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**An *in vivo* cytochrome P450<sub>cin</sub> (CYP176A1) catalytic system for metabolite production**

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**Abstract**

Cytochrome P450<sub>cin</sub> (CYP176A1) is a bacterial P450 isolated from *Citrobacter braakii* that catalyses the hydroxylation of 1,8-cineole to (1*R*)-6 $\beta$ -hydroxycineole. P450<sub>cin</sub> uses two redox partners *in vitro* for catalysis: cindoxin, its physiological FMN-containing redox partner, and *Escherichia coli* flavodoxin reductase. Here we report the construction of a tricistronic plasmid that expresses P450<sub>cin</sub>, cindoxin and *E. coli* flavodoxin reductase and a bicistronic plasmid that encodes only P450<sub>cin</sub> and cindoxin. *E. coli* transformed with the bicistronic vector effectively catalysed the oxidation of 1,8-cineole, with the endogenous *E. coli* flavodoxin reductase presumably acting as the terminal electron transfer protein. This *in vivo* system was capable of producing enantiomerically pure (1*R*)-6 $\beta$ -hydroxycineole in yields of ~1 g/L culture, thus providing a simple, one-step synthesis of this compound. In addition, the metabolism of (1*R*)- and (1*S*)-camphor, structural homologues of 1,8-cineole was also evaluated in order to investigate the ability of this *in vivo* system to produce compounds for mechanistic studies. Significant quantities of five of the six possible secondary alcohols arising from methylene oxidation of both (1*R*)- and (1*S*)-camphor were isolated and structurally characterised. The similarity of the (1*R*)- and (1*S*)-camphor product profiles highlight the importance of the inherent reactivity of the substrate in determining the regiochemistry of oxidation in the absence of any specific enzyme-substrate binding interactions.

**Keywords**

cytochrome P450, *in vivo*, *Escherichia coli*, cineole, camphor

## 1. Introduction

Cytochromes P450 enzymes (P450s) form a super family of versatile catalytic hemoproteins that can activate molecular oxygen, allowing them to catalyse a remarkable array of interesting oxidative transformations. These include reactions such as the hydroxylation of non-activated hydrocarbons, epoxidation, heteroatom oxidation and carbon-carbon bond cleavage.[1] The ability of P450s to catalyse the often regio-, stereo- and enantiospecific hydroxylation of non-activated hydrocarbons, a significant challenge for traditional synthetic means, makes them attractive targets both as biocatalysts for organic synthesis and for mechanistic investigations.[2-4] Despite their oxidative potential, the application of P450s as biocatalysts is limited for a number of reasons. These include: substrate specificity, reaction rates, the required multi-component electron transfer proteins and the stoichiometric amounts of expensive reducing cofactors (NAD(P)H) needed when using purified or semi-purified enzymes. Heterologous coexpression of the P450 and its redox partners in whole cells can be employed to overcome some of these barriers.

Over-expression of both the P450 and its reductase partners in whole cells has been extensively explored with a variety of P450s from a number of different organisms.[5-13] Two bacterial examples that investigate the bio-catalytic potential of these whole cell systems include P450<sub>cam</sub>[5, 8, 12] and P450<sub>BM-3</sub>[10, 11, 14, 15]. Cytochrome P450<sub>cam</sub> catalyses the hydroxylation of (1*R*)-camphor to 5-exo-hydroxycamphor. It has been demonstrated that the expression of P450<sub>cam</sub>, putidaredoxin (Pdx) and putidaredoxin reductase (PdR) in whole cells either individually (tricistronic plasmid)[5, 8] or as a fusion-protein[12] can effectively catalyse camphor oxidation. This P450<sub>cam</sub>/Pd/PdR whole-cell system has been investigated with non-natural substrates including limonene[8]. P450<sub>BM-3</sub>, a natural fusion of a P450 and its

reductase components, has also been demonstrated to convert a variety of long-chain saturated fatty acids to  $\omega$ -1,  $\omega$ -2 and  $\omega$ -3 hydroxy fatty acids in whole cells.[10, 11] Such systems have also been used to produce minor metabolites for structure elucidation, leading to important insights into the mechanism of P450 catalysed oxidations.[16] *In vivo* systems of both P450<sub>cam</sub> and P450<sub>BM-3</sub> have also been engineered to enhance oxidation of non-natural substrates by introducing specific mutations.[5, 16] For example, the P450<sub>cam</sub> Y96F-V247L mutant *in vivo* system was observed to successfully catalyse the turnover of styrene and ethylbenzene.[5]

P450<sub>cin</sub> (CYP176A1) is a soluble, microbial P450 isolated from *Citrobacter braakii* that catalyses the hydroxylation of cineole **1** to (1*R*)-6 $\beta$ -hydroxycineole **2** (Scheme 1).[17] This hydroxylation step is believed to be the first in the metabolism of cineole **1**[18] by *C. braakii* allowing it to survive on cineole as its sole carbon and energy source.[17] Reconstitution of P450<sub>cin</sub> activity *in vitro* has been achieved with cindoxin (Cdx), its native redox partner, and *Escherichia coli* flavodoxin reductase (Fpr).[19] We report here the construction of polycistronic plasmids encoding the genes of this P450<sub>cin</sub> system. These constructs enabled the preparative scale bioproduction of enantiopure (1*R*)-6 $\beta$ -hydroxycineole **2** in a single oxidative step from cineole **1**, mimicking the natural transformation catalysed by P450<sub>cin</sub>. We also demonstrate that this system has potential for the *in vivo* transformation of compounds that are much poorer substrates for P450<sub>cin</sub> than cineole, which should be of significant value for mechanistic investigation. The *in vivo* oxidation of the (1*R*)- and (1*S*)-camphor **3** (Fig. 1) was demonstrated to yield sufficient quantities of metabolites for structural elucidation, even in cases where the non-natural substrates produced complex metabolite mixtures. Development of this *in vivo* P450<sub>cin</sub> system may also be

a useful tool in producing antimicrobial, bactericidal agents,[20] or fragrances in the perfume industry.[21]

## 2. Experimental

**2.1 Construction of pCW-P450<sub>cin</sub>/CdR/Cdx** – pUC19-Cdx[19] was digested with *SphI* and *BamHI*, effectively removing one *PstI* site from the multiple cloning site, and retaining the second *PstI* site found within the Cdx gene. This plasmid was treated with DNA polymerase I (Klenow fragment) to create blunt ends and re-ligated. A *PstI-PstI* 4 kb fragment generated from pC1[17] (containing P450<sub>cin</sub>, CdR and 98% of the Cdx gene) was ligated into the altered *PstI* cut pUC19-Cdx to form pUC19-C3. The *PstI* fragment was oriented using *PpuMI/HindIII* which removes a 3 kb fragment if inserted correctly. This 3 kb *PpuMI/HindIII* fragment was then inserted into pUCPKS-*cinA*[17] which was similarly digested with *PpuMI/HindIII* to create pUC-PHC3. pUC-PHC3 was then digested with *NdeI/HindIII* and the fragment cloned into similarly cut pCW to yield the tricistronic plasmid pCW-P450<sub>cin</sub>/CdR/Cdx.

**2.2 Construction of pCW-P450<sub>cin</sub>/Cdx** - pCW-P450<sub>cin</sub>/CdR/Cdx was digested with *BstBI* and the 800 bp fragment containing the CdR gene discarded. The vector was treated with DNA polymerase I (Klenow fragment) to create blunt ends and re-ligated to form pCW-P450<sub>cin</sub>/Cdx.

**2.3 Construction of pCW-P450<sub>cin</sub>/Fpr/Cdx** - pET11a-Fpr[22] was digested with *XbaI* and *BamHI* to excise the Fpr gene. The fragment was treated with DNA polymerase I and ligated into pCW-P450<sub>cin</sub>/Cdx which had been digested with *BstBI* and similarly treated with DNA polymerase I. Correct orientation of the Fpr gene

within the construct was achieved using *Hind*III digests to give pCW-P450<sub>cin</sub>/Fpr/Cdx.

**2.4 Evaluation of polycistronic constructs: Expression** – A polycistronic plasmid (either pCW-P450<sub>cin</sub>/CdR/Cdx, pCW-P450<sub>cin</sub>/Cdx or pCW-P450<sub>cin</sub>/Fpr/Cdx) was transformed into *E. coli* DH5 $\alpha$ F'IQ and used to inoculate Terrific broth (1 L in 2.8 L Fernbach flask) containing ampicillin (50  $\mu$ g/mL). This culture was incubated at 37°C (approx. 180 rpm; Innova 4000, New Brunswick Scientific) until an OD<sub>600</sub> of approximately 0.6 was attained and protein expression was induced with isopropyl  $\beta$ -D-1-thiogalactopyranoside (1 mM). The culture was incubated at 27.5°C for a further 18 h and the cells harvested by centrifugation (4000 g). To determine the relative amounts of each protein a crude purification was performed. The cells were lysed by sonication (Branson sonifier 450; 6 x 1 min; 50% output) in Buffer A (50 mM Tris.HCl pH 7.4, 50 mM KCl, 1 mM EDTA and 0.5 mM DL-dithiothreitol) containing 0.1 mM phenylmethylsulfonyl fluoride and the cellular debris removed (15 000 g). The supernatant was loaded onto a DEAE ion-exchange column, washed with Buffer A and each protein eluted during a 50-500 mM KCl gradient in Buffer A. The individual proteins were pooled and the amount of protein estimated by UV absorption. P450<sub>cin</sub> 415 nm ( $\epsilon = 150 \text{ cm}^{-1} \text{ mM}^{-1}$ )[17], Cdx 456 nm ( $\epsilon = 10825 \text{ cm}^{-1} \text{ M}^{-1}$ )[19] and Fpr at 456 nm ( $\epsilon = 7100 \text{ cm}^{-1} \text{ M}^{-1}$ ).[23]

**2.5 Evaluation of polycistronic constructs: Cineole oxidation** –Protein expression was performed as outlined above with the following modifications. The cell culture (1 L in 2.8 L Fernbach flask) was supplemented with cineole **1** (0.5 mL/L) concurrently to induction with isopropyl  $\beta$ -D-1-thiogalactopyranoside (1 mM). The cultures were incubated at 27.5°C for 43 h (approx. 180 rpm; Innova 4000, New Brunswick



Scientific). The cells were removed by centrifugation, the supernatant extracted with ethyl acetate and dried over  $\text{MgSO}_4$ . This was followed by GC-MS analysis as has previously been described.[17]  $\alpha$ -Terpineol was used as an internal standard to examine the progress of the oxidation over time.

**2.6 *In vitro* catalytic turnover** –A solution of P450<sub>cin</sub> (0.5 mM), Cdx (4 mM) and Fpr (1 mM) in Tris.HCl buffer (50 mM, pH 7.4) was prepared and the substrate was added in excess (5 mM in ethanol). Turnover was initiated by the addition of NADPH (0.2 mM – 5 mM) and the reaction incubated at room temperature with stirring for 30-60 min. The solution was then extracted into ethyl acetate, dried over  $\text{MgSO}_4$  and analysed by GC/MS.[17] Calculation of the dissociation constant ( $K_d$ ), NADPH consumption and coupling have all been outlined elsewhere.[17, 19, 24] In this study they were all performed in 100 mM potassium phosphate, pH 7.4. The percentage spin state change was calculated from the maximum absorbance shift observed in the presence of substrate (5 mM final concentration; 417 -392 nm) and standardized against P450<sub>cin</sub> with cineole.

**2.7 Large scale oxidation of cineole 1 (pCW-P450<sub>cin</sub>/Cdx)** – P450<sub>cin</sub> and Cdx were expressed as outlined above with the plasmid pCW-P450<sub>cin</sub>/Cdx and the following modifications. Cineole **1** (0.6 mL/L broth) was added to the culture (1 L in 2.8 L Fernbach flask) approximately every 12 h (approx. 180 rpm; Innova 40, New Brunswick Scientific). After three days of expression, a total of 11.6 mL of **1** had been added to 3.5 L of culture. The cells were pelleted at 4000 g for 30 min and the supernatant collected. NaCl was added to the supernatant, extracted with ethyl acetate, filtered through celite and dried over  $\text{MgSO}_4$ . The solvent was removed *in vacuo* and the crude material purified by flash chromatography (30% ethyl acetate in hexane) to

yield (1*R*)-6 $\beta$ -hydroxycineole **2** (3.1 g, 26%). Spectral data matched the literature.[24] mp. 91-92 C (Lit.[24] mp. 90-91 C) [ $\alpha$ ]<sub>D</sub> +29.1° (Lit.[24] [ $\alpha$ ]<sub>D</sub> -26.0° for enantiomer).

### 2.8 Large scale oxidation of (1*R*) and (1*S*)-camphor **3** (pCW-P450<sub>cin</sub>/Cdx) -

Oxidation of (1*R*) and (1*S*)-**3** were performed in an identical fashion. Camphor **3** (0.3 g/L broth) was added to the culture (1 L in 2.8 L Fernbach flask) approximately every 12 h (approx. 180 rpm; Innova 40, New Brunswick Scientific). After three days of expression, a total of 1 g/L of **3** had been added to 3.5 L of culture. **(1*R*)-camphor**: The crude material from ethyl acetate extraction of the culture was purified by flash chromatography in sequential stages to yield the hydroxycamphors. Firstly, a gradient of 5-100% ethyl acetate in hexane was used to fractionate the crude mixture. Two pools were collected: Pool 1 contained (1*R*)-**3** (1.1 g, 31% recovery of starting material); Pool 2 contained several hydroxycamphors. These were further purified by flash chromatography (30% ethyl acetate in hexane). Three mixed fractions containing hydroxycamphors were isolated and the structures of their components identified by GC/MS, <sup>1</sup>H and <sup>13</sup>C NMR. Fraction 1 (70 mg) contained a 4:1 mixture of 3-*exo*-hydroxycamphor **5** to 3-*endo*-hydroxycamphor **6**. Fraction 2 (2 mg) was identified as 6-*endo*-hydroxycamphor **7**. Fraction 3 (85 mg) contained 5-*exo*-hydroxycamphor **8**, 5-*endo*-hydroxycamphor **9** and 6-*endo*-hydroxycamphor **7** in a 3:3:1 ratio respectively. The yield of hydroxycamphors was approximately 6% based on recovered starting material.

Key data for metabolite identification:

3-*exo*-hydroxycamphor **5**: <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>)  $\delta$  0.93 (3H, s), 0.95 (3H, s), 0.99 (3H, s), 3.74 (1H, s). This is consistent with the literature.[25] 3-*endo*-

hydroxycamphor:  $^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ )  $\delta$  0.88 (3H, s), 0.94 (3H, s), 1.01 (3H, s), 4.20 (1H, dm,  $J = 5.0$  Hz). This is consistent with the literature.[26] **5** and **6** co-eluted by GC. MS (70 eV)  $m/z$  (%) 168 ( $\text{M}^+$ , 1.5), 153 (1.3), 140 (2.9), 125 (21), 100 (10), 84 (61), 83 (70), 55 (88), 41 (100).

5-*endo*-hydroxycamphor **9**:  $^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ )  $\delta$  0.82 (3H, s), 0.83 (3H, s), 0.97 (3H, s), 4.60 (1H, apparent dtd,  $J = 10$  Hz, 4 Hz, 2 Hz).  $^{13}\text{C}$  NMR (125MHz,  $\text{CDCl}_3$ )  $\delta$  69.3. MS (70 eV)  $m/z$  (%) 168 ( $\text{M}^+$ , 2), 153 (37), 150 (0.5), 135 (6), 125 (2), 108 (41), 107 (39), 93 (42), 70 (15), 55 (36), 41 (100). This is consistent with literature data.[27]

6-*endo*-hydroxycamphor **7**:  $^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ )  $\delta$  0.80 (3H, s), 0.95 (6H, s), 4.15 (1H, dd,  $J = 9.5$  Hz, 2.5 Hz).  $^{13}\text{C}$  NMR (125 MHz,  $\text{CDCl}_3$ )  $\delta$  63.9. MS (70 eV)  $m/z$  (%) 168 ( $\text{M}^+$ , 5), 153 (29), 135 (6), 125 (5), 108 (33), 107 (33), 93 (29), 81 (14), 69 (24), 55 (55), 41 (100). This is consistent with the literature.[28]

5-*exo*-hydroxycamphor **8**:  $^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ )  $\delta$  0.81 (s, 3H), 0.89 (s, 3H), 1.22 (s, 3H), 3.98 (1H, dd,  $J = 3.5$  Hz, 7.5 Hz).  $^{13}\text{C}$  NMR (125 MHz,  $\text{CDCl}_3$ )  $\delta$  74.3. MS (70 eV)  $m/z$  (%) 168 ( $\text{M}^+$ , 2), 153 (7), 125 (18), 111 (49), 83 (20), 69 (33), 55 (56), 41 (100). This is consistent with the literature.[27]

### 3. Results and Discussion

Three polycistronic plasmids were constructed that contained combinations of the P450<sub>cin</sub>, Cdx, cindoxin reductase (CdR) and *E. coli* Fpr genes (Table 1). The pCW vector was chosen to contain the polycistronic constructs as it has previously been shown to be effective for heterologous P450 expression in *E. coli*. In particular, it proved effective for the expression of both P450<sub>cin</sub> and Cdx.[17, 19] The first

construct investigated, pCW-P450<sub>cin</sub>/CdR/Cdx, was arranged identically to the native arrangement of the operon in *C. braakii* with expression of the encoded proteins inducible by the addition of IPTG.[17] It was hoped that this arrangement, using the natural upstream ribosome binding site and in the presence of P450<sub>cin</sub> and Cdx, may enable the expression of active CdR. CdR is the proposed natural terminal redox partner which has yet to be expressed in an active form despite significant effort.[19] However, SDS-PAGE analysis of crude cell lysates of *E. coli* transformed with this plasmid and induced with IPTG, indicated expression of the CdR gene only resulted in polypeptide that accumulated in the insoluble fraction. Despite this, the construct was able to oxidise cineole **1** to (1R)-6 $\beta$ -hydroxycineole **2** effectively as determined by GC/MS analysis of culture extracts (Table 1). We hypothesise that this oxidation is supported by endogenous Fpr in *E. coli*, mimicking the *in vitro* system we had previously reported.[19]

To establish that a small amount of CdR was not being expressed in active form and hence supporting the observed P450<sub>cin</sub> activity, a second plasmid, pCW-P450<sub>cin</sub>/Cdx, was constructed in which approximately 75% of the CdR gene had been deleted. Expression in *E. coli* produced a system that was again capable of cineole oxidation and with a similar efficiency to that observed with the pCW-P450<sub>cin</sub>/CdR/Cdx tricistronic construct. Thus, it appeared that cineole **1** oxidation observed *in vivo* is supported by the endogenous Fpr from the *E. coli* host.

With this result in mind, a third plasmid, pCW-P450<sub>cin</sub>/Fpr/Cdx, was constructed in order to supplement native Fpr expression in *E. coli* and ensure that P450 activity was not limited by low levels of endogenous Fpr within the cell. Surprisingly, however, poor oxidation of **1** was observed in this whole-cell system (Table 1). Employing

both SDS-PAGE and estimating protein content by UV it was observed that over-expression of Fpr had occurred. However, it effectively diminished Cdx expression (Table 1), presumably in part by competition for flavin cofactors. Interestingly, P450<sub>cin</sub> concentration was also reduced during expression using the pCW-P450<sub>cin</sub>/Fpr/Cdx construct. Hence, the native Fpr expression in *E. coli* must be produced at the correct concentration to adequately support efficient *in vivo* oxidation.

The bicistronic pCW-P450<sub>cin</sub>/Cdx construct was then used to evaluate the biocatalytic viability of this system to produce useful quantities of oxidised metabolites. Naturally, the oxidation of cineole **1** to (1*R*)-6 $\beta$ -hydroxycineole **2** was investigated initially. Due to the poor aqueous solubility and volatility of **1**, aliquots were added at approximately 12 hour intervals to an *E. coli* culture expressing both P450<sub>cin</sub> and cindoxin from pCW-P450<sub>cin</sub>/Cdx to ensure that an adequate concentration of substrate was maintained. The production of 6 $\beta$ -hydroxycineole **2** was monitored by GC/MS analysis of culture extracts, employing  $\alpha$ -terpineol as an internal standard (Fig. 2). This revealed that the bicistronic system was actively oxidising substrate for approximately 40 hours, after which oxidation ceased. The 6 $\beta$ -hydroxycineole **2** was isolated and purified by extraction and subsequent column chromatography to yield approximately 1 g per litre of bacterial culture (26% based upon added **1**). The <sup>1</sup>H and <sup>13</sup>C NMR data for 6 $\beta$ -hydroxycineole **2** generated enzymatically matched that of the authentic standard synthesised previously.[24] Enantioselective GC analysis revealed that only the (1*R*)-**2** enantiomer had been formed. A small amount of (1*R*)-ketocineole **4** was also observed during the *in vivo* turnover of **1**, which presumably results from the oxidation of **2** (Scheme 1). This type of over-oxidation has also been observed in an analogous *in vivo* system using P450<sub>cam</sub>/PdR/Pdx where 5-oxocamphor was produced ultimately from camphor (1*R*)-**3**.[5]

(1*R*)-6 $\beta$ -hydroxycineole **2** can be synthesised chemically by the treatment of  $\alpha$ -(*S*)-terpineol with *m*-CPBA followed by treatment with an acid catalyst.[24] However, the somewhat variable enantiomeric purity of the starting material and the formation of unwanted side products make large scale production via this route unattractive. The use of P450<sub>cin</sub> as a biocatalyst shows significant potential as a new synthetic tool to generate enantiomerically pure (1*R*)-6 $\beta$ -hydroxycineole **2**. Although cineole **1** itself is a *meso* compound, hydroxylation by P450<sub>cin</sub> occurs only at the pro-*R* carbon generating enantiomerically pure (1*R*)-6 $\beta$ -hydroxycineole **2** with three new stereogenic centres.

### 3.1 Oxidation of unnatural substrates: (1*R*)- and (1*S*)-camphor

It was also important to determine if this *in vivo* P450<sub>cin</sub> catalytic system could be useful for compounds that were much poorer substrates than cineole **1**. Although not valuable synthetically, this would be useful for metabolite production for structural elucidation in the investigation of P450 mechanism and structural determinants of oxidation. This possibility was evaluated by exploring the *in vivo* oxidation of (1*R*)- and (1*S*)-camphor **3**, structural homologues of the natural substrate cineole **1**, as model systems. We had previously reported that the enantioselectivity of cineole oxidation was largely controlled by a hydrogen bond from the ethereal oxygen of **1** to asparagine 242 of P450<sub>cin</sub>. [29, 30] The regiochemistry of oxidation of (1*R*)-camphor **3** by P450<sub>cam</sub> is similarly controlled by a hydrogen bond between the camphor carbonyl and a protein residue, tyrosine 96.[31] This similarity in mechanism of regiocontrol led us propose that there may be interesting differential selectivity in the oxidation of (1*R*)- and (1*S*)-camphor by P450<sub>cin</sub>.

We first evaluated the interaction of (1*R*)- and (1*S*)-camphor **3** with P450<sub>cin</sub> *in vitro* and showed that they had the ideal characteristics for further work *in vivo*. Both (1*R*)- and (1*S*)-camphor **3** are able to access the active site of P450<sub>cin</sub> with reduced affinity for the enzyme as compared to cineole **1** (Over 100 fold difference in  $K_d$ ; Table 2). (1*R*)- and (1*S*)-**3** were also found to partially displace the water molecule normally coordinated to the heme in the resting active site of P450<sub>cin</sub> as monitored by the change in the Soret absorbance (from 417 nm to 392 nm);  $27 \pm 2\%$  ((1*R*)-**3**) or  $32 \pm 1\%$  ((1*S*)-**3**) efficient as cineole **1**. Catalytic turnover was investigated and it was shown that the rate of NADPH consumption by P450<sub>cin</sub> in the presence of (1*R*)- and (1*S*)-**3** was approximately a third of that observed with cineole **1** (Table 2). Additionally, only a quarter to a third of the NADPH consumed during (1*R*)- or (1*S*)-**3** oxidation by P450<sub>cin</sub> was directed to the production of a somewhat surprisingly complex mixture of hydroxycamphors. In sum, both (1*R*)- and (1*S*)-**3** was clearly a much poorer substrate for P450<sub>cin</sub> than cineole **1**. Based on these observations the catalytic turnover of camphor **3** appeared ideal for investigation with the *in vivo* P450<sub>cin</sub> system both to test whether it could produce metabolites in sufficient quantities for structure elucidation and to provide information as to the selectivity of camphor oxidation.

Oxidation of camphor **3** via a typical P450 catalysed oxidation reaction, oxygen insertion into a C-H bond, has the potential to generate ten different hydroxyl camphors. There are three methyl groups, three methylene groups with six diastereomeric positions and a single methine position that can be hydroxylated. Oxidation of (1*R*)-camphor **3** by P450<sub>cin</sub> using the whole cell oxidation system described above generated at least seven different oxidised (1*R*)-camphor **3** metabolites (Data not shown). The GC/MS trace of the crude extract revealed six

different metabolites with  $m/z$  168, which is expected for a hydroxycamphor as well as a peak with  $m/z$  166, required for an oxocamphor; the latter would result from a second oxidation of a hydroxycamphor. The products of *in vivo* transformation were extracted from the culture medium into ethyl acetate and partially purified by column chromatography. This resulted in the isolation of the five major (1*R*)-camphor **3** metabolites (Fig. 3: **5-9**) in three different pools in sufficient quantities to be unambiguously identified by a combination of GC/MS, GC and NMR.

GC/MS analysis of Pool 1 revealed only one peak with an  $m/z$  168. However, by  $^1\text{H}$  NMR it was apparent that two different hydroxycamphors were present in a 4:1 ratio: there were six signals corresponding to methyl groups and two downfield signals ( $\delta$  4.17 and 3.70 ppm) for hydroxymethine positions. The simplicity of the downfield signals suggested that hydroxylation had occurred on the parent camphor at C3, as this has few available vicinal coupling partners. The major isomer was 3-*exo*-hydroxycamphor **5**, providing the resonance at  $\delta$  3.70 ppm (*s*, H3); the absence of coupling is in accord with the dihedral angle of almost  $90^\circ$  to H4. The minor isomer was identified as 3-*endo*-hydroxycamphor **6** and corresponded to the resonance at  $\delta$  4.17 ppm (*dd J* 5.2, 1 Hz, H3). These assignments were confirmed by comparison of full NMR data with the literature[32, 33] (ESI). The epimeric 3-hydroxycamphors **5** and **6** were shown to be resolved when analysed by enantioselective GC ( $\beta$ -cyclodextrin), with **5** eluting first.

Pool 2 was found to contain only 6-*endo*-hydroxycamphor **7**, characteristically displaying a hydroxymethine at  $\delta_{\text{H}}$  4.14 ppm (*ddd J* 9.5, 2.5, 1 Hz, H6), the smallest coupling corresponding to long range coupling to H4. This assignment was confirmed by the correspondence of  $^1\text{H}$  and  $^{13}\text{C}$  data with literature data (ESI).[28, 34]



Three different hydroxycamphors were found in Pool 3, including a portion of **7** identified above. The remaining two compounds were readily identified by their diagnostic NMR data (ESI) as 5-*exo*-hydroxycamphor, **8** and 5-*endo*-hydroxycamphor, **9**. In particular, **8** exhibits a hydroxymethine resonance at  $\delta_{\text{H}}$  3.96 (*dd J* 7.2, 4.4 Hz) whilst **9** is characterized by a complex signal at  $\delta$  4.58 (*dddd J* 9.5, 3.5, 3.5, 1.5 Hz). Full NMR data of each compound determined on the mixture was in good agreement with that reported previously (ESI).[27]

With these results in hand, we investigated the P450<sub>cin</sub>-catalysed oxidation of (1*S*)-camphor **3**. The hydroxycamphors **5-9** isolated above would of course provide standards for (1*S*)-camphor metabolites analysed under non-enantioselective conditions. (1*S*)-**3** was oxidised under *in vitro* and *in vivo* conditions as described above and the products analysed by GC/MS. Unexpectedly, the product profile was remarkably similar to that observed with (1*R*)-**3** (Fig. 4). The major site for hydroxylation is at C3 in both (1*R*)- and (1*S*)-camphor **3** (approximately 50% of total oxidation) with the 3-*exo*-product ((1*R*)- and (1*S*)-**5**) the dominant isomer in both instances. The remainder of the oxidation products were mainly 5- and 6-*endo*-hydroxycamphors ((1*R*)- and (1*S*)-**7** and **9**) with a small amount of 5-*exo*-hydroxycamphor ((1*R*)- and (1*S*)-**8**). (Fig. 5)

The similarity in the P450<sub>cin</sub> metabolite profiles from (1*R*)- and (1*S*)-camphor and the large number of products formed strongly suggest that these substrates are highly mobile in the active site of the enzyme. Clearly, the H-bond formed to the ethereal oxygen of cineole bound to P450<sub>cin</sub> cannot be replicated in a similar interaction with the carbonyl of camphor, despite such an interaction controlling the regiochemistry of (1*R*)-camphor oxidation by P450<sub>cam</sub>.

The regiochemical outcome of P450 catalysed oxidation is dependent not only on the substrate's orientation in the active site but also on the inherent reactivity of the substrate itself (essentially C-H bond strength), or, the relative stabilities of the radicals formed from hydrogen atom abstraction. The major site of hydroxylation of both enantiomers of camphor is C3 and one rationalisation for this lies in the stabilities of the radicals produced: hydrogen abstraction at C3 is expected to generate the most stable carbon radical due to delocalisation of the unpaired electron into the neighbouring carbonyl group.[35] In contrast, the hydroxylation of (1*R*)- and (1*S*)-norcamphor hydroxylation by P450<sub>cam</sub>[36] primarily occurs at C5 in the (1*R*)-isomer and at C6 in the (1*S*)-isomer. This is proposed to be due to a hydrogen bond between the substrate ketone and Y96 of P450<sub>cam</sub>, controlling the substrate orientation in the enzyme-substrate complex. The apparent absence of such an orienting H-bond in P450<sub>cin</sub> means the reactivity of the substrate and stability of the intermediates become more important in determining the regiochemical outcome of the reaction.

#### 4. Conclusion

Several vectors for the polycistronic coexpression of P450<sub>cin</sub> and cindoxin with or without a terminal redox partner were constructed. The bicistronic plasmid containing only P450<sub>cin</sub> and cindoxin proved to be the most effective in the whole cell oxidation of cineole. When expressed in *E. coli* this plasmid was capable of oxidising cineole **1** to (1*R*)-6β-hydroxycineole **2** presumably utilising *E. coli*'s native flavodoxin reductase as the terminal redox protein. This system could produce approximately 1 g/L culture over a 48 h incubation and thus provides a simple and convenient way to make enantiomerically pure (1*R*)-6β-hydroxycineole **2** on a preparative scale. The oxidation of unnatural substrates was also investigated with a

view to developing this *in vivo* P450<sub>cin</sub> system as a biocatalyst and a tool for investigating P450 mechanism. P450<sub>cin</sub> oxidised both (1*R*)- and (1*S*)-camphor with little regioselectivity but sufficient material was isolated from *in vivo* transformation to allow structural characterisation of the products. The metabolite profiles from the oxidation of (1*R*)- and (1*S*)-camphor were surprisingly similar. This highlights the importance of the inherent reactivity of the molecule in determining the regiochemical outcome of P450 catalysed oxidation when substrate orientation within the active site is not controlled.

In the future, construction of a bicistronic vector with the recently described P450<sub>cin</sub>-N242A mutant,[37] which introduces oxygen at the pro-*S* carbon (90% *ee*), may enable the preparative scale production of the enantiomeric (1*S*)-6 $\beta$ -hydroxycineole **2**. The ease with which preparative amounts of enantiopure hydroxycineole from the original P450<sub>cin</sub> bicistronic system can be generated provides easy access to a large range of cineole derivatives via functionalization of the newly added hydroxyl moiety. We plan to make use of such derivatives as probes for studying the mechanism of P450s but they may also be useful as antimicrobial or bactericidal agents,[20] or as fragrances in the perfume industry.[21]

## Acknowledgements

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**Table 1** Evaluation of polycistronic constructs

Construct	P450 <sub>cin</sub> (nmol/L <sup>a</sup> )	CdR/Fpr (nmol/L)	Cdx (nmol/L)	Cineole oxidation
pCW-P450 <sub>cin</sub> /CdR/Cdx	560	NM <sup>b</sup>	1700	Yes
pCW-P450 <sub>cin</sub> /Cdx	510	NA <sup>c</sup>	2000	Yes
pCW-P450 <sub>cin</sub> /Fpr/Cdx	380	4000	NM	Poor

<sup>a</sup> nmol per litre of culture expressed; <sup>b</sup> NM –Not measurable; <sup>c</sup> NA –Not applicable

**Table 2** *In vitro* comparison of catalytic turnover of cineole and (1*R*)- and (1*S*)-camphor by P450<sub>cin</sub><sup>a</sup>

	Spin change <sup>b</sup> (%)	K <sub>d</sub> ( $\mu$ M)	NADPH consumption (%)	Coupling <sup>c</sup> (%)
Cineole	100	0.7[1]	100	80 $\pm$ 2
(1 <i>R</i> )-camphor	27 $\pm$ 2	101 $\pm$ 4	33 $\pm$ 1	35 $\pm$ 2
(1 <i>S</i> )-camphor	32 $\pm$ 1	81 $\pm$ 5	29 $\pm$ 1	25 $\pm$ 3

<sup>a</sup> See Experimental Section for condition details; <sup>b</sup> Both the percentage spin state and the rate of NADPH consumption were standardised against cineole with P450<sub>cin</sub> (Assuming P450<sub>cin</sub>:cineole is 100%); <sup>c</sup> Ratio of the amount of NADPH consumed compared to the amount of product formed.

[1] D.B. Hawkes, G.W. Adams, A.L. Burlingame, P.R. Ortiz de Montellano, J.J. De Voss, *J. Biol. Chem.* 277 (2002) 27725-27732.

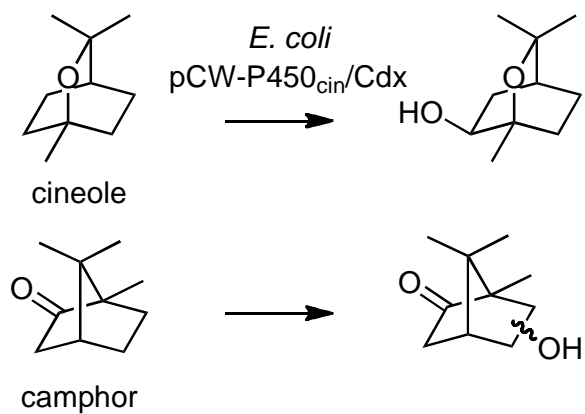


## Highlights

1. *In vivo* system for utilising P450cin (CYP176A1) as oxidative catalyst
2. Enantiomerically pure (1*R*)-6 -hydroxycineole (1 g/L culture) produced
3. (1*R*)- and (1*S*)-camphor metabolites also generated for structure elucidation

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## Graphical Abstract



**Scheme 1** P450<sub>cin</sub> oxidation of cineole **1** to produce (1*R*)-6β-hydroxycineole **2**. Under certain conditions, over-oxidation by P450<sub>cin</sub> can result in the production of ketocineole **4**.

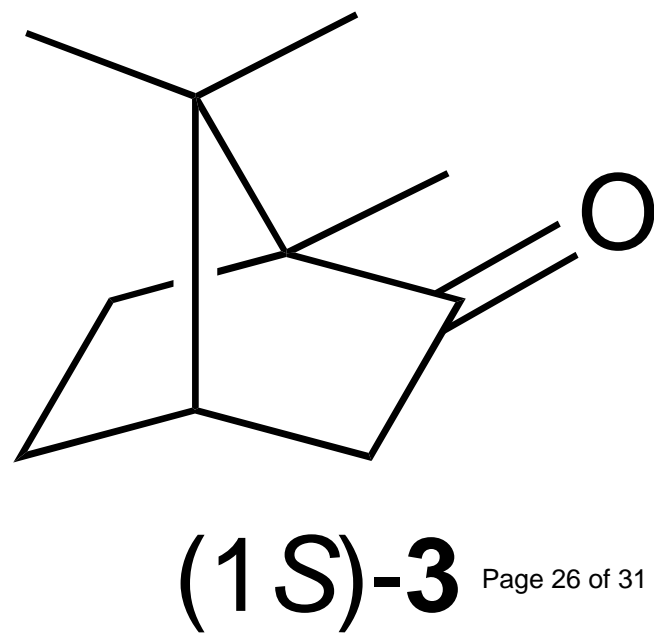
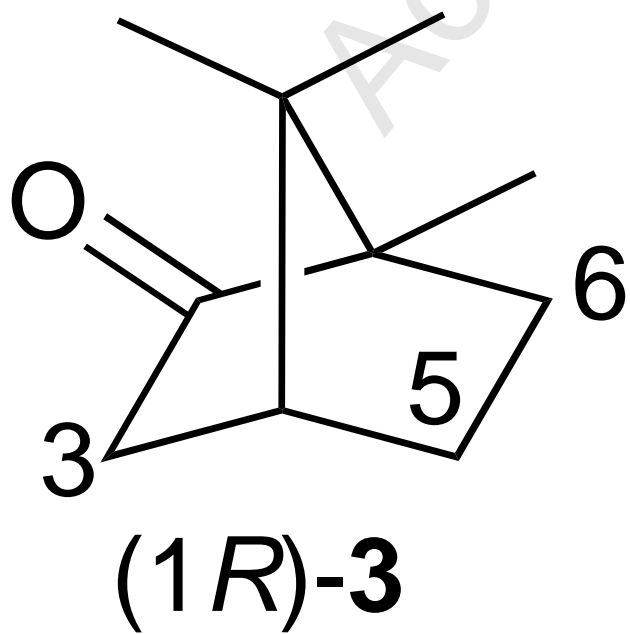
**Figure 1** The two enantiomers of camphor (1*R*) and (1*S*)-**3** used in this study.

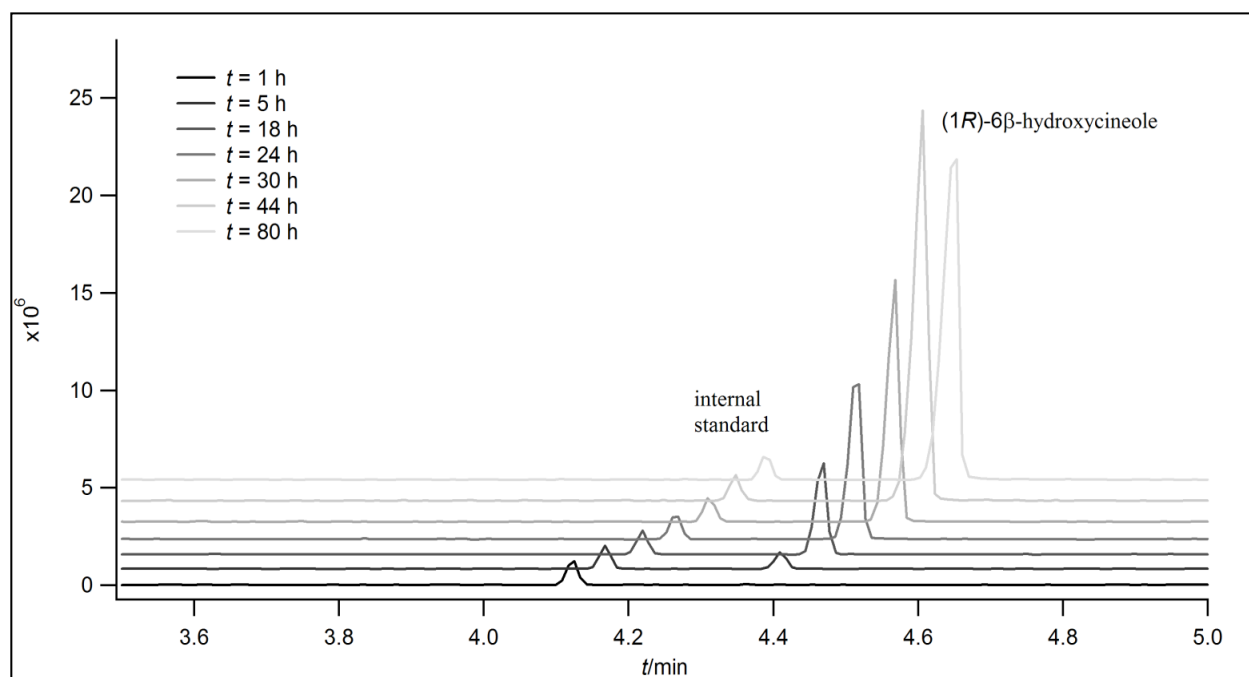
**Figure 2** Monitoring (1*R*)-6β-hydroxycineole **2** production by GC/MS using the *in vivo* system expressing both P450<sub>cin</sub> and Cdx.

**Figure 3** Hydroxycamphors ((1*R*)-**5-9**) identified from P450<sub>cin</sub> *in vivo* turnover of (1*R*)-camphor **3**.

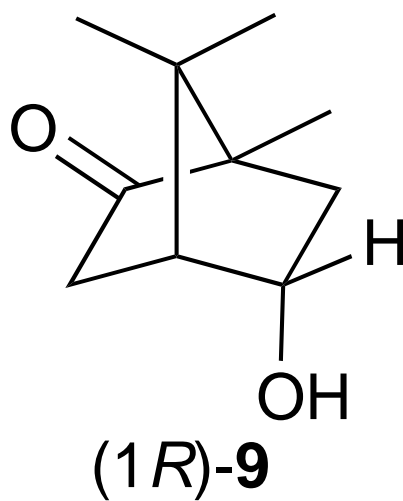
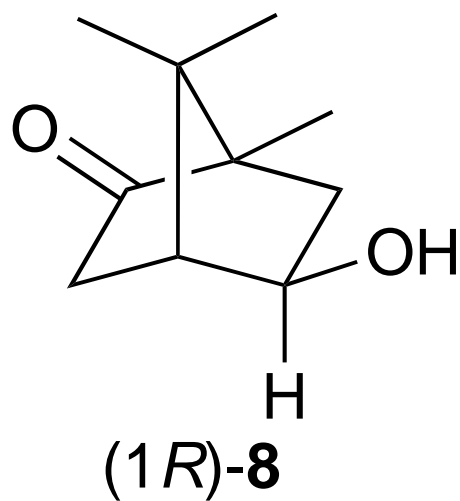
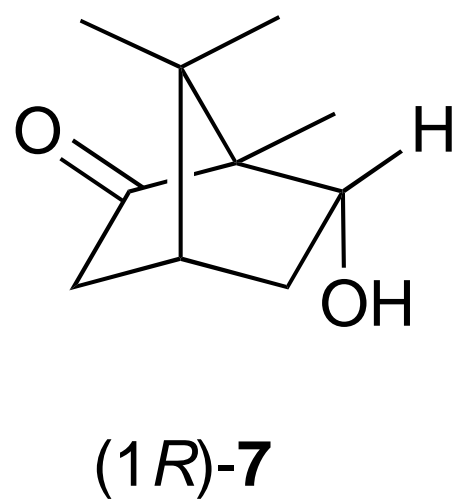
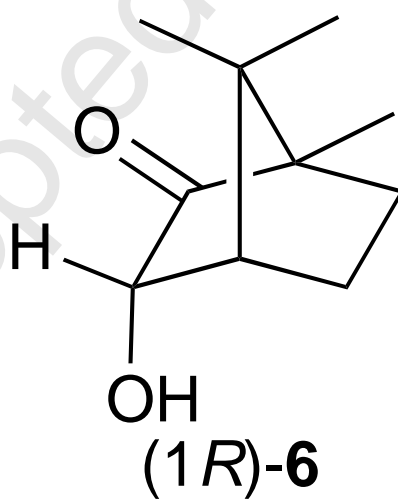
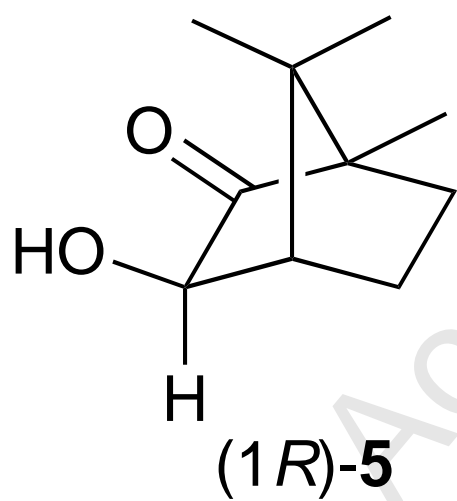
**Figure 4** GC/MS trace comparing the oxidation products of (1*R*)- and (1*S*)-camphor **3** of catalytic turnover by P450<sub>cin</sub>.

**Figure 5** Oxidation profile of both (1*R*)- and (1*S*)-camphor by the *in vivo* P450<sub>cin</sub> expression system.

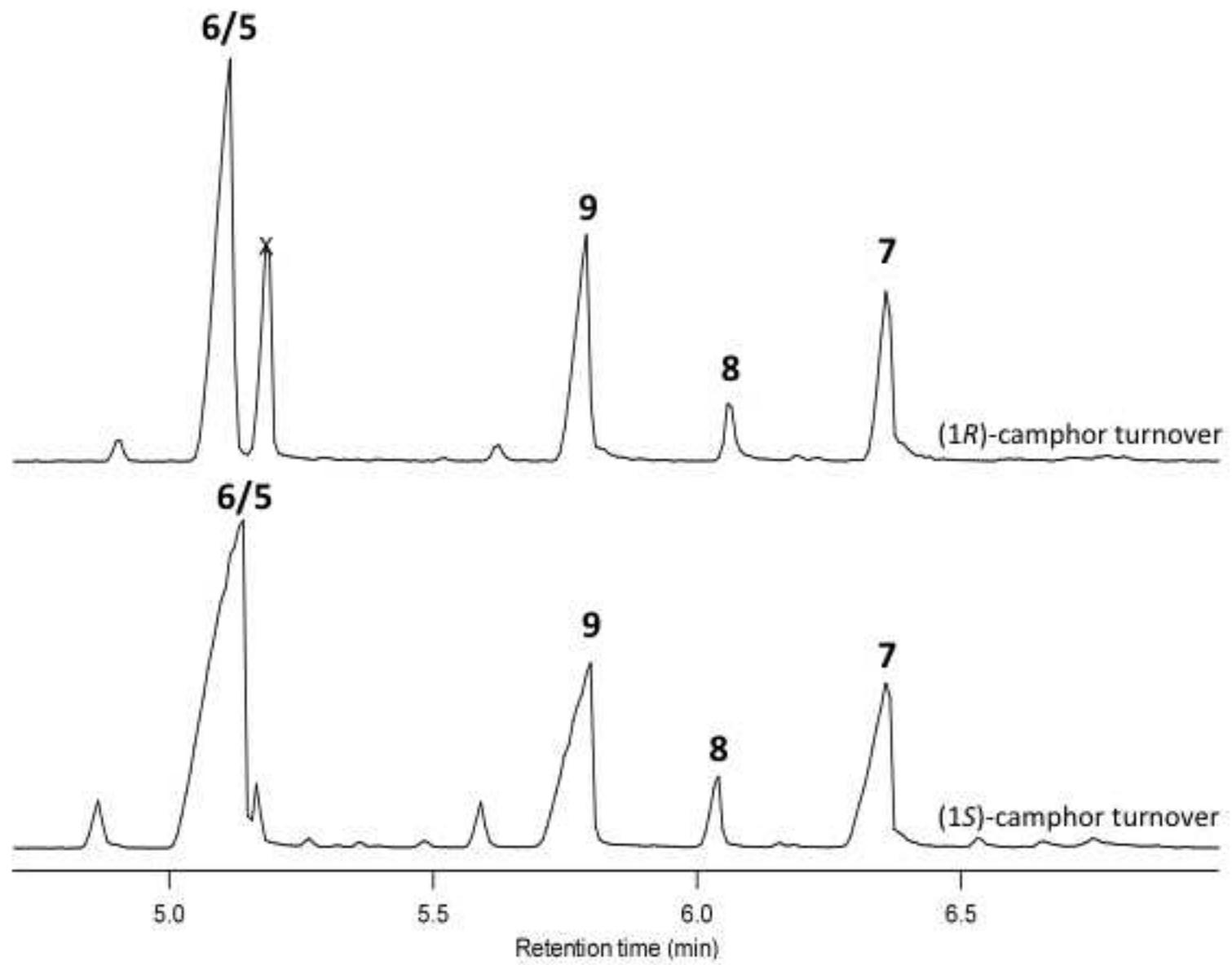


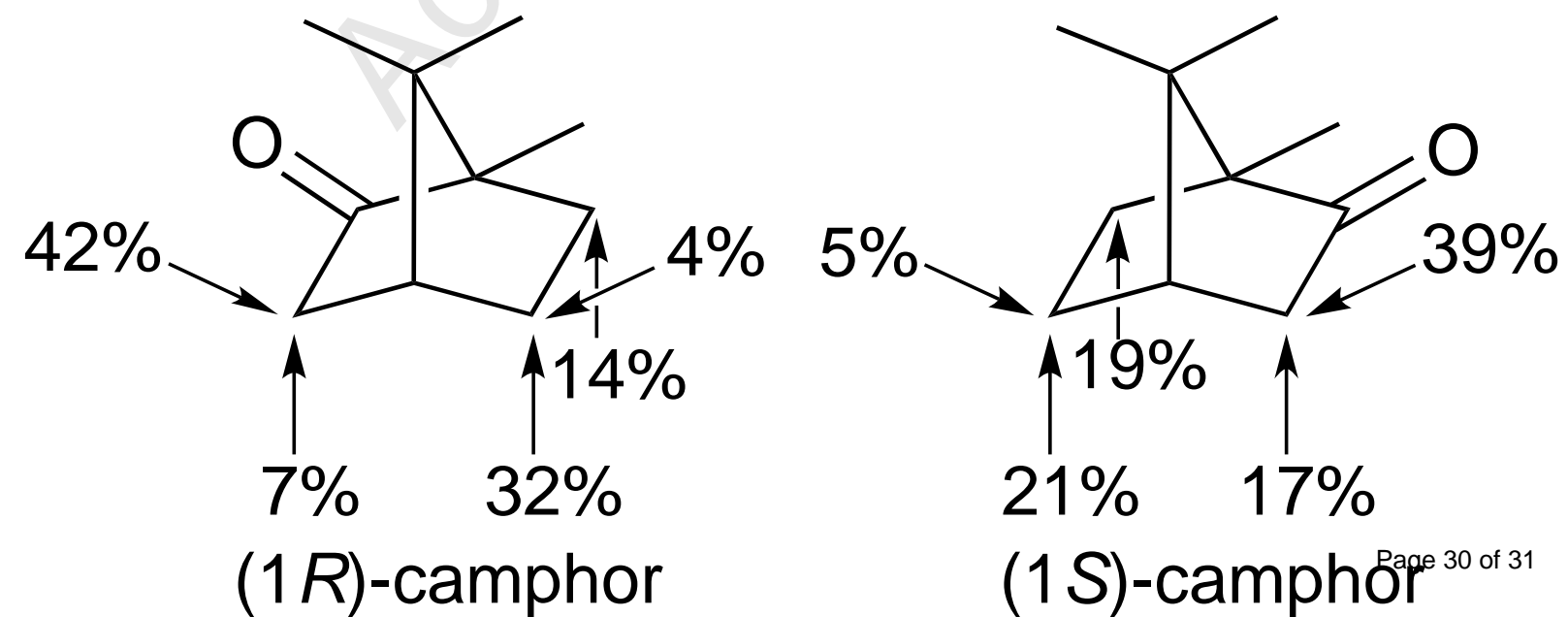


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