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Bacterial Metabolism of α-Pinene: Pathway from α-Pinene Oxide to Acyclic Metabolites in *Nocardia* sp. Strain P18.3

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Over 20 gram-positive bacteria were isolated by elective culture with (±)-α-pinene as the sole carbon source. One of these strains, *Nocardia* sp. strain P18.3, was selected for detailed study. α-Pinene-grown cells oxidized, without lag, α-pinene, α-pinene oxide (epoxide), and the *cis* and *trans* isomers of 2-methyl-5-isopropylhexa-2,5-dienal. No other tested terpene was oxidized at a significant rate. α-Pinene was not metabolized by cell extracts in the presence or absence of NADH or NADPH. Cell extracts catalyzed a rapid decyclization of α-pinene oxide, in the absence of added cofactors, with the formation of *cis*-2-methyl-5-isopropylhexa-2,5-dienal. Further oxidation of the aldehyde to the corresponding acid occurred in the presence of NAD. Both activities were induced by growth with α-pinene. A rapid, nonenzymic transformation of the *cis* aldehyde into the *trans* isomer occurred in glycine buffer. The *trans* isomer was also a substrate for the NAD-linked aldehyde dehydrogenase. The distribution of the α-pinene oxide lyase in α-pinene-utilizing *Pseudomonas* spp. was also investigated and was compatible with the two alternative ring-cleavage sequences that have been proposed on the basis of accumulated metabolites.

The growth of microorganisms with α-pinene as the sole source of carbon has been reported by several workers (2, 9, 10, 16, 17, 19; N. C. Floyd, A. Burfield, A. Magalhaes, P. M. Rhodes, and D. J. Best, EMBO Workshop, Geneva, 1986). Although many accumulated neutral and acidic metabolites have been isolated from culture media and identified, the sequence of steps leading to ring cleavage and the pathways whereby the terpene yields central metabolites and biosynthetic precursors have not been established.

Cleavage of the cyclobutane ring appears to be a broadly distributed transformation accompanied or followed by cleavage of the cyclohexane ring at one of two alternative bonds. The identification of perillic acid, phellandric acid, and the acyclic compounds β-isopropylpimelic acid and β-isopropenylpimelic acid as metabolites formed by *Pseudomonas* sp. strain PL (16, 17) is indicative of the second ring cleavage occurring between carbon atoms 2 and 3 of the α-pinene. In contrast, accumulation of the *cis* isomer of 2-methyl-5-isopropylhexa-2,5-dienoic acid by *Pseudomonas putida* PX1 (9, 10) and the *trans* isomer of the same compound by a mutant of *P. putida* PIN11 (19) is indicative of the second ring cleavage occurring between carbon atoms 3 and 4 (Fig. 1).

With the exception of some whole cell oxidation studies and limited characterization of perillaldehyde and perillyl alcohol dehydrogenases from α-pinene-grown *Pseudomonas* sp. strain PL (2, 16, 17), the pathways proposed for cleavage of α-pinene have not been substantiated at the subcellular level.

In a recent short communication (Floyd et al., EMBO Workshop) a strain of *Pseudomonas fluorescens* was reported to catalyze the NADH-linked oxygenation of α-pinene to form the epoxide (α-pinene oxide), and then cleavage of both rings of the epoxide by a decyclase with the formation of a novel aldehyde.

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In this paper we report studies of α-pinene metabolism by bacteria capable of growth on α-pinene as the sole carbon source, detailed examination of the products of the α-pinene oxide decyclase step in *Nocardia* sp. strain P18.3, and evidence for the enzyme catalyzing a key reaction of α-pinene oxidation in this and other organisms.

MATERIALS AND METHODS

Sources of bacteria. *P. putida* PX1 (NCIB 10684), *Pseudomonas* sp. strain PIN18 (NCIB 10687), and *P. fluorescens* NCIB 11671 were obtained from the National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland. *Nocardia* sp. strain P18.3 was isolated from mixed coniferous woodland soil, obtained in the Aberystwyth area, by enrichment culture with (±)-α-pinene provided as the sole carbon source from a vapor tube. It was one of over 20 gram-positive organisms that were isolated in this manner. *Pseudomonas* sp. strain PL was a generous gift from D. W. Ribbons.

Identification of *Nocardia* sp. strain P18.3. The branched filamentous gram-positive organism was identified as a species of *Nocardia* at the National Collection of Industrial and Marine Bacteria by analysis of the extracted fatty acids. Gas-liquid chromatographic (GLC) analysis of the fatty acid methyl esters showed the presence of tuberculostearic, palmitic, and palmitoleic acids, characteristic of *Mycobacterium*, *Rhodococcus*, and *Nocardia* species. However, thin-layer chromatography of the methyl esters also showed the presence of mycolic acid; this, taken in conjunction with routine diagnostic tests and the morphology of the organism, identified it as a *Nocardia* sp.

Growth of organisms. Bacteria were maintained on nutrient agar slants. Liquid culture medium contained the following (per liter): KH_2PO_4, 2 g; Na_2HPO_4, 4 g; $(NH_4)_2SO_4$, 1 g; and trace element solution, 4 ml (14). Sodium succinate hexahydrate (4.6 g) was added where stated. For growth of *Pseudomonas* strains on α-pinene the substrate was added

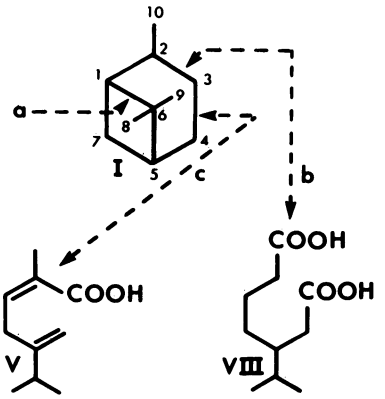

FIG. 1. Ring cleavage points in catabolism of the bicyclic terpene α-pinene (I) suggested by the structures of the identified acyclic metabolites 3-isopropylpimelic acid (VIII) and *cis*-2-methyl-5-isopropylhexa-2,5-dienoic acid (V).

directly to the sterilized growth medium at 3 g per liter. *Nocardia* sp. strain P18.3 was grown with α-pinene supplied from a vapor tube as described by Claus and Walker (4).

All cultures were grown in Erlenmeyer flasks on a gyratory shaker at 150 rpm and 30°C. Typically 20 ml of medium in a 100-ml Erlenmeyer flask was inoculated from a succinate- or nutrient broth-grown starter culture and grown for 48 h (*Pseudomonas* strains) or 120 h (*Nocardia* sp. strain P18.3). Subsequently, 10% (vol/vol) inocula were transferred to 50- and 500-ml volumes of medium at 24- or 120-h intervals as appropriate. Cultures were harvested by centrifugation at 10,000 × *g* (average) and 4°C for 20 min, and the cell pellets were washed once by suspension in 42 mM phosphate buffer (pH 7.1) followed by centrifugation. The cell paste was suspended in 1 volume of 42 mM phosphate buffer (pH 7.1) and either used directly or stored at −20°C until required.

For assessment of growth specificity with a range of terpenes and related volatile compounds, petri dishes containing basal medium solidified with 1.5% purified agar were inoculated with bacteria from nutrient agar slants or broth-grown cultures, and the substrate was provided in the vapor phase from a capillary tube placed in the lid of the inverted dish.

Cell extracts. Cell suspensions of *Nocardia* sp., frozen in situ, were routinely broken by a single passage through a Hughes press at −15°C, although for specific experiments the French press and ultrasonic treatment were also used. Cell suspensions of the *Pseudomonas* strains were thawed at room temperature and disrupted by ultrasonic treatment at 0°C twice for 30 s, with a 1-min cooling interval, per 10 ml of cell suspension with a 23-kHz MSE Soniprep (type 150; MSE Scientific Instruments, Crawley, United Kingdom [U.K.]) at a probe amplitude setting of 18 to 20 μm. Each 10 ml of disintegrated preparation was incubated with approximately 0.5 mg of crystalline DNase for 15 min at 3°C and then centrifuged at 27,000 × *g* (average) for 45 min to remove cell debris.

Protein estimation. The protein content of crude cell extracts was routinely measured by the biuret method (11) with crystalline bovine serum albumin as the standard.

Preparation of terpene "solutions." Most of the terpenes used were of low aqueous solubility. Such compounds were used as aqueous or buffered emulsions (nominally 20 mM) prepared by ultrasonication. Typically, 5-ml samples were treated for 30 s at 23 kHz and a probe amplitude of 10 to 12 μm. Emulsions were normally stable for several hours. Compounds with oxygen functional groups typically yielded true solutions when emulsions were added to assay systems to give a final concentration of 0.5 to 2 mM.

Measurement of oxygen consumption. Oxygen consumption by washed cell suspensions or cell extracts was measured either by Warburg manometry or polarographically at 30°C with an oxygen monitor (Yellow Springs Instrument Co., Yellow Springs, Ohio) fitted with a Clark-type electrode. Volatile terpene substrates were introduced into Warburg flasks in the gas phase by applying 50 μl of the compound to a roll of filter paper placed in the sidearm immediately before assembling the flasks on the manometers.

Enzyme assays. Alcohol and aldehyde dehydrogenase activities were assayed spectrophotometrically in a 1-ml reaction by following the formation of NADH at 340 nm and 30°C in the presence of cell extract, a suitable buffer, 2 μmol of NAD, and 1 μmol of the substrate. Attempts to detect α-pinene monooxygenase activity included searching for α-pinene-stimulated NADPH oxidation spectrophotometrically at 340 nm or oxygen consumption polarographically with α-pinene presented as an emulsion or, in the case of Warburg flasks, in the vapor phase. α-Pinene oxide lyase (decyclizing) was routinely assayed by following the formation of either the *cis* or the *trans* isomer (depending on the buffer system used) of 2-methyl-5-isopropylhexa-2,5-dienal at 235 nm. A typical reaction system contained (in 1 ml) 100 μmol of appropriate buffer and protein (0.025 to 0.15 U of activity). After preincubation at 30°C the reaction was initiated by the addition of 1 μmol of α-pinene oxide.

Enzyme units. All enzyme units are defined as the activity required to convert 1 μmol of substrate into product per min.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis under nondenaturing conditions was carried by the general procedure of Davies (6) on 1.5-mm slab gels. Gels were routinely stained with Coomassie brilliant blue R-250 and cleared by washing in ethanol-acetic acid-water (3:1:6, vol/vol).

Detection of α-pinene oxide lyase activity on polyacrylamide gels. Polyacrylamide gels run under nondenaturing conditions were immersed in an emulsion (20 mM) of α-pinene oxide in 0.1 M glycine–NaOH buffer (pH 9.0). Precipitation of a white band of the less soluble *trans*-2-methyl-5-isopropylhexa-2,5-dienal in the polyacrylamide gel marked the position of the lyase band.

GLC. A Carlo-Erba HRGC 5300 gas-liquid chromatograph (Mega Series) with flame ionization detector equipped for cold on-column injection was used for establishing a library of retention time data, for analysis of diethyl ether extracts of culture supernatants, and for monitoring reactions with subcellular systems. GLC was performed on a WCOT fused silica CP-wax-52 CB capillary column (0.32 mm by 25 m; Chrompack UK Ltd., London). The operating parameters were as follows: air (flame ionization detector), 600 ml/min; hydrogen (flame ionization detector), 30 ml/min; hydrogen (carrier gas), 1.5 ml/min. The temperature program used for routine GLC analysis was 45 to 80°C at 25°C/min, 80 to 150°C at 3°C/min, and 150 to 200°C at the maximum rate (approximately 30°C/min). The final temperature was then maintained for 10 min.

GLC analysis of free acids was performed with the same temperature program but with a final temperature of 245°C.

Methyl esters of acidic metabolites were formed by incubating material extracted from acidified reaction mixtures for

2 to 15 min at 100°C with 1 ml of boron trichloride-methanol reagent (10%, wt/vol). The reactions were cooled, 5 ml of H_2O was added, and the product was extracted with diethyl ether for GLC analysis.

GLC-MS analysis. GLC-mass spectral (MS) analyses were performed on a 25-m polar BP20 fused silica capillary column programmed from 50°C (3 min) to 250°C at 10°C/min. Helium was used as carrier gas at a flow rate of 2 ml/min. Electron impact spectra were recorded on a VG 12F mass spectrometer (VG Analytical Ltd.) with a beam energy of 70 eV, source temperature of 150°C, 100-μA emission, and a scan cycle time of 2.7 s. Chemical ionization spectra were obtained by using isobutane as the reagent gas at a source housing pressure of 2×10^{-5} bar and 200-μA emission on a VG 70 HSE mass spectrometer. This instrument was also used to obtain elemental compositions of molecular ion species at a resolution of 1 in 5,000.

NMR spectroscopy. Proton nuclear magnetic resonance (NMR) spectra were obtained with a Bruker AM-200SY spectrometer at 300 MHz, and ^{13}C nuclear magnetic resonance (^{13}C-NMR) spectra were obtained with a Bruker AM-200SY spectrometer at 50.32 MHz or a Bruker CXP-300 spectrometer at 75.46 MHz. Data were interpreted by a combination of inverse gated broad-band decoupling edited spectra (8) and distortion enhancement by polarization transfer edited spectra (7).

Infrared Spectra. Infrared spectra were recorded as cast films on a Bruker IFS-88 spectrometer.

Chemicals. (*S*)-(+)-Carvone was supplied by Ralph Emanuel, Wembley, U.K. (+)-β-Citronellene and the four isomers of pinane were from Fluka, Buchs, Switzerland. (+)-Camphor was supplied by Sigma (London) Chemical Co., Poole, Dorset, U.K. All other commercially supplied terpenes were from Aldrich Chemical Co., Gillingham, Dorset, U.K. (−)-Myrtenol, *cis*-pinan-2-ol, and (1*S*)-(−)-verbenone were generous gifts from Unilever Research, Bedford, U.K. (±)-Pinan-3-one (isopinocamphone) was synthesized by direct oxidation of (±)-pinan-3-ol (isopinocarveol).

Silica gel 40 (Merck 10181) was supplied by BDH Ltd., Poole, U.K. NAD^+, $NADP^+$, NADH, and NADPH were from Park Scientific Ltd., Northampton, U.K.

All other reagents were of analytical grade and supplied either by BDH or Sigma. All solvents were distilled before use.

Purification of α-pinene. Samples (25 ml) of α-pinene were passed through a dry silica gel 40 column (10 g, 0.2- to 0.5-mm beads) which increased the purity to >99% as judged by GLC.

RESULTS

Growth of (±)-α-pinene isolates on terpenes. The range of terpenes capable of supporting growth of nine morphologically distinct strains chosen from our collection was very limited. All grew well with (±)-α-pinene, but of the other tested terpenes only citronellol was capable of supporting growth of all of the investigated strains. Two organisms, including the *Nocardia* strain that was chosen for detailed study, were also capable of growth with α-terpineol, (*R*)-(+)-limonene, (*S*)-(−)-perillyl alcohol, and (*S*)-(−)-perillaldehyde. They did not grow with (−)-borneol, (+)-camphor, carvone, (+)-β-citronellene, (−)-fenchone, geraniol, (±)-linalool, (−)-menthol, β-myrcene, (−)-myrtenol, (±)-pinan-3-ol or (1*S*)-(−)-verbenone. This general lack of a broad terpene growth capability when organisms are selected with (±)-α-pinene may indicate that the induced route(s) for α-pinene oxidation used by our isolates has little in common with the degradative pathways for other monoterpenes.

Growth on (±)-α-pinene. *Nocardia* sp. strain P18.3, like our other gram-positive isolates, would not grow when α-pinene was added directly to basal salts culture medium. Provision of α-pinene to Erlenmeyer flask cultures from vapor tubes (4) resulted in rapid linear growth of *Nocardia* sp. strain P18.3. A_{600} values of 3.5 to 4.0 were reached after 100 to 120 h of growth, at which point cultures were harvested. In contrast the *Pseudomonas* strains (NCIB 10684, 10687, and 11671 and PL) grew rapidly when (±)-α-pinene (0.3%, vol/vol) was included in the growth medium, although an acclimatization period in which α-pinene was supplied from a vapor tube was of advantage for good growth to be obtained subsequently.

Metabolite accumulation during growth of *Nocardia* sp. strain P18.3 with (±)-α-pinene. GLC analysis of unconcentrated diethyl ether extracts of culture medium, which is capable of detecting <10 μg of terpenes per ml in the original culture, showed that there was no significant accumulation of neutral or acidic metabolites in any of the α-pinene cultures of the *Nocardia* sp. tested. Analysis of concentrated diethyl ether extracts of a 120-h culture showed the presence of traces of a number of compounds, which were tentatively identified by GLC-MS as *iso*-borneol, 1,8-cineole, menth-1-en-6-one, menthone, pinocamphone, *iso*-pinocamphone, thujone, verbenol, and verbenone. None of these compounds is acyclic, and, as observed by other workers (16, 17), the diversity of accumulated structures is such that they cannot be accommodated within a unified catabolic sequence.

Additional experiments, in which (±)-α-pinene-grown *Nocardia* sp. strain P18.3 was suspended in buffers covering a range of pHs and under conditions of full or limited aeration, did not result in significant metabolite accumulation when α-pinene was supplied either as a vapor or by direct addition to the medium.

Oxidation of compounds by intact cells. Washed *Nocardia* cell suspensions grown with (±)-α-pinene as the sole carbon source oxidized rapidly (±)-α-pinene, α-pinene oxide, and metabolites III and IV (see Fig. 3) without any significant lag (Fig. 2). Metabolites III and IV were obtained in milligram amounts from incubation of α-pinene oxide with cell extracts of *Nocardia* sp. strain P18.3 as described in Results. Oxidation of (−)-myrtenol, (+)-limonene, (*S*)-(−)-perillaldehyde, and α-terpineol (the last three compounds are growth substrates for the *Nocardia* strain) was initially very slow, with induction occurring after about 60 min, indicative that metabolism of these compounds is not associated with α-pinene degradation. Carvone, geraniol, *cis*-pinan-2-ol, (±)-pinan-3-ol (*iso*-pinocampheol), β-pinene oxide, (1*S*)-(−)-verbenone, and the four isomers of pinane were not significantly oxidized during the experimental period. Succinate-grown cells did not oxidize any of the tested terpenes.

Studies with subcellular fractions. (i) Activity toward (±)-α-pinene. No enzyme-mediated transformation of α-pinene was detected in 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, high-speed supernatants, and membrane preparations were all inactive toward (±)-α-pinene, irrespective of the electron donor employed or the analytical method used. In contrast to the results reported for *P. fluorescens* (Floyd et al.,

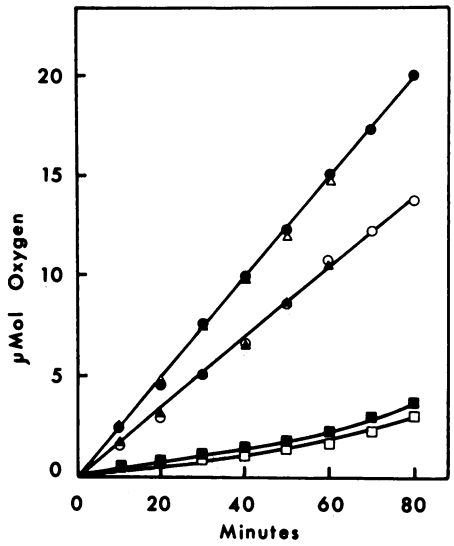

FIG. 2. Substrate oxidation by whole cells of α-pinene-grown *Nocardia* sp. strain P18.3. Warburg flasks contained (in a total volume of 2 ml) 14 mg (dry weight) of bacteria and 74 μmol of phosphate buffer (pH 7.1). Center wells of flasks contained 0.1 ml of 20% KOH and a rolled strip of filter paper to facilitate absorption of CO_2. Substrates were supplied in the vapor phase as described in Materials and Methods, and reactions at 30°C were followed as soon as temperature equilibration had been achieved. Symbols: ○, α-pinene; ●, α-pinene oxide; ▲, metabolite III; △, metabolite IV; ■, α-terpineol; □, (+)-limonene, (−)-myrtenol, and (*S*)-(−)-perillaldehyde. Endogenous oxygen consumption (0.12 μmol/min) has been subtracted.

EMBO Workshop), oxygenase activity toward α-pinene could not be detected either in spectrophotometric or polarographic assays performed in a variety of buffers covering a range of pH values and with either NADH or NADPH as the electron donor.

(ii) Enzyme activity toward α-pinene oxide. The involvement of α-pinene oxide as an intermediate in α-pinene oxidation by *Nocardia* sp. strain P18.3 was suggested by the selective oxidation of this compound by α-pinene-grown cells, and a more broadly based role as an intermediate is also supported by the recent studies of Floyd et al. (EMBO Workshop).

However, a preliminary study of the stability of α-pinene oxide in buffered aqueous solution at 30°C demonstrated the rapid decomposition of the compound at pH 7 or lower, with the formation of four major metabolites that were identified by GLC-MS as campholenic aldehyde, *cis*- and *trans*-carveol, and sobrerol. Decomposition was suppressed with increasing alkalinity of the incubation buffer; the compound was stable for several hours in 0.1 M glycine–NaOH buffer (pH 9). These observations were compatible with the reported pH-dependent stability profile of α-pinene oxide under rather different conditions (13, 15). Initial biochemical studies were therefore performed in 0.1 M glycine–NaOH buffer to suppress nonenzymic transformation.

GLC analysis of diethyl ether extracts of reactions in which α-pinene oxide was incubated with Hughes press extracts of α-pinene-grown *Nocardia* sp. strain P18.3 in 0.1 M glycine–NaOH buffer (pH 9.0) showed that the disappearance of α-pinene oxide was concomitant with the sequential appearance of two neutral metabolites, which were subsequently identified as compounds III and IV. The latter of these did not appear to be further metabolized at a significant rate under the assay conditions used. Neither of these compounds correlated chromatographically with any of over 30 standard monoterpene hydrocarbons, alcohols, ketones, and related compounds or with the four major products of spontaneous α-pinene oxide decomposition.

The two metabolites bear a precursor-product relationship, and this transformation of α-pinene oxide also occurred with dialyzed cell extract in the absence of added cofactors. Boiled cell extracts and extracts of succinate-grown *Nocardia* sp. strain P18.3 were inactive.

Identity of metabolites formed from α-pinene oxide by extracts of *Nocardia* sp. strain P18.3. Metabolite IV was accumulated in a larger-scale preparation when 156 mg of extract protein was incubated with 279 mg (1.67 mmol) of α-pinene oxide in 100 ml of 0.1 M glycine–NaOH buffer (pH 9.0) for 30 min at 30°C. The product was extracted into diethyl ether and yielded 200 mg of viscous oil on removal of solvent. GLC analysis of a suitable dilution confirmed compound IV to be the only significant neutral metabolite. In ethanolic solution the compound gave a single absorbance peak (λ_{max}, 229 nm), which shifted to 235 nm in aqueous solution. The addition of sodium borohydride abolished this absorbance, and the addition of acidic 2,4-dinitrophenylhydrazine (0.1% [wt/vol] in 2 M HCl) to a sample of metabolite IV resulted in the rapid formation of an insoluble yellow 2,4-dinitrophenylhydrazone.

GLC-MS analysis of metabolite IV. Chemical ionization MS gave a pseudomolecular ion at *m/e* 153 (25%) with a base ion at *m/e* 135 ($-H_2O$). Electron impact MS confirmed the molecular ion at *m/e* 152 with associated ions at *m/e* 137 ($M^+ - CH_3$), 134 ($M^+ - H_2O$), 123 ($M^+ - HC{=}O$), 119 [$M^+ - (CH_3 + H_2O)$], and 109 (base ion; $M^+ - C_3H_7$). Analysis of the molecular ion at 1-in-5,000 resolution gave an elemental composition of $C_{10}H_{16}O$. Loss of discrete HC=O indicates the presence of a carbonyl group.

^{13}C-NMR analysis of metabolite IV. If we ignore the solvent peak at 77 ppm, nine peaks were observed; one was a double-intensity peak (21.5 ppm), showing that this was a C_{10} compound. A distortion enhancement polarization transfer editing of the spectrum was used to assign peaks (Fig. 3), and metabolite IV was identified as the *trans* isomer of 2-methyl-5-isopropylhexa-2,5-dienal. The downfield shift of the vinyl methyl resonance from 16.4 to 9.1 ppm resulting from shielding of the vinyl methyl of the *trans* isomer relative to the *cis* isomer can be compared with the observations made by Tudroszen et al. (19) in ^{13}C-NMR studies of the 2-methyl-5-isopropylhexa-2,5-dienoic acids, where a

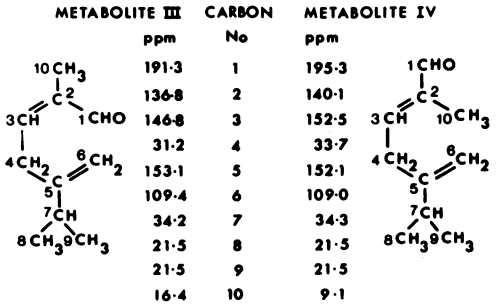

FIG. 3. Structures of metabolites III and IV and the assignment of ^{13}C-NMR resonances to individual carbon atoms. Compounds: III, *cis*-2-methyl-5-isopropylhexa-2,5-dienal; IV, *trans*-2-methyl-5-isopropylhexa-2,5-dienal.

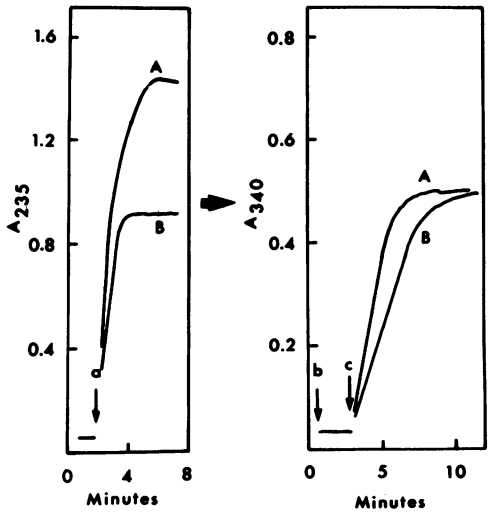

FIG. 4. Spectrophotometric analysis of the formation of the *cis* and *trans* isomers of 2-methyl-5-isopropylhexa-2,5-dienal (metabolites III and IV) in situ and of the NAD-linked aldehyde dehydrogenase. The reaction mixtures contained (in a total volume of 1 ml) 180 μmol of glycine-NaOH buffer (A) or pyrophosphate-orthophosphoric acid buffer (B) at pH 9.0 and 35 μg of cell extract. Reactions were started by the addition of 0.1 μmol of α-pinene oxide (a), and formation of the *trans* (A) or *cis* (B) isomer of the aldehyde was followed at 235 nm. When aldehyde formation was complete, dehydrogenase activity was followed at 340 nm in the presence of an additional 112 μg of extract protein (b) and 0.5 μmol of NAD^+ (c).

similar shift was observed and the *cis* and *trans* isomeric assignments could be made unequivocally on other grounds.

Spectrophotometric investigation of α-pinene oxide metabolism. The established spectral characteristics of *trans*-2-methyl-5-isopropylhexa-2,5-dienal (λ_{max}, 235 nm; Σ, 13,000), coupled with the lack of absorbance at 235 nm by α-pinene oxide, provided a system whereby the decyclization of the compound could be investigated spectrophotometrically, although amounts of protein that could be added to cuvettes were, of necessity, limited at this wavelength.

When crude extract of α-pinene-grown *Nocardia* sp. strain P18.3 was incubated with 0.1 M glycine–NaOH buffer (pH 9) in a 1-ml assay the addition of α-pinene oxide resulted in a rapid increase in A_{235} (Fig. 4). From the total absorbance change observed after the addition of limited amounts of α-pinene oxide (100 to 250 nmol), it was established that its conversion into *trans*-2-methyl-5-isopropylhexa-2,5-dienal was stoichiometric. A specific activity of 15 to 20 μmol of product formed per min per mg of extract protein was typically found when assays were performed in the presence of an excess (1 mM) of α-pinene oxide. Extracts of succinate-grown cells gave no significant enzyme activity.

When assays were performed in buffers other than glycine-NaOH with limited amounts of α-pinene oxide, the observed increase in A_{235} terminated at a lower value (Fig. 4). GLC analysis of the diethyl ether extract of a reaction performed in 42 mM Na^+-K^+-phosphate buffer (pH 7.0) showed the presence of only metabolite III. Isolation of the compound from a larger-scale reaction, performed as described above for the production of metabolite IV (the *trans* isomer of 2-methyl-5-isopropylhexa-2,5-dienal) but in 42 mM Na^+-K^+-phosphate buffer (pH 7.0), allowed the molecule to be spectroscopically characterized, (λ_{max}, 235 nm in ethanol and 242 nm in aqueous solution; Σ, 9,000). GLC-MS analyses gave the same fragmentation patterns as reported for metabolite IV, with only minor differences in relative intensities, and the same empirical formula. ^{13}C-NMR and distortion enhancement by polarization transfer editing of the spectrum showed a shift in vinyl methyl resonance from 9.1 to 16.4 ppm (Fig. 3), which again, by comparison with the results of Tudroszen et al. (19) obtained with the corresponding acids, allowed metabolite III to be identified as the *cis* isomer of 2-methyl-5-isopropylhexa-2,5-dienal.

Search for intermediates in α-pinene oxide decyclization. Short-term reactions were used to search for intermediates in the decyclization sequence that might lie between α-pinene oxide and *cis*-2-methyl-5-isopropylhexa-2,5-dienal. GLC analysis of diethyl ether extracts of short-term reactions did not reveal the presence of any additional metabolites. None of the following compounds, *cis*-pinan-2-ol, (±)-pinan-3-ol (*iso*-pinocarveol), pinan-3-one (*iso*-pinocamphone), pinocarveol, limonene oxide, or β-pinene oxide, was converted into the aldehyde in spectrophotometric assays when incubated with extract of α-pinene-grown *Nocardia* sp. strain P18.3.

Conversion of the *cis* isomer of 2-methyl-5-isopropylhexa-2,5-dienal into the *trans* isomer. Spectrophotometric assays coupled with GLC analysis established that the *trans* isomer of 2-methyl-5-isopropylhexa-2,5-dienal was formed only when assays were conducted in glycine-NaOH buffer. The addition of 0.1 ml of 1 M glycine-NaOH buffer (pH 9.0) to a 1-ml assay in Na^+-K^+-phosphate buffer (pH 7.0) in which 200 nmol of α-pinene oxide had been transformed into the *cis* isomer resulted in a rapid increase in A_{235} concomitant with formation of the *trans* isomer. Extraction of the *cis* isomer from a similar initial reaction into diethyl ether, removal of the ether under a stream of nitrogen, addition of 0.1 M glycine–NaOH buffer (pH 9), incubation for 5 min at 30°C, and GLC analysis of a diethyl ether extract of this solution also showed the transformation of *cis*-2-methyl-5-isopropylhexa-2,5-dienal into the *trans* isomer. The isomerization reaction therefore occurs in the absence of extract protein. It is specifically catalyzed by glycine since, of a range of buffers tested that also included Tris hydrochloride and pyrophosphate-orthophosphoric acid, only glycine-NaOH, and, to a lesser extent, glycylglycine-NaOH buffers, mediated a significant rate of isomerization.

In this context we have also observed that, in alkaline buffers, the *trans* isomer of 2-methyl-5-isopropylhexa-2,5-dienal also undergoes a slow isomerization (double bond shift) to form an approximately 50:50 equilibrium mixture with the *trans* isomer of 2-methyl-5-isopropylhexa-2,4-dienal (λ_{max}, 295 nm). Fortunately this transformation is very slow in glycine-NaOH buffer at pH 9.0 and does not interfere with enzymic studies.

Further metabolism of the *cis* and *trans* isomers of 2-methyl-5-isopropylhexa-2,5-dienal. When either isomer of the aldehyde, formed in situ from α-pinene oxide by crude extract of α-pinene-grown *Nocardia* sp. strain P18.3, was incubated with additional crude extract and NAD^+ the aldehyde-dependent formation of NADH was observed. The results of an experiment in which decyclization and dehydrogenase activities were followed sequentially are shown in Fig. 4. The formation of NADH was approximately stoichiometric, with 0.8 μmol of NADH formed for each molecule of α-pinene oxide introduced into the assay. Rates of NADH formation in sequential reactions performed in pyrophosphate and glycine-NaOH buffers demonstrated that, under these assay conditions, the *trans* isomer of the aldehyde (formed nonbiologically) is a marginally better substrate than the *cis* isomer (Fig. 4).

TABLE 1. ^{13}C-NMR peaks of the products formed by incubating cell extracts of α-pinene-grown *Nocardia* sp. strain P18.3 with α-pinene oxide and NAD^+ at pH 9 in glycine-NaOH buffer

^{13}C-NMR peaks (ppm)			
Reaction product		2-Methyl-5-isopropylhexa-2,5-dienoic acid[a]	
Major	Minor	*cis*	*trans*
171.6	171.8	173.7	
154.2	152.3	154.1	
144.1	142.9	144.6	
126.6	127.6	126.9	
107.9	108.7	107.9	
34.6	33.6	35.2	
34.2	29.7	34.6	
21.6	21.6	21.6	
20.6	12.0	20.5	12.1

[a] Values reported by Tudroszen et al. (19).

No significant dehydrogenase activity toward either aldehyde isomer was detected in extracts of succinate-grown cells.

Identity of the product of 2-methyl-5-isopropylhexa-2,5-dienal dehydrogenase activity. GLC analysis of diethyl ether extracts of reactions followed to completion by spectrophotometric analysis at 340 nm showed that both isomers of the aldehyde disappeared from the reaction concomitant with NADH formation.

Attempts to identify the product from small-scale reactions after acidification with 5 M HCl, diethyl ether extraction, methyl esterification, and GLC-MS analysis yielded inconclusive results with very low yields of a compound that was identified as the methyl ester of 2-methyl-5-isopropylhexa-2,5-dienoic acid.

In an alternative experiment the product was obtained from a larger-scale reaction which contained, in 100 ml of 0.1 M glycine–NaOH buffer (pH 9.0), 100 μmol of α-pinene epoxide, 105 μmol of NAD, and 200 mg of crude extract protein. The α-pinene oxide was omitted from a smaller-scale (10-ml) control flask. The flasks were incubated at 30°C, and 1-ml samples were removed, extracted with an equal volume of diethyl ether, and monitored by GLC at timed intervals. After 2 h of incubation, when metabolites III and IV had disappeared, the reaction was acidified to pH 1 with 5 M HCl and extracted with two 200-ml batches of diethyl ether. The ether phases were pooled and dried over anhydrous sodium sulfate, and the ether was removed by rotary evaporation.

The reaction product (distinguished from endogenous material by thin-layer chromatography of the diethyl ether extracts of the control and test flasks) was purified by preparative thin-layer chromatography on 1-mm-thick Kieselgel plates developed with toluene-acetone (4:1, vol/vol), detected by spraying with bromocresol green reagent (R_f, 0.7), and eluted from the support with diethyl ether.

GLC analysis (45-to-245°C temperature program) revealed a rather broad peak with a longer retention time than even the polar neutral terpene standards. Diethyl ether was removed from the product under a stream of nitrogen to yield 5 mg of an acidic residue.

The identity of this product as 2-methyl-5-isopropylhexa-2,5-dienoic acid was established by GLC-MS and infrared analysis. Electron impact MS gave a molecular ion at *m/e* 168 and ions at 153 ($M^+ - CH_3$), 150 ($M^+ - H_2O$), 135 $M^+ - (CH_3+H_2O)$], 125 [$M^+ - C_3H_7$], 123 [M^+ - COOH], and 107 (*m/e* 125, $-H_2O$). The elemental composition of the molecular ion at 1/5,000 resolution was $C_{10}H_{16}O_2$. The infrared spectrum was compatible with the presence of a carboxyl group. The ^{13}C-NMR spectrum showed what appeared to be two sets of peaks in a 3:2 ratio. These correspond to the reported resonance spectra of *cis* and *trans* isomers of 2-methyl-5-isopropylhexa-2,5-dienoic acid (19), with the *cis* isomer predominating (Table 1).

α-Pinene oxide lyase activity in extracts of α-pinene-grown *Pseudomonas* sp. The *Pseudomonas* strains were grown with α-pinene and succinate as sole carbon sources, and cell extracts were assayed for α-pinene oxide lyase spectrophotometrically at 235 nm. Induced lyase activity was found in extracts of α-pinene-grown *P. putida* PX1 and *P. fluorescens* NCIB 11671, but the enzyme was absent from extracts of *Pseudomonas* sp. strains PIN18 and PL (Table 2). In organisms where the lyase was present the *cis* isomer of 2-methyl-5-isopropylhexa-2,5-dienal was the primary product, yielding the *trans* isomer only in glycine-NaOH buffer.

Polyacrylamide gel electrophoresis of the crude cell extracts of α-pinene- and succinate-grown organisms, followed by detection of lyase activity as described in Materials and Methods and staining of the gels with Coomassie brilliant blue, confirmed the distribution of the enzyme reported in Table 2 and indicated that in organisms where the enzyme is induced it is a major component of the soluble cell protein.

Specificity of dehydrogenase activity induced by growth with α-pinene. Assay of cell extracts of α-pinene- and succinate-grown *Pseudomonas* strains demonstrated the presence of induced NAD-linked dehydrogenase activity toward the *cis* and *trans* isomers of 2-methyl-5-isopropylhexa-2,5-dienal in all organisms, which contrasted with the limited distribution of α-pinene oxide lyase. However, investigation of the substrate range of NAD-linked dehydrogenase activity in *Nocardia* sp. strain P18.3 (lyase positive) and *Pseudomonas* sp. strain PL (lyase negative) showed that although both organisms displayed broad substrate specificity the *cis* and *trans* isomers of 2-methyl-5-isopropylhexa-2,5-dienal were oxidized most rapidly by extract of *Nocardia* sp. strain P18.3, whereas, as previously reported (2), perillyl alcohol was the preferred substrate for *Pseudomonas* sp. strain PL.

DISCUSSION

Isolation of organisms that are capable of growth with (±)-α-pinene as a sole carbon source provided in the vapor phase resulted in the predominant selection of gram-positive bacteria. This contrasts with the selection of *Pseudomonas* strains by other workers, who added α-pinene directly to the elective media (9, 10, 16, 17, 19; Floyd et al., EMBO

TABLE 2. Distribution of α-pinene oxide lyase activity in cell extracts of α-pinene- and succinate-grown bacteria

Strain	α-Pinene oxide lyase (U/mg of protein) in cells grown on:	
	α-Pinene	Succinate
Nocardia sp. strain P18.3[a]	16	0.2
Pseudomonas sp. strains		
NCIB 10684	5.3	0.3
NCIB 11671	3.9	0.09
NCIB 10687	0.01	0.001
PL	0.01	0.001

[a] Assays performed on a large number of cell extracts gave values ranging from 10 to 20 U/mg protein.

Workshop). These results may be a consequence of the differing cell wall structures of the two groups of organisms (the greater structural complexity of the gram-negative cell wall makes the *Pseudomonas* spp. less susceptible to invasive damage at the water-pinene interface) and are supported by the work of Andrews et al. (1), who have reported that *Bacillus* spp. are much more susceptible to inhibition of growth by α-pinene than is *Escherichia coli*.

Previous workers, using *Pseudomonas* spp., have suggested that either α-pinene oxidation is integrated into the pathway for limonene oxidation by *Pseudomonas* PL (9, 16, 17), although the pathway is obscured by the wide range of metabolites accumulated in growth media, or a second ring cleavage between carbon atoms 3 and 4 of the α-pinene skeleton yields novel branched-chain acids not encountered in established routes for the degradation of other mono- or bicyclic monoterpenes (9, 10, 19; Floyd et al., EMBO Workshop). The very restricted terpene growth spectra of our gram-positive isolates would suggest the involvement of a novel catabolic route rather than one that makes use of components of more general application.

The exclusive nature of the route used by *Nocardia* sp. strain P18.3 for α-pinene oxidation was also suggested by the ability of α-pinene-grown cells to oxidize only the growth substrate, α-pinene oxide, and the *cis* and *trans* isomers of 2-methyl-5-isopropylhexa-2,5-dienal. None of a wide range of other available putative intermediates was oxidized at a significant initial rate.

We have been unable to find any subcellular enzyme activity toward (±)-α-pinene in cell extracts of the *Nocardia* strain. The anticipated formation of the epoxide from α-pinene, presumably catalyzed by a monooxygenase, could not be demonstrated. Such a monooxygenase is likely to be a multiprotein complex (3, 5, 18), possibly containing a cytochrome P_{450}-oxygenating component. It is possible that disruption of a structurally sensitive complex, concomitant with cell breakage, may provide an explanation for our failure. Gentle methods of cell disruption, including the Hughes press, coupled with high protein concentrations and reduced NADP generating systems in, for example, manometric assays failed to circumvent these problems.

In contrast, Floyd et al. (EMBO Workshop) recently reported that cell extracts of an α-pinene-grown *P. fluorescens* contained an NADH-linked monooxygenase which, in the presence of atebrin, formed a α-pinene oxide.

Because of the reported instability of α-pinene oxide in neutral and acidic aqueous solution, which was confirmed in this study, initial subcellular investigations were performed in glycine-NaOH buffer (pH 9.0), conditions in which α-pinene oxide is reasonably stable. GLC analysis showed that cell extracts of the α-pinene-grown *Nocardia* sp. strain P18.3 catalyzed a rapid transformation of the epoxide into *cis*-2-methyl-5-isopropylhexa-2,5-dienal, which was followed by rapid isomerization to the *trans* isomer (Fig. 3). Although the amount of protein that could be included in spectrophotometric assays at 235 nm was very restricted, the high Σ values of the aldehyde isomers coupled with the high specific activity of the lyase in cell extracts (10 to 20 U/mg of protein) enabled assays to be performed with ease. As a consequence, reactions could be run to completion rapidly, in a range of buffers and at pH values as low as 7, without significant nonenzymic epoxide decomposition.

When reactions were performed in buffers other than glycine-NaOH and over a pH range from 7 to 10, only the *cis* isomer of 2-methyl-5-isopropylhexa-2,5-dienal was detected as the ring cleavage product. This isomer would be expected as the initial cleavage product on the basis of the reaction mechanism proposed for the epoxide lyase (12). Isomerization of the aldehyde to the *trans* isomer occurs nonenzymically in the presence of glycine, and to a lesser extent glycylglycine, although the mechanism of catalysis is not understood.

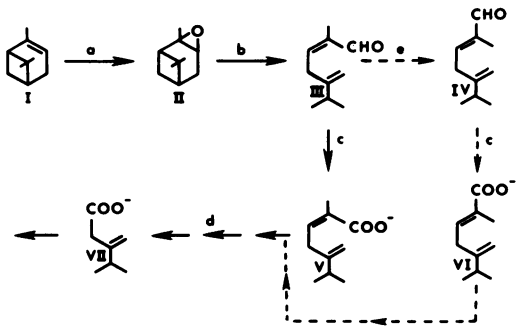

FIG. 5. Pathway proposed for the partial metabolism of (±)-α-pinene by *Nocardia* sp. strain P18.3. Compounds: I, α-pinene; II, α-pinene oxide; III and IV, *cis*- and *trans*-2-methyl-5-isopropylhexa-2,5-dienal, respectively; V and VI, *cis*- and *trans*-2-methyl-5-isopropylhexa-2,5-dienoic acid, respectively; VII, 3-isopropylbut-3-enoic acid. Reactions and enzymes: a, α-pinene monooxygenase (Floyd et al., EMBO Workshop); b, α-pinene oxide lyase; c, 2-methyl-5-isopropylhexa-2,5-dienal dehydrogenase; d, postulated β-oxidation leading to VII; e, spontaneous isomerization in glycine-NaOH buffer (pH 9.0).

Further metabolism of the aldehyde isomers is indiscriminate, since cell extracts of α-pinene-grown *Nocardia* sp. strain P18.3 possess broad dehydrogenase capability which, in the presence of NAD^+, oxidizes a variety of terpene aldehydes and ketones, although the isomers of 2-methyl-5-isopropylhexa-2,5-dienal are the preferred substrates. Ironically, if glycine buffer at pH 9.0 had not been used to suppress spontaneous α-pinene oxide decomposition, the nonenzymic formation of the *trans* isomer of the aldehyde would probably not have been encountered.

Because we have been unable to demonstrate the anticipated epoxidation of α-pinene by cell extracts of the *Nocardia* strain or to accumulate relevant intermediates in whole cell systems, the evidence for α-pinene oxide being an intermediate in α-pinene oxidation by this organism is circumstantial and consists of the following.

(i) *Nocardia* sp. strain P18.3 will not grow with any isomer of pinane, nor is any isomer oxidized by (±)-α-pinene-grown cells, suggesting that the double bond of α-pinene plays a key role in degradation.

(ii) α-Pinene oxide and the *cis* and *trans* isomers of 2-methyl-5-isopropylhexa-2,5-dienal were the only tested compounds that were oxidized rapidly by α-pinene-grown cells.

(iii) Enzymes for the cleavage of α-pinene oxide and for further metabolism are induced by growth with α-pinene.

(iv) The organism makes a very significant commitment to protein synthesis (6% of the soluble cell protein) with the induced formation of the lyase. The purification and properties of this novel enzyme are reported by Griffiths et al. (12).

(v) A monooxygenase forming α-pinene oxide from α-pinene has been reported to be formed by an α-pinene-grown strain of *P. fluorescens* (Floyd et al., EMBO Workshop).

A proposed pathway for the conversion of α-pinene into acyclic metabolites which takes into account our own results and observations made by other workers is shown in Fig. 5.

A brief examination of the distribution of α-pinene oxide lyase in available *Pseudomonas* strains supports the provisional suggestion, drawn from metabolite accumulation studies, that two distinct pathways for α-pinene oxidation exist.

The reports by Gibbon et al. (9, 10) with *P. putida* NCIB 10684 (PX1) and of Tudroszen et al. (19) with *Pseudomonas* sp. strain PIN11 in which the *cis* and *trans* isomers of 2-methyl-5-isopropylhexa-2,5-dienoic acid were accumulated in culture media are compatible with the reaction sequence established for *Nocardia* sp. rather than the pathway originally proposed (19). This proposal involved a biological Baeyer-Villiger reaction; although such reactions are widely distributed in catabolic sequences (18), in this instance there was no supporting evidence from work with cell-free systems. We would anticipate that *P. fluorescens* NCIB 11671 uses the same metabolic route (Table 2), whereas, in contrast, the absence of an induced α-pinene oxide lyase in cell extracts of α-pinene-grown *Pseudomonas* sp. strain NCIB 10687 (PIN18) and *Pseudomonas* sp. strain PL is compatible with the alternative catabolic route proposed (9, 16, 17), in which an initial isolated cleavage of the cyclobutane ring leads to limonene and a sequence of common catabolic intermediates.

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