

## Metabolism of 1,8-Cineole by a *Rhodococcus* Species: Ring Cleavage Reactions

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A *Rhodococcus* sp. (strain C1) was isolated by elective culture with 1,8-cineole as sole carbon source. 6-*endo*-Hydroxycineole and 6-oxocineole accumulated transiently during the latter part of the exponential growth phase and, together with 1,8-cineole, were oxidized rapidly by 1,8-cineole-grown cells. Although a putative 1,8-cineole monooxygenase was not detected in cell-free systems an induced 6-*endo*-hydroxycineole dehydrogenase and an induced NADPH-linked 6-oxocineole oxygenase were readily demonstrated. The lactone 5,5-dimethyl-4-(3'-oxobutyl)-4,5-dihydrofuran-2(3H)-one was isolated from oxygenation reactions with 6-oxocineole as substrate. This was not the immediate product of oxygenation but resulted from non-enzymic lactonization of the ring cleavage intermediate 3-(1-hydroxy-1-methylethyl)-6-oxoheptanoic acid during extraction procedures. 2,5-Diketocamphane 1,2-monooxygenase purified from (+)-camphor-grown *Pseudomonas putida* ATCC 17453 was also able to utilize 6-oxocineole as a substrate with formation of the same isolated product. The established oxygen-insertion specificity of this enzyme coupled with an unequivocal absence of esterase activity allowed the nature of the oxygen insertion into 6-oxocineole by the enzyme from *Rhodococcus* C1 to be inferred and a reaction sequence for cleavage of both rings of 1,8-cineole to be proposed. It provides an explanation for the reported isolation of (*R*)-5,5-dimethyl-4-(3'-oxobutyl)-4,5-dihydrofuran-2(3H)-one from culture media of *Pseudomonas flava* grown with 1,8-cineole.

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### INTRODUCTION

Although the degradation of cyclic monoterpenes by bacteria growing with them as sole carbon sources has been studied for more than 25 years the ring cleavage reactions are understood for a very limited number of compounds and in no instance has the complete pathway to central metabolites been fully documented. Degradation of naturally occurring (+)-camphor is the most thoroughly understood and involves an initial hydroxylation at carbon-5 by *Pseudomonas putida* ATCC 17453 (Katagiri *et al.*, 1968) or at carbon-6 by a *Rhodococcus* sp. (Chapman *et al.*, 1966). In the case of *P. putida* cleavage of the two carbocyclic rings is catalysed by discrete 'Baeyer-Villiger' monooxygenases (Ougham *et al.*, 1983; Taylor & Trudgill, 1986). Although the ring cleavage product formed by the *Rhodococcus* sp. has been unequivocally identified the cleavage reactions have not been investigated in detail. In neither case have the post-cleavage reactions, leading to central metabolites, been established.

Degradation of the bicyclic hydrocarbon  $\alpha$ -pinene has also attracted significant attention and at least two distinct cleavage pathways have been described. One of these almost certainly involves initial cleavage of the four-membered carbon ring with the formation of the monocyclic monoterpene skeleton and integration of this into a proposed pathway for limonene degradation (Shukla *et al.*, 1968) while the other is quite novel. Initial monooxygenation forms  $\alpha$ -pinene epoxide (Best *et al.*, 1987) and ring cleavage in both *Nocardia* and some *Pseudomonas* spp. is mediated by  $\alpha$ -pinene oxide lyase (EC 4.99.-.-) which catalyses the concerted cleavage of all three rings without the requirement for any additional non-protein components (Griffiths *et al.*, 1987*a,b*).

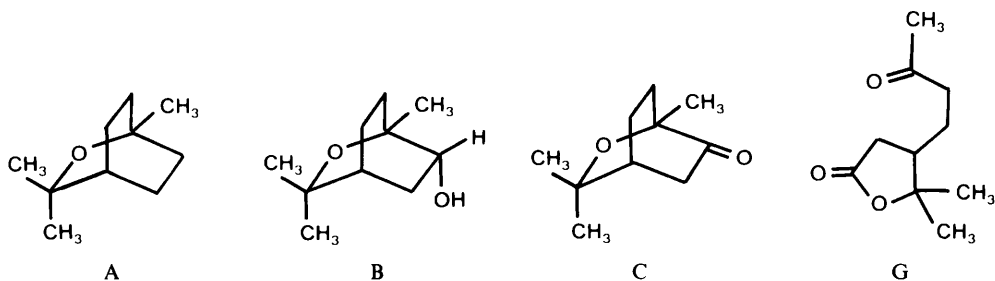


Fig. 1. The structure of 1,8-cineole and relevant reported metabolites. A, 1,8-cineole; B, 2-*endo*-hydroxycineole; C, 2-oxocineole; G, (*R*)-5,5-dimethyl-4-(3'-oxobutyl)-4,5-dihydrofuran-2(3H)-one.

There is good evidence that *Pseudomonas* spp., capable of utilizing the monocyclic monoterpene limonene as sole carbon source, degrade it by initial oxidation of a methyl group (carbon-7) to carboxyl followed by a  $\beta$ -oxidation cycle which achieves ring cleavage (Dhavalikar & Bhattacharyya, 1966; Dhavalikar *et al.*, 1966) although, once again, the route to central metabolites is obscure.

1,3,3-Trimethyl-2-oxabicyclo(2,2,2)octane [1,8-cineole] is a component of many essential oils and is the dominant component of the oil from leaves of *Eucalyptus radiata* var. *Australiana* (Nishimura *et al.*, 1982). It is of particular interest in that while its carbocyclic skeleton is that of the monocyclic monoterpenes the additional structural complication of a 1,8-ether linkage turns it into a bicyclic compound. Reports of 1,8-cineole metabolism are rudimentary, although analytically thorough, and include the isolation of (1*S*,4*R*,6*R*)-1,3,3-trimethyl-2-oxabicyclo(2,2,2)octan-6-ol [2-*endo*-hydroxycineole], (1*S*,4*R*,6*S*)-1,3,3-trimethyl-2-oxabicyclo(2,2,2)octan-6-ol [2-*exo*-hydroxycineole], (1*S*,4*R*)-1,3,3-trimethyl-2-oxabicyclo(2,2,2)octan-6-one [2-oxocineole] and the lactone (*R*)-5,5-dimethyl-4-(3'-oxobutyl)-4,5-dihydrofuran-2(3H)-one from the culture medium of a strain of *Pseudomonas flava* isolated by elective culture with, and grown upon, 1,8-cineole as sole carbon source (MacRae *et al.*, 1979; Carman *et al.*, 1986) (Fig. 1) and the formation of the analogous C-3-substituted 1,8-cineole derivatives by broth-grown *Aspergillus niger* (Nishimura *et al.*, 1982). It should be noted that strict application of chemical nomenclature rules does not allow the use of *endo* and *exo* in the (2,2,2)bicyclo system. In common with other authors in this field (MacRae *et al.*, 1979) we have designated compound B as the *endo* isomer because the hydroxyl substitution is on the opposite side of the reference plane from the lowest priority bridge. We have also made use of the ring numbering system consistent with the trivial name 1,8-cineole. In this paper we describe the isolation of a *Rhodococcus* sp. capable of growth with 1,8-cineole as sole carbon source, identify two enzymes involved in the initial stages of oxidation, and propose a reaction sequence for the concerted cleavage of the carbocyclic ring and the ether linkage.

A preliminary report of a section of this work has already appeared (Taylor & Trudgill, 1988).

#### METHODS

**Bacterial strains and culture conditions.** *Pseudomonas putida* ATCC 17453 was obtained from the American Type Culture Collection. *Rhodococcus* sp. strain C1 was isolated from soil obtained from beneath a *Eucalyptus* sp. growing on the College campus at Aberystwyth. Stock cultures were grown on nutrient agar (Oxoid) at 30 °C, stored at 3 °C and subcultured at regular intervals. For experimental purposes *P. putida* was grown on (+)-camphor as previously described (Taylor & Trudgill, 1986) and *Rhodococcus* C1 was grown at 30 °C in a medium that contained (g l<sup>-1</sup>):KH<sub>2</sub>PO<sub>4</sub>, 2; Na<sub>2</sub>HPO<sub>4</sub>, 4; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1. Trace element solution (Rosenberger & Elsdon, 1960) was added at 0.4% (v/v) before the medium was sterilized by autoclaving. When required sodium succinate hexahydrate (4.6 g l<sup>-1</sup>) was added prior to autoclaving. Sterile 1,8-cineole (1 g l<sup>-1</sup>) was added aseptically to the autoclaved basal medium. For growth with 1,8-cineole a 20 ml starter culture, with succinate as carbon source, was inoculated from a slope and grown on a gyratory shaker at 150 r.p.m. and 30 °C for 48 h. Subsequently 10% (v/v) inocula were transferred to 100 ml and 900 ml volumes of media, with 1,8-cineole as substrate, in 250 ml and 2 l

Erlenmeyer flasks respectively and grown for 18–24 h. Cells were then either harvested for experimental purposes (10000 g, 30 min, 4 °C) or used to inoculate 9 l of 1,8-cineole medium in a New Brunswick Microferm laboratory fermenter which was stirred at 500 r.p.m. and aerated by the passage of sterile air at the rate of 1 l min<sup>-1</sup>. After 15–18 h growth the culture was either harvested in a continuous flow centrifuge (Alfa Laval) at a rate of 0.4–0.5 l min<sup>-1</sup> or used to inoculate 30 l of medium in a 50 l plastic vessel adapted to fit the fermenter which was stirred at 600 r.p.m., aerated by the passage of sterile air at 6 l min<sup>-1</sup>, and the culture harvested after ≈ 10 h growth. Cell pellets were routinely resuspended in an equal volume of 42 mM-KH<sub>2</sub>PO<sub>4</sub>/Na<sub>2</sub>HPO<sub>4</sub> buffer (pH 7.1) and either used immediately or stored at -20 °C until required.

*Preparation of cell extracts.* Washed resuspended cells were disrupted by either (a) passage through a French pressure cell with a pressure difference at the orifice of 138 MPa, (b) passage of the frozen cell paste through a Hughes press at -15 °C or (c) by sonication of 10 ml samples in an ice-bath for two 30 s periods, with a 1 min cooling interval, at an amplitude of 18–20 μm (MSE Soniprep). Broken cell suspensions were incubated with approximately 0.5 mg of DNAase (EC 3.1.21.1) per 10 ml for 15 min at 4 °C and then centrifuged at 27000 g and 4 °C for 45 min to remove cell debris. The crude cell extract was decanted and stored at -20 °C until required. Membrane preparations were obtained by centrifugation of crude cell extract at 100000 g and 4 °C for 60 min. The pink gelatinous pellet was resuspended in 42 mM-KH<sub>2</sub>PO<sub>4</sub>/Na<sub>2</sub>HPO<sub>4</sub> buffer (pH 7.1), centrifuged as above for a further 60 min and again resuspended in the same phosphate buffer before use.

*Protein measurement.* The biuret assay, as modified by Gornall *et al.* (1949), was used routinely for the estimation of protein in solution. A crude estimate of the protein content of column fractions was made by measuring absorbance at 280 nm.

*Measurement of oxygen uptake.* Oxygen consumption by whole cell suspensions or crude cell extracts was measured either by conventional Warburg manometry or polarographically using an oxygen monitor (Yellow Springs Instrument Co.).

*Extraction of metabolites.* Bacteria were removed from culture media either by passage of small volumes (<20 ml) through a membrane filter (0.22 μm) or by continuous flow centrifugation of larger volumes (Alfa Laval). Neutral metabolites were extracted by shaking the aqueous medium with an equal volume of redistilled diethyl ether which was separated and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. When necessary the diethyl ether was removed by rotary evaporation under vacuum. Acidic metabolites were similarly extracted into diethyl ether after first acidifying the aqueous medium to ≈ pH 1 with 5 M-HCl.

*GLC analysis of metabolites.* Samples (1 μl) of diethyl ether extracts containing not more than 100 μg ml<sup>-1</sup> of individual metabolites were analysed by GLC on a Carlo Erba HRGC 5300 instrument fitted with a WCOT fused silica CP-wax-52 CB capillary column (0.32 mm × 25 m) and a flame ionization detector. Hydrogen was used as the carrier gas at 1.5 ml min<sup>-1</sup>. The temperature programme was 45–80 °C at 25 °C min<sup>-1</sup>; 80–150 °C at 3 °C min<sup>-1</sup>; 150–245 °C at 35 °C min<sup>-1</sup>. The final temperature was maintained for 10 min.

*GLC-MS analysis.* GLC-mass spectral analyses were done on a 25 m polar BP20 fused silica capillary column programmed from 50 °C (3 min) to 250 °C at 10 °C min<sup>-1</sup>. Helium was used as the carrier gas at a flow rate of 2 ml min<sup>-1</sup>. Electron impact spectra were recorded on a VG 12F mass spectrometer (VG Analytical) with a beam energy of 70 eV, source temperature of 150 °C, 100 μA emission and scan cycle time of 2.7 s. Chemical ionization spectra were obtained with a VG 70 HSE instrument using isobutane as the reagent gas at a source housing pressure of 2 Pa and 200 μA emission.

*NMR spectra.* Proton nuclear magnetic resonance (<sup>1</sup>H-NMR) spectra were recorded on a JOEL FX60Q Fourier transform spectrometer in CDCl<sub>3</sub> at 60 MHz with tetramethylsilane as internal standard.

*Measurement of optical rotation.* Optical rotations of isolated metabolites were measured in ethanolic solution with a 20 mm light-path cell in a Bendix NPL Automatic Polarimeter at 589.5 nm (sodium D line).

*Estimation of lactones.* The alkaline hydroxamate procedure of Cain (1961) was used for the detection and assay of lactones.

*Measurement of changes in proton concentration.* The consumption of protons in enzyme-catalysed reactions was followed in near-buffer-free aqueous reaction systems at 20 °C. The pH was maintained at a constant value by the controlled addition of 2 mM-HCl from a burette attached to an autotitrator (Pye-Unicam).

*Enzyme units.* All enzyme activities are expressed as μmol substrate used or product formed min<sup>-1</sup>. Unless otherwise stated all assays were done at 30 °C.

*Enzyme assays.* 2,5-Diketocamphane 1,2-monooxygenase was routinely assayed, using (+)-camphor as the substrate, either by measuring substrate-stimulated NADH oxidation spectrophotometrically or by following oxygen consumption polarographically. A typical reaction mixture contained, in a total volume of 3 ml, 120 μmol KH<sub>2</sub>PO<sub>4</sub>/Na<sub>2</sub>HPO<sub>4</sub> buffer (pH 7.1), 0.4 μmol NADH and 0.025–0.50 units of enzyme. Reactions were initiated by the addition of 1 μmol (+)-camphor. 6-*endo*-Hydroxycineole dehydrogenase was assayed either (a) by measuring substrate-stimulated NAD<sup>+</sup> reduction spectrophotometrically at 340 nm when 1 μmol 6-*endo*-hydroxycineole was added to a reaction mixture that contained, in a total volume of 1 ml, 80 μmol glycine/NaOH buffer or H<sub>3</sub>PO<sub>4</sub>/Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub> buffer (pH 10.5), 2 μmol NAD<sup>+</sup> and 0.01–0.25 units of enzyme, or (b) by following the

substrate-stimulated oxidation of NADH when 1  $\mu\text{mol}$  6-oxocineole was added to a cuvette that typically contained in 1 ml, 80  $\mu\text{mol}$   $\text{H}_3\text{PO}_4/\text{Na}_4\text{P}_2\text{O}_7$  buffer (pH 7.5), 0.15  $\mu\text{mol}$  NADH and 0.01–0.25 units of enzyme. 6-Oxocineole oxygenase was assayed either by measuring substrate-stimulated NADPH oxidation spectrophotometrically or oxygen uptake polarographically. A typical reaction mixture contained, in a total volume of 3 ml, 250  $\mu\text{mol}$   $\text{H}_3\text{PO}_4/\text{Na}_4\text{P}_2\text{O}_7$  buffer (pH 9), 0.5–1  $\mu\text{mol}$  NADPH and 0.07–0.7 units of enzyme. Reactions were initiated by the addition of 2  $\mu\text{mol}$  6-oxocineole.

*Enzyme purification.* 2,5-Diketocamphane 1,2-monooxygenase was partially purified by  $(\text{NH}_4)_2\text{SO}_4$  fractionation and anion exchange chromatography essentially as described previously (Taylor & Trudgill, 1986). The final preparation was dialysed against 1 mM- $\text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$  buffer (pH 7.1) and stored at  $-20^\circ\text{C}$  until required.

*Chemical synthesis.* The lactone 5,5-dimethyl-4-(3'-oxobutyl)-4,5-dihydrofuran-2(3H)-one was prepared from  $\alpha$ -terpineol, essentially as described by MacRae *et al.* (1979) and recrystallized from diethyl ether. 3-(1-Hydroxy-1-methylethyl)-6-oxoheptanoic acid was prepared as a 20 mM buffered solution from the lactone. The lactone (20 mg) was dissolved in 2 ml 1 M-NaOH and incubated at  $100^\circ\text{C}$  for 20 min. The solution was cooled on ice, 1 ml 0.1 M-Tris/HCl buffer (pH 8) added and, after adjustment of the pH to 8 with 2 M-HCl, made up to 5 ml with  $\text{H}_2\text{O}$  and stored at  $-20^\circ\text{C}$ .

*Sources of other chemicals.*  $\alpha$ -Terpineol and magnesium monoperoxyphthalate were supplied by Aldrich. 1,8-Cineole and (+)-camphor were supplied by Sigma.  $\text{NAD}^+$ ,  $\text{NADP}^+$ , NADH and NADPH were from Park Scientific. DEAE-Sephacel was supplied by Pharmacia, DEAE-cellulose (DE52) by Whatman and DNAase by Fluka. 2,5-Diketocamphane was a generous gift from Dr I. C. Gunsalus, Department of Biochemistry, University of Illinois, Champaign-Urbana, Illinois, USA. All other reagents were of high purity, the majority being of AnalaR grade from BDH.

## RESULTS

### *Identification of Rhodococcus sp. strain C1*

Strain C1 is a Gram-positive non-motile rod (average size  $4.0 \times 1.3 \mu\text{m}$ ) with limited mycelial development, rudimentary branching and early (15 h) fragmentation into rods and coccobacilli. Colonies on nutrient agar and glucose-nutrient agar (72 h) were soft butyrous, 3 mm diameter, coral-red, shiny and opaque with a crenate edge. The organism grew at temperatures up to  $47^\circ\text{C}$ , was catalase and oxidase positive and an obligate aerobe. In sugar broths there was no acid production from glucose, sucrose or lactose. Tests for lecithinase, indole formation, starch hydrolysis,  $\text{H}_2\text{S}$  formation (cysteine agar) and the Voges-Proskauer and methyl red tests were all negative. Nitrate was reduced only to nitrite in peptone water, urease was produced on Christensen's medium and the organism was sensitive to a wide range of antibiotics. These properties are consistent with the organism being a *Rhodococcus sp.* (Goodfellow, 1986).

### *Growth of organism and metabolite accumulation*

*1,8-Cineole.* *Rhodococcus* C1 grew on 1,8-cineole in shake-flask culture with a doubling time of approximately 8 h. GLC analysis of unconcentrated neutral diethyl ether extracts of culture media, taken throughout the growth phase, showed the transient accumulation of two compounds that reached maximum concentrations after about 16–17 h growth (Fig. 2). Yields were quantified (no correction for structure-related variation in flame-ionization detector response) by reference to (+)-camphor as the internal standard, added to the cell-free medium at a concentration of  $40 \mu\text{g ml}^{-1}$  before diethyl ether extraction. No acidic metabolites were detected.

Sufficient quantities of the two metabolites for unequivocal identification and for use in metabolic studies were obtained by extraction of a 40 l culture. The diethyl ether extract was dried, evaporated to a volume of 4 ml under reduced pressure and stored at  $-20^\circ\text{C}$  for 72 h. The copious crystalline white solid that had formed was filtered off, washed with cold dry diethyl ether, dried under vacuum and recrystallized from saturated solution in boiling diethyl ether. GLC analysis gave a single peak, retention time 12.2 min, m.p.  $93\text{--}95^\circ\text{C}$ . Mass spectral analysis gave a molecular ion at  $m/e$  170 (22%) with associated ions at 126 (93%), 111 (48%), 108 (100%), 93 (43%), 83 (40%), 71 (81%), 69 (52%) and 43 (77%) – a fragmentation pattern almost identical with that reported for the two isomers of 2-hydroxycineole (MacRae *et al.*, 1979). The  $^1\text{H-NMR}$  spectrum ( $\delta$  3.53, broad multiplet that collapsed to a triplet on adding  $\text{D}_2\text{O}$ ,  $J_{6,5\text{endo}} = J_{6,5\text{exo}} = 6 \text{ Hz}$ ; three methyl groups at  $\delta$  1.27, 1.20 and 1.10) was also identical with that

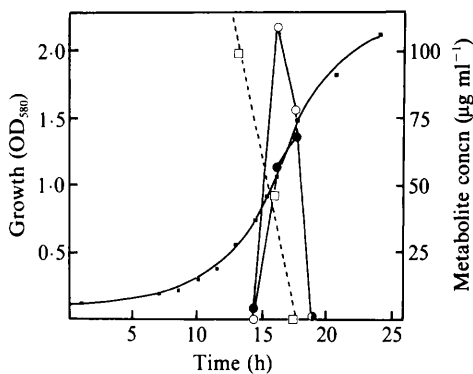


Fig. 2

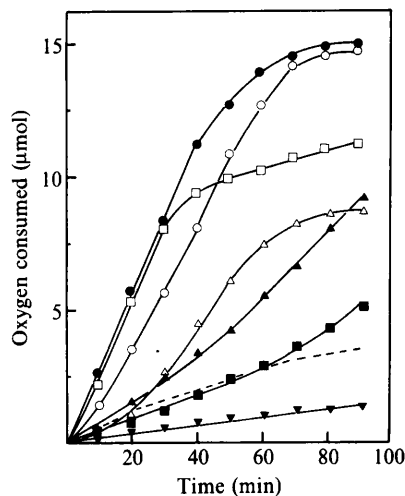


Fig. 3

Fig. 2. Transient accumulation of metabolites in the culture medium during growth of *Rhodococcus* C1 on 1,8-cineole. ■, Growth (OD<sub>580</sub>); □, 1,8-cineole; ○, 6-endo-hydroxycineole; ●, 6-oxocineole.

Fig. 3. Oxidation of 1,8-cineole and putative catabolic intermediates by *Rhodococcus* C1. Warburg flasks contained, in a total volume of 2 ml, 5 mg dry wt of 1,8-cineole-grown *Rhodococcus* C1 and 80 μmol phosphate buffer, pH 7.1. Reactions, at 30 °C, were started by the addition of substrates (3 μmol) from side-arms. Centre wells contained 0.1 ml 20% (w/v) KOH and a roll of filter paper to facilitate absorption of CO<sub>2</sub>. ○, 1,8-Cineole; ●, 6-endo-hydroxycineole; □, 6-oxocineole; ■, 5,5-dimethyl-4-(3'-oxobutyl)-4,5-dihydrofuran-2(3H)-one; ▣, 3-(1-hydroxy-1-methylethyl)-6-oxoheptanoic acid; △, levulinic acid; ▲, acetone. Endogenous respiration (----) has not been subtracted.

previously reported for 2-endo-hydroxycineole (MacRae *et al.*, 1979). However, while MacRae *et al.* (1979) reported that 2-endo-hydroxycineole was laevorotatory,  $[\alpha]_D -26^\circ$  ( $c = 0.2$  in ethanol), the accumulated metabolite was dextrorotatory,  $[\alpha]_D +27.5^\circ$  ( $c = 0.2$  in ethanol). These results are only compatible with the compound being the optical isomer 6-endo-hydroxycineole.

The mother liquor was greatly enriched in the second, more volatile metabolite which was further purified, after removal of solvent, by chromatography on Brockmann activity III, alumina, developed with n-hexane. Removal of the eluting solvent left a soft white crystalline solid which was shown by GLC (retention time 11 min) to be contaminated with traces of 6-endo-hydroxycineole. The residual impurity was removed from a small sample of the compound by sublimation, in a closed vessel maintained at 55 °C, onto a cold finger. This gave 150 mg of chromatographically pure material. This compound, m.p. 47–49 °C, reacted with acidic 2,4-dinitrophenylhydrazine reagent (Friedemann & Haugen, 1943) to form a bright-orange insoluble 2,4-dinitrophenylhydrazone. Mass spectral analysis of the ketonic compound gave a spectrum almost identical with that previously reported (Nishimura *et al.*, 1982) for 2-oxocineole with a molecular ion at  $m/e$  168 (10%) and major associated ions at 140 (21%), 111 (7%), 97 (9%), 82 (100%), 69 (17%), 67 (14%) and 43 (22%). The <sup>1</sup>H-NMR spectrum ( $\delta$  2.92 and 2.71 triplets, H5 *exo* and *endo*,  $J_{5,5} = 20$  Hz,  $J_{5,4} = 3$  Hz, three methyl groups at 1.40, 1.25 and 1.15) also displayed all the diagnostic features attributable to 2-oxocineole (MacRae *et al.* 1979; Nishimura *et al.*, 1982). However, although MacRae *et al.* (1979) reported that 2-oxocineole was dextrorotatory,  $[\alpha]_D +70.2^\circ$ , the accumulated ketone was laevorotatory,  $[\alpha]_D -42.5^\circ$  ( $c = 0.2$  in ethanol), again suggesting that the compound is predominantly the optical isomer 6-oxocineole.

*Putative catabolic intermediates.* In addition to growth with 1,8-cineole *Rhodococcus* C1 was also able to utilize 6-endo-hydroxycineole, 6-oxocineole, 3-(1-hydroxy-1-methylethyl)-6-oxoheptanoic acid, levulinic acid and acetone as sole carbon sources for growth. The synthetic lactone 5,5-dimethyl-4-(3'-oxobutyl)-4,5-dihydrofuran-2(3H)-one did not support growth.

*Oxidation of 1,8-cineole and related compounds by whole cells*

Washed cell suspensions of 1,8-cineole-grown *Rhodococcus* C1 oxidized 1,8-cineole, 6-*endo*-hydroxycineole and 6-oxocineole rapidly with no significant lag period while levulinic acid, acetone and synthetic 3-(1-hydroxy-1-methylethyl)-6-oxoheptanoic acid were oxidized more slowly (Fig. 3). Succinate-grown cells did not oxidize any of these compounds significantly during the experimental period (80 min).

*Studies with subcellular systems*

*1,8-Cineole as substrate.* No enzyme-mediated transformation of 1,8-cineole was detected with subcellular systems obtained from cells disrupted in the Hughes press, in the French press or by ultrasonication whether or not reducing agents were included with the cell suspension. Crude cell crushes and membrane preparations were all inactive towards 1,8-cineole, irrespective of the buffer system, the electron donor employed, or the analytical method used.

*6-endo-Hydroxycineole dehydrogenase.* An NAD<sup>+</sup>-dependent enzyme, catalysing the dehydrogenation of 6-*endo*-hydroxycineole, was detected spectrophotometrically in extracts of 1,8-cineole-grown cells. The pH optimum of the reaction was 10.5 and the product was identified as 6-oxocineole by GLC analysis and comparison with the authentic ketone. The reverse reaction, pH optimum 7.5 with 6-oxocineole as substrate and NADH as electron donor, yielded 6-*endo*-hydroxycineole exclusively. Some activity (<10%) was also detected when NADPH was substituted as the electron donor but, again, only 6-*endo*-hydroxycineole was detected. Enzyme activity was negligible in extracts of succinate-grown cells (Table 1).

*6-Oxocineole oxygenase.* When crude cell extracts were incubated with 6-oxocineole and NADPH in the oxygen monitor a substrate-stimulated consumption of oxygen occurred. The induced enzyme responsible was distinguished from the low level of NADPH-linked 6-*endo*-hydroxycineole dehydrogenase activity by doing spectrophotometric assays at pH 9; under these conditions the dehydrogenase activity was almost completely suppressed (Table 1). Oxygen monitor assays done in the presence of limited amounts of either 6-oxocineole or NADPH

Table 1. *Properties of 6-endo-hydroxycineole dehydrogenase and 6-oxocineole oxygenase in extracts of Rhodococcus C1*

*(a) 6-endo-Hydroxycineole dehydrogenase*

Substrate	6- <i>endo</i> -Hydroxycineole
Substrate specificity	Not known
Electron acceptor	NAD <sup>+</sup> (NADP <sup>+</sup> ?)
pH optimum (ol → one)	10.5
pH optimum (one → ol)	7.5
Specific activity (ol → one)	
1,8-Cineole-grown	0.62 (pH 10)
Succinate-grown	0.01
Specific activity (one → ol)	
1,8-Cineole-grown	1.08 (pH 7.5)
	0.09 (pH 7.5)
	0.01 (pH 9)

} NADH  
 } NADPH

*(b) 6-Oxocineole oxygenase*

Substrate	6-Oxocineole
Substrate specificity*	Absolute?
pH optimum	9
Specific activity	
1,8-Cineole-grown	0.03-0.04
Succinate-grown	0.001

\* No significant activity with (+)-camphor, (*R*)-(-)-carvone, (*S*)-(+)-carvone, cyclohexanone, 1,3-cyclohexanedione, dihydrocarvone, 2-,3- and 4-methylcyclohexanone, fenchone, menthone, pinan-3-one, pulegone or verbenone.

allowed a crude substrate stoichiometry ( $O_2$ :6-oxocineole:NADPH) of 1:0.83:0.90 to be determined. Assays done in anaerobic Thunberg cuvettes as previously described (Donoghue *et al.*, 1976) confirmed that NADPH oxidation was linked to oxygen consumption. GLC analysis of diethyl ether extracts of reactions with limited 6-oxocineole that had been allowed to run to completion at pH 9 and adjusted to pH 1 with 5 M-HCl before extraction showed that the 6-oxocineole had been converted into a single more polar metabolite (retention time 29 min). GLC-MS analysis gave a spectrum - *m/e* 166 (37%,  $M - H_2O$ ), 111 (56%), 98 (85%), 82 (37%), 55 (30%), 43 (100%) - identical with that of the lactone (*R*)-5,5-dimethyl-4-(3'-oxobutyl)-4,5-dihydrofuran-2(3H)-one isolated by MacRae *et al.* (1979) from culture medium of *P. flava* growing on 1,8-cineole. As the route whereby this lactone might arise from either 2-oxocineole (MacRae *et al.*, 1979) or 6-oxocineole was not immediately apparent, attempts were made to purify 6-oxocineole oxygenase so that the initial oxygenation product could be isolated and identified.

#### *Stability and attempted purification of 6-oxocineole oxygenase*

Crude extract of 1,8-cineole-grown *Rhodococcus* C1, diluted to 4 mg protein ml<sup>-1</sup> in 42 mM-KH<sub>2</sub>PO<sub>4</sub>/Na<sub>2</sub>HPO<sub>4</sub> buffer (pH 7.1), lost all oxygenase activity when maintained at 4 °C for 24 h. Reducing agents were not effective as stabilizers but addition of 5 or 10% (v/v) ethanol to the buffer resulted in the loss of <15% of the activity over the same time-period. Chromatography of 800 mg of crude extract protein on a 2.5 × 14 cm DEAE-Sephacel or DEAE-cellulose column, developed with a linear gradient (700 ml, 0-0.5 M-KCl in 42 mM-phosphate buffer, pH 7.1, containing 5%, v/v, ethanol) separated the oxygenase from 6-*endo*-hydroxycineole dehydrogenase activities with 2.5 to 7-fold increase in specific activity and ≈40% recovery. All attempts at additional purification, including taking advantage of the short time scale allowed by FPLC, led to extremely poor recovery of enzyme units and a decrease in specific activity. When 15 mg of partially purified enzyme (DEAE-cellulose or DEAE-sephacel stage), concentrated in an Amicon ultrafiltration cell, was incubated in a Warburg with 60 μmol 6-oxocineole and an excess of NADPH in H<sub>3</sub>PO<sub>4</sub>/Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub> buffer (pH 9), 50 μmol of oxygen were consumed. Direct ferric hydroxamate assay for the presence of lactones (Cain, 1961) showed them to be absent. However, when the remainder of the reaction mixture was acidified with 5 M-HCl, followed by extraction of the product into diethyl ether, and a sample of this again tested (after removal of solvent) the positive response indicated the presence of a lactone. GLC analysis confirmed that this was the compound also obtained following the extraction of product formed by crude cell extract. GLC-MS analysis again gave a spectrum identical with that reported for the lactone (*R*)-5,5-dimethyl-4-(3'-oxobutyl)-4,5-dihydrofuran-2(3H)-one accumulated by *P. flava* (MacRae *et al.*, 1979).

The cleavage of saturated cyclic ketones by micro-organisms is most frequently accomplished by biological Baeyer-Villiger oxygenation, followed by enzyme-mediated hydrolysis of the lactone thus formed (Trudgill, 1984, 1986). Since the lactone isolated from incubations of crude extract or partially purified 6-oxocineole oxygenase with NADPH and 6-oxocineole is a substituted 2-oxodihydrofuran (Fig. 1) and cannot be formed by direct oxygen insertion into the substrate it appears to be an artifact of the acidification and extraction procedure.

An investigation of the substrate specificity of the partially purified oxygenase, in which a wide range of cyclic and acyclic ketones was used, failed to reveal a single alternative substrate. This, unfortunately, excluded the exploitation of substrate analogues as investigative tools. In addition, our failure to purify the 6-oxocineole oxygenase to homogeneity necessitated an alternative approach to understanding the reaction sequence. This was sought on the working assumption that Baeyer-Villiger oxygenation was, nevertheless, the most likely initial step (Trudgill, 1984, 1986).

#### *Studies with 2,5-diketocamphane 1,2-monooxygenase from P. putida*

This Baeyer-Villiger monooxygenase has a broad ketone substrate specificity and initial trials with the partially purified enzyme from (+)-camphor-grown *P. putida* ATCC 17453 showed that oxygen consumption was stimulated by 6-oxocineole in the presence of NADH, the electron

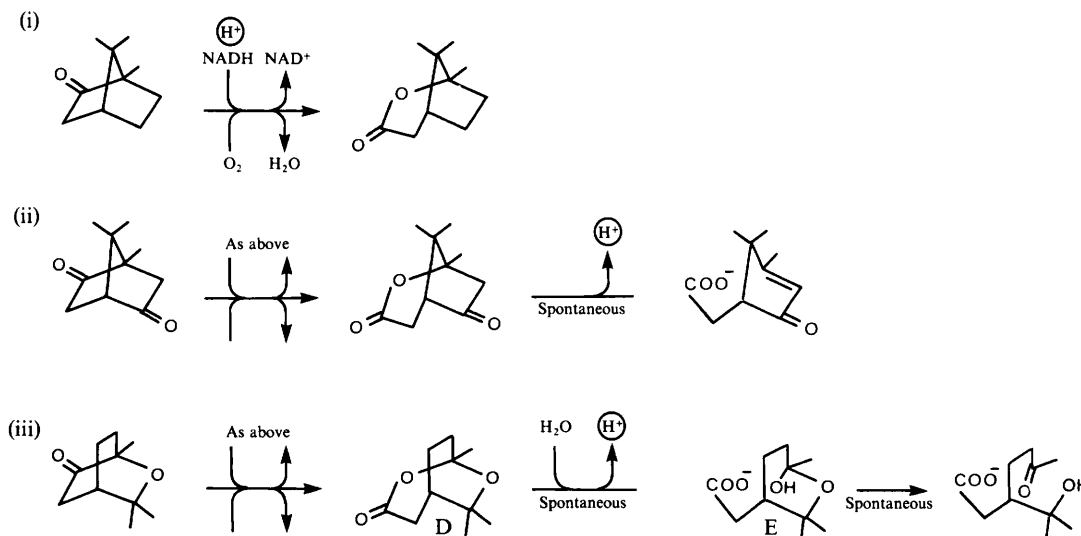


Fig. 4. Reactions catalysed by 2,5-diketocamphane 1,2-monooxygenase from *P. putida* with (i) (+)-camphor and (ii) 2,5-diketocamphane as substrates. The proposed oxygenation reaction with 6-oxocineole as substrate (iii) is followed by the spontaneous cleavage of the oxygenation product (compound D) 1,6,6-trimethyl-2,7-dioxo(3,2,2)bicyclononan-3-one and the hemiacetal (compound E) 2,6,6-trimethyl-5-acetyltetrahydropyran-2-ol.

donor for this enzyme. Reactions in which 0.65 units of enzyme were incubated with 10  $\mu$ mol 6-oxocineole and 15  $\mu$ mol NADH in 42 mM-KH<sub>2</sub>PO<sub>4</sub>/Na<sub>2</sub>HPO<sub>4</sub> buffer (pH 7.1) were used for product accumulation and analysis. Direct hydroxamate assay for lactones showed them to be absent. However, acidification of the reaction mixture and diethyl ether extraction again generated a lactone which was identical with that formed with the crude and partially purified oxygenating system from 1,8-cineole-grown *Rhodococcus* C1.

This observation was of particular interest since (+)-camphor-grown *P. putida* does not produce a detectable lactone hydrolase (Taylor & Trudgill, 1986). When either crude cell extract or the purified enzyme complex is incubated with (+)-camphor as ketonic substrate the stable 1,2-lactone accumulates. In contrast 2,5-diketocamphane yields an unstable lactone that spontaneously cleaves without enzymic assistance (Taylor & Trudgill, 1986). The consequences of this, in terms of proton balance during the reaction, can be clearly observed in a pH stat. With (+)-camphor as substrate the proton consumed in oxygenation is not regenerated upon lactone cleavage and proton consumption can be followed. In contrast, oxygenation of 2,5-diketocamphane is followed by spontaneous lactone cleavage, regeneration of a proton, and consequent maintenance of neutrality. When 6-oxocineole was used as substrate the preservation of near neutrality observed was also indicative of proton regeneration subsequent to oxygenation (Figs 4 and 5).

#### Chemical Baeyer-Villiger oxygenation of 6-oxocineole

6-Oxocineole (15 mg) was incubated with 110 mg magnesium monoperoxyphthalate in 5 ml dimethylformamide for 16 h at 20 °C followed by addition of 5 ml of water and acidification. The product, extracted into diethyl ether, co-chromatographed with the detected reaction product of the biological Baeyer-Villiger oxygenation of 6-oxocineole and with the authentic lactone 5,5-dimethyl-4-(3'-oxobutyl)-4,5-dihydrofuran-2(3H)-one formed from  $\alpha$ -terpineol as described in Methods.

#### DISCUSSION

Other workers have reported that hydroxylation of 1,8-cineole either in preparation for ring cleavage (MacRae *et al.*, 1979; Carman *et al.*, 1986) or in non-assimilatory fermentation



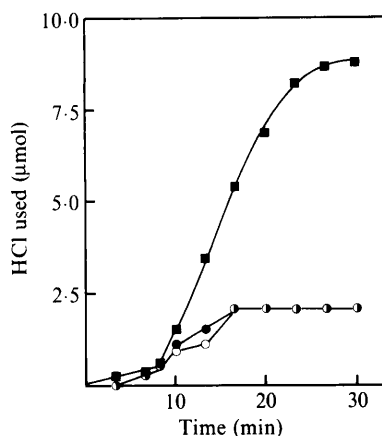


Fig. 5. A comparison of proton balance in the oxygenation of (+)-camphor, 2,5-diketocamphane and 6-oxocineole by partially purified 2,5-diketocamphane 1,2-monooxygenase from *P. putida* ATCC 17453 by pH-stat assay. The stirred reaction vessel contained, in 11 ml of distilled water (adjusted to pH 6.7 by the addition of a minimum amount of 42 mM-KH<sub>2</sub>PO<sub>4</sub>/Na<sub>2</sub>HPO<sub>4</sub> buffer), 10 μmol NADH and 0.5 units of oxygenase. The reaction pH was maintained at 6.7 by the controlled addition of 2 mM-HCl. After the establishment of endogenous rates (8 min) substrates (10 μmol) were added and proton consumption measured by monitoring the controlled 2 mM-HCl additions required to maintain the preset pH. ■, camphor; ●, 2,5-diketocamphane; ○, 6-oxocineole.

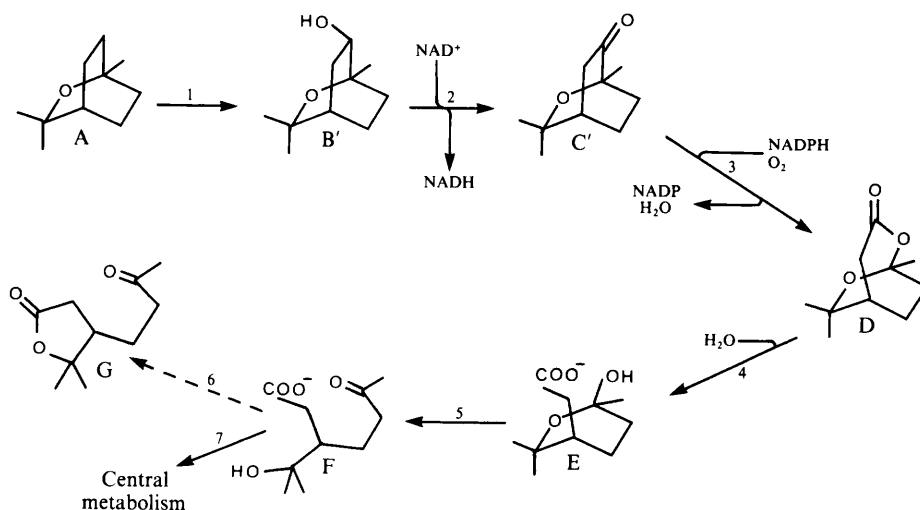


Fig. 6. Proposed ring cleavage reactions in the metabolism of 1,8-cineole by *Rhodococcus* C1. Compounds are (A) 1,8-cineole; (B') 6-*endo*-hydroxycineole; (C') 6-oxocineole; (D) 1,6,6-trimethyl-2,7-dioxabicyclo(3,2,2)nonan-3-one; (E) 2,6,6-trimethyl-5-acetyltetrahydropyran-2-ol; (F) 3-(1-hydroxy-1-methylethyl)-6-oxoheptanoic acid; (G) 5,5-dimethyl-4-(3'-oxobutyl)-4,5-dihydrofuran-2(3H)-one.

(Nishimura *et al.*, 1982) involved attack exclusively at the C-2 and C-3 positions. Indeed, in our preliminary report (Taylor & Trudgill, 1988) which was published before we obtained information on the optical rotation of the accumulated hydroxycineole and oxocineole we assumed, on the basis of <sup>1</sup>H-NMR and mass spectral results, that this organism also attacked the terpene at the 2 position. It is of interest, but for no logical reason less likely, that *Rhodococcus* C1 initiates attack on 1,8-cineole at carbon-6. The proposed sequence of steps involved in the cleavage of 1,8-cineole is shown in Fig. 6.

The reaction sequence has both commonplace and novel features. Initial hydroxylation of the terpene, although not demonstrated in cell-free systems, was clearly supported by metabolite accumulation studies and the induced dehydrogenation of 6-*endo*-hydroxycineole has been established. Our failure to detect this initial step is puzzling but not unique since a number of workers have been unable to demonstrate oxygenation steps in the initial activation of other terpene hydrocarbons (Griffiths *et al.*, 1987*a*; Trudgill, 1984, 1986). The majority of identified biological Baeyer–Villiger monooxygenases are stable and relatively easy to purify. Unfortunately, the instability and apparent absolute specificity of the 6-oxocineole oxygenase made investigation of the detailed sequence of ring cleavage steps difficult. The observation that 2,5-diketocamphane 1,2-monooxygenase from *P. putida* [a biological Baeyer–Villiger oxygenase that inserts a single atom of oxygen into (+)-camphor and related compounds between the ketonic carbon and the bridgehead] formed the same detected product provided strong circumstantial evidence that the oxygenase from strain C1 catalyses an identical reaction and (i) is a monooxygenase, (ii) catalyses a biological Baeyer–Villiger reaction and (iii) inserts an oxygen atom between the ketonic carbon and the adjacent carbon carrying a methyl group (Fig. 6). Other examples of alicyclic ketone metabolism in which biological Baeyer–Villiger oxygenation leads to the formation of an unstable lactone without a consequent requirement for a lactone hydrolase include 2,5-diketocamphane oxygenation, already mentioned, in which the presence of the second keto group initiates ring cleavage through loss of a proton from an adjacent carbon atom (Taylor & Trudgill, 1986) and, more directly relevant to 1,8-cineole oxidation, hemiacetal formation by oxygenation of 2-hydroxycyclohexanone by an *Acinetobacter* sp. (Davey & Trudgill, 1977). Spontaneous cleavage of the lactone 1,6,6-trimethyl-2,7-dioxabicyclo(3,2,2)nonan-3-one, which we propose as the immediate product of 6-oxocineole oxygenation, is a property of this very highly strained molecule and the spontaneous cleavage of the hemiacetal thus formed is a direct parallel to the spontaneous cleavage of other hemiacetals formed as intermediates in catabolic pathways (Bernhardt *et al.*, 1973; Davey & Trudgill, 1977), and yields the acyclic compound 3-(1-hydroxy-1-methylethyl)-6-oxoheptanoic acid. One of the characteristics of 4-hydroxy acids is the ease with which they form lactones in acid solution and dry solvents. Ring closure of the acid would yield the detected product of 2- or 6-oxocineole oxygenation: the respective isomer of 5,5-dimethyl-4-(3'-oxobutyl)-4,5-dihydrofuran-2(3H)-one. Indeed, precisely this spontaneous ring closure was exploited by Wallach (1895) in his investigation into the structure of  $\alpha$ -terpineol.

MacRae *et al.* (1979) established the absolute configuration of the lactone and other intermediates formed by *P. flava* and suggested that they were all derived by microbial oxidation at the same prochiral carbon of 1,8-cineole. Although we have not established the absolute configuration of 5,5-dimethyl-4-(3'-oxobutyl)-4,5-dihydrofuran-2(3H)-one formed by *Rhodococcus* C1 we have provided a logical explanation for its production. In principle, this sequence of ring cleavage reactions is also applicable to the other, less widely distributed and even more strained, natural isomer 1,4-cineole, with the parallel formation of the acyclic 3-hydroxy-(1-methylethyl)-6-oxoheptanoic acid. In this context the recently reported formation of the 2-*endo*-hydroxy and 2-oxo derivatives as products of 1,4-cineole fermentation by a variety of bacteria and fungi grown in a rich medium is of interest (Rosazza *et al.*, 1987).

The further metabolism of the acyclic catabolic intermediate of 1,8-cineole metabolism, 3-(1-hydroxy-1-methylethyl)-6-oxohexanoic acid is currently under investigation. A mechanistically acceptable route for its degradation can be formulated in which levulinic acid and acetone are intermediates. In this context the ability of *Rhodococcus* C1 to grow with acetone as sole carbon source is of interest since the ability to utilize the compound is not universally distributed (Taylor *et al.*, 1980).

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