to enhance their

*Abbreviations:* P450, Cytochrome P450; $P450_{cam}$, CYP101A1; $P450_{BM3}$, CYP102A1; Pdx, putidaredoxin; PdR, putidaredoxin reductase; $P450_{cin}$, CYP176A1; Cdx, cindoxin; FdR, *Escherichia coli* flavodoxin reductase

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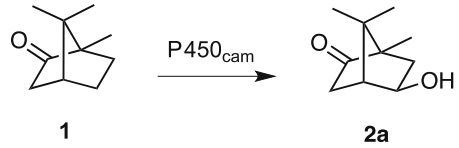
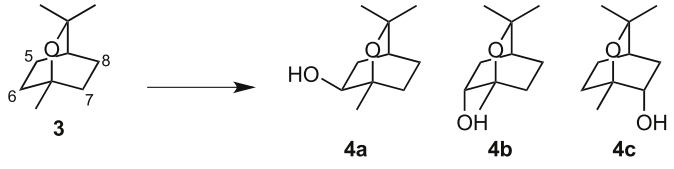



**Fig. 1.** The catalytic oxidation of camphor **1** to 5-*exo*-hydroxycamphor **2a** by $P450_{cam}$ (CYP101A1) [5].

biocatalytic properties and catalyse the oxidation of a broad range of substrates, including terpenes, are $P450_{cam}$ and $P450_{BM3}$ (CYP102A1) [3,7]. $P450_{cam}$ from *Pseudomonas putida* is responsible for the oxidation of the monoterpene (1*R*)-camphor **1** and allows *P. putida* to utilize camphor as its sole carbon and energy source (Fig. 1) [5]. A number of studies have employed site-directed mutagenesis to modify $P450_{cam}$ permitting it to catalyse the oxidation of monoterpenes other than camphor [8–12]. Additionally, $P450_{cam}$ requires two auxiliary proteins, putidaredoxin (Pdx) and putidaredoxin reductase (PdR), and an electron source (NADH) in order to facilitate oxidation. To improve the biocatalytic potential of $P450_{cam}$ whole-cell *in vivo* systems, which include its redox partners Pdx and PdR, can be used to simplify the process and make it more appealing for biotechnological applications [10,13].

In contrast to $P450_{cam}$, $P450_{BM3}$ does not naturally catalyse the oxidation of terpenes but rather catalyses the oxidation of long-chain fatty acids at the ω-1, ω-2 and ω-3 positions [14]. However, unlike $P450_{cam}$, $P450_{BM3}$ does not require auxillary proteins to deliver electrons as it is a natural fusion of the P450 and its redox partner(s) in one polypeptide. Thus, the fact that $P450_{BM3}$ can perform oxidations without any additional proteins, together with its substrate promiscuity, makes it is an attractive P450 to engineer to accept a range of alternative substrates [15]. A number of mutagenesis studies have specifically explored terpene oxidation catalysed by $P450_{BM3}$ and have included monoterpene substrates such as limonene and pinene [16–20].

$P450_{cin}$ (CYP176A1) is another bacterial P450 with potential biocatalytic capabilities for the oxidation of terpenes. $P450_{cin}$ (CYP176A1) was originally isolated from an organism that can live on cineole as its sole carbon source and catalyses the enantiospecific hydroxylation of a monoterpene 1,8-cineole **3** to produce (1*R*)-6β-hydroxycineole **4a** (Fig. 2). This is the first step in the biodegradation of cineole [21]. $P450_{cin}$ was found to have an asparagine residue (N242) in the position where most other P450s have a mechanistically important active site threonine [21]. Typically, P450s have been shown to use this conserved threonine to ensure the protonation of the distal oxygen of a hydroperoxy intermediate as protonation of the proximal oxygen leads to hydrogen peroxide formation (uncoupling the electron consumption without product formation) [22]. However, studies indicated that the asparagine in $P450_{cin}$ was not a functional replacement for threonine, but instead was observed to control the stereo- and regioselectivity of the oxidation [23,24]. Additionally, the N242A mutant was found to




| | | | |
|---|---|---|---|
| **$P450_{cin}$** | 100 | | |
| **N242A** | 5 | 5 | 90 |
| **N242T** | 47 | 22 | 31 |

**Fig. 2.** The catalytic oxidation of cineole **3** by $P450_{cin}$ (CYP176A1) and its mutants N242A and N242T. Numbers indicate the percentage of the hydroxycineole **4** formed from the reaction [23,24].

catalyse the selective oxidation of cineole **3** to (1*S*)-6α-hydroxycineole **4b** (90% of total products formed) rather than (1*R*)-6β-hydroxycineole **4a**, the isomer that had originally been observed with the wildtype enzyme (Fig. 2) [23]. When the asparagine was replaced with a threonine (N242T) the product profile was altered further resulting in three hydroxycineole isomers: **4a** (47%), **4b** (22%) and **4c** (31%) [24].

The aim of this study was to examine a range of compounds, primarily terpenes, to determine their potential as substrates for wildtype $P450_{cin}$, in addition to the N242A and N242T mutants. Since previous reports had indicated that N242A could alter the product profile as compared to the wildtype $P450_{cin}$, we were interested in determining whether this extended to other non-natural $P450_{cin}$ substrates, especially other terpenes. $P450_{cin}$ and N242A have previously been shown to oxidise camphor **1**, which is a bicyclic monoterpene similar to cineole **3** [25,26]. The N242A mutant catalysed oxidation of camphor resulted in comparatively different product profiles for each isomer of camphor [26], whereas $P450_{cin}$ gave a similar profile for both (1*R*) and (1*S*)-camphor **1** [25]. Therefore, we initially screened a library of terpene and terpenoid compounds (**1**, **5–26**; Fig. 3) to determine whether they could be catalytically oxidised by $P450_{cin}$, N242A and/or N242T. Once we had determined if these compounds were substrates for these enzymes, a number of catalytic parameters were determined. It is anticipated that this study may highlight pathways for further diversification of the substrate range of $P450_{cin}$ and its mutants in the oxidation of terpenes.

## 2. Materials and methods

### 2.1. Chemicals

Valencene **16**, guaiazulene **17**, α-bisabolol **19**, (1*R*)-nopol **21**, *cis*-jasmone **22**, isophorone **23**, β-ionone **25** and α-ionone **26** were gifts from Dr. Steven G. Bell. Camphane **24** has been synthesised and reported previously [27].

A mixture of *cis* and *trans* (*R*)-limonene 1,2-epoxide **31** was prepared according to previously published methods [28]. Briefly, (*R*)-limonene **7** was stirred with *meta*-chloroperbenzoic acid with sodium bicarbonate in dichloromethane. GC-MS data obtained for the (*R*)-limonene 1,2-epoxide isomers was consistent with literature values [29].

All other chemicals used in this study were analytical grade reagents.

### 2.2. Purification of enzymes: $P450_{cin}$ and mutants

$P450_{cin}$, mutants N242A and N242T, and the redox partners were all expressed and purified using previously published methods [21,30,31].

### 2.3. Catalytic turnover

The catalytic turnover of a number of substrates with $P450_{cin}$ and the N242A and N242T mutants were performed employing the following method. A solution of Buffer A (50 mM Tris, pH 7.4, 100 mM KCl; 1 mL) containing the appropriate enzyme (0.5 μM), cindoxin (Cdx) (4 μM), *Escherichia coli* flavodoxin reductase (FdR) (1 μM), catalase (1 μM), NADPH (1–2 mM) and relevant substrate (5 mM) were incubated at room temperature (approximately 25 °C). The reaction time was altered depending on the enzyme used ($P450_{cin}$, 30 min; N242A or N242T, 1 h). The reaction mixture was extracted using ethyl acetate and dried over $MgSO_4$. The products were characterised by GC-MS (Econo-cap capillary column EC-1) as previously described [21,23] using the following conditions: 50 °C for 2 min, 16 °C/min until 250 °C, hold for 20 min. Products observed during the turnovers were identified by comparison to the MS library or authentic standards. Product ratios of structurally similar compounds were determined via integration of the GC-MS Total ion current trace.

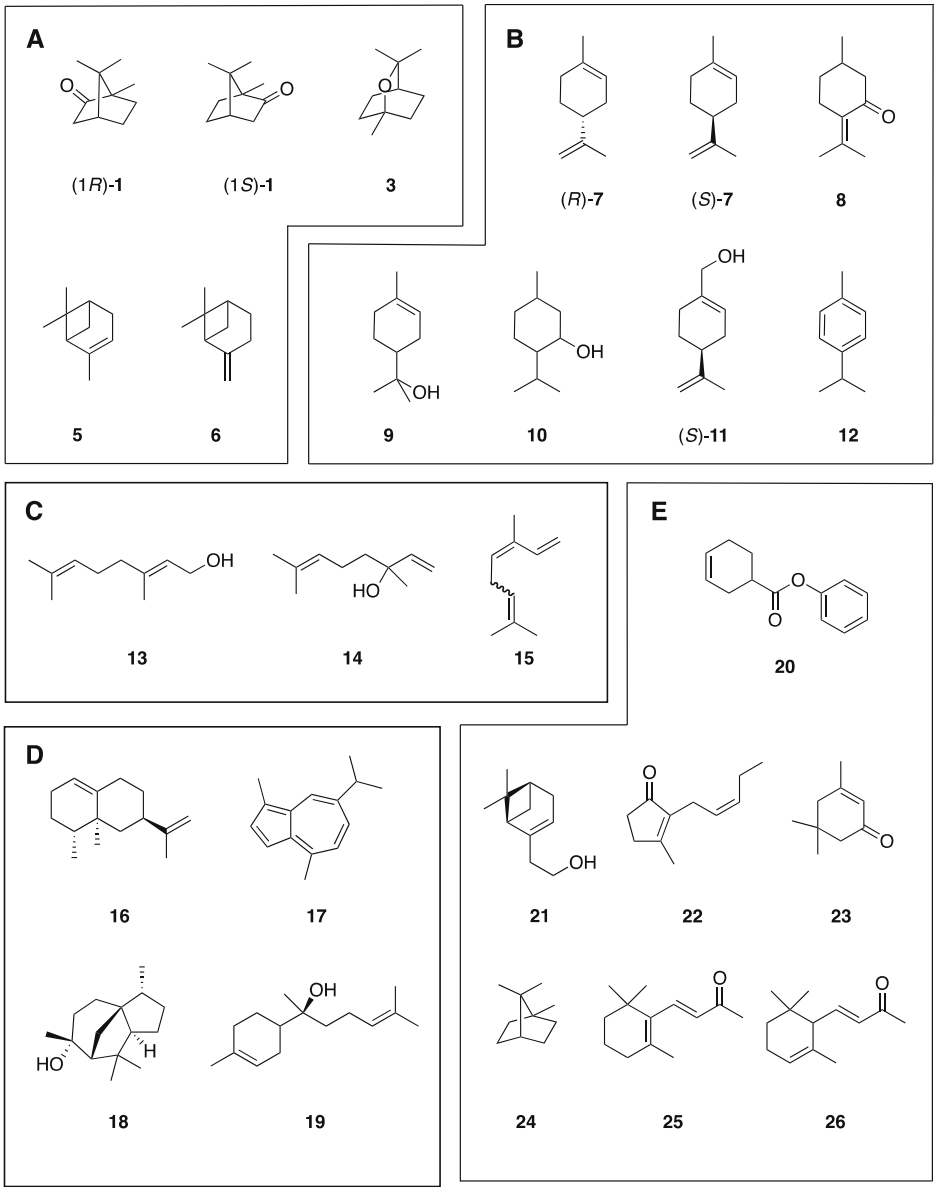



**Fig. 3.** Structures of all compounds used in this study. Letters (**A-E**) indicate each group type: **A**. Bicyclic monoterpenes (**1,3,5,6**); **B**. Monocyclic monoterpenes (**7-12**); **C**. Acyclic monoterpenes (**13–15**); **D**. Sesquiterpenes (**16–19**); and **E**. Compounds $C_9$–$C_{13}$ (**20–26**).

### 2.4. Spin state change

The percentage spin state change was calculated for $P450_{cin}$ and the N242A and N242T mutants with a number of compounds (**1**, **5–8**, **10–14**, **19**, **21–25**) [25]. Briefly, the compound (5 mM, final concentration) was added to a solution of the P450 (2 μM in Buffer A) and the difference in absorbance ($A_{392}$ –$A_{417}$) measured. The percentage spin state change was calculated from the difference observed and standardised against $P450_{cin}$ with cineole **3**.

### 2.5. Dissociation constant

The dissociation constants ($K_d$) for $P450_{cin}$, N242A and N242T with a variety of compounds (**5–7** and **12**) were determined using a previously reported method [21]. Briefly, the appropriate compound (final concentration dependent on the compound and enzyme) was added to a solution of P450 (2 μM) in Buffer A and the absorbance difference measured ($A_{392}$-$A_{417}$). The $A_{392}$-$A_{417}$ difference was then plotted against the concentration of the compound. A typical hyperbolic equation was employed if the affinity of the substrate for the enzyme satisfied the assumptions the equation. If the compound displayed high affinity for the enzyme the dissociation constant was calculated using a tight binding quadratic equation [21].

### 2.6. Rate of NADPH consumption

The rate of NADPH consumption was measured using the following procedure [23]. Enzyme (0.5 μM), Cdx (4 μM), *E. coli* flavodoxin reductase (1 μM), catalase (1 μM) and substrate (5 mM) were all combined in Buffer A. The reaction was initiated with NADPH (200 μM) and the rate of NADPH consumption recorded over time via the decrease in absorbance at 340 nm. The background was determined by measuring the rate of the reaction in the absence of the P450. NADPH consumption was standardised against $P450_{cin}$ with cineole and reported as a percentage.

**Table 1**
Catalytic oxidation and percentage high spin (HS) data ($P450_{cin}$ low spin Fe(III)·$H_2O$ to high spin Fe(III)) of a small library of terpene/terpenoid compounds with $P450_{cin}$, N242A and N242T. Letters (**A-E**) indicate each group type: **A**. Bicyclic monoterpenes; **B**. Monocyclic monoterpenes; **C**. Acyclic monoterpenes; **D**. Sesquiterpenes; and **E**. Compounds $C_9$–$C_{13}$.P = Products detected, ND = Not Detected.

| | | $P450_{cin}$ | | N242A | | N242T | |
|---|---|---|---|---|---|---|---|
| | | Products | HS | Products | HS | Products | HS |
| **A** | **3** | P [25] | 100 [21] | P [26] | 30 [23] | P | 11 [24] |
| | (1*R*)-**1** | P [25] | 27 ± 2 [25] | P [26] | 84 ± 1 [26] | P | 8 ± 1 |
| | (1*S*)-**1** | P [25] | 32 ± 1 [25] | P [26] | 89 ± 1 [26] | P | 5 ± 3 |
| | **5** | P | 70 ± 6 | P | 79 ± 1 | ND | 36 ± 5 |
| | **6** | P | 56 ± 1 | P | 71 ± 1 | P | 16 ± 3 |
| **B** | (*R*)-**7** | P | 32 ± 3 | P | 64 ± 1 | P | 17 ± 1 |
| | (*S*)-**7** | P | 60 ± 3 | P | 52 ± 4 | P | 18 ± 2 |
| | **8** | P [38] | 11 ± 1 [38] | P | 30 ± 1 | ND | 13 ± 1 |
| | **9** | ND | | ND | | | |
| | **10** | P | 5 ± 1 | P | 33 ± 4 | P | 8 ± 1 |
| | **11** | P | 11 ± 1 | ND | 8 ± 1 | | 6 ± 1 |
| | **12** | P | 21 ± 1 | ND | 23 ± 1 | | ND |
| **C** | **13** | P | 6 ± 1 | P | 10 ± 1 | | 2 ± 1 |
| | **14** | P | 6 ± 1 | P | 16 ± 1 | ND | 8 ± 1 |
| | **15** | ND | | ND | | | |
| **D** | **16** | ND | | ND | | | |
| | **17** | ND | | ND | | | |
| | **18** | ND | | ND | | | |
| | **19** | ND | 4 ± 2 | ND | 9 ± 1 | ND | ND |
| **E** | **20** | ND | | ND | | | |
| | **21** | P | 11 ± 1 | ND | 2 ± 1 | | 5 ± 1 |
| | **22** | P | 5 ± 1 | P | 34 ± 2 | | 10 ± 1 |
| | **23** | P | 7 ± 1 | P | 32 ± 1 | | 10 ± 1 |
| | **24** | P [24] | 59 [24] | P | 110 ± 3 | | ND |
| | **25** | ND | 25 ± 1 | ND | 29 ± 2 | | 36 ± 1 |
| | **26** | P | | ND | | | |

### 2.7. Coupling (NADPH consumed: product formed)

The percentage of coupling (NADPH consumed: product formed) was determined employing a previously published method [23]. A catalytic turnover reaction (see Section 2.3) was performed with the following modifications. An internal standard, α-pulegone (1 mM) was added once the reaction was completed before extraction and GC-MS analysis. Catalytic oxidation products of **5**, **6**, and (*R*) and (*S*)-**7**, were compared to a standard curve (mixture of *cis*- and *trans*-limonene oxide **31**) and the amount of product was determined. Coupling was then calculated as a percentage of product formed vs NADPH consumed.

### 2.8. $P450_{cin}$: catalysed oxidation of p-cymene **12** in vivo and product identification

A previously constructed bicistronic plasmid (pCW.$P450_{cin}$.Cdx) [25] was transformed into *E. coli* DH5αF'IQ and used to inoculate Terrific Broth (500 mL in a 1.8 L Fernbach flask) containing ampicillin (50 μg/mL). This culture was incubated at 37 °C (approx. 180 rpm; Innova 4000, New Brunswick Scientific) until an $OD_{600}$ of approximately 0.6 was attained. Protein expression was then induced with isopropyl β-D-1-thiogalactopyranoside (1 mM) and the incubation continued at 27.5 °C for 17 h. The culture was then pelleted via centrifugation (5000 *g*; 20 min) to remove the supernatant and the cells were re-suspended in 4 × 250 mL minimal medium (per L: 6.4 g $Na_2HPO_4{\cdot}7H_2O$, 1.5 g $KH_2PO_4$, 0.5 g NaCl, 1 g $NH_4Cl$, 0.24 g $MgSO_4$, 0.01 g $CaCl_2$ and 0.4% glucose). *p*-Cymene **12** (20 μL) was added to each flask at the following intervals: 30 min; 60 min; 2 h; and 3 h (Total amount of **12**: 275 mg). After the second hour, glucose (final concentration 0.2%) was added and the cultures incubated for a further 17 h. The cells were pelleted at 5000 *g* for 20 min and the supernatant collected. The supernatant was extracted with ethyl acetate, washed with saturated NaCl and dried over $MgSO_4$. The solvent was removed *in vacuo* giving a yield of 90 mg of multiple oxidation products (33%), which were characterised by NMR. Trifluoroacetic anhydride derivatisation was used to differentiate between the multiple products by GC-MS (See Section 2.3 for details).

#### 2.8.1. Key data for metabolite identification

$^1$H NMR: *p*-cymene oxidation products.

4-Hydroxycymene **36**: $^1$H NMR (500 MHz, $CDCl_3$) δ 7.03 (d, 1H), 6.72 (dd, 1H), 6.65 (d, 1H), 2.81 (m, 1H), 1.21 (d, 6H). This is consistent with the literature [32].

1-Hydroxycymene **37**: $^1$H NMR (500 MHz, $CDCl_3$) δ 7.29 (d, 2H), 4.66 (s, 2H), 2.90 (m, 1H), 1.25 (d, 6H). This is consistent with the literature [33].

8-Hydroxcymene **40**: $^1$H NMR (500 MHz, $CDCl_3$) δ 7.38, 7.16 (2 x m, 2 × 2H), 2.30 (s, 3H), 1.60 (s, 6H). This is consistent with the literature [34].

MS fragmentation patterns of TFA-derivatised *p*-cymene oxidation products

10-Hydroxycymene **35** *m/z* (EI, 70eV) 91 (42), 115 (52), 117 (46), 133 (18), 149 (24), 185 (10), 213 (51), 231 (100), 246 ($M^+$; 52).

4-Hydroxycymene **36**: *m/z* (EI, 70eV) 91 (32), 115 (37), 117 (41), 133 (15), 105 (10), 185 (4), 213 (11), 231 (100), 246 ($M^+$; 39).

1-Hydroxycymene **37**: *m/z* (EI, 70eV) 91 (22), 115 (29), 117 (36), 133 (12), 185 (3), 213 (7), 231 (100), 246 ($M^+$; 33).

2-Methyl-4-isopropylphenol **38**: *m/z* (EI, 70eV) 91 (26), 115 (24), 117 (95), 132 (22), 133 (21), 213 (13), 231 (100), 246 ($M^+$; 41).

6-Hydroxycymene **39**: *m/z* (EI, 70eV) 65 (22), 91 (81), 115 (61), 131 (74), 159 (71), 213 (26), 228 (100), 246 ($M^+$; 12).

8-Hydroxcymene **40**: *m/z* (EI, 70eV) 69 (11), 91 (25), 117 (83), 132 (21), 133 (22), 203 (7), 231 (100), 246 ($M^+$; 45).

## 3. Results

### 3.1. Catalytic turnover/spin state change

Previous experiments with $P450_{cin}$ catalysed oxidation of camphor **1** indicated that $P450_{cin}$ could catalyse the oxidation of substrates other

than cineole **3**, although with reduced specificity and efficiency (coupling of substrate oxidation to NADPH consumption) [24,25]. Thus, a number of additional terpenes/terpenoid compounds (Fig. 3: **5–26**) were screened to assess the ability of $P450_{cin}$ and/or its mutants N242A and N242T to catalytically oxidise alternative substrates (Table 1). In general, both $P450_{cin}$ and N242A were found to oxidise the same compounds (**5–8**, **10, 13, 14, 23, 24**) with four compounds (**11, 12, 21**, and **26**) only oxidised by $P450_{cin}$ and not the mutants. Due to its poor catalytic turnover rates in our initial screen (with **1**, **3**, **5–8** and **10**) N242T was not further evaluated for product formation with other substrates and its kinetic parameters were not determined. (The coupling of N242T during cineole **3** oxidation was previously determined [24] to be 17% that of the wildtype enzyme.) The product yield from $P450_{cin}$ and N242A catalysed oxidation of **8**, **10, 13, 14**, **22–24** was poor and this was also reflected in their limited ability to convert $P450_{cin}$ low spin Fe(III)·$H_2O$ to high spin Fe(III), a well-known indicator of substrate binding at the active site of a P450 (Table 1). In general, the percentage high spin conversion was determined with all isoforms for compounds that had generated products following catalytic turnover with wildtype $P450_{cin}$, but was also determined for a small number of compounds that were not detectably oxidised. Interestingly, a number of terpenes were found to give a significantly higher percentage high spin value for N242A compared to the wildtype enzyme; these included (*R*)-limonene **7**, pulegone **8**, (−)-menthol **10**, **22** and **23**. $P450_{cin}$ failed to catalyse the oxidation of a number of other substrates including all four sesquiterpenes ($C_{15}$) **16–19**. This observation suggested that the active site of $P450_{cin}$ may be too small to accommodate the extra bulk of the larger sesquiterpene substrates.

### 3.2. $P450_{cin}$: Unsaturated monoterpene hydrocarbons: α-pinene 5, β-pinene 6 and limonene 7

Pinenes **5** and **6**, and limonene **7** all gave sufficient quantities of products to justify further detailed analysis of the interaction of these terpenes with $P450_{cin}$ and N242A. For $P450_{cin}$, the ability for **5**, **6** and (*S*)-**7** to shift the resting heme iron spin state from low spin Fe(III)·$H_2O$ (417 nm) to high spin Fe(III) (392 nm) upon binding was relatively high (Table 1: 56–70%). The change in the spin state observed for (*R*)-**7** (32 ± 3%) was similar to that previously reported for (1*R*) and (1*S*)-camphor **1** (27–32%). $P450_{cin}$ was observed to have dissociation constants ($K_d$) with pinenes **5** and **6** (Table 2) of 1.2 ± 0.2 and 2.6 ± 0.3 μM, respectively, similar to those reported for cineole **3** with $P450_{cin}$ (0.7 μM) [21]. However, the dissociation constants found for $P450_{cin}$ with (*R*)- and (*S*)-limonene **7** (Table 2: 27 ± 3 and 17 ± 2 μM, respectively) were approximately an order of magnitude higher than $P450_{cin}$ with the pinenes **5** and **6**. The rate of NADPH consumption was determined for $P450_{cin}$ with **5**, **6** and (*R*)- and (*S*)-**7** and found to be approximately the same, if not faster than the rate reported for $P450_{cin}$ with cineole **3** (Table 2: 83–140%). However, the coupling of reducing equivalents (NADPH) to the generation of terpene oxidation products (Table 2: 18–44%) was much lower than with the natural substrate cineole **3** but very similar to that which had been observed previously for $P450_{cin}$ with camphor **1** (25–35%) [25].

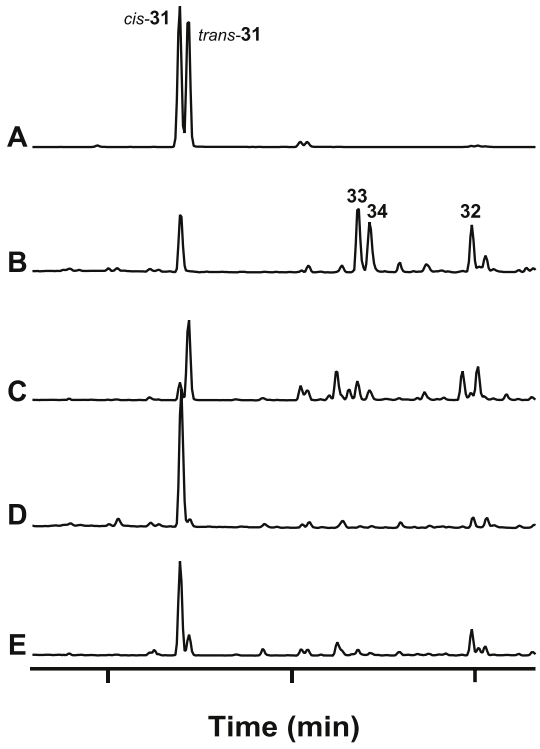



**Fig. 4.** GC-MS trace comparing the oxidation products of the catalytic turnover of (*R*)- and (*S*)-limonene **7** with $P450_{cin}$ WT and N242A, and the *cis* and *trans* (*R*)-limonene 1,2-epoxide **31** standards. **A**. *cis* and *trans* (*R*)-limonene 1,2-epoxide; **B**. WT with (*R*)-limonene; **C**. WT with (*S*)-limonene; **D**. N242A with (*R*)-limonene; and **E**. N242A with (*S*)-limonene. Other products (RT = 7–8 min) include: carveol **33**, carvone **34** and perillyl alcohol **32**.

$P450_{cin}$ oxidation of **5**, **6**, (*R*)- and (*S*)-**7** were all found to produce a mixture of oxygenated products (Fig. 4: **B** and **C**; Data for **5** and **6** not shown). Comparison to a commercial MS fragmentation library and authentic standards revealed that the products generated from oxidation of **5**, **6**, (*R*)- and (*S*)-**7** were mainly alcohols, carbonyl compounds and epoxides (Fig. 5). For both pinenes **5** and **6** these products did not include myrentol **27**, but mainly pinocarveol **28** and the epoxides **29** and **30**. In addition to *cis* and *trans*-limonene 1,2-epoxide **31**, $P450_{cin}$ oxidation of (*R*)- and (*S*)-**7** also generated: perillyl alcohol **32**; carveol **33**; and carvone **34**. It was observed that the stereochemistry of **7** dictated whether $P450_{cin}$ produces the *cis* or *trans*-limonene 1,2-epoxide

**Table 2**
Kinetic constants determined for $P450_{cin}$ and N242A. $K_d$ (μM): Dissociation constants ($K_d$) were calculated by measuring the absorbance difference from $A_{392}$-$A_{417}$ upon substrate binding. **Rate** (%): The rate of NADPH consumption is given as a percentage of the rate observed for $P450_{cin}$ in the presence of cineole, and was linear over the time of the experiment. The rate of NADPH consumption by $P450_{cin}$ with cineole is typically 218 μM $min^{-1}$ per μM P450 when the ratio of enzymes are 1:2:8 (P450:FdR; Cdx) [30]. **Coupling** (%): The coupling of NADPH to the generation of products was determined using a mixture of *cis/trans* limonene oxide **31** as the product standard. See Materials and Methods for experimental detail.

| Substrate | $P450_{cin}$ | | | N242A | | |
|---|---|---|---|---|---|---|
| | $K_d$ (μM) | Rate (%) | Coupling (%) | Kd (μM) | Rate (%) | Coupling (%) |
| **3** [21,23,30] | 0.7 | 100 | 80 ± 2 | 0.3 | 25 | 49 ± 3 |
| (1*R*)-**1** [25,26] | 101 ± 4 | 33 ± 1 | 35 ± 2 | 1.2 ± 0.1 | 55 ± 5 | 3.5 ± 0.5 |
| (1*S*)-**1** [25,26] | 81 ± 5 | 29 ± 1 | 25 ± 3 | 1.5 ± 0.2 | 55 ± 6 | 3.4 ± 0.6 |
| **5** | 1.2 ± 0.2 | 138 ± 14 | 18 ± 2 | 0.09 ± 0.05 | 19 ± 2 | 38 ± 5 |
| **6** | 2.6 ± 0.3 | 118 ± 4 | 27 ± 2 | 0.08 ± 0.04 | 18 ± 1 | 20 ± 3 |
| (*R*)-**7** | 27 ± 3 | 83 ± 4 | 44 ± 6 | 4.6 ± 0.5 | 21 ± 2 | 30 ± 1 |
| (*S*)-**7** | 17 ± 2 | 140 ± 10 | 24 ± 1 | 6.6 ± 0.7 | 18 ± 2 | 5.8 ± 0.2 |

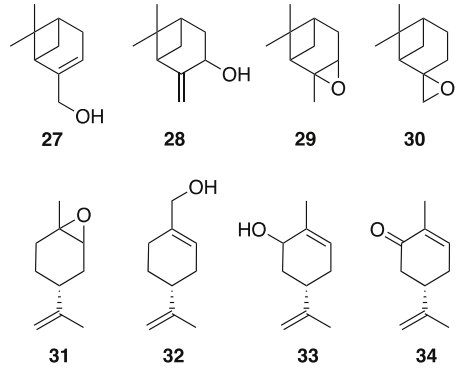



**Fig. 5.** Possible oxidation products from the catalytic turnover of α-pinene **5**, β-pinene **6** (*R*)- and (*S*)-limonene **7**.

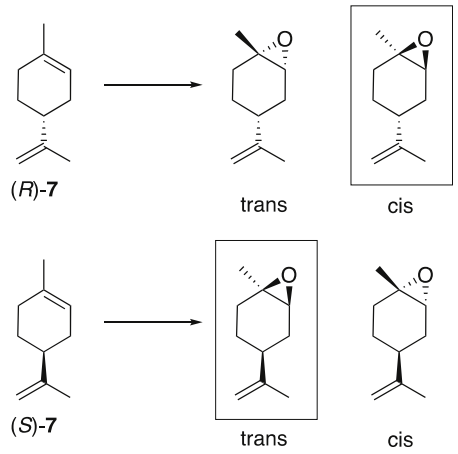



**Fig. 6.** P450$_{cin}$ catalysed oxidation of limonene **7** to *cis* or *trans*-limonene 1,2-epoxide **31**; *cis*-(4*R*)-limonene 1,2-epoxide from (*R*)-limonene, *trans*-(4*S*)-limonene 1,2-epoxide from (*S*)-limonene.

**31**; (*R*)-limonene produced the *cis*-(4*R*)-limonene 1,2-epoxide, whereas the (*S*)-limonene gave predominantly the *trans*-(4*S*)-limonene 1,2-epoxide (Fig. 6).

### 3.3. N242A: Unsaturated monoterpene hydrocarbons: α-pinene *5*, β-pinene *6* and limonene *7*

Binding of pinenes **5, 6** or (*S*)-limonene **7** to N242A produced a spin state change of a similar magnitude to that of these compounds with P450$_{cin}$ (Table 1). However, there was a two-fold increase in the spin state change produced by (*R*)-**7** with N242A when compared to wildtype (Table 1: N242A 64 ± 1% vs P450$_{cin}$ 32 ± 3%). The dissociation constants obtained for N242A with **5, 6** and (*R*)- and (*S*)-**7** were approximately an order of magnitude smaller than those obtained with the same substrates with P450$_{cin}$ (Table 2) indicating a significant increase in binding affinity with the mutant. The rate of NADPH consumption of **5, 6** and (*R*)- and (*S*)-**7** with N242A dropped considerably when compared with P450$_{cin}$ and the same substrates. However, coupling of reducing equivalents to the generation of products remained similar to that observed with the wildtype enzyme (Table 2) with the exception of (*S*)-limonene **7**, which was considerably lower than that observed for P450$_{cin}$.

The catalytic turnover of (*R*)- and (*S*)-**7** with N242A produced predominantly the *cis*-limonene 1,2-epoxide **31** (Fig. 4: **D** and **E**). The *cis*-limonene 1,2-epoxide **31** comprised 80% of the products following (*R*)-limonene **7** oxidation and 57% of the products during (*S*)-limonene **5** oxidation. This suggests that the N242A mutant is more selective for the generation of a single product that the wildtype enzyme where the catalytic turnover of (*R*)- and (*S*)-**7** gave a much more complex mixture of different oxidation products (Fig. 4: **B** and **C**). For example, *cis*-(*R*)-limonene 1,2-epoxide **31** was only 23% of the total products following the catalytic turnover of (*R*)-**7** with P450$_{cin}$ compared to 80% with N242A.

### 3.4. P450$_{cin}$: Aryl monoterpene: p-cymene *12*

P450$_{cin}$ oxidation of *p*-cymene was also analysed using an *in vivo* system [25] that generated sufficient quantities of the products for characterisation. $^{1}$H NMR of the crude product mixture allowed the identification of the three major oxidation products by comparison to reported literature values: 4-hydroxycymene **36**; 1-hydroxycymene **37**; and 8-hydroxcymene **40** (Fig. 7). Derivatisation with trifluoroacetic anhydride and analysis by GC-MS confirmed these assignments. Three other minor products were observed in the GC-MS (Fig. 7B) and were tentatively assigned as, 10-hydroxycymene **35**, 2-methyl-4-isopropylphenol **38** and 6-hydroxycymene **39**, based on their *m/z* 246 (TFA protected monohydroxylated *p*-cymene) and by comparison with a commercial MS fragmentation library. Oxidation products **35** and **39** were expected, as they are direct hydroxylation products of *p*-cymene. However, **38** was unexpected, as only five possible hydroxylation products can be generated. It is thought that **38** may be produced from a rearrangement resulting in a methyl migration analogous to the NIH shift [35,36]. Unusual binding spectra were observed by UV/Visible spectroscopy, preventing simple $K_d$ determination: low to high spin conversion was seen at low concentrations of *p*-cymene (< 150 μM) but the reverse was observed at higher concentrations (0.2–1 mM) with wildtype P450$_{cin}$. The percentage high spin conversion for *p*-cymene (determined at 100 μM final concentration) with P450$_{cin}$ was 21 ± 1%. A moderate rate of NADPH consumption of P450$_{cin}$ with *p*-cymene **12** (41 ± 4% when compared to P450$_{cin}$) with **3** was also observed.

### 3.5. N242T: camphor *1*

The catalytic turnover of (1*R*)- and (1*S*)-camphor **1** with wildtype P450$_{cin}$ and N242A has been previously analysed [25,26]. To complete this series, the catalytic turnover of both (1*R*)- and (1*S*)-**1** with N242T was performed. During catalytic turnover of (1*R*)-**1** with the N242T mutant it was observed to produce predominantly 5-*endo*-hydroxycamphor **2b** (77% of the products formed; Fig. 8) Additionally, the oxidation of (1*S*)-**1** by N242T also yielded mainly 5-*endo*-hydroxycamphor **2b** (65% of the products formed) with a significant amount of the 3-*exo* and 3-*endo*-hydroxycamphors **2c-d** also present (28%).

## 4. Discussion

P450$_{cin}$ and the available mutants N242A and N242T were screened to determine their ability to catalyse the oxidation of a range of terpenes and terpenoid compounds. It was demonstrated that these enzymes were limited to the oxidation of terpene and terpenoids that were similar in size to P450$_{cin}$'s natural $C_{10}$ substrate, cineole **3,** rather than the larger sesquiterpenes ($C_{15}$). Interestingly, nonpolar monoterpene hydrocarbons, such as *p*-cymene **12**, limonene **7**, α-pinene **5** and β-pinene **6** that all lack an oxygen in their structure, were found to be relatively good substrates for wildtype P450$_{cin}$ (18–44% coupling for **5–7**; Table 2). These results were similar to those previously observed for P450$_{cin}$ catalysed oxidation of camphor ((1*S*)-camphor 25%; (1*R*)-camphor 35%) [25]. However, both the rate of reaction and binding constants for pinenes **5–6** were found to be more analogous to the interaction between P450$_{cin}$ and cineole than to P450$_{cin}$ and camphor. Therefore, this provides some preliminary evidence indicating that P450$_{cin}$ may be a useful enzyme for modification of other monoterpenes, especially bicyclic monoterpenes. The *in vitro* binding behaviour of P450$_{cin}$ with *p*-cymene was found to be unusual but a number of products were observed to be produced by catalytic oxidation with

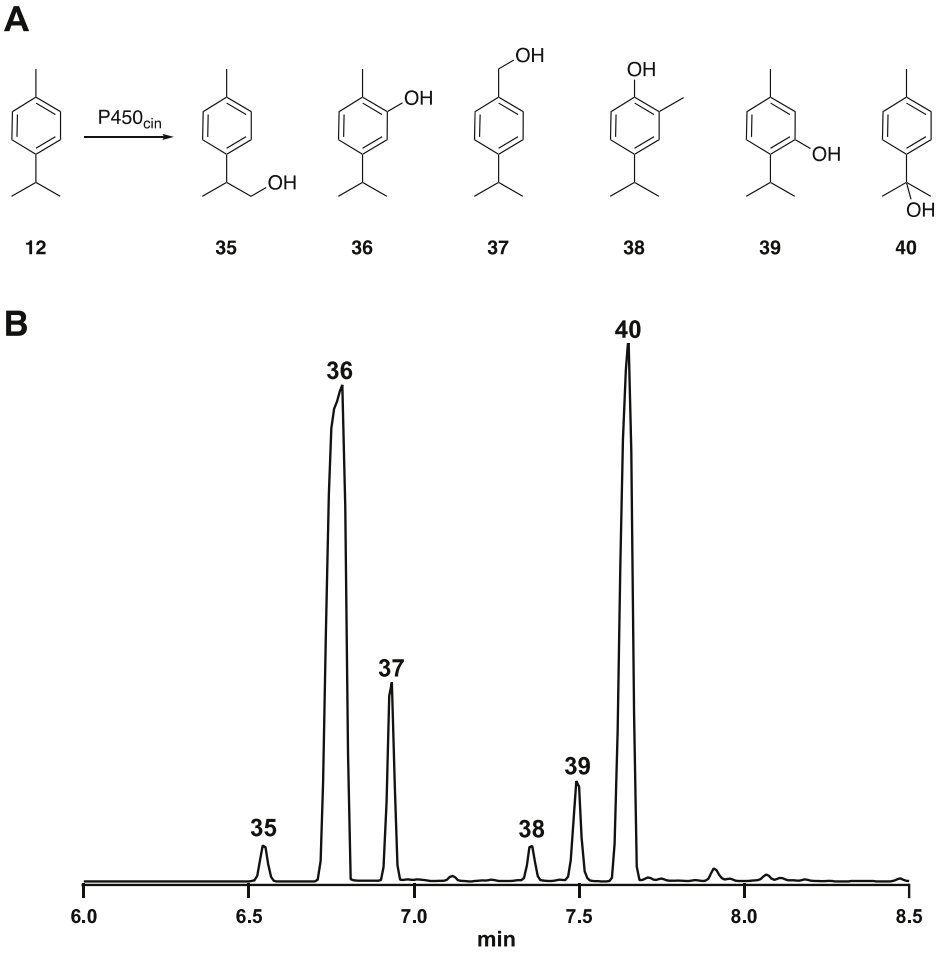



**Fig. 7. A**. Possible oxidation products (**35**–**40**) formed from a $P450_{cin}$ *in vivo* oxidation of *p*-cymene **12** in minimal medium. **B.** GC-MS *m/z* 246 ion trace of the TFA-derivatised oxidation products from an *in vivo* turnover of *p*-cymene **12**.

$P450_{cin}$. Thus, the *in vivo* metabolism of this substrate by a bicistronic $P450_{cin}$ system was characterised to elucidate the nature of the products formed. The three major products formed from $P450_{cin}$ catalysed oxidation of *p*-cymene were **36**, **37** and **40** in a ratio of 2:1:6. These correspond to hydroxylation at the easily oxidised benzylic methine, the slightly less easily oxidised benzylic methyl and the least sterically encumbered aromatic position. Careful analysis of the GC-MS trace of the derivatised products showed the remaining two expected products **35** and **39**, corresponding to oxidation at the less reactive methyl and hindered aromatic position, were present at low levels (Fig. 7). Interestingly, a sixth product was identified by comparison of its MS fragmentation pattern with a commercial library as **38**. This would arise from *ipso* attack on the methyl bearing aromatic carbon followed by an NIH shift of the methyl group and subsequent rearomatisation. Such a shift has previously been reported as a minor product in the $P450_{BM-3}$ catalysed oxidation of xylene [36] but, interestingly, was found to occur more in *o*-xylene and not at all in *p*-xylene.

In general, it was observed that the percentage high spin was not a satisfactory indication of the potential for a compound to be a substrate for the enzyme. The percentage high spin shows whether the compound has displaced the resting water in the active site by shifting the heme iron from low (417 nm) to high spin (392 nm). Frequently, the percentage high spin enzyme was observed to increase with the N242A mutant as compared to wildtype $P450_{cin}$ (**8**, **10**, **22**, **23**; Table 1), but this increase did not coincide with an increased amount of product observed in the N242A catalysed oxidation of these compounds when compared to $P450_{cin}$ (Table 1). In the case of β-ionone **25**, N242T was observed to have a 35% spin state change but no products were detected from the catalytic turnover. It therefore appears that the coupling of reducing equivalents to the production of oxidation products may be the most important indicator when optimising these oxidations. Consequently, screening compounds by catalytic activity together with the coupling information appears to be the best strategy to determine which enzyme/substrate combinations could be the most viable for further modification.

Both the N242A and N242T mutants of $P450_{cin}$ were explored as potential catalysts for monoterpene oxidation. It has previously been observed that amino acid substitutions at this position (N242) have resulted in significantly altered product profiles for the oxidation of both cineole and camphor [23,24,26]. In a similar fashion to N242A [26], N242T catalysed oxidation of (1*R*)-camphor was observed to increase the production of the 5-*endo*-hydroxycamphor **2b** and decrease the oxidation at the 3-position to **2c**/**d**. However, unlike the N242A catalysed oxidation of (1*S*)-camphor **1**, this preference for the production of **2b** was maintained during the oxidation of the (1*S*)-camphor by N242T. This further suggests that amino acid substitutions at the N242 position are important in directing the regioselectivity of the products generated and that the type of amino acid can also change the expected stereoselectivity of the oxidation. Thus, subtle changes in the active site induced by the N242A mutation may be an important starting point by which to produce new oxygen containing monoterpenes. Interestingly, wildtype $P450_{cin}$ was also found to differentiate between different isomers of monoterpenes such as limonene **7**. $P450_{cin}$ predominantly converted (*R*)-**7** to *cis*-limonene 1,2-epoxide **31**, whereas *trans*-**31** was produced from the oxidation of (*S*)-**7**. This again demonstrates the importance of the stereochemistry of the substrate itself in dictating the

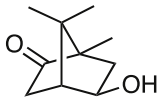
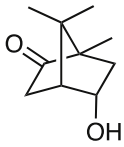
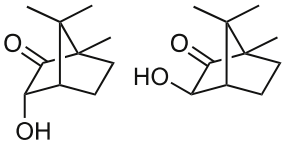
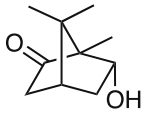
| | N242T | | $P450_{cin}$ [25] | | N242A [26] | |
|---|---|---|---|---|---|---|
| | (1*R*)-**1** | (1*S*)-**1** | (1*R*)-**1** | (1*S*)-**1** | (1*R*)-**1** | (1*S*)-**1** |
|  **2a** | 3 | 1 | 4 | 5 | 4 | 1 |
|  **2b** | 77 | 65 | 32 | 21 | 73 | 35 |
|  **2c** **2d** | 15 | 28 | 49 | 56 | 13 | 14 |
|  **2e** | 5 | 6 | 14 | 19 | 11 | 50 |

**Fig. 8.** Oxidation products of the catalytic turnover of (1*R*)- and (1*S*)-**1** with N242T, and for comparison, previously published results for $P450_{cin}$ [25] and N242A [26]. Only the *R* stereochemistry is shown. Numbers indicate the amount (%) of each isomer formed from both (1*R*)- and (1*S*)-**1** as determined by GC-MS.

outcome of the oxidation.

Furthermore, in contrast to wildtype $P450_{cin}$ and with the exception of cineole, oxidation of monoterpenes by N242A often generates mainly one product. For example, N242A catalysed oxidation of (*R*)-limonene **7** produced *cis*-(*R*)-limonene 1,2-epoxide **31**, which comprised 80% of the products of the oxidation. This is similar to the selectivity previously observed with (1*R*)-camphor **1** where N242A converted (1*R*)-**1** to predominantly 5-*endo*-hydroxycamphor **2b** (73% of the total products formed) [26]. It therefore appears that the substitution at the N242 position not only significantly alters the product profile, but can potentially direct the oxidation towards a single product. However, critically, there are a number of factors that must be improved for the potential use of the N242A mutant in biocatalytic applications. The rate of the reaction and coupling efficiency were found to be compromised by the introduction of this mutation. Therefore, other modifications would be required to improve the viability of N242A, such as the introduction of "accelerator" mutations that could increase the rate of the reaction without disturbing the specificity. These types of accelerator mutations have been employed in other P450s such as $P450_{BM3}$ [37].

In sum, this study has shown that $P450_{cin}$ is a good scaffold to explore for the oxidation of a variety of $C_{10}$ bicyclic monoterpenes. The N242 residue has also been shown to be a useful site to alter when attempting to control the specificity of the oxidation. Future work will examine both other amino acid substitutions at this site, in addition to other mutations that can refine the specificity and maintain the rate and coupling efficiency of the original $P450_{cin}$ oxidation.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.abb.2019.07.025.

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