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Cineole biodegradation: Molecular cloning, expression and characterisation of (1*R*)-6β-hydroxycineole dehydrogenase from *Citrobacter braakii*

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ABSTRACT

The first steps in the biodegradation of 1,8-cineole involve the introduction of an alcohol and its subsequent oxidation to a ketone. In *Citrobacter braakii*, cytochrome $P450_{cin}$ has previously been demonstrated to perform the first oxidation to produce (1*R*)-6β-hydroxycineole. In this study, we have cloned *cinD* from *C. braakii* and expressed the gene product, which displays significant homology to a number of short-chain alcohol dehydrogenases. It was demonstrated that the gene product of *cinD* exhibits (1*R*)-6β-hydroxycineole dehydrogenase activity, the second step in the degradation of 1,8-cineole. All four isomers of 6-hydroxycineole were examined but only (1*R*)-6β-hydroxycineole was converted to (1*R*)-6-ketocineole. The (1*R*)-6β-hydroxycineole dehydrogenase exhibited a strict requirement for NAD(H), with no reaction observed in the presence of NADP(H). The enzyme also catalyses the reverse reaction, reducing (1*R*)-6-ketocineole to (1*R*)-6β-hydroxycineole. During this study the N-terminal His-tag used to assist protein purification was found to interfere with NAD(H) binding and lower enzyme activity. This could be recovered by the addition of Ni^{2+} ions or proteolytic removal of the His-tag.

1. Introduction

Terpenes constitute a large class of natural products and play important roles in chemical ecology including acting as repellents, attractants, toxins and sex or alerting pheromones [1]. Monoterpenes (C_{10}) are major components of plant oils and are structurally diverse, encompassing acyclic, monocyclic and bicyclic compounds. The generation of oxygenated terpenoid derivatives is usually initiated by a cytochrome P450 monooxygenase, with this functionalization forming part of either biosynthetic or biodegradative pathways [2]. Cineole, a monoterpene, is one of the more widespread and abundant chemicals in the Australian bush. It is a component of many essential oils and is the dominant component of the oil from many eucalypts. It is estimated that more than 500,000 tonnes of cineole are produced and released into the Australian environment annually by the eucalypt population [3]. The role of cineole remains unclear, although it is believed to have a number of different functions including as a deterrent to herbivores and pathogens, and attracting pollinators. A large proportion of the cineole released into the environment is believed to be removed by microbial degradation, with lesser quantities consumed by native wildlife (koalas, possums) and bushfires [4,5]. The pathways and enzymology of cineole biodegradation however, are not well documented.

A metabolic pathway for the degradation of cineole was proposed [6] following the analysis of cineole metabolites from two soil microbes: *Pseudomonas flava* [4] and a *Rhodococcus* species [7] (Fig. 1). Interestingly, the two species are believed to metabolize cineole (Fig. 1, **1**) via chemically identical but enantiomerically different pathways. Thus, the initial step is believed to be hydroxylation at either the *pro-S* or *pro-R* carbon of cineole to furnish 6β-hydroxycineole[1] (Fig. 1, **2a**). This is then followed by oxidation to give the corresponding ketone, 6-ketocineole (Fig. 1, (1*R*) **3a**). Fur-

Abbreviations: PAGE, Polyacrylamide gel electrophoresis; His-tag, Hexahistidine-tag; P450, Cytochrome P450.

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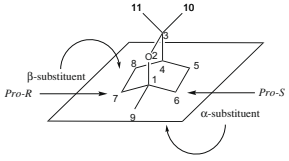
[1] Nomenclature footnote: The nomenclature of the hydroxy cineoles is complex and there is no consistency in the literature for naming such compounds. We use the descriptors α and β in order to discuss stereochemistry. The α descriptor describes substituents below the plane passing through C5, C6, C7 and C8 while β describes substituents above this plane. As cineole is an achiral, meso compound hydroxylation at either of the carbons flanking the C1 bridgehead leads to enantiomeric products and the creation of three new stereogenic centres at C1, C4 and C6. We therefore have defined the carbon atom that, following oxidation, leads to the *R*-C1 isomer as the *pro-R* carbon and the carbon atom that leads to the *S*-C1 isomer as the *pro-S* carbon.



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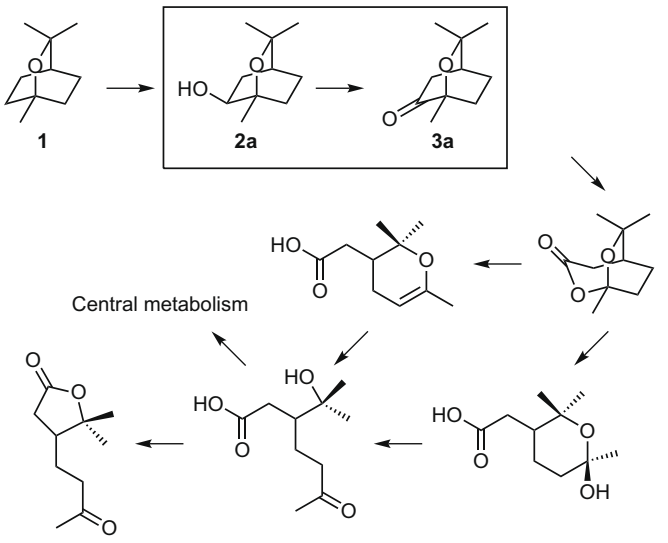

Fig. 1. Proposed pathway of 1,8-cineole degradation [6]. The first step has shown to be catalysed by $P450_{cin}$ in *Citrobacter braakii*. The step that (1*R*)-6β-hydroxycineole dehydrogenase (CinD) is proposed to catalyse is boxed.

ther transformations are somewhat speculative but are believed to produce a highly functionalized hydroxy keto acid that can enter central metabolism, thereby enabling the organism to live on cineole as its sole source of carbon and energy [7].

To date, only two enzymes implicated in cineole biodegradation have been successfully cloned, expressed and purified: cytochrome $P450_{cin}$ and its immediate FMN-containing redox partner, cindoxin. In the presence of an exogenous electron transport partner $P450_{cin}$ and cindoxin catalyze the hydroxylation of cineole to (1*R*)-6β-hydroxycineole (**2a**) [8,25]. The genes encoding these proteins were isolated from the CIN operon of the soil microbe, *Citrobacter braakii*, which is capable of living on cineole as its sole source of carbon and energy. The next step in cineole degradation is believed to be oxidation of the secondary alcohol to the corresponding ketone (Fig. 1, boxed). It has previously been observed that proteins encoded by both the CAM operon from *Pseudomonas putida* and the TERP operon from *Pseudomonas* sp. are responsible for the initial degradation of camphor and α-terpineol, respectively [9–11]. In both these operons an open reading frame encoding an alcohol dehydrogenase was found which is responsible for the oxidation of the alcohol produced following the initial P450 reaction. We thus set out to determine whether a gene encoding a dehydrogenase was also present in, or adjacent to, the CIN operon and, once isolated, clone the gene and express and characterise the gene product in order to investigate its role in cineole metabolism.

2. Materials and methods

2.1. Chemicals

The four isomers of 6-hydroxycineole (**2**) and two isomers of 6-ketocineole (**3**) were prepared from enantiomerically enriched α-terpineol according to the method of Carman and Fletcher [12] with the stereochemistry of the starting α-terpineol determining which enantiomer is formed. All other chemicals used in this study were analytical grade reagents.

2.2. Sequencing of cinD

Sequence analysis of clone pC1 (pUC19 containing the original 4 kb *Pst*I fragment isolated from *C. braakii* [8]) was carried out via subcloning small internal fragments and sequencing using M13 forward and reverse primers. Any gaps that remained in the sequence were determined by using appropriate synthetic primers. All sequencing was analysed using the BLAST algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The open reading frame *cinD* was sequenced completely in both directions.

2.3. Cloning cinD into proEX HTa

Primers were designed to either end of *cinD* containing EcoRI and *Nde*I sites at the 5′ end (5′-CAGAATTCCATATGACCGGCAGAC-TCGAAGG-3′) and EcoRI and HindIII sites at the 5′ end (5′-AAGAATT-CAAGCTTCCTACCCGTCGACGACAGC-3′). *cinD* was amplified from pC1 employing the following conditions: 94 °C for 30 s, 59 °C for 45 s, 72 °C for 90 s. The 0.7 kb DNA fragment was digested with EcoRI and HindIII and cloned into similarly cut pPROEX HTa (Invitrogen, Mount Waverley, VIC) to give pPROEX-*cinD*. This construct was verified by sequencing (AGRF, Brisbane QLD).

2.4. Expression and purification of CinD ((1R)-6β-hydroxycineole dehydrogenase)

Terrific broth containing ampicillin (50 μg/mL) was inoculated with pPROEX-*cinD*-transformed *E. coli* DH5α cells. The culture was incubated at 37 °C until an OD_{600} of 0.6 was attained. Isopropyl β-D-thiogalactopyranoside (IPTG; 1 mM final concentration) was then added and the culture incubated at 27 °C for 18 h. All subsequent procedures were performed at 4 °C unless otherwise stated. The cells were harvested by centrifugation at 4000*g* for 20 min and resuspended in Buffer A (50 mM Tris–HCl, pH 7.4, 1 mM EDTA, 0.5 mM DL-dithiothreitol). Lysis was carried out by sonication on ice (Branson sonifer 450; 6 × 1 min intervals, 30% output) and the cellular debris was removed by centrifugation (10,000g, 1 h). The supernatant was loaded onto a Ni^{2+}-chelating column (1 mL, HiTrap Chelating HP; GE Healthcare, Rydalmere, NSW) that had been equilibrated with Buffer B (20 mM phosphate buffer, 0.5 M NaCl, 10 mM imidazole, pH 7.4). The column was washed with 10 column volumes of Buffer B and the dehydrogenase eluted in Buffer B containing 100–250 mM imidazole. Fractions were analysed by SDS–PAGE (NuPage 4–12% Bis–Tris gels; Invitrogen) and those containing a single band at ~30 KDa were combined and concentrated. The dehydrogenase was loaded onto a G-25-desalting column (1 × 6 cm) and eluted in buffer (50 mM Tris–HCl pH 7.4). The eluted protein was stored at −70 °C. Protein concentration was determined using the Bio-Rad Protein Assay (Bio-Rad, Gladesville, NSW) with bovine serum albumin as the standard.

2.5. Cleavage of His-tag from (1R)-6β-hydroxycineole dehydrogenase

(1*R*)-6β-hydroxycineole dehydrogenase (34 μM) was added to a solution containing Buffer A and AcTEV™ protease (200 U; Invitrogen) and incubated at 4 °C for 16 h. The solution was then diluted 5-fold in Buffer B before loading onto a Ni^{2+}-chelating column (1 mL, HiTrap Chelating HP) that had been equilibrated with Buffer B. The flow-through (containing cleaved native enzyme) was collected and concentrated. Excess NaCl/imidazole was removed by loading the protein onto a G-25-desalting column (1 × 6 cm) and eluting in buffer (50 mM Tris–HCl pH 7.4). The cleaved protein was stored at −70 °C.

2.6. Assay for (1R)-6β-hydroxycineole dehydrogenase activity: NAD(H) detection/consumption

Oxidation of (1*R*)-6β-hydroxycineole (**2a**) was detected by measuring the rate of NADH formation. The assay, containing mixture of the dehydrogenase (0.5–10 μM) and (1*R*)-6β-hydroxycineole

(**2a**) (60 mM stock solution in ethanol, final concentration: 600 μM) in buffer (50 mM Tris–HCl pH 7.4), was initiated by the addition of NAD^+ (6 mM; $\varepsilon_{340nm} = 6200\ M^{-1}cm^{-1}$). The reaction was monitored by UV spectroscopy, measuring the appearance of NADH at 340 nm. All assays were performed at room temperature (typically 25 °C) and ethanol concentration never exceeded 1%. The oxidation of other substrates, such as androsterone was measured in an analogous fashion. To measure the reverse reaction (reduction) the rate of NADH consumption was monitored by the decrease in absorbance at 340 nm.

The NADH-detection assay was used to examine the effect of metal ions on the dehydrogenase activity. The assay was run as described above with the inclusion of the following metal ions at 1 mM: Zn^{2+} ($ZnCl_2$), Cu^{2+} ($CuCl_2$), Co^{2+} ($CoCl_2$), Ca^{2+} ($CaCl_2$), Mg^{2+} ($MgCl_2$), Mn^{2+} ($MnCl_2$) and Ni^{2+} ($NiSO_4$).

2.7. Assay for (1R)-6β-hydroxycineole dehydrogenase activity: enantioselective GC

2.7.1. Oxidation of 6-hydroxycineole (**2**) to 6-ketocineole (**3**)

All four isomers of 6-hydroxycineole (**2a–d**) were assayed. 6-hydroxycineole (**2**) (6 mM) was added to a solution of (1*R*)-6β-hydroxycineole dehydrogenase (8 μM) in buffer (50 mM Tris–HCl pH 7.4) and stirred at room temperature for 10 min before the addition of NAD^+ (1 mM). The reaction was left stirring for 2 h, extracted with ethyl acetate and the organic extract dried over $MgSO_4$. The ethyl acetate was removed *in vacuo* and the product resuspended in dichloromethane. Several drops of trifluoroacetic anhydride were added to the solution and the sample was then concentrated *in vacuo*. The derivatized product was dissolved in diethyl ether and analysed by enantioselective GC. Briefly, this was performed on a gas chromatograph fitted with a β-cyclodextrin enantioselective column under the following conditions: 120 °C for 2 min, 1 °C/min to 200 °C. Retention times were compared with synthetic standards [12] that had been identically derivatized.

2.7.2. Reduction of 6-ketocineole (**3**) to 6-hydroxycineole (**2**)

(1*R*)- and (1*S*)-6-ketocineole (**3a** and **3b**) were assayed in an analogous fashion to that described for 6-hydroxycineole (**2**), employing NADH (rather than NAD^+) to initiate the reaction.

3. Results

3.1. Operon analysis

Following the sequencing of pC1, a fourth open reading frame (*cinD*; Genbank ID: GQ849481) was identified. The *cinD* gene is divergently transcribed with respect to *cinA* ($P450_{cin}$), *cinB* and *cinC* (cindoxin), three ORFs that were identified during previous analysis of the CIN operon (Fig. 2) [8]. This ORF (*cinD*) displays significant homology to a number of short-chain alcohol dehydrogenases (Table 1).

3.2. Subcloning and expression of CinD ((1R)-6β-hydroxycineole dehydrogenase)

Initially, expression of CinD in *E. coli* as the wild type enzyme (i.e. with no His-tag present) was attempted. These efforts were unsuccessful and consequently, an alternative strategy to express CinD was evaluated. PCR was used to amplify *cinD* employing primers that introduced EcoRI and HindIII restriction sites and facilitated subsequent cloning of the gene into the expression vector pPROEX HTa. pPROEX HTa includes a coding region for a hexahistidine N-terminal affinity tag (His-tag) to assist in protein purification. pPROEX-*cinD* was transformed into *E. coli* DH5α cells and protein expression was induced with 1 mM isopropyl β-D-thiogalactpyranoside (IPTG). The best expression was obtained at between 27–30 °C following induction in terrific broth (data not shown).

3.3. Purification and characterisation of the (1R)-6β-hydroxycineole dehydrogenase

Purification of (1*R*)-6β-hydroxycineole dehydrogenase was facilitated by the addition of an N-terminal His-tag, enabling purification of the protein in a single step. The recombinant protein was purified by immobilized metal affinity chromatography (IMAC) employing a HiTrap Chelating column (GE Healthcare) loaded with Ni^{2+}. This resulted in a single band as evaluated by SDS–PAGE at approximately 30 KDa (theoretical mass including His-tag = 29.3 KDa). The purification typically yielded 50 mg of purified protein per litre of original culture.

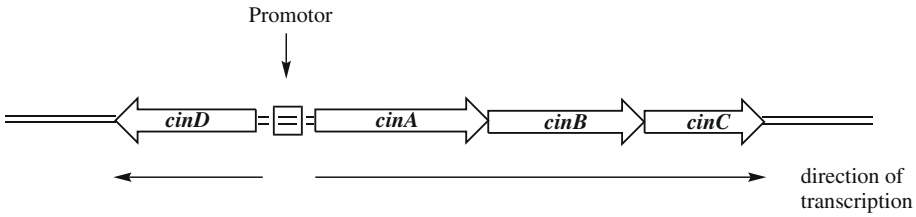

Fig. 2. Map of the CIN operon region that contains four open reading frames, *cinA*, *cinB*, *cinC* and *cinD*.

Table 1
Sequence homology of *cinD* open reading frame.

Protein	Organism	%I[a] (%S)[b]	Genbank ID	Refs.
Putative 20-β-hydroxysteroid dehydrogenase FabG3_1	*Mycobacterium marinum M*	60 (73)	YP_001851274	[20]
3α, 20β-Hydroxysteroid dehydrogenase	*M. tuberculosis*	59 (72)	P69167	[13]
Putative short-chain dehydrogenase	*Nocardia facinica IFM 10152*	50 (67)	YP_121022	[21]
Putative oxidoreductase	*Streptomyces coelicolor A3(2)*	47 (62)	NP_628345	[22]
R-specific alcohol dehydrogenase	*Lactobacillus brevis*	37 (55)	CAD66648	[23]
3-Ketoacyl reductase	*M. tuberculosis* H37Rv	32 (50)	AAC69639	[24]

[a] Percent identity.
[b] Percent similarity (identity + substitutions with an amino acid with similar properties).

To establish the pH profile of (1*R*)-6β-hydroxycineole dehydrogenase, specific activity was measured every 0.2 pH units using 50 mM Tris–HCl from pH 7.0 to 8.4 employing the NADH-detection assay. Below pH 7.2 the enzyme was found to significantly drop in activity (approximately 30% of maximum activity). Between pH 7.2 and 8.2 only a slight increase in activity was observed. A high concentration of sodium chloride is present in the elution buffer for the Ni^{2+}-chelating column used to purify the His-tagged dehydrogenase. To determine whether the sodium chloride concentration had any effect on activity the introduction of sodium chloride at various concentrations (20–200 mM) was examined. No significant effects were observed with increased sodium chloride (data not shown).

3.4. Substrate and cofactor specificity

It was postulated that the *cinD* gene product may be responsible for catalysing the oxidation of 6-hydroxycineole (**2**) to 6-ketocineole (**3**) (Fig. 1, boxed). Individually, all four isomers of 6-hydroxycineole (Fig. 3, **2a–d**) were incubated with (1*R*)-6β-hydroxycineole dehydrogenase and NAD^+ and the products analysed by enantioselective GC. Only (1*R*)-6β-hydroxycineole (**2a**) was observed to be converted to (1*R*)-6-ketocineole (Fig. 4, **3a**); (**2b–d**) were unreactive. To establish if the dehydrogenase could also catalyse the reverse reaction (reduction), both isomers of the 6-ketocineole (**3**) were incubated with NADH and the enzyme. Again only one isomer, (1*R*)-6-ketocineole (**3a**) was converted to the corresponding alcohol: (1*R*)-6β-hydroxycineole (**2a**). Cofactor specificity was also examined by incubating the dehydrogenase and (1*R*)-6β-hydroxycineole (**2a**) with either NAD^+ or $NADP^+$. No product could be observed with $NADP^+$ as the cofactor. In addition, no products were observed when attempting the reduction of (1*R*)-6-ketocineole (**3a**) with the dehydrogenase and NADPH.

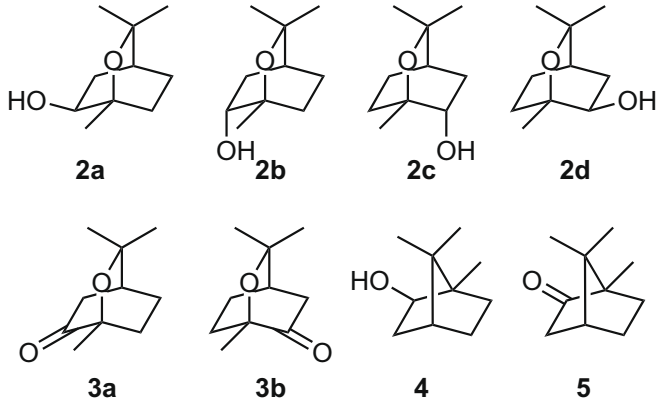

Fig. 3. The four isomers of 6-hydroxycineole (**2a–d**), the two enantiomers of 6-ketocineole (**3a–b**), isoborneol (**4**) and camphor (**5**).

Other structurally related terpenes, isoborneol (**4**) and camphor (**5**) (Fig. 3) were incubated with the dehydrogenase to gain insight into the substrate specificity of the enzyme. Neither (**4**) nor (**5**) were metabolised. Because of the high degree of similarity of the (1*R*)-6β-hydroxycineole dehydrogenase to a 3α, 20β-hydroxysteroid dehydrogenase (Genbank ID: P69167, 59% identity, 72% similarity; [13]), both androsterone and progesterone were examined as possible substrates. Androsterone was found to be oxidised in the presence of CinD and NAD^+ and have a V_{max} of 23 ± 2 min^{-1} (rate measured in the presence of Ni^{2+} *vide infra*). Progesterone was not observed to be reduced by (1*R*)-6β-hydroxycineole dehydrogenase.

3.5. Effect of metal ions

To examine whether metal ions are required for activity a number of divalent metal ions were incubated with (1*R*)-6β-hydroxycineole dehydrogenase (with the His-tag still present), NAD^+ and (1*R*)-6β-hydroxycineole (**2a**). Both the nickel ($NiSO_4$) and cobalt ($CoCl_2$) ions increased the activity of the dehydrogenase by 10 to 15-fold. Activity did not increase any further with both Ni^{2+} and Co^{2+} present together. All other metal ions tested (Zn^{2+}, Cu^{2+}, Ca^{2+}, Mg^{2+}, Mn^{2+}) had no effect on the enzyme activity. For maximal activity the optimum concentration of nickel was observed

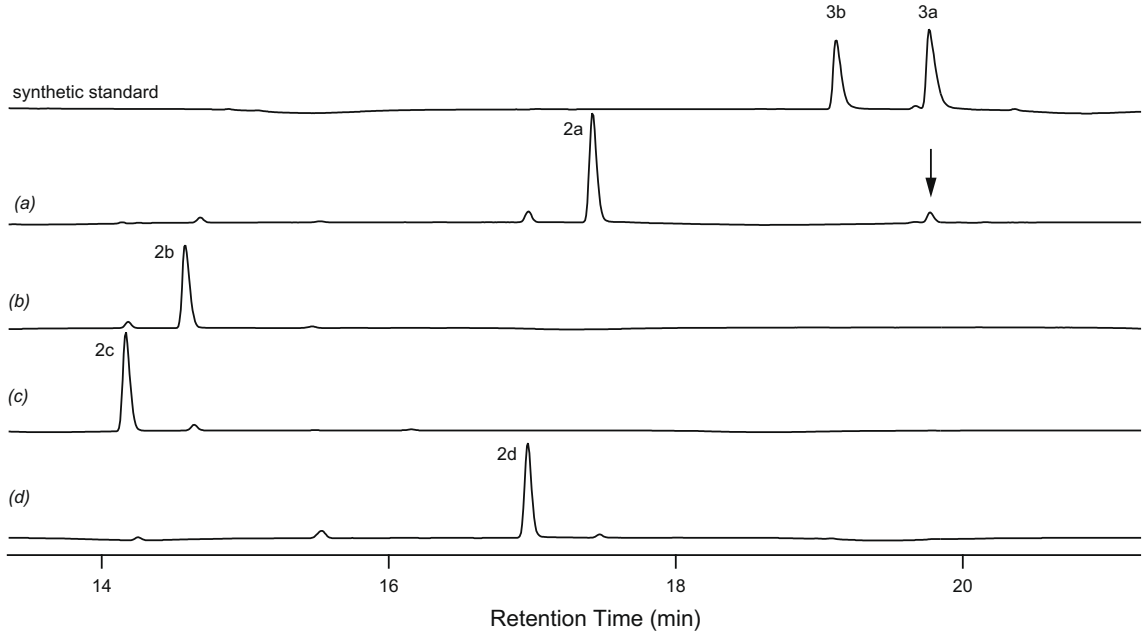

Fig. 4. Enantioselective GC analysis of products formed following incubation of 6-hydroxycineole (**2a–d**) with (1*R*)-6β-hydroxycineole dehydrogenase. Arrow indicates the formation of (1*R*)-6-ketocineole (**3a**) from (**2a**) by CinD catalysed oxidation in the presence of NAD^+. (Traces of the other isomers are present in each of **2a–2d** due to the synthetic route employed [12]).

to be between 0.5 and 5 mM metal ion/μM dehydrogenase. Concentrations above 5 mM had a similar dehydrogenase activity to that when no nickel is present. Assays were routinely run at 2 mM metal ion/μM dehydrogenase.

3.6. Removal of the His-tag from (1R)-6β-hydroxycineole dehydrogenase

To examine whether the His-tag was interfering with the natural activity of the dehydrogenase, AcTEV™ protease was employed to remove the tag. This protease recognises a seven amino acid sequence (ENLYFQG) and cleaves the peptide bond between Q and G [14]. Following the cleavage only eight amino acids remained before the native start site of the protein. The successful removal of the His-tag was confirmed by SDS–PAGE, in addition to the dehydrogenase's behaviour on the Ni^{2+}-chelating column.

3.7. (1R)-6β-hydroxycineole dehydrogenase-kinetic analysis

Kinetic constants (K_m and V_{max}) were measured for the His-tagged-dehydrogenase and the dehydrogenase with the His-tag removed with both the substrate (1*R*)-6β-hydroxycineole (**2a**) and the cofactor NAD^+ (Table 2). Reliable K_m values could not be determined for the His-tagged-dehydrogenase because Michaelis–Menten conditions could not be satisfied (i.e. substrate concentration = $10 \times K_m$ could not be attained). Only with the addition of divalent nickel ions could accurate K_m and V_{max} values be measured. In contrast, the addition of nickel to the dehydrogenase with the His-tag removed did not change the enzyme activity. The K_m for NAD^+ with the dehydrogenase is 3-fold higher than that of the His-tagged-dehydrogenase in the presence of nickel. The improvement in K_m seen for the His-tagged dehydrogenase in the presence of nickel is mirrored in the observed V_{max} for both these forms of the dehydrogenase with NAD^+ (3-fold increase for His-tagged-dehydrogenase with nickel). The opposite trend was observed when comparing K_m values for (1*R*)-6β-hydroxycineole (**2a**). The K_m for the cleaved-dehydrogenase with (1*R*)-6β-hydroxycineole (**2a**) was 2-fold lower than for the His-tagged-dehydrogenase with nickel.

4. Discussion

Subsequent to the isolation of the genes encoding $P450_{cin}$ (*cinA*) and its two redox partners (*cinB* and *cinC*) from *C. braakii* [8], a fourth gene (*cinD*) divergently transcribed with respect to *cinA*, *cinB* and *cinC* was discovered. The *cinA*, *cinB* and *cinC* genes encode the suite of enzymes responsible for the hydroxylation of cineole to (1*R*)-6β-hydroxycineole (**2a**) [8,25]. The *cinD* gene displays significant homology to a number of short-chain dehydrogenases/oxidoreductases (Table 1). The second step in cineole degradation is believed to be oxidation of the initially formed secondary alcohol to the corresponding ketone (Fig. 1). Because the sequence alignment indicated that the *cinD* gene product was likely to be a short-chain dehydrogenase it was postulated that the *cinD* gene product may be responsible for catalysing the oxidation of (1*R*)-6β-hydroxycineole (**2a**) to (1*R*)-6-ketocineole (**3a**) in *C. braakii*.

Following the purification of the His-tagged-dehydrogenase, incubation of (1*R*)-6β-hydroxycineole (**2a**) with the enzyme and NAD^+ as a cofactor indicated (1*R*)-6β-hydroxycineole dehydrogenase did catalyse its oxidation producing (1*R*)-6-ketocineole (Fig. 3, **3a**). This demonstrates that the *cinD* gene product is involved in the second step of cineole degradation in *C. braakii*. As expected, the reverse reaction, the reduction of (1*R*)-6-ketocineole (**3a**) to (1*R*)-6β-hydroxycineole (**2a**), is also catalysed by the (1*R*)-6β-hydroxycineole dehydrogenase in the presence of NADH. The dehydrogenase was found to be highly stereoselective. Of the four isomers that exist for 6-hydroxycineole (**2**), only (1*R*)-6β-hydroxycineole (**2a**) is oxidised to the corresponding ketone (**3a**) (Figs. 3 and 4). Of the two enantiomeric ketocineoles (**3**), only (1*R*)-6-ketocineole (**3a**) is reduced, specifically yielding **2a**. $NADP^+$/NADPH could not be used as a cofactor in these reactions. These results are similar to that observed in a *Rhodococcus* species reported by Williams et al. [7]. NAD^+-dependent '6-*endo*-hydroxycineole dehydrogenase' activity was detected in extracts of cells grown in the presence of cineole, although the enzyme was not purified to homogeneity. These extracts were found to catalyse the dehydrogenation of (1*R*)-6β-hydroxycineole (**2a**) to (1*R*)-6-ketocineole (**3a**) and the corresponding reverse reaction. They report a small amount of activity (<10%) when NADPH is substituted as the electron donor. This activity is not observed with (1*R*)-6β-hydroxycineole dehydrogenase isolated from *C. braakii*. Interestingly, GC analysis of extracts of *P. flava*, another soil microbe capable of employing cineole as an energy source, revealed the formation of both (1*S*)-6α- and (1*S*)-6β-hydroxycineole (**2c** and **2d**) [4]. This indicates that either the enzymes responsible for the initial steps in the oxidative biodegradation of cineole in *P. flava* are not as stereoselective as those in *C. braakii* or that there are other, parallel activities in *P. flava* that account for the production of both **2c** and **2d**.

The (1*R*)-6β-hydroxycineole dehydrogenase shares a high degree of similarity to a group of enzymes called 3α, 20β-hydroxysteroid dehydrogenases (Table 1). Some of these enzymes have been shown to be capable of the oxidation/reduction of the 3α and 20β-hydroxyl/ketone groups of steroids such as progesterone [15,16]. One such enzyme from *Mycobacterium tuberculosis* was found to have 59% identity and 72% similarity [13] with CinD and has been demonstrated to oxidise androsterone (V_{max} = 7.6 min^{-1}) and reduce progesterone (V_{max} = 1.2 min^{-1}). (1*R*)-6β-hydroxycineole dehydrogenase was also found to oxidise androsterone (V_{max} = 23 ± 2 min^{-1}) but not reduce progesterone. Considering the highly selective requirements of the dehydrogenase for its proposed substrates ((1*R*)-6β-hydroxycineole **2a**/(1*R*)-6-ketocineole **3a**) this result was unexpected. This confirms that assigning function simply based on sequence alignment will not always be reflective of *in vivo* function.

Initially, (1*R*)-6β-hydroxycineole dehydrogenase was found to have very low rates of activity (1–10 min^{-1}). Two different approaches were found to be successful in improving the activity. It was thought that the His-tag could be interfering with the NAD^+ binding to the enzyme due to the inability to accurately measure the K_m for NAD^+ (Table 2). Two distinctive sequences that consti-

Table 2
Kinetic constants determined for the purified (1*R*)-6β-hydroxycineole dehydrogenase.

	NAD^+		(1*R*)-6β-hydroxycineole[a]	
	K_m (μM)	V_{max} (min^{-1})	K_m (μM)	V_{max} (min^{-1})
HCDH[b]	537 ± 39	26 ± 1[c]	11 ± 2	12 ± 1[c]
HIS-HCDH	<6000[d]	9.5 ± 0.3[d]	~56[d]	~1.8[d]
HIS-HCDH (Ni)	175 ± 6	88 ± 1[c]	21 ± 2	101 ± 7[c]

[a] Kinetic constants for the conversion of (1*R*)-6β-hydroxycineole to the (1*R*)-6-ketocineole were calculated from the production of NADH from NAD^+ (see Section 2).

[b] Abbreviations used in this table: HCDH = cleaved (1*R*)-6β-hydroxycineole dehydrogenase; HIS-HCDH = His-tagged (1*R*)-6β-hydroxycineole dehydrogenase; HIS-HCDH (Ni) = His-tagged (1*R*)-6β-hydroxycineole dehydrogenase in the presence of 2 mM $NiSO_4$/μM enzyme.

[c] Ideally, the V_{max} values should be the same for each form of the enzyme under the same conditions. The observed differences may be due to variations in temperature and/or the instability of the enzyme. In addition, the high K_m for the HCDH (NAD^+) means that the conditions for measuring V_{max} for this form of the enzyme only just satisfy Michaelis–Menten conditions.

[d] K_m or V_{max} could not be measured accurately as Michaelis–Menten conditions could not be satisfied.

tute the Rossmann Fold [GXX(X)GX(X)G and YXXXK] were found in the protein sequence of (1*R*)-6β-hydroxycineole dehydrogenase. The Rossmann Fold allows for the binding of NADH/NAD^+ during the reduction/oxidation of the substrate [17,18]. The first (GAAQGMG (14-20) is very close to the N-terminus to which the His-tag was attached. His-tags have previously been demonstrated to affect enzyme activity in some cases [19]. For example, glucosamine-6-phosphate synthase from *Candida albicans* was expressed in *E. coli* with a His-tag at the N-terminus. It was found to lose activity completely. Once the His-tag was placed at the C-terminus rather than the N-terminus the enzyme regained activity [19]. We hypothesised that divalent nickel ions could coordinate to the His-tag and remove it from the NAD(H) binding site. It was also possible that the enzyme may require a metal-ion for catalysis. Another dehydrogenase, 5-*exo*-hydroxycamphor dehydrogenase from *P. putida*, that catalyses a similar reaction, converting 5-*exo*-hydroxycamphor to 2,5-diketocamphane is believed to have two firmly bound zinc atoms per subunit [10]. In an attempt to improve the rates of turnover of (1*R*)-6β-hydroxycineole dehydrogenase, various divalent metal ions were added to the reaction. Interestingly, only nickel and cobalt were observed to increase the activity (10–15-fold). This, together with the observation that nickel did not improve the activity of (1*R*)-6β-hydroxycineole dehydrogenase without the His-tag (*vide infra*), suggested that the His-tag had been coordinated by the nickel or cobalt, exposing the NAD(H) binding site. In addition, (1*R*)-6β-hydroxycineole dehydrogenase does not have any significant homology with 5-*exo*-hydroxycamphor dehydrogenase, or the zinc-containing dehydrogenases in general.

The second approach to improving the activity was to remove the His-tag enzymatically using AcTEV™ protease. This approach also resulted in an enzyme with increased activity (Table 2; approximately 2-fold). The additional overnight incubation and purification of the enzyme is thought to be responsible for the only moderate increase in activity compared to that observed with the His-tagged (1*R*)-6β-hydroxycineole dehydrogenase in the presence of nickel as we had previously observed loss of activity in the enzyme upon storage at room temperature. Interestingly, the K_m (NAD^+) for the cleaved-dehydrogenase was not as low as that for the nickel-coordinated His-tagged-dehydrogenase (Table 2). This perhaps suggests that the eight amino acids left at the N-terminus following cleavage are still large enough to interfere with the optimal binding of NAD^+ to the enzyme.

In summary, we have demonstrated that the gene product of *cinD* from *C. braakii* exhibits (1*R*)-6β-hydroxycineole dehydrogenase activity and also catalyses the reverse reaction, reducing (1*R*)-6-ketocineole (**3a**) to (1*R*)-6β-hydroxycineole (**2a**). During this study it was observed that the N-terminal His-tag used to assist purification interfered with the NAD(H) binding site and lowered the activity of the enzyme. This activity could be recovered via the addition of Ni^{2+} ions, postulated to coordinate to the His-tag and thus allow NAD(H) to access its binding site. (1*R*)-6β-hydroxycineole dehydrogenase is stereo- and enantioselective, only accepting (1*R*)-6β-hydroxycineole (**2a**). Importantly, this is the same enantiomer produced by $P450_{cin}$ during the oxidation of 1,8-cineole linking these two activities in cineole biodegradation [8].

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