Biochem. J. (1977) **168**, 315–318
Printed in Great Britain

α-Pinene Metabolism by *Pseudomonas putida*

By NEVILLE J. TUDROSZEN, DAVID P. KELLY* and NANCY F. MILLIS
Department of Microbiology, University of Melbourne, Parkville, Vic. 3052, Australia

(*Received* 22 *August* 1977)

By using metabolically altered mutants and acrylate, novel putative intermediates of α-pinene metabolism by *Pseudomonas putida* PIN11 were detected. They were characterized as 3-isopropylbut-3-enoic acid and (*Z*)-2-methyl-5-isopropylhexa-2,5-dienoic acid.

Previous workers have described the characterization of three acids accumulated by cultures of a soil pseudomonad PX1 grown with α-pinene as the sole source of carbon and energy. These acids were identified as 3,4-dimethylvaleric acid, 2,5,6-trimethylhept-3-enoic acid and 2-methyl-5-isopropylhexa-2,5-dienoic acid (Gibbon & Pirt, 1971; Gibbon *et al.*, 1972). The present study examines α-pinene metabolism in a similar organism, *Pseudomonas putida* PIN11, and is concerned with the chemical characterization of two novel metabolites.

Materials and Methods

Organism

The parent *Pseudomonas putida* PIN11 was obtained from Professor S. J. Pirt, Queen Elizabeth College, London W.8, U.K.

Media and growth of organisms

The basic mineral salts (minimal) medium used was that described by Gibbon & Pirt (1971). (+)-α-Pinene ($[\alpha]_D^{25}$ +45.94°) (Bush Boake Allen, London E.8, U.K.) was purified by distillation under reduced pressure before use. Where solid medium was required 1% (w/v) Ionagar no. 2 (Oxoid, London E.C.4, U.K.) was added. All organisms were grown aerobically at 30°C. Liquid cultures (500ml) of *Ps. putida* PIN11 were grown in 2-litre Erlenmeyer flasks on a rotary shaker. α-Pinene (final concentration 1%, w/v) was added in three equal portions over 24h and incubation continued until cultures reached stationary phase. Sodium acetate when used as a carbon source was at a final concentration of 0.2%.

Extraction procedures and preparation of derivatives

Cells were removed by centrifugation, and the supernatant was acidified and extracted as described by Gibbon & Pirt (1971). Methylation of extracted carboxylic acids (0.1–0.2g) was performed by using BF_3 and methanol (Metcalf & Schmitz 1961). Methyl esters of carboxylic acids in culture supernatants were prepared by treating the supernatants with methanol and H_2SO_4 (Holdeman & Moore 1972). Pyrrolidine derivatives were prepared by the method of Andersson & Holman (1974). Hydrogenation was performed with Adam's catalyst (PtO_2) at 138kPa for 5h in a Parr hydrogenator.

Spectral analyses

A Perkin–Elmer R12 n.m.r. (nuclear-magnetic-resonance) spectrometer was used to obtain ^1H n.m.r. spectra (60MHz). Tetramethylsilane was used as an internal reference and [^2H]chloroform as the solvent. I.r. spectra were recorded by using NaCl plates in a Perkin–Elmer 457 grating i.r. spectrophotometer. A Perkin–Elmer 402 u.v.–visible spectrophotometer was used to record u.v. spectra. Mass spectra were obtained on an EAI Quad 300 mass spectrometer interfaced to a Nova 1200 computer with an EAI 130 data incremental plotter. ^{13}C n.m.r. spectra of C^2HCl_3 solutions in 10mm tubes were measured on a hybrid Varian HA-60-Digilab-DEC PDP-15 spectrometer operating at 15.085MHz. Chemical shifts are ±0.1p.p.m downfield from tetramethylsilane (Dewar *et al.*, 1975).

Thin-layer chromatography

Analytical and preparative t.l.c. were performed on glass plates (20cm×20cm) coated to a thickness of 0.25mm with silica gel GF_{254} (E. Merck, Darmstadt, Germany) impregnated with 5% (w/v) $AgNO_3$ as described by Morris (1966). The developing solvent system was hexane/diethyl ether (18:1, v/v). Compounds were detected under u.v. light at 254nm.

Gas–liquid chromatography

A Pye–Unicam series 104 model 24 chromatograph with a flame-ionization detector was used as a routine. The glass column (152cm×6.4mm) was

* Present address: Department of Organic Chemistry, University of Melbourne, Parkville, Victoria, Australia.

packed with 10% (w/w) Carbowax 20M on Diatomite AW (80–100 mesh) (Supelco, Bellefonte, PA, U.S.A.). The carrier-gas (N_2) flow rate was 40ml/min. The operation for methylated samples was isothermal at 80°C for 2min followed by programming at 5°C/min to 180°C. This column was also used for preparative g.l.c. The carrier-gas (N_2) flow rate was 60ml/min, operation was at 190°C isothermally.

For mass-spectral analysis, a 5720A gas chromatograph (Hewlett–Packard) with a flame-ionization detector was interfaced to a Quad 300 mass spectrometer through an all-glass jet separator. Methylated samples were separated by using a glass column (366cm×3.2mm) packed with 5% (w/w) Carbowax 20M TPA on Chromosorb G AW-DMCS (80–100 mesh). Separation was achieved by programming from 70 to 200°C at 8°C/min. The carrier-gas (He) flow rate was 20ml/min.

A glass column (213cm×3.2mm) packed with 3% (w/w) OV-1 on Chromosorb W AW-DMCS (80–100 mesh) was used for the separation of the pyrrolidine derivatives of carboxylic acids. The carrier-gas flow rate was 30ml/min. Operation was at 100°C for 15min followed by programming at 20°C/min to 140°C and isothermal operation thereafter.

Mutant selection and examination

Mutants capable of utilizing acetate but not α-pinene (designated *pin*$^-$) were obtained by treating an exponentially grown culture of the parent *Ps. putida* PIN11 with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (180μg/ml of 0.1M-potassium phosphate buffer, pH5.5). The *pin*$^-$ mutants were enriched by growing the *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine-treated culture in α-pinene minimal medium containing penicillin G at 10^4i.u./ml (Lederberg & Zinder, 1948). Colonies of surviving cells were grown on acetate minimal agar and screened for their ability to grow on α-pinene.

The *pin*$^-$ mutants were examined for the accumulation of metabolites. They were grown in acetate minimal medium, α-pinene (0.3%) was added at stationary phase and incubation continued for 24h. Culture supernatants were subsequently methylated, extracted and analysed by g.l.c. or g.l.c.–mass spectrometry.

Inhibition by acrylate

The effect of acrylate on α-pinene metabolism was investigated by using conditions similar to those of Thijsse (1964) when studying alkane oxidation. A suspension of α-pinene-grown PIN11 cells (0.4mg dry wt./ml in 0.1M-phosphate buffer, pH7.0) was used and α-pinene (0.33%) was the carbon source.

Results and Discussion

Intermediates accumulated by PIN11 cells were similar to those of *Pseudomonas* PX1. 3,4-Dimethylvaleric acid, 2,5,6-trimethylhept-3-enoic acid and 2-methyl-5-isopropylhexa-2,5-dienoic acid were characterized by Gibbon & Pirt (1971) from PX1 cells. Extraction of the supernatant of a PIN11 culture yielded 0.5g of mixed acids/l. After methylation and examination by g.l.c.–mass spectrometry, this fraction was found to consist of the methyl esters of 3,4-dimethylvaleric acid (95%), 3-isopropylbut-3-enoic acid (1%) and 2-methyl-5-isopropylhexa-2,5-dienoic acid (3%).

Metabolites accumulated by mutants

After treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and penicillin selection approx. 10% of the surviving organisms were *pin*$^-$ mutants. Only 5% of these displayed accumulation patterns significantly different from the wild-type *Ps. putida* PIN11. The majority of these *pin*$^-$ mutants accumulated the same compounds as the wild-type organism, but in greater overall yield and different relative amounts.

Mutant 51A differed, however, by accumulating a novel metabolite. When methylated, the acid fraction (1.0g/l) of the culture supernatant of mutant 51A contained the methyl esters of 3,4-dimethylvaleric acid (21%), 2,5,6-trimethylhept-3-enoic acid (30%) and an unknown compound (*a*). The mass spectrum of the methyl ester of compound (*a*) showed a molecular ion (*M*) at *m/e* 142. The peak due to the McLafferty rearrangement (*m/e* 74) represented 21.0% of the base peak, which was *m/e* 83 ($M-CO_2CH_3$). Hydrogenation of compound (*a*) resulted in the formation of a compound identical in retention time and mass spectrum with 3,4-dimethylvaleric acid. The mass spectrum of the pyrrolidine derivative of compound (*a*) was consistent with these observations, illustrating *m/e* 181 (*M*) and *m/e* 113 (McLafferty rearrangement, 12.5%). Interpretation of this mass spectrum did not unequivocally indicate the unsaturated position, despite the simplified fragmentation pattern (Andersson & Holman, 1974).

Resolution of the methyl esters of 0.5g of mixed acids by preparative argentation t.l.c. yielded 68mg of methylated compound (*a*) [R_F 0.33 in the solvent hexane/diethyl ether (18:1, v/v)].

The i.r. spectrum of the methyl ester of compound (*a*) revealed moderate absorption at 3094cm^{-1} (olefinic group), strong absorption at 1740cm^{-1} (C=O stretching) and strong absorption at 1165cm^{-1} (C–O stretching). The n.m.r. spectrum of the methyl ester revealed an isopropyl group (doublet, δ 1.03, 6H, $J=6$Hz; septet, δ 2.27, 1H, $J=6$Hz), a methylene group in the α position to a carbonyl

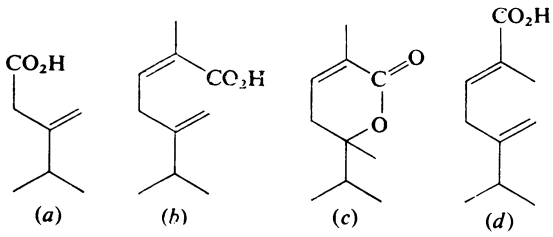

Fig. 1. *Structures of compounds characterized* (*a*) 3-Isopropylbut-3-enoic acid; (*b*) (*Z*)-2-methyl-5-isopropylhexa-2,5-dienoic acid; (*c*) 2,5,6-trimethylhept-2-en-5-olide; (*d*) (*E*)-2-methyl-5-isopropylhexa-2,5-dienoic acid.

group (singlet, δ 3.02, 2H), a methoxyl group (singlet, δ 3.62, 3H) and a terminal methylene group (doublet, δ 4.84, 2H, $J = 4$ Hz). From this information the methyl ester of compound (*a*) was identified as the methyl ester of 3-isopropylbut-3-enoic acid (Fig. 1). Trace amounts of 3-isopropylbut-3-enoic acid were subsequently identified by g.l.c.–mass spectrometry in cultures of various mutants and the wild-type organism.

Effect of acrylate

Compound (*b*), a novel intermediate, accumulated during α-pinene metabolism by PIN11 cells in the presence of acrylate. Purification was achieved by preparative g.l.c.; 430mg of the acid fraction yielded 60mg of pure compound (*b*) in the form of a colourless oil. Elemental analysis was as follows. Found: C, 71.4; H, 9.4; $C_{10}H_{16}O_2$ requires C, 71.4; H, 9.5%. The i.r. spectrum displayed absorption bands at 2960cm^{-1} (broad intense, O–H stretching), 1687cm^{-1} (C=O stretching), 1255 and 1410cm^{-1} (C–O stretching and O–H-bending vibrations respectively). The mass spectrum showed peaks at *m/e* 153 ($M-CH_3$), 125 ($M-C_3H_7$) and 41 (base peak). The u.v. spectrum in ethanol showed $\lambda_{max.}$ 215nm ($\varepsilon_{max.} = 6014$ litre·mol^{-1}·cm^{-1}). The ^{1}H n.m.r. revealed an isopropyl group (doublet, δ 1.03, 6H, $J = 7$ Hz; septet δ 2.26, 1H, $J = 7$Hz), a vinyl methyl group (doublet, δ 1.94, 3H, $J = 2$Hz), a methylene group between two vinyl carbons (doublet, δ 3.30, 2H, $J = 7$Hz), a vinyl methylene group (multiplets, δ 4.67, 1H, δ 4.79, 1H) and a vinyl proton (triplet of quartets, δ 6.11, 1H, $J = 7$Hz, 1.5Hz). The ^{13}C n.m.r. spectrum showed the following peaks: δ 173.7 (singlet, CO_2H); δ 154.1 (singlet); δ 144.6 (doublet, =CH); δ 126.9 (singlet); δ 107.9 (triplet, $=CH_2$); δ 35.2 (triplet, CH_2); δ 34.6 (doublet $\underline{C}H(CH_3)_2$); δ 21.6 (quartet, $(CH_3)_2$) and δ 20.5 (quartet, CH_3). This information suggested that the structure of compound (*b*) was (*Z*)-2-methyl-5-isopropylhexa-2,5-dienoic acid. We were able to assign the *Z*-configuration to compound (*b*) from the difference between its ^{13}C n.m.r. spectrum and that for compound (*d*) (see below) and because treatment of compound (*b*) with acidic methylating agents yields not the methyl ester but a δ-lactone, identified as 2,5,6-trimethylhept-2-en-5-olide (compound *c*) on the basis of the following data. Elemental analysis: Found C, 71.1; H 9.5; $C_{10}H_{16}O_2$ requires C, 71.4; H, 9.5%. The i.r. spectrum showed intense absorptions at 1710 and 1117cm^{-1} (C=O and C–O respectively). The ^{1}H n.m.r. spectrum showed non-equivalent isopropyl methyl groups (doublet, δ 0.94, 3H, $J = 6$Hz; doublet, δ 1.00, 3H, $J = 6$ Hz), an isopropyl methine group (multiplet, δ 1.8–2.2, 1H), a tertiary methyl group (singlet, δ 1.31, 3H), a vinyl methyl group (doublet, δ 1.93, 3H, $J = 2$Hz), a methylene group α to a vinyl carbon (doublet, δ 2.38, 2H, $J = 8$Hz) and an olefinic proton (multiplet, δ 6.5, 1H). No absorption of a methoxyl group was observed. The mass spectrum showed peaks at *m/e* 153 ($M-CH_3$) and 150 ($M-H_2O$). Further evidence for the presence of a δ-lactone was afforded by a base peak at *m/e* 125 ($M-C_3H_7$) (Honkanen *et al.*, 1965). The purified compound showed very little optical activity ($[\alpha]_D^{25}$ +0.57°).

Compound (*d*) also accumulated when acrylate was used as a metabolic inhibitor. Its spectral characteristics were remarkably similar to those of compound (*b*), the only significant difference being the vinyl methyl chemical shifts in the ^{13}C n.m.r. Whereas this carbon resonates at 20.5 p.p.m. in compound (*b*), that of compound (*d*) resonates at 12.1 p.p.m., i.e. 8.4 p.p.m. to higher field. Such shielding of methyl groups is due to steric buttressing by other groups (Stothers, 1970). Compound (*d*) was thus identified as (*E*)-2-methyl-5-isopropylhexa-2,5-dienoic acid. This structure is confirmed by the ready conversion of compound (*d*), by using BF_3 and methanol, into the methyl ester of 2-methyl-5-isopropylhexa-2,5-dienoic acid (Gibbon & Pirt, 1971) and not to a δ-lactone.

This study suggests an isomerization step within the pathway of α-pinene metabolism. The acid described by Gibbon & Pirt (1971) can now be assigned the structure (*E*)-2-methyl-5-isopropylhexa-2,5-dienoic acid, whereas that detailed in this report is the *Z*-isomer. The *Z*-isomer has also been found in the acid fraction (0.1 g/l) of the culture supernatant of mutant 33 exposed to α-pinene in the absence of acrylate; it constituted 50% of the fraction. The pathway of α-pinene metabolism remains uncertain. Gibbon & Pirt (1971) offered a tentative scheme, including a number of hypothetical intermediates that have yet to be isolated. Their scheme does not include 3-isopropylbut-3-enoic acid nor the *Z*- and *E*-isomers of 2-methyl-5-isopropylhexa-2,5-dienoic acid.

We thank Mr. John Fisher, Royal Melbourne Institute of Technology, Melbourne, Victoria, Australia, who performed the mass-spectral analyses.

References

Andersson, B. A. & Holman, R. T. (1974) *Lipids* **9**, 185–190
Dewar, M. K., Johns, R. B., Kelly, D. P. & Yates, J. F. (1975) *Aust. J. Chem.* **28**, 917–924
Gibbon, G. H. & Pirt, S. J. (1971) *FEBS Lett.* **18**, 103–105
Gibbon, G. H., Millis, N. F. & Pirt, S. J. (1972) *Proc. Int. Fermentation Symp. 4th*, 609–612
Holdeman, L. V. & Moore, W. E. C. (1972) *Anaerobe Laboratory Manual*, p. 113, Virginia Polytechnic Institute and State University, Blacksbury
Honkanen, E., Moisio, T. & Karvonen, P. (1965) *Acta Chem. Scand*, **19**, 370–374
Lederberg, J. & Zinder, N. (1948) *J. Am. Chem. Soc.* **70**, 4267–4268
Metcalf, L. D. & Schmitz, A. A. (1961) *Anal. Chem.* **33**, 363–364
Morris, L. J. (1966) *J. Lipid Res.* **7**, 717–732
Stothers, J. B. (1970) *Org. Chem.* **24**, 404–411
Thijsse, G. J. E. (1964) *Biochim. Biophys. Acta* **84**, 195–197