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CYTOCHROME P450$_{cin}$ (CYP176A): ISOLATION, EXPRESSION AND CHARACTERISATION

David B. Hawkes[¶], Gregory W. Adams[§], Alma L. Burlingame[§], Paul R. Ortiz de Montellano[§] and James J. De Voss[*¶]

[¶]*Department Of Chemistry, University Of Queensland, Brisbane Queensland Australia 4067*

[§] *Department Of Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco California 94143-0446*

Address Editorial Correspondence to:

Dr. James J. De Voss

Department of Chemistry

University of Queensland

St. Lucia Queensland Australia 4067

Email: devoss@chemistry.uq.edu.au

FAX: 61 7 3365 4299

Telephone 61 7 3365 3825

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[*] To whom correspondence should be addressed. Email: devoss@chemistry.uq.edu.au

[1] The abbreviations used are: P450, cytochrome P450; Cd, cindoxin; CPR, cytochrome P450 reductase; $P450_{cin}$, CYP176A1; $P450_{cam}$, CYP101; $P450_{terp}$, CYP108; $P450_{BioI}$, CYP107H; *RZ*, (A_{415nm}/A_{280nm}) used when determining the purity of P450 samples (> is better); ORF, open reading frame; K_D, dissociation constant; IPTG, isopropyl-β-D-thiogalactopyranoside; PMSF, phenylmethylsulfonyl fluoride; DTT, DL-Dithiotheitol; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; Amp, ampicillin; TB, Terrific broth; cineole, 1,8-cineole. Degenerate DNA code used: R=A or G; K=G or T; H=A, C or T; M=A or C; N=A, T, C or G.

Abstract: Cytochromes P450 are members of a superfamily of hemoproteins involved in the oxidative metabolism of various physiologic and xenobiotic compounds in eukaryotes and prokarayotes. Studies on bacterial P450s, particularly those involved in monoterpene oxidation, have provided an intergral contribution to our understanding of these proteins, away from the problems encountered with eukaryotic forms. We report here a novel cytochrome P450 ($P450_{cin}$, CYP176A1) purified from a strain of *Citrobacter braakii* which is capable of using cineole **1** as its sole source of carbon and energy. This enzyme has been purified to homogeneity and the amino acid sequences of three tryptic peptides determined. Using this information, a PCR-based cloning strategy was developed that allowed the isolation of a 4kb DNA fragment containing the cytochrome $P450_{cin}$ gene (*cinA*). Sequencing revealed three open reading frames which were identified on the basis of sequence homology as a cytochrome P450, an NADPH dependent flavodoxin/ferrodoxin reductase and a flavodoxin. This arrangement suggests that $P450_{cin}$ may be the first isolated P450 to use a flavodoxin as its natural redox partner. Sequencing also identified the unprecedented substitution of a highly conserved catalytically important, active site threonine with an asparagine residue. The P450 gene was subcloned and heterologously expressed in *E. coli* at approximately 2000 nmol/l of original culture and purification was achieved by standard protocols. Postulating the native *E. coli* flavodoxin/flavodoxin reductase system might mimic the natural redox partners of $P450_{cin}$, it was expressed in *E. coli* in the presence of cineole **1**. A product was formed *in vivo* that was tentatively identified by GCMS as 2-hydroxycineole **2**. Examination of $P450_{cin}$ by UV/visible spectroscopy revealed typical spectra characteristic of P450s, a high affinity for cineole **1** (K_D = 0.7 μM), and a large spin state change of the heme iron associated with binding of cineole **1**. These facts support the hypothesis that cineole **1** is the natural substrate for this enzyme and that P450cin catalyses the initial monooxygenation of cineole **1** biodegradation. This constitutes the first characterization of an enzyme involved in this pathway.

INTRODUCTION

The cytochrome P450s (P450s) are a superfamily of oxidative hemoproteins (1,2) that carry out an enormous variety of oxidative transformations. These range from simple alkene epoxidation and heteroatom oxidation all the way through oxygen insertion into unactivated C-H bonds and C-C bond cleavage (3). P450s are broadly categorized as either soluble, e.g., most bacterial P450s, or as membrane bound/microsomal monooxygenases, e.g., most eukaryotic P450s. All P450s, however, contain a prosthetic heme group that is ligated to the protein backbone by a cysteinyl sulfur coordinated to the heme iron. It is this heme-thiolate moiety that is responsible for much of the chemistry carried out by these enzymes. The typical reaction catalyzed by a P450 is given by the following equation:

$$RH + NAD(P)H + H^+ + O_2 \rightarrow ROH + NAD(P)^+ + H_2O$$

The exact mechanism of the oxidation reaction is still the subject of much debate, despite an enormous amount of work (3,4). The electrons derived from the nicotinamide cofactors are generally transferred to the P450 one at a time by auxiliary redox protein(s) that are characteristic of the origin of the enzymes. Thus, bacterial P450s generally employ an FAD dependent ferrodoxin reductase and an iron sulfur protein to mediate this transfer while microsomal P450s employ a single protein that contains both an FAD and an FMN domain.

Investigations of bacterial P450s have provided much of our current understanding of P450 mechanism and structure. This is particularly true of $P450_{cam}$ isolated from *Pseudomonas putida*, in part because it was the first P450 available in relatively large amounts (5). However, some features of $P450_{cam}$ appear to be unique to this enzyme, e.g., a potassium binding site (6), and not typical of this enzyme class. $P450_{cam}$ still remains the most studied of all P450s and one of the few bacterial enzymes of this family that has been heterologously expressed with its redox partners as a catalytically active system (7). It is a biodegradative enzyme that catalyses the conversion of camphor to 5-*exo*-hydroxycamphor, which initiates a cascade of biodegradative reactions, that allow *P. putida* to live on camphor as its sole source of carbon and energy (8). $P450_{cam}$ and others involved in such pathways e.g. $P450_{lin}$ (9), $P450_{terp}$ (10) are generally

characterized by high substrate specificity and high rates of turnover. As such, they make attractive starting points for attempting to design biocatalysts that can carry out synthetically useful oxidative transformations. We wished to isolate a new bacterial biodegradative P450 system that could be i) used for comparison with $P450_{cam}$; ii) heterologously expressed as a catalytically active system and iii) used as the basis for constructing libraries of P450s that would be explored for their ability to catalyze useful oxidative transformations. Such a P450 system was implicated in the bacterial utilization of 1,8-cineole (cineole, **1,** Scheme 1).

Eucalypt trees produce a mixture of nearly 50 hydrocarbons termed the "essential oil" which contains extremely high concentrations of the monoterpene cineole **1**. The role of cineole **1** is somewhat unclear, though it is believed to be involved in a number of functions including defense against herbivores (11,12) and pathogens (13), attracting pollinators and fruit-dispersing animals (14) and/or as an allelopathic agent (15,16). The Australian eucalypt population collectively produces and releases an estimated 500,000 tons of cineole **1** into the environment annually (17). Studies indicate that the major part of the released cineole **1** is rapidly removed by microbial oxidation (18), with lesser quantities consumed by native wildlife (19) and bushfires.

In 1979 R. MacRae isolated a soil microorganism identified as *P. flava*, which could utilize cineole **1** as it sole source of carbon and energy (18) and oxidized derivatives of cineole **1** were identified in the growth medium. Subsequently, Trudgill *et al* identified a *Rhodococcus* species also capable of using cineole **1** as its sole carbon source, and similarly identified oxygenated cineole derivatives (20). Interestingly, it was found that the initial oxidation of cineole **1** by the two organisms produced enantiomeric forms of 2-hydroxycineole **2**. A metabolic pathway for the catabolism of cineole **1** in *Rhodococcus sp.*C1 was proposed, based on the isolated metabolites (Scheme 1). Thus, cineole **1** is initially oxidized to 2-hydroxycineole **2** by a monooxygenase and further transformations lead to a highly functionalized hydroxyketo acid **3** that may spontaneously form the isolated dihydrofuranone **4** or be incorporated into central metabolic pathways.

Despite the relatively large amount of information concerning the chemistry of the cineole **1** biodegradative pathway, nothing was known about the enzymes involved. Early attempts at isolating the proposed monooxygenase that catalyses cineole **1** oxidation failed (21). Several lines of evidence suggested, however, that the monooxygenase might be a P450. The biodegradative utilization of a number of monoterpenes, including camphor, linalool and terpineol have all been shown to be initiated by oxidations catalyzed by P450s (21). Additionally, CYP3A4 was recently identified as being the main P450 isoform responsible for cineole **1** metabolism in humans, with the primary product, 2-hydroxycineole **2**, being the same as the one implicated in bacterial biodegradation (22). Finally, the relative scarcity of other enzymes e.g. non-heme iron oxygenases capable of carrying out such an oxidation also suggested a P450 would be involved.

We thus set out to isolate and characterize a monooxygenase system involved in the bacterial utilization of cineole **1**. Reported here is the isolation, over-expression and purification of a P450, $P450_{cin}$, believed to be responsible for cineole **1** oxidation. In addition, the sequences of two genes that may encode the required redox partners for $P450_{cin}$ are described along with preliminary characterization of the P450 that supports its involvement in the biodegradation of cineole **1**.

[INSERT SCHEME 1 HERE]

EXPERIMENTAL PROCEDURES

Materials-Yeast extract and Bacto-tryptone were obtained from Becton Dickinson and Co. (Cockeysville, MD). Reagent grade 1,8-cineole was obtained from Aldrich. Sodium dithionite (sodium hydrosulfite) was purchased from Aldrich Chemical Co. Inc. (Milwaukee, WI). Nylon hybridization transfer membranes (GeneScreen Plus) were obtained from NEN™ Life Science Products (Boston, MA). All other chemicals used were purchased from commercial sources and were of the highest grade generally available. The restriction enzymes and polynucleotide kinase were obtained from New England Biolabs, Inc. (Beverly MA), and Boehringer Mannheim Corp. Biochemical Products (Indianapolis, IN) based on availability. Terminal deoxynucleotidyl transferase was obtained from Promega Corporation (Madison, WI). *Escherichia coli* strain DH5αF' ϕ80d*lac*ZΔM15 Δ(l*ac*ZYA-*arg*F) U169 *deo*R *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44 λ^- *thi*-1 *gyr*A96 *rel*A1, was obtained as frozen competent cells, and from Life Technologies. Inc. (Gaithersburg. MD). Sonication was achieved using a Branson Sonicator set to 50% duty cycle.

Isolation of the microorganism- Soil samples were collected from several sites beneath eucalypt trees within a small eucalypt forest at the University of California San Francisco, USA. A dirt/water slurry prepared from each sample was transferred to minimal medium (23) and bacteria selected for an ability to use 1,8-cineole as the sole carbon source. Following several rounds of serial culturing (1 into 50 dilutions) at 30°C, the cell culture was spread onto minimal medium plates (1.5% agar plates) with 0.5 g of cineole in the lid, yielding a single pure strain according to the protocol of Peterson *et al*. (23). The strain was propagated in 10-15 ml of minimal medium with 20μl of 1,8-cineole at 30 °C.

Tryptic Digest of $P450_{cin}$ - Internal amino acid sequence for $P450_{cin}$ was obtained from tryptic peptides of $P450_{cin}$. These were purified by microbore HPLC and sequenced using automated Edman degradation protein sequencer. This data was used in concert with that obtained via MALDI mass spectrometry to yield the sequence of three fragments (Table I).

[INSERT TABLE 1 HERE]

Preparation of Whole Cell DNA– Cells were grown on minimal medium (23) with 1,8-cineole as the sole carbon source at 30 °C. Whole cell DNA was prepared according to Peterson (24).

Generation of an oligonucleotide probe to cytochrome P450$_{cin}$- A 92 bp PCR product was amplified from whole cell DNA (*C. braakii*) using degenerate primers (5'-ATAT GAATTC TGG GC**N** AT**M** CAN CA**H** GT-3' and 5'-TATC GGATCC C**K** **N**GC **R**TG **N**GC **N**AC-3') designed based upon five amino acids at either end of Fragment 1 (Fig. 1). PCR was performed according to the following protocol; 94 °C 3 min; [94 °C 1 min, ramp to 37 °C over 2 min, 72 °C 1 min] x 5 cycles; [94 °C 1 min, 55 °C 1 min, 72 °C 1 min] x 25 cycles; 72 °C 10 min; 4 °C. The product was treated with *Bam*HI and *Eco*RI, and cloned into similarly cut pCRII™ (Invitrogen). The insert sequenced on an automated sequencer using M13 forward and reverse primers, confirming the central non-degenerate 42 bp coded for the same fourteen amino acids seen in Fragment 1 (Table 1). The 92 bp insert was labeled with [α-^{32}P] dCTP using terminal deoxynucleotidyltransfease (25) and denatured at 100 °C prior to use in subsequent hybridizations.

[INSERT FIGURE 1 HERE]

Library Construction and Screening – DNA isolated from *C. braakii* was digested with a bank of restriction enzymes (*Kpn*I, *Pst*I, *Sal*I, *Pst*I/*Hind*III, *Sal*I/*Hind*III, *Xho*I) and separated by electrophoresis on an agarose gel (0.7% w/v, 0.5 x TBE). The DNA was transferred to a nylon membrane (GeneScreen Plus NEN™) by capillary transfer, dried and probed with labeled oligonucleotide (26). Autoradiography revealed bands of suitable size in *Pst*I (4 kb) and *Sal*I (3 kb) digests. These latter digests were repeated, separated by electrophoresis and the bands of appropriate size recovered by gel extraction.

The recovered DNA fragments were ligated into *Pst*I or *Sal*I digested pUC19 and transformed individually into library efficiency DH5α cells producing *Pst*I and *Sal*I libraries. Approximately 4,000-5,000 recombinant cells for each fragment were plated onto 2 x YT plates containing ampicillin (50 μg/ml) and incubated overnight. Colonies were lifted onto nylon

membranes and their DNA fixed by successive treatments with 0.5 M NaOH, 1.5 M NaCl (4 min), 1 M TrisHCl, pH 7.4, 1.5 M NaCl (4 min) and 50 mM TrisHCl, pH 8.5, 50 mM EDTA, 100 mM NaCl, 1% sodium laurylsarcosine and 250 μg/ml proteinase K (20 min). Membranes were dried and the DNA crosslinked via UV irradiation. The membranes were screened by hybridization using the 92 bp oligonucleotide probe, labeled with ^{32}P as described above. Putative positive clones were picked from the original plates, grown overnight in 2 x YT medium containing ampicillin (50 μg/ml). The identities of the clones were confirmed by restriction analysis and southern hybridization. A positive *Pst*I (4 kb) clone (designated pC1) was identified. Restriction analysis of pC1 revealed the *Sal*I (3 kb) fragment (cloned as pC2) was contained entirely within the *Pst* (4 kb) fragment and so its isolation was not pursued further. To gain further sequence information, probes generated by the random primer method (27) using the pC1 insert as template were used to probe an *Eco*RI/*Sph*I digest of *C. braakii* DNA. An *Sph*I (2.9 kb) and *Eco*RI/*Sph*I (5.7 kb) fragment were isolated and cloned into pUC19 to give pC3 and pC4 respectively (Fig. 2).

Sequencing- Careful restriction analysis of clones pC1 and pC4 identified a series of small internal fragments that were subcloned and sequenced using the M13 forward and reverse primers. Gaps remaining in the sequence were filled by employing suitable synthetic primers. Sequencing data were analyzed using the BLAST algorithm (NCBI) and three open reading frames (*cinA*, *cinB* and *cinC*) were identified which were sequenced completely in both directions.[†]

Cloning of P450$_{cin}$ – Primers were designed to either end of *cin*A containing suitable terminal restriction sites (*Eco*RI and *Nde*I for the N-terminal primer – 5'-CC GATTC CATATG AC TGC GAC AGT CGC-3', and *Xba*I and *EcoR*I for the C-terminal primer –5'-CC GAATTCTAGA GGA GCT TGC TCA TTC CG-3'). A PCR experiment was performed with pC1 as template DNA according to the following parameters: 94 °C for 3 min; 30 cycles of 94 °C for 45 sec, 52 °C for 1 min, 72 °C for 1 min 30 sec. The single 1.2 kb product was digested

[†] Sequence data has been submitted to Genebank: Acession No. AF456128

with *Eco*RI and inserted into the *Eco*RI site of pUCPKS (28). To ensure no errors had been introduced by PCR the majority of the internal sequence was replaced according to the following procedure: the clone was cut with *Nco*I, treated with DNA polymerase (Klenow) in a fill-in reaction and religated to produce a selectable $P450_{cin}$ mutant. Both the mutant and pC1 were digested with *Ppu*MI/*Sph*I. The wild-type 1.2 kb *Ppu*MI/*Sph*I insert from pC1 was used to replace the mutant insert in pUCPKS. An *Nco*I digest was performed to confirm the wild-type fragment had indeed been inserted, and sequencing (using M13 forward and reverse primers) confirmed no PCR errors were incorporated into either terminus of *cinA*. An *Eco*RI fragment was excised from pUCPKS-*cin*A and cloned into pUC19. This allowed convenient excision with *Nde*I/*Hind*III for insertion into pCW, producing pCW-$P450_{cin}$.

Purification of recombinant Cytochrome $P450_{cin}$ – E. coli (DH5α) cells freshly transformed with expression vector pCW-$P450_{cin}$ were cultured overnight in 2 ml of 2 x YT/Amp (50 μg/ml ampicillin) medium. This was used to inoculate four 2.8 L fernbach flasks containing 1 L of TB/Amp (50 μg/ml ampicillin) medium. The flasks were incubated at 37 °C (4.5 h approx.) until an OD_{600} of 0.6 was attained. The cells were then induced with IPTG (1 mM) and incubated at 27 °C for 18 h. Cells were harvested via centrifugation (6000g) for 20 min at 4 °C and then resuspended in 80 ml of 50 mM Tris.HCl pH 7.4, 50 mM KCl, 1 mM EDTA, 0.1 mM PMSF, 0.5 mM DTT, cineole (750 μM). An indication of purity of various cytochrome $P450_{cin}$ preparations was obtained by measuring the ratio of the absorbance at 415 nm to that at 280 nm. Lysis was carried out by adding lysozyme (40 mg) and stirring the suspension for 1 hr at 4 °C, followed by sonication on ice (6 x 1 min intervals, 50% output). The lysate was centrifuged (15000g) for 1 hr to remove cellular debris. The supernatant was loaded onto a DEAE ion exchange column which was then washed with 50 mM Tris.HCl pH 7.4, 50 mM KCl, 0.5 mM DTT (Buffer A). Protein was then eluted from the column by running a KCl gradient (50-500 mM KCl) in Buffer A. Fractions containing $P450_{cin}$ were detected by its characteristic soret absorbance at 415 nm and were combined. $P450_{cin}$ eluted at approximately 180 mM KCl. Pooled fractions were concentrated in an Amicon ultrafiltration cell containing a YM-30 membrane.

The concentrate was then separated on an S-300 Sephacryl gel filtration column using Buffer A. $P450_{cin}$ containing fractions were pooled, concentrated to approximately 3-5mls using an Amicon ultrafiltration cell (YM-30 membrane), then loaded onto a hydroxyapatite column equilibrated with 5 mM KH_2PO_4 pH 7.2. A phosphate gradient (5-300 mM KH_2PO_4) was run (0.5 ml/min). $P450_{cin}$ eluted at ~120 mM KH_2PO_4. Fractions with an R/Z of >1.2 were combined. The cytochrome P450 content was determined from the difference spectrum between samples which had been reduced with sodium dithionite and carbon monoxide saturated versus a sodium dithionite treated reference sample using an ε_{450} of 125 $mM^{-1}cm^{-1}$. Purified protein was snap frozen in liquid nitrogen and stored at –80 °C. The procedure produced 42 mg of $P450_{cin}$ per liter of culture.

Calculation of Molar Extinction Coefficients (ε) for $P450_{cin}$ – A dilute solution of $P450_{cin}$ was prepared and characterized by UV in the presence of cineole. The protoheme content of the solution was determined as the pyridine haemochromogen, which was prepared as follows. Pyridine (80µl) was added to the $P450_{cin}$ solution (272µl) and gently mixed. 10% SDS (40ul) was added and the volume brought to 400µl. The solution was then divided equally between two cuvettes (reference and sample). Sodium dithionite (0.5-1mg) was added to the sample cuvette and the difference spectra recorded on a dual beam spectrophotometer. The absorbance at 556.5 nm ($A_{556.5nm}$-A_{570nm}) was measured and the haemochromogen content estimated using an ε value of 33.2 mM^{-1} (29). The following molar extinction coefficients were calculated for $P450_{cin}$: Ferric, substrate bound, ε_{392} = 132 $mM^{-1}cm^{-1}$; Ferric, substrate free, ε_{415} = 150 $mM^{-1}cm^{-1}$; Ferrous, (substrate present), ε_{411} = 106 $mM^{-1}cm^{-1}$.

In vivo oxidation of cineole by $P450_{cin}$ – *E. coli* (DH5α) cells freshly transformed with expression vector pCW-$P450_{cin}$ were cultured overnight in 2 ml of 2 x YT/Amp medium. This was used to inoculate 500ml of TB/Amp (100 µg/ml ampicillin) medium. The flasks were incubated at 37 °C (4.5 h approx.) until an OD_{600} of 0.6 was attained. The cells were then induced with IPTG (1 mM) and cineole (250 µl) was added. The culture was incubated at 27 °C for 36 h. Cells were removed via centrifugation (6000g) for 20 min at 4 °C and the supernatant

extracted with ethyl acetate (2 x 100 ml). The combined organic extracts were dried over magnesium sulfate and concentrated *in vacuo* and the residue analyzed by GCMS (BP-5 column (30 m x 0.25mm); Temperature program: 60°C, 2min; 16°C min^{-1} to 250 °C). A peak was observed with a mass spectrum consistent with that reported for 2-hydroxycineole **2** (20) [GCMS m/z: 170 ($M^{+\cdot}$, 6%), 155 (M-CH_3, 1), 126 (37), 108 (70), 93(34), 71 (59), 69 (37), 43 (100)].

RESULTS

Isolation of Citrobacter braakii – A microorganism capable of utilizing 1,8-cineole **1`** as its sole carbon source was isolated through culture enrichment (23) of bacteria from the cineole **1** enriched soils beneath eucalypt trees. The procedure yielded a pure strain identified using the Biolog Identification System (Biolog, Inc., Hayward, Calif.) (30) as *C. braakii*. This strain grew best at room temperature on a variety of nutrient rich media. However, use of such media resulted in loss of the ability to grow on cineole **1** as a sole carbon source, and thus the organism was generally maintained on minimal medium (23) in the presence of cineole**1**. Examination of CO difference spectra of cell lysates from these cultures indicated that a P450 was present, albeit at low concentrations.

Purification of $P450_{cin}$ from C. braakii-A P450 was purified from a large scale culture of *C. braakii* grown on minimal medium in the presence of cineole **1**. The purification protocol used was based on those published for $P450_{cam}$, $P450_{terp}$ and $P450_{BioI}$ ((9,10,31)). A refined version of this protocol is currently used for purification of recombinant $P450_{cin}$ (*vide infra*). The procedure yielded purified protein with a peak fraction possessing an R/Z (A415/A280) of ~1.28. The protein isolated was clearly a P450 by CO difference spectroscopy and had a molecular weight of approximately 45 kDa by SDS PAGE analysis. Importantly, the heme Soret absorbance at 415 nm shifted to 392 nm in the presence of cineole **1**. This is characteristic of the spin state change of the heme iron (low to high spin) seen upon binding of substrate to a P450 and strongly suggested that the isolated $P450_{cin}$ was involved in cineole **1** metabolism.

Tryptic Digestion and Sequencing of Protein Fragments - N-terminal sequencing of $P450_{cin}$ (purified from *C. braakii*) using automated Edman degradation failed to produce useful data, presumably due to N-terminal blocking. However, tryptic digestion of the protein followed by HPLC purification of the resultant fragments gave several peptides amenable to Edman degradation. A combination of automated Edman degradation sequencing and mass spectrometry yielded the sequences of three fragments which are shown in Table 1. Fragments 1 and 2 are clearly related, differing only in the identity of the first amino acid. However, as their

sequences were independently obtained we were confident of their reliability and this sequence was chosen as the basis upon which to design oligonucleotides for use in isolation of the gene encoding $P450_{cin}$. Fragment 3 was not useful for probe design due to the preponderance of amino acids with highly degenerate codons. We chose to amplify a larger segment of the P450 gene by PCR in order to generate a better probe, rather than simply employ short degenerate oligonucleotides. Thus, degenerate primers were designed to either end of coding sequence for Fragment 1 and were chosen to minimize their inherent degeneracy (Fig 1). These were used in a PCR experiment with genomic DNA from *C. braakii* as template. The product from this PCR was sequenced and shown to encode the peptide Fragment 1, providing the key, unique 92 bp probe for $P450_{cin}$.

Southern Hybridization-Probing a series of restriction enzyme digests of *C. braakii* genomic DNA with the 92 bp probe labeled with ^{32}P by reaction with [γ-^{32}P]-ATP in the presence of polynucleotide kinase failed to yield useable results. Believing that sensitivity was the source of our problems we chose to label the probe with [α-^{32}P]-dCTP in the presence of terminal deoxynucleotidyl transferase. TdT is known to transfer up to 30 nucleotides to the 3' end of single stranded DNA, which we hoped would result in an order of magnitude increase in the specific activity, and thus sensitivity, of the probe. With dCTP labeled oligonucleotide, southern hybridization experiments led to the isolation of a 4 kB *Pst*I fragment that was cloned into pUC19 to give pC1. Initial results revealed that this *Pst*I fragment did not contain a stop codon for the final gene in the putative operon (*vide infra*). Thus, random priming using pC1 insert was employed to isolate two further genomic fragments which were cloned into pUC19 as pC3 and pC4, such that the entire operon containing $P450_{cin}$ could be sequenced (Fig 2).

Operon Analysis. Sequencing of the above DNA fragments revealed a putative operon consisting of three open reading frames with overlapping stop and start codons (Fig. 2). All orf's were analyzed using the BLAST algorithm (32) and the Genbank database. The first orf (*cinA*) encoded a 405 amino acid protein with a predicted molecular weight of 45 kDa. Homology analysis indicated that it was clearly a P450, being similar to a number of bacterial P450s (Table

2), including $P450_{cam}$ (27% identity, 46% similarity) but different enough to be classified as the sole member of a new P450 family CYP176A1 (2). All of the peptide fragments that had been characterized from the tryptic digest of the isolated P450 were encoded by this gene.

The two other orf's (*cinB* and *cinC*) appeared to encode the expected redox partners for a catalytically functional P450 system. The second orf (*cinB*) encoded a 49 kDa, 452 amino acid protein that was apparently an NADPH dependent ferrodoxin reductase on the basis of sequence homology (Table 2). The final gene (*cinC*) encoded a protein (16 kDa, 155 amino acids) that was most similar to the FMN containing domain of cytochrome P450 reductase (Table 2). The participation of a dedicated flavodoxin as a P450 redox partner has not previously been described but *E. coli* flavodoxin has been reported to provide an unnatural redox partner for a variety of heterologously expressed cytochromes (33).

[INSERT TABLE 2 HERE]

Subcloning and Expression of $P450_{cin}$. A PCR based approach was used to clone the *cinA* gene into the expression vector pCWori (34). Expression of the encoded protein after induction with IPTG was monitored by SDS PAGE and CO difference spectroscopy of cell lysates. It was found that while polypeptide production occurred at temperatures from 25 to 37 °C, P450 holoprotein was only observed when the growth temperature was below 28 °C. CO difference spectroscopy indicated that P450 was produced at concentrations of approximately 2000 nmol/l of original culture. Characterization of the purified protein by N-terminal sequencing and electrospray mass spectrometry gave similar results. Both techniques clearly identified the protein as $P450_{cin}$ and indicated that some degree of N terminal processing of the protein had occurred. The degree of processing varied between different preparations, with some having the major species present derived from loss of the N-terminal methionine, while others had the predominant protein derived from loss of the first six amino acids of the predicted sequence.

In vivo oxidation of cineole by $P450_{cin}$ – When $P450_{cin}$ was expressed in *E. coli* in the presence of cineole **1** a metabolite was produced with mass spectral characteristics consistent

with that of 2-hydroxycineole **2**. This compound was not produced when *E. coli* not expressing $P450_{cin}$ was grown in the presence of cineole **1**. This result strongly supports the hypothesis that $P450_{cin}$ catalyses the first monooxgenation in the biodegradation of cineole **1**.

Spectral Characteristics of Cytochrome $P450_{cin}$ - The protein was first examined by UV/visible spectroscopy (Fig. 3) which revealed the typical characteristics of a bacterial P450: a heme soret at 415 nm in the absence of substrate which shifted to 392 nm upon the addition of cineole **1**. This spectral change is correlated with a change in the heme iron from low to high spin and is associated with the binding of substrate to bacterial P450s and as such was the first evidence that cineole **1** was indeed the natural substrate of this enzyme. Reduction of the protein with dithionite and treatment with CO resulted in the formation of a CO complex with the typical 449 nm absorbance of a P450.

[INSERT FIGURE 3 HERE]

1,8-Cineole Binding by Cytochrome $P450_{cin}$ –The addition of 1,8-cineole **1** to the substrate free form of the enzyme results in a decrease in absorbance at 415 and 568 nm and an increase in absorbance at 392 and 643 nm, indicating the conversion of low-spin hemoprotein to the high-spin form (35). Difference spectroscopy (Fig. 4) was used to calculate a dissociation constant for the enzyme-substrate complex (K_D) by adding cineole **1** to a solution of $P450_{cin}$ and monitoring the absorbance at 415 and 392 nm. The difference (measured as A_{392}-A_{415}) was plotted against the cineole **1** concentration to produce a substrate-binding curve (Fig. 4) from which a K_D value of 0.7 μM was determined using the following equation:

$$[S]_{TOT} = (K_D\ \Delta A)/(\Delta Amax - \Delta A) + \Delta A/\varepsilon$$

where: $[S]_{TOT}$ is the concentration of added ligand, K_D is the dissociation constant of ligand-P450 complex, ΔA is the measured peak to trough difference in absorbance between 415nm and 392 nm, ΔAmax is the maximal ΔA value and ε is the molar absorptivity constant for the difference in absorbance at 415 nm and 392 nm of the high and low spin forms of the P450 i.e. $((\varepsilon_{415}LS - \varepsilon_{415}HS) + (\varepsilon_{392}HS - \varepsilon_{392}LS))$. This calculation was made with the following assumptions (1) $[S]_{TOT} = [S]_{FREE} + [ES]$ and (2) $[E]_{TOT} = [E]_{FREE} + [ES]$ where [ES] is the

concentration of the P450-ligand complex, $[S]_{TOT}$ and $[S]_{FREE}$ are the total and free ligand concentration respectively and $[E]_{TOT}$ and $[E]_{FREE}$ are the total and free P450 concentration respectively. Because of the high affinity of the enzyme for its ligand, the assumption that $[ES] << [S]_{TOT}$, which would greatly simplify the calculation, is not valid.

[INSERT FIGURE 4 HERE]

DISCUSSION

Prior to the beginning of this work, much was known about the chemistry of cineole **1** biodegradation. Two organisms had previously been independently isolated that could live on cineole **1** as their sole source of carbon and energy (18,20). From an investigation of the oxidized cineole metabolites isolated from growth media, a consensus biodegradative pathway was proposed (Scheme 1), albeit proceeding via intermediates with different absolute stereochemistry in each of the two organisms. Nothing was known about the enzymes involved in the proposed pathway, despite some early investigations (21). Our results support the hypothesis that a P450 is responsible for the initial step in the biodegradation of cineole **1**.

We isolated from the soil of a eucalypt forest a strain of *C. braakii* that could utilize cineole **1** for growth and energy. *C. braakii* is a gram negative motile rod that is a common environmental organism and which has recently been reclassified from *C. freundi* (36). *P. flava* and *Rhodococcus*, in which cineole **1** utilization had previously been found, are also common gram negative organisms. Given the quantity of cineole **1** that is believed to be released into the environment by eucalypts, we were not surprised to find that a third genus of bacteria has developed the ability to grow on this compound. The organism grew poorly on minimal medium in our hands when cultured on a large scale and although it grew well on nutrient rich media, such growth resulted in loss of its ability to utilize cineole **1**. This latter fact perhaps suggests that the genes responsible for cineole **1** metabolism are plasmid borne as has been shown for the P450s involved in camphor and terpineol utilization (10,37). The difficulty in growing the organism, clearly meant that isolation of the gene encoding the cineole monooxygenase would be essential for its characterization.

Initial approaches to isolating the P450 gene focused on its expected homology with other P450s, since this seemed to offer the most direct route to $P450_{cin}$. Homology based methodology in bacterial P450 systems is particularly difficult, however, since most of these proteins reside in sparsely populated families with only limited regional homology to one another. However, with this in mind, a degenerate oligonucleotide was constructed based upon the conserved region that

includes the cysteine that ligates the heme iron and used to probe genomic DNA from *C. braakii*. A second protocol used this oligonucleotide in a genomic PCR with another degenerate primer based upon a conserved region seen in the redoxin reductase partners of P450cam and P450terp. The latter experiment was based on the hypothesis that the $P450_{cin}$ gene would be followed directly by those of its redox partners and that the first of these would encode a redoxin reductase as seen in the $P450_{cam}$ and $P450_{terp}$ operons. Both approaches were unsuccessful, in part due to the limited sensitivity of the degenerate oligonucleotides. In addition, although the architecture of the $P450_{cin}$ operon was as expected, the proteins encoded constitute an entirely novel redox system for P450s with no homology to those found with other bacterial P450s.

More protein specific information was thus required and this was obtained by isolating native $P450_{cin}$ from *C. braakii*. Protein purification proved remarkably straightforward, with essentially homogenous protein obtained in three steps using a protocol based upon that published for $P450_{cam}$ and $P450_{BioI}$. Protein sequence, obtained via trypsinolysis and peptide sequencing, was used to design degenerate PCR primers to either end of a large peptide fragment (Fig. 1). A 92bp oligonucleotide probe for $P450_{cin}$ was then generated via genomic PCR. This oligonucleotide was labeled with ^{32}P by reaction with [γ-^{32}P]-ATP in the presence of polynucleotide kinase but probing a series of restriction enzyme digests of *C. braakii* genomic DNA failed to yield useable results. Believing that sensitivity was the source of our problems we chose to label the probe with [α-^{32}P]-dCTP in the presence of terminal deoxynucleotidyl transferase (25). TdT is known to transfer up to 30 nucleotides to the 3' end of single stranded DNA, which results in an order of magnitude increase in the specific activity, and thus sensitivity, of the probe. Utilization of [α-^{32}P]-dCTP labeled oligonucleotide resulted in the eventual isolation of DNA fragments containing the $P450_{cin}$ gene.

Sequencing of the isolated DNA revealed a putative $P450_{cin}$ operon that contained three open reading frames with overlapping start and stop codons (Fig. 2). The first of these genes clearly encoded a P450, as seen by its strong similarity to a number of other bacterial P450s (Table 2). The encoded protein was classified as CYP176A1 and trivially denoted $P450_{cin}$.

Although most of the conserved residues found in P450s were also seen in $P450_{cin}$, there was one striking exception (Fig. 5). Threonine 252 in $P450_{cam}$ is located on the I-helix, a conserved structural feature of P450s, and is believed to hydrogen bond to a water molecule during the oxygen activation process and thus direct delivery of a proton to the distal oxygen in the iron-dioxygen complex (38,39). This specific delivery is believed to result in cleavage of the O-O bond to yield the active ferryl species; delivery of a proton to the proximal oxygen is thought to result in the dissociation of hydrogen peroxide (so called uncoupling). Given the essential role of this residue in catalysis it is not surprising that a threonine or serine is seen in this position in essentially all known P450s. An exception occurs in P450eryF (CYP107A1), in which it is replaced by an alanine; in this enzyme a hydroxyl group located on the substrate is believed responsible for the positioning of the required water molecule. In $P450_{cin}$, however, sequence alignments suggest that the threonine has been replaced with an asparagine. Clearly, the amide functionality of the asparagine could serve to hydrogen bond to a water molecule and thus direct proton delivery and cleavage of the dioxygen bond. However, this would be the first time that a hydroxyl group has not filled this role and further experimentation will be required to validate this hypothesis.

[INSERT FIGURE 5 HERE]

The two further genes discovered during the sequencing of the putative operon appear to encode potential redox partners for $P450_{cin}$. While this arrangement is identical to that seen in the $P450_{cam}$ and $P450_{terp}$ operons, the identity of these redox partners is quite different. In general there are two types of P450, as classified by their auxiliary electron transport partners. In bacteria and mitochondria (Class I P450s) there are two proteins that mediate the transfer of electrons from the pyridine nucleotide to the P450, one is an FAD containing ferrodoxin reductase and the other is an iron sulfur protein, either 2Fe-2S or 3Fe-4S. In eukaryotes (Class II P450s), there is generally a single flavoprotein, cytochrome P450 reductase that contains both an FAD and an FMN cofactor, which mediates P450 reduction. Other, less common, arrangements also exist for electron transfer. $P450_{nor}$ (40) accepts electrons directly from NADH while $P450_{BM-3}$

(41), a bacterial P450, incorporates both a Class II like reductase and a P450 domain into a single polypeptide. Class III P450s e.g. CYP74A do not require exogenous redox partners as they act upon peroxide containing substrates(42-44). Analysis of the second gene in the $P450_{cin}$ operon revealed that it had strong homology with adrenoredoxin reductase, the ferrodoxin reductase that functions in the adrenal mitochondrial system (Table 2). However, the third gene of the operon appeared to be a flavodoxin and not an iron sulfur containing protein as a BLAST search indicated that it was most homologous to the FMN domain of CPR (Table 2). This suggests that $P450_{cin}$ utilizes a novel, natural redox system for electron transfer, which consists of an FAD dependent flavodoxin reductase and an FMN containing flavodoxin. This system is intermediate between the classical Class I bacterial P450 that utilizes two proteins, but which includes one with an Fe-S cluster, and the Class II P450 which only employs a single protein but utilizes FAD and FMN cofactors. While there is no precedent for this type of system occurring naturally, it has been reported that flavodoxins can act as "unnatural" redox partners for P450s. Waterman has shown that the flavodoxin reductase and flavodoxin systems from both *E. coli* and *Anabaena* will serve as functional redox partners for a variety of (heterologous) mitochondrial P450s (33,45). It should be noted that there is as yet no firm experimental evidence that links $P450_{cin}$ with the two proteins as its redox partners. However, as mentioned, the arrangement of the genes within the operon is identical to that seen with both $P450_{cam}$ and $P450_{terp}$ and their redox partners.

It is interesting to note that while the biodegradative P450s such as $P450_{cam}$, $P450_{terp}$ and $P450_{cin}$ occur in an operon closely associated with their redox partners, this is not the case for many other bacterial P450s uncovered by genome sequencing projects. For example, in *Mycobacterium tuberculosis* of the approximately 20 P450s identified, at least 14 have no obvious operon associated redox partners. Perhaps this implies that biodegradative P450s are necessarily organized into operons with their electron transfer proteins to ensure coordinated expression and efficient functioning. Other bacterial P450s, which may not be required to operate as efficiently as biodegradative ones that supply the cell carbon and energy, may employ general redox partners such as cellular flavodoxins or ferrodoxins.

$P450_{cin}$ was heterologously expressed in *E. coli* at a level of approximately 2000 nmol/l of culture. Purification, including a hydroxyapatite column (46) as a final polishing step, gave essentially homogenous protein (~1000 nmol/l) after three steps. Its identity was confirmed by N-terminal sequencing and electrospray mass spectrometry, which also indicated that some N-terminal processing (truncation of up to six amino acids) had occurred in a batch dependent manner. However, spectral analysis of the protein (*vide infra*) revealed no effect of this inhomogeneity of the protein. While catalytic activity remains to be explored, it is not expected that this variation will significantly alter its efficiency, given that the N-terminus is not implicated in either the mechanism of oxygen activation or the interaction with redox partners in any other bacterial P450 (47).

We postulated that as $P450_{cin}$ was believed to have a flavodoxin as a redox partner, it might utilize the endogenous *E. coli* flavodoxin/flavodoxin reductase system. Waterman had reported that this system was responsible for the observed catalytic activity in a variety of heterologously expressed P450s (33,45). Thus, we expressed $P450_{cin}$ in the presence of cineole **1** and careful GCMS analysis of the organic extracts of the growth medium revealed the presence of a metabolite tentatively identified as 2-hydroxycineole **2**. This compound was not produced when cineole **1** was added to the growth medium of *E. coli* not expressing $P450_{cin}$. Whilst not conclusive, this result clearly demonstrates that $P450_{cin}$ is capable of cineole **1** hydroxylation and strongly suggests that it is involved in its biodegradation.

UV/visible analysis of the heterologously expressed $P450_{cin}$ revealed the typical characteristics of a bacterial P450 (Fig. 3). Significantly, a spin state change of the heme iron from low spin to high spin was observed in the presence of cineole **1**, typical of a bacterial P450 in the presence of its substrate. This, and the tight binding of cineole **1** to $P450_{cin}$ (K_D = 0.7 μM) strongly support our hypothesis that the natural substrate for this enzyme is cineole **1**, as it mirrors exactly the behavior of other terpene degrading P450s with their substrates, e.g., $P450_{cam}$ (5), $P450_{terp}$ (10), and $P450_{lin}$ (9). These observations combined with our *in vivo* turnover results also provide the first real evidence that a P450 is involved in the biodegradation of cineole **1**. It

will be of interest to determine the way in which this strong, specific binding of cineole **1** is achieved as it presents little, if any, opportunity for H-bonding. Such interactions are known to play an important role in the specific binding of other terpene substrates with their biodegradative P450 (48). The oxygen in cineole **1** is present as a ditertiary ether, and as such unlikely to participate in any H-bonding. It is hoped that crystallization trials currently underway may shed light on the specificity of this enzyme substrate interaction.

Conclusion. A new bacterial P450 system has been isolated and the P450 cloned and over-expressed. The high level of expression, as well as the ease of purification, encourage us in the belief that this P450 may serve as a useful model for P450s in general and bacterial P450s in particular. It contains a number of unique features, such as an unusual asparagine substitution for a catalytically essential threonine, which should help to increase our fundamental understanding of P450 mechanism. In addition, it appears that $P450_{cin}$ is the first known P450 to utilize a flavodoxin as its natural redox partner. Experiments designed to further explore the catalytic activity and the interactions of $P450_{cin}$ with both its substrate and redox partners are currently in progress.

REFERENCES

1. Nelson, D. R., Kamataki, T., Waxman, D. J., Guengerich, F. P., Estabrook, R. W., Feyereisen, R., Gonzalez, F. J., Coon, M. J., Gunsalus, I. C., Gotoh, O., Okuda, K., and Nebert, D. W. (1993) *DNA Cell Biol* **12**, 1-51
2. Nelson, D. R., Koymans, L., Kamataki, T., Stegeman, J. J., Feyereisen, R., Waxman, D. J., Waterman, M. R., Gotoh, O., Coon, M. J., Estabrook, R. W., Gunsalus, I. C., and Nebert, D. W. (1996) *Pharmacogenetica* **6**, 1-41
3. Ortiz de Montellano, P. R. (1995) in *Cytochrome P450 : Structure, Mechanism and Biochemistry* (Ortiz de Montellano, P. R., ed), Second Edition Ed., pp. 245-303, Plenum Press, New York
4. Ortiz de Montellano, P., and De Voss, J. J. (2002) *Nat. Prod. Rep.*, In Press
5. Katagiri, M., Ganguli, B. N., and Gunsalus, I. C. (1968) *J. Biol. Chem.* **243**, 3543
6. Peterson, J. A. (1971) *Arch. Biochem. Biophys.* **144**, 678-693
7. Sibbesen, O., De Voss, J. J., and De Montellano, P. R. O. (1996) *J. Biol. Chem.* **271**, 22462-22469
8. Gunsalus, I. C., and Wagner, G. C. (1978) *Meth. Enzymol.* **52**, 166-188
9. Ropp, J. D., Gunsalus Irwin, C., and Sligar Stephen, G. (1993) *J. Bacteriol.* **175**, 6028-6037
10. Peterson, J. A., Lu, J., Geisselsoder, J., Graham-Lorence, S., Carmona, C., Witney, F., and Lorence, M. C. (1992) *J. Biol. Chem.* **267**, 14193-14203
11. Morrow, P. A., Belas, T. E., and Eisner, T. (1976) *Oecologia* **24**, 193-206
12. Morrow, P. A., and Fox, L. R. (1980) *Oecologia* **45**, 209-214
13. Hattori, Y. (2001) in *Jpn. Kokai Tokkyo Koho*, JP2001031508, Japan
14. Penfold, A. R., and Willis, J. L. (1955) *Australasian J. Pharm.* **36**, 992-995
15. Al-Mousawi, A. H., and Al-Naib, F. A. G. (1975) *J. Univ. Kuwait, Sci.* **2**, 59-65
16. Del Moral, R., and Muller, C. H. (1969) *Bull. Torrey Bot. Club* **96**, 467-475
17. Carman, R., and Fletcher, M. (1983) *Aust. J. Chem.* **36**, 1483-1493
18. MacRae, I., Vincent, A., Carman, R., and Shaw, I. (1979) *Aust. J. Chem.* **32**, 917-922

19. Flynn, T. M., and Southwell, I. A. (1979) *Aust. J. Chem.* **32**, 2093
20. Williams, D. R., Trudgill, P. W., and Taylor, D. G. (1989) *J. Gen. Microbiol.* **135**, 1957-1967
21. Trudgill, P. W. (1994) in *Biochemisty of Microbial Degradation* (Ratedge, C., ed), Netherlands
22. Miyazawa, M., Shindo, M., and Shimada, T. (2001) *Drug Metab Dispos* **29**, 200-205
23. Peterson, J. A., and Lu, J. Y. (1991) *Methods Enzymol.* **206**, 612-620
24. Peterson, J. A., Lorence, M. C., and Amarneh, B. (1990) *J. Biol. Chem.* **265**, 6066-6073
25. Kato, K. (1967) *J. Biol. Chem.* **242**, 2780
26. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989), 2nd Ed., pp. 9.38, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N. Y.
27. Feinberg, A., and Vogelstein, B. (1983) *Anal. Biochem.* **132**, 6-13
28. Schweizer, H. P. (1991) *Gene* **97**, 109-112
29. Appleby, C. A., and Morton, R. K. (1959) *Biochem. J.* **73**, 539-550
30. Miller, J. M., and Rhoden, D. L. (1991) *J. Clin. Microbiol.* **29**, 1143-1147
31. Yu, C. A., Gunsalus, I. C., Katagiri, M., Suhara, K., and S., T. (1974) *J. Biol. Chem.* **249**, 94-101
32. Gish, W., and States, D. J. (1993) *Nature Genet.* **3**, 266-272
33. Jenkins, C. M., and Waterman, M. R. (1994) *J. Biol. Chem.* **269**, 27401-27408
34. Barnes, H. J., Arlotto, M. P., and Waterman, M. R. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 5597-5601
35. George, P., Bettlestone, J., and Griffith, J. S. (1961) in *Haematin Enzymes* (Falk, J. E., Lemberg, R., and Morton, R. K., eds) Vol. 1, pp. 105, Pergamon, London
36. Brenner, D., Grimont, P., Steigerwalt, A., Fanning, G., Ageron, E., and Riddle, C. (1993) *International Journal of Systematic Bacteriology* **43**, 645-658
37. Koga, H., Rauchfuss, B., and Gunsalus, I. C. (1985) *Biochem. Biophys. Res. Commun.* **130**, 412-417
38. Poulos, T. L., Cupp-Vickery, J., and Li, H. (1995) in *Cytochrome P450 - Structure, Mechanism, and Biochemistry Second Edition* (Ortiz de Montellano, P., ed), pp. 125-150, Plenum Press, New York

39. Schlichting, I., Berendzen, J., Chu, K., Stock, A. M., Maves, S. A., Benson, D. E., Sweet, R. M., Ringe, D., Petsko, G. A., and Sligar, G. S. (200) *Science* **287**, 1615-1622
40. Kudo, T., Tomura, D., Liu, D. L., Dai, X. Q., and Shoun, H. (1996) *Biochimie* **78**, 792-799
41. Ruettinger, R. T., Wen, L. P., and Fulco, A. J. (1989) *J. Biol. Chem.* **264**, 10987-10995
42. Song, W. C., Funk, C. D., and Brash, A. R. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 8519-8523
43. Hecker, M., Haurand, M., Ullrich, V., and Terao, S. (1986) *Eur. J. Biochem.* **157**, 217-223
44. Miyata, A., Hara, S., Yokoyama, C., Inoue, H., Ullrich, V., and Tanabe, T. (1994) *Biochem. Biophys. Res. Commun.* **200**, 1728-1734
45. Waterman, M. R. (1998) *Biochemistry* **37**, 6106-6113
46. Stok, J. E., and De Voss, J. J. (2000) *Arch. Biochem. Biophys.* **384**, 351-360
47. Graham, S. E., and Peterson, J. A. (1999) *Arch. Biochem. Biophys.* **369**, 24-29
48. Peterson, J. A., and Graham, S. E. (1995) in *Cytochrome P450 : Structure, Mechanism and Biochemistry* (Ortiz de Montellano, P. R., ed), Second Edition Ed., pp. 151-180, Plenum Press, New York
49. Cole, S. T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S. V., Eiglmeier, K., Gas, S., Barry, C. E. I., Tekaia, F., Badcock, K., Basham, D., Brown, D., Chillingworth, T., Connon, R., Davies, R., Devlin, K., Feltwell, T., Gentles, S., Hamlin, N., Holroyd, S., Hornsby, T., Jagels, K., Krogh, A., McLean, J., Moule, S., Murphy, L., Oliver, S., Osborne, J., Quail, M. A., Rajandream, M. A., Rogers, J., Rutter, S., Seeger, K., Skelton, S., Squares, S., Squares, R., Sulston, J. E., Taylor, K., Whitehead, S., and Barrell, B. G. (1998) *Nature* **393**, 537-544
50. Inouye, M., Takada, Y., Muto, N., Horinouchi, S., and Beppu, T. (1994) *Mol. Gen. Genet.* **245**, 456-464
51. Ahn, K. S., and Wake, R. G. (1991) *Gene* **98**, 107-112
52. Merson-Davies, L. A., and Cundliffe, E. (1994) *Mol. Microbiol.* **13**, 349-355
53. Haydock, S. F., Dowson, J. A., Dhillon, N., Roberts, G. A., Cortes, J., and PLeadlay, P. F. (1991) *Mol. Gen. Genet.* **230**, 120-128
54. Unger, B. P., Gunsalus, I. C., and Sligar, S. G. (1986) *J. Biol. Chem.* **261**, 1158-1163

55. Bower, S., Perkins, J. B., Yocum, R. R., Howitt, C. L., Rahaim, P., and Pero, J. (1996) *J. Bacteriol.* **178**, 4122-4130
56. Takada, Y., Y., S., Kono, A., Sekimizu, K., and Horiuchi, T. (1993) *Biol. Pharm. Bull.* **16**, 1200-1206
57. Bobe, J., Goetz, F. W., and Langenau, D. (2000) *unpublished data*
58. Lin, D., Shi, Y., and Miller, W. L. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 8516-8520
59. Zhao, Q., Modi, S., Smith, G., Paine, M., McDonagh, P. D., Wolf, C. R., Tew, D., Lian, L.-Y., Roberts, G. C. K., and Driessen, H. P. C. (1999) in *Protein Sci.* Vol. 8, pp. 298-306
60. Kuroda, M., Ohta, T., Uchiyama, I., Baba, T., Yuzawa, H., Kobayashi, I., Cui, L., Oguchi, A., Aoki, K. I., Nagai, Y., Lian, J., Ito, T., Kanamori, M., MAtsumaru, H., Maruyama, A., Murakami, H., Hosoyama, A., Mizutani-Ui, Y., Takahashi, N. K., Sawano, T., Inoue, R. I., Kaito, C., Sekimizu, K., Hirakawa, H., Kumhara, S., Goto, S., Yabuzaki, J., Kanehisa, M., Yamashita, A., Oshima, K., Furuya, K., Yoshino, C., Shiba, T., Hattori, M., Ogasawara, N., Hayashi, H., and Hiramatsu, K. (2001) *Lancet* **357**, 1225-1240
61. Ohgiya, S., Goda, T., Hoshino, T., Shinriki, N., and Kamataki, T. (1996) *unpublished data*
62. Porter, T. D., and Kasper, C. B. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 973-977
63. Helms, L. R., Krey, G. D., and Swenson, R. P. (1990) *Biochem. Biophys. Res. Commun.* **168**, 809-817

FIGURE CAPTIONS

Figure 1 – PCR primer design based on tryptic peptide Fragment 1. (a) forward PCR primer (b) Fragment 1 (c) reverse PCR primer. The restriction sites engineered into the 5' ends of the primers are indicated. Amino acid residues in parentheses are portions of the peptides not encoded by the oligonucleotide primers. Degenerate nucleotide codes are shown in bold. F1PCR-F and F1PCR-R are 72- and 128-fold degenerate, respectively.

Figure 2 – Map of sequenced DNA from *C. braakii*. C1-C4 represent the DNA fragments isolated via southern hybridization experiments and cloned as pC1-pC4. Note only selected restriction sites are indicated.

Figure 3 – Absorbance spectra of: ¨ oxidised $P450_{cin}$; ----- oxidised $P450_{cin}$ plus cineole **1**; ¨¨¨¨ $P450_{cin}$ reduced by addition of sodium dithionite in the presence of cineole **1**; . . . carbon monoxide complex of reduced $P450_{cin}$. The oxidised substrate free form has absorbance maxima at 415 (ε = 150 mM^{-1} cm^{-1}), 535, 568 nm. Addition of 1,8-cineole **1** cases a shift in the Soret absorbance maximum to 392 (ε = 132 mM^{-1} cm^{-1}) and 643 nm; the sodium dithionite reduced form has maxima at 411 (ε = 106 mM^{-1} cm^{-1}) and 541 nm; and the reduced carbon monoxide complex form at 449 (ε = 125 mM^{-1} cm^{-1}) and 547 nm.

Figure 4 – UV/Visible difference spectra of $P450_{cin}$ with increasing concentrations of cineole **1** (0.25 to 16 μM) versus $P450_{cin}$ alone. Protein concentration was approximately 2 μM in 50 mM Tris-HCl, pH 7.4. An isobestic point is seen at 404 nm. These data were used to calculate a K_D for the enzyme substrate complex by plotting (see Inset) absorbance difference between 392 and 415nm versus cineole **1** concentration.

Figure 5 – Clustal X alignment of bacterial P450s, showing the I-Helix and conserved heme binding domains.

TABLE I

Sequences of Tryptic Peptides of Cytochrome P450$_{cin}$

Fragment	Sequence	Mol. Wt.	Technique
1	WAITHVENPEEGAEIFAELVAHAR	2690.4	Auto/MS[a]
2	VWAITHVENPEEGAEIFAELVAHAR	2788.8	Auto/MS
3	GEGDAATWLANEIPAR	ND	Auto

[a] Auto – Automated Edman Sequencing; MS – mass spectrometry.

TABLE 2

Sequence homology of the cinABC open reading frames

Orf	Protein	Organism	%I[a] (%S)[b]	Accession no.[c]	ref.
cinA	CYP143	*M. tuberculosis*	33 (52)	O53936	(49)
	CYP107E1 ($P450_{mycG}$)	*M. griseorubida*	32 (52)	Q59523	(50)
	CYP130	*M. tuberculosis*	30 (51)	Q11062	(49)
	CYP109	*B. subtilis*	30 (50)	P27632	(51)
	CYP113B1 ($P450_{tylI}$)	*S. fradiae*	30 (49)	Q59910	(52)
	CYP107A1 ($P450_{EryF}$)	*S. erythraea*	29 (45)	Q00441	(53)
	CYP101 ($P450_{cin}$)	*P. putida*	27 (46)	P00183	(54)
	CYP107H ($P450_{BioI}$)	*B. subtilis*	27 (50)	P53554	(55)
	CYP101 ($P450_{cam}$)	*P. putida*	27 (46)	P00183	(54)
	CYP108 ($P450_{terp}$)	*Pseudomonas sp.*	25 (38)	P33006	(10)
cinB	Adrenodoxin Reductase	*B. taurus*	31 (47)	P08165	(56)
	Adrenodoxin Reductase	*S. fontinalis*	31 (45)	P82861	(57)
	Adrenodoxin Reductase	*H. sapiens*	31 (47)	P22570	(58)
	Ferredoxin-NAD(P+) Reductase	*M. tuberculosis*	30 (46)	Q10547	(49)
cinC	CPR (FMN-Binding Domain)	*H. sapiens*	37 (56)	P16435	(59)
	Sulfite reductase flavoprotein	*S. aureus*	35 (50)	Q99R17	(60)
	NADPH-CPR	*C. griseus*	33 (51)	Q60451	(61)
	NADPH-CPR	*R. norvegicus*	33 (51)	P00388	(62)
	Flavodoxin domain of $P450_{BM-3}$	*B. megaterium*	31 (44)	P14779	(41)
	Flavodoxin	*D. salexigens*	29 (49)	P18086	(63)

[a] Percent indentity

[b] Percent similarity (indentity + substitutions with an amino acid with similar properties)

[c] SWISS-PROT Accession Number

(a) *Eco*RI

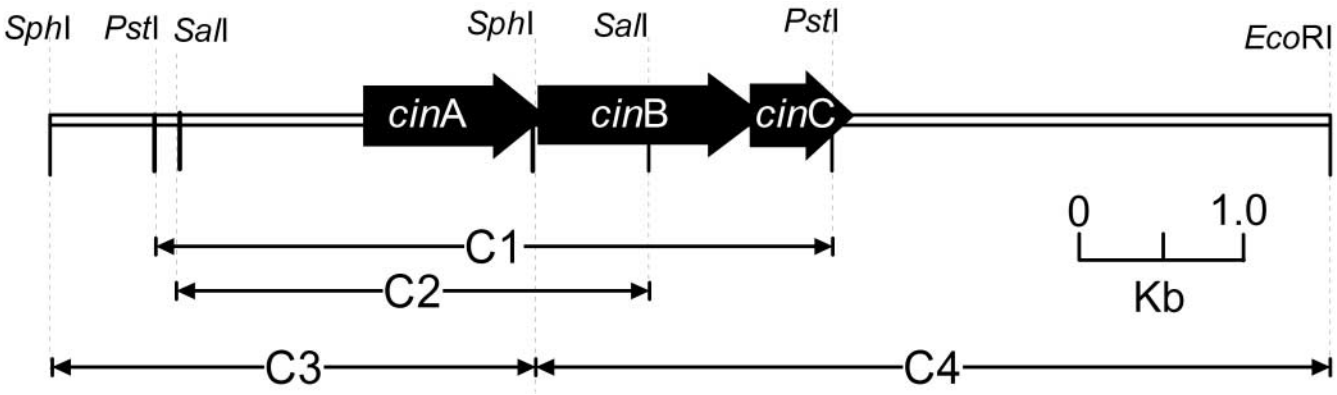
5′-ATAT GAATTC TGG GC**N** AT**M** AC**N** CA**H** GT-3′

(b) NH_2-val-trp-ala-ile-tyr-his-val-(glu-asp-pro-glu-glu-gly-ala-glu-ile-phe-ala-glu-lue)-val-ala-his-ala-arg-COOH

(c) 3′-CA**N** CG**N** GT**R** CG**N** **K**C CCATGG TATC-5′

*Bam*HI

Fig. 1



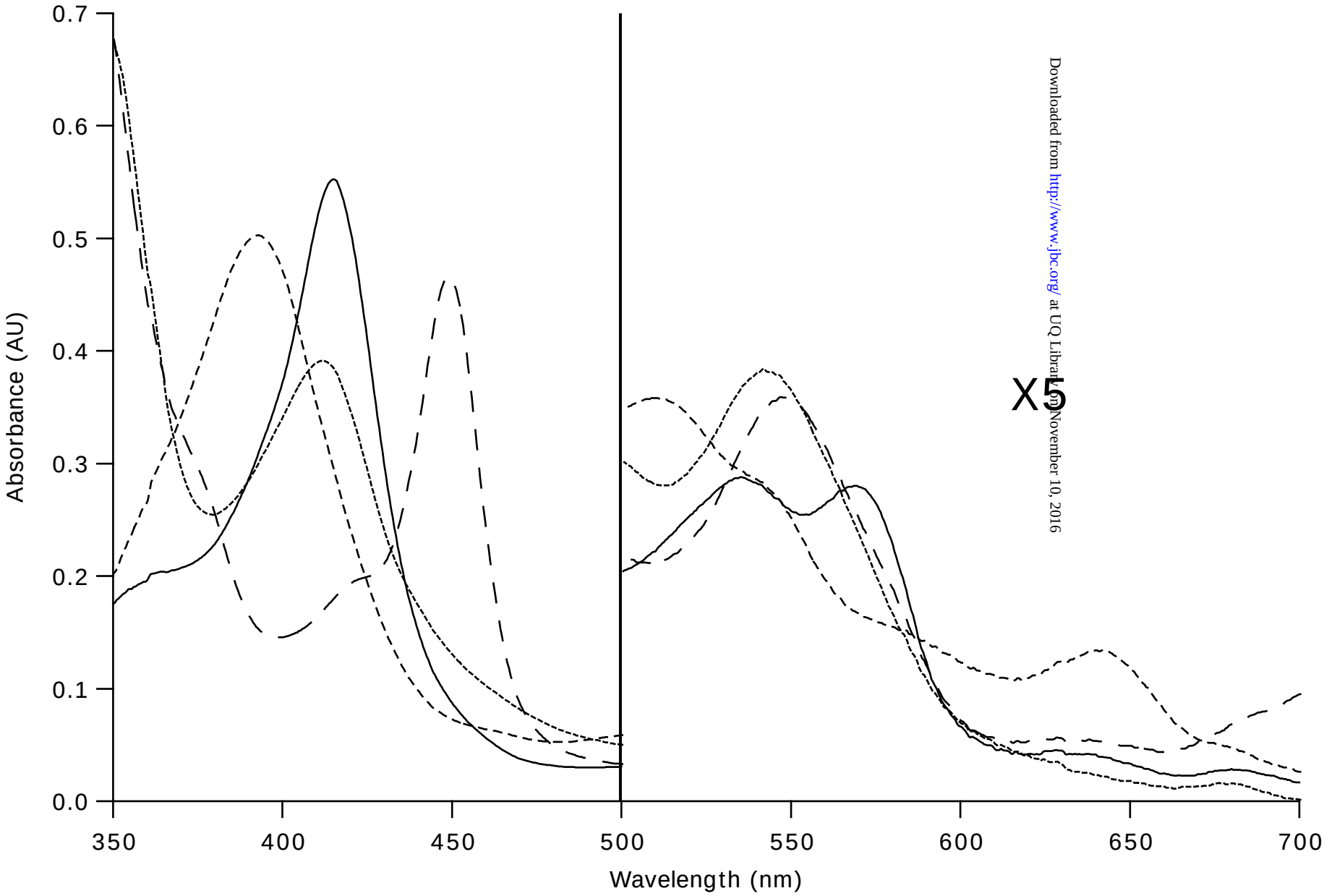


Fig. 3

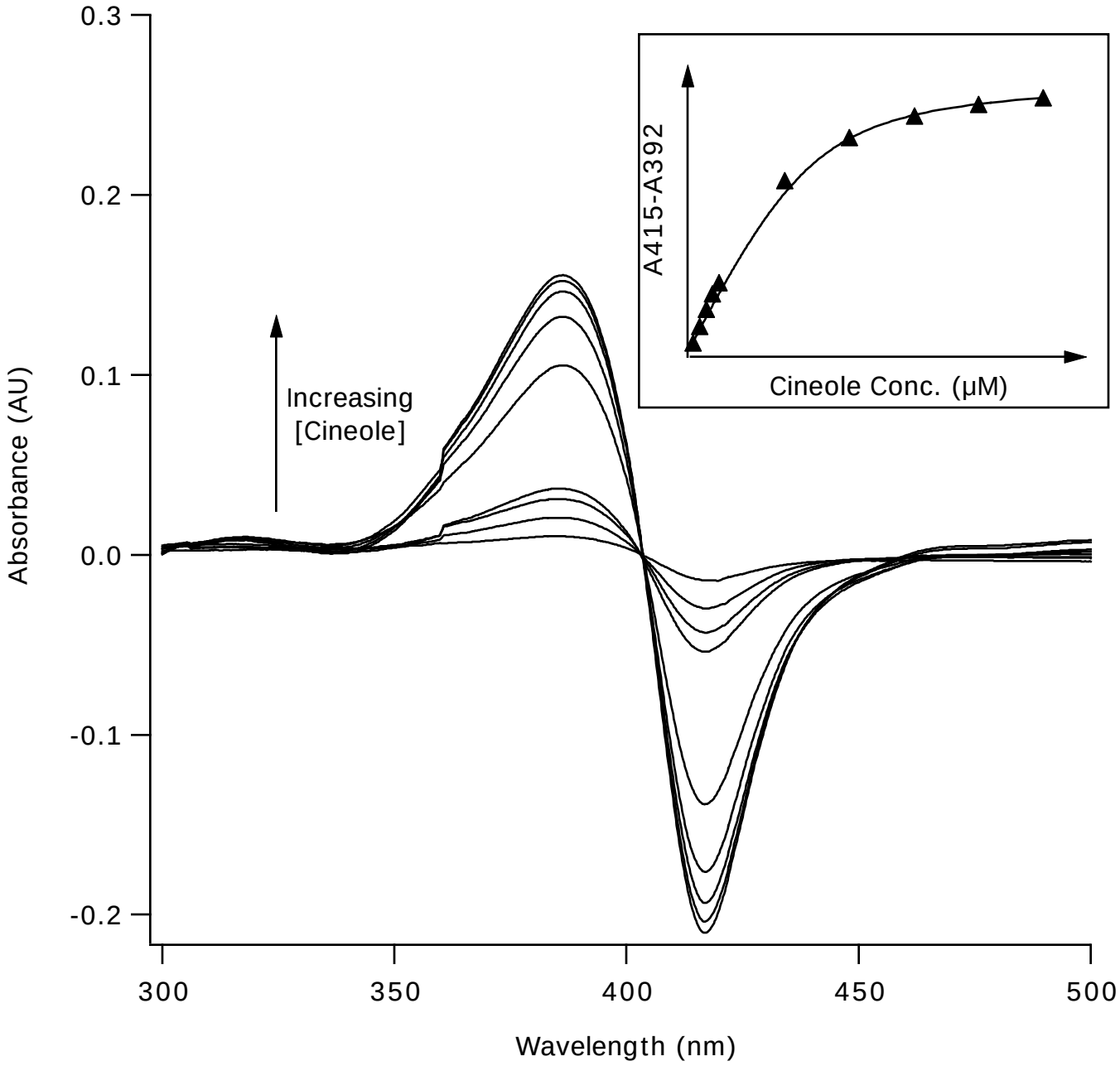


Fig. 4

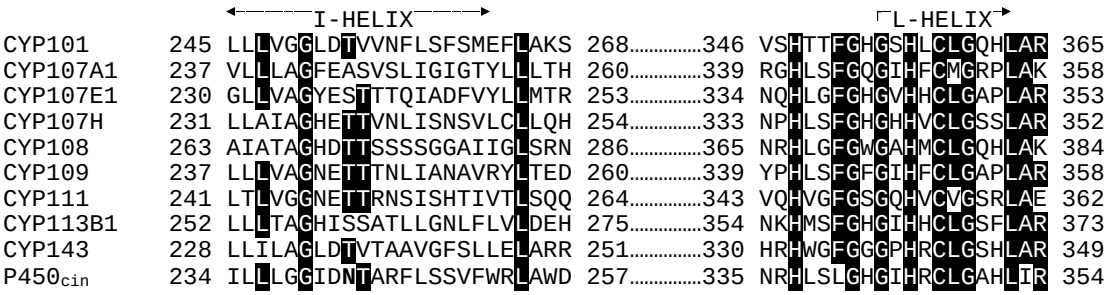
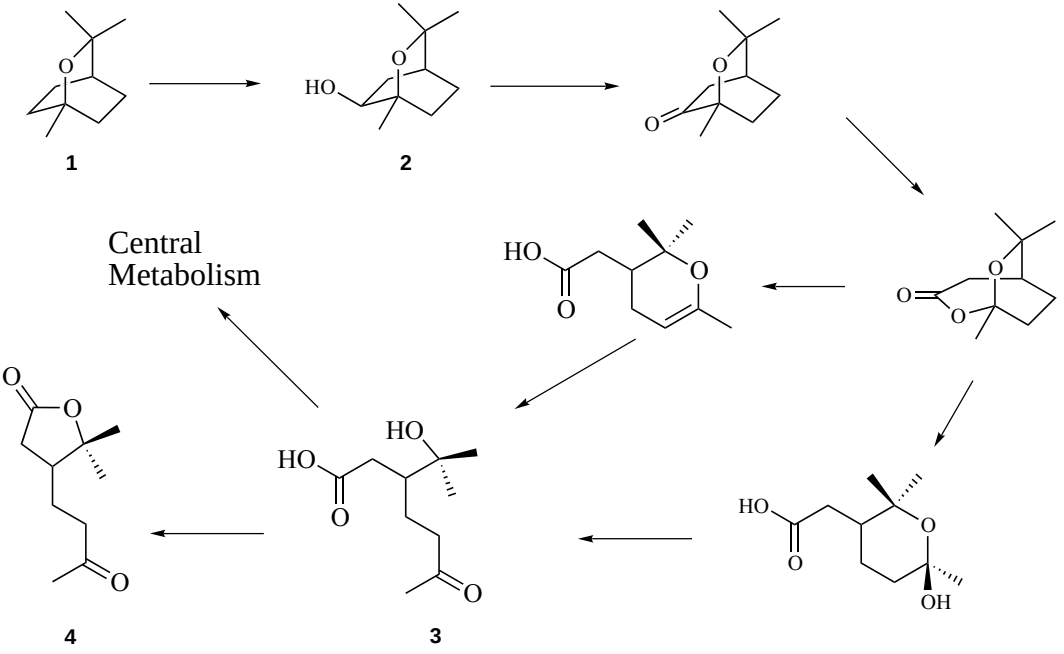


Fig. 5


Central
Metabolism
HO
O
1
2
3
4
OH

Cytochrome P450cin (CYP176A): Isolation, expression and characterisation

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