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# An *in vivo* cytochrome $P450_{cin}$ (CYP176A1) catalytic system for metabolite production

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ABSTRACT

Cytochrome $P450_{cin}$ (CYP176A1) is a bacterial P450 isolated from *Citrobacter braakii* that catalyses the hydroxylation of 1,8-cineole to (1*R*)-6β-hydroxycineole. $P450_{cin}$ 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 $P450_{cin}$, cindoxin and *E. coli* flavodoxin reductase and a bicistronic plasmid that encodes only $P450_{cin}$ 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*)-6β-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.



## 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 $P450_{cam}$ [5,8,12] and $P450_{BM-3}$ [10,11,14,15]. Cytochrome $P450_{cam}$ catalyses the hydroxylation of (1*R*)-camphor to 5-exo-hydroxycamphor. It has been demonstrated that the expression of $P450_{cam}$, 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 $P450_{cam}$/Pd/PdR whole-cell system has been investigated with non-natural substrates including limonene [8]. $P450_{BM-3}$, 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 ω-1, ω-2 and ω-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 $P450_{cam}$ and $P450_{BM-3}$ have also been engineered to enhance oxidation of non-natural substrates by introducing specific mutations [5,16]. For example, the $P450_{cam}$ Y96F-V247L mutant *in vivo* system

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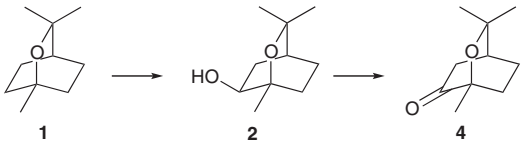


**Scheme 1.** $P450_{cin}$ oxidation of cineole **1** to produce (1*R*)-6β-hydroxycineole **2**. Under certain conditions, over-oxidation by $P450_{cin}$ can result in the production of ketocineole **4**.

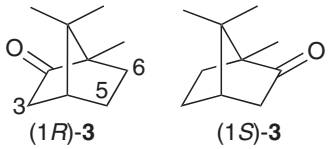


**Fig. 1.** The two enantiomers of camphor (1*R*) and (1*S*)-**3** used in this study.

was observed to successfully catalyse the turnover of styrene and ethylbenzene [5].

$P450_{cin}$ (CYP176A1) is a soluble, microbial P450 isolated from *Citrobacter braakii* that catalyses the hydroxylation of cineole **1** to (1*R*)-6β-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 $P450_{cin}$ 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 $P450_{cin}$ system. These constructs enabled the preparative scale bioproduction of enantiopure (1*R*)-6β-hydroxycineole **2** in a single oxidative step from cineole **1**, mimicking the natural transformation catalysed by $P450_{cin}$. We also demonstrate that this system has potential for the *in vivo* transformation of compounds that are much poorer substrates for $P450_{cin}$ 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* $P450_{cin}$ 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-$P450_{cin}$/CdR/Cdx

pUC19-Cdx [19] was digested with *Sph*I and *Bam*HI, effectively removing one *Pst*I site from the multiple cloning site, and retaining the second *Pst*I site found within the Cdx gene. This plasmid was treated with DNA polymerase I (Klenow fragment) to create blunt ends and re-ligated. A *Pst*I–*Pst*I 4 kb fragment generated from pC1 [17] (containing $P450_{cin}$, CdR and 98% of the Cdx gene) was ligated into the altered *Pst*I cut pUC19-Cdx to form pUC19-C3. The *Pst*I fragment was oriented using *Ppu*MI/*Hind*III which removes a 3 kb fragment if inserted correctly. This 3 kb *Ppu*MI/*Hind*III fragment was then inserted into pUCPKS-*cinA* [17] which was similarly digested with *Ppu*MI/*Hind*III to create pUC-PHC3. pUC-PHC3 was then digested with *Nde*I/*Hind*III and the fragment cloned into similarly cut pCW to yield the tricistronic plasmid pCW-$P450_{cin}$/CdR/Cdx.

### 2.2. Construction of pCW-$P450_{cin}$/Cdx

pCW-$P450_{cin}$/CdR/Cdx was digested with *Bst*BI 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-$P450_{cin}$/Cdx.

### 2.3. Construction of pCW-$P450_{cin}$/Fpr/Cdx

pET11a-Fpr [22] was digested with *Xba*I and *Bam*HI to excise the Fpr gene. The fragment was treated with DNA polymerase I and ligated into pCW-$P450_{cin}$/Cdx which had been digested with *Bst*BI 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-$P450_{cin}$/Fpr/Cdx.

### 2.4. Evaluation of polycistronic constructs: expression

A polycistronic plasmid (either pCW-$P450_{cin}$/CdR/Cdx, pCW-$P450_{cin}$/Cdx or pCW-$P450_{cin}$/Fpr/Cdx) was transformed into *E. coli* DH5αF′IQ and used to inoculate Terrific broth (1 L in 2.8 L Fernbach flask) containing ampillicin (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 and protein expression was induced with isopropyl β-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 × 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. $P450_{cin}$ 415 nm ($\varepsilon = 150\ cm^{-1}\ mM^{-1}$) [17], Cdx 456 nm ($\varepsilon = 10{,}825\ cm^{-1}\ M^{-1}$) [19] and Fpr at 456 nm ($\varepsilon = 7100\ cm^{-1}\ M^{-1}$) [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 β-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 centifugation, the supernatant extracted with ethyl acetate and dried over $MgSO_4$. This was followed by GC/MS analysis as has previously been described [17]. α-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 $P450_{cin}$ (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 (0.2–5 mM) and the reaction incubated at room temperature with stirring for 30–60 min. The solution was then extracted into ethyl acetate, dried over $MgSO_4$ 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 standardised against $P450_{cin}$ with cineole.