THE DEGRADATION OF α-PINENE BY *PSEUDOMONAS* PX1

G.H. GIBBON and S.J. PIRT
Microbiology Department, Queen Elizabeth College
London W8 7AH, England

Received 9 August 1971

1. Introduction

Previous studies [1, 2] on the fermentation of α-pinene (I) by a soil pseudomonad (PL strain) have indicated that a complex system of pathways exists through which this hydrocarbon is degraded and transformed into different products.

In this communication we report the isolation of another α-pinene utilizing bacterium (*Pseudomonas* PX1) and describe the identification of some of the organic acids produced during the fermentation of α-pinene by this organism. Our present findings suggest that the degradation of α-pinene by *Pseudomonas* PX1 is not similar to that exhibited by the PL strain and that the degradation proceeds via a novel pathway.

2. Materials and methods

Pseudomonas PX1 was grown in an ammonia-mineral salts medium with (+)-α-pinene (Bush Boake Allen Ltd., Carpenters Road, London, E15; $[\alpha]_D^{25}$ = + 26°) as the sole carbon source. Batch culture fermentations were carried out in a stirred fermenter (CECA, A. Gallenkamp Ltd., Christopher Street, London EC2) using 3 l culture volumes. Culture conditions were temperature 25°; pH 7, automatically controlled; stirrer speed 1400 rpm; air-flow rate 12 l hr^{-1}. The medium contained 2 g l^{-1} $(NH_4)_2SO_4$ giving 2.5 g l^{-1} cell dry wt. The initial α-pinene concentration was 0.33% v/v and similar aliquots were added at 18 hr intervals. Fermentations were continued until growth became nitrogen limited (usually after 48 hr).

At the end of the fermentaiton the whole culture (3 l) was acidified to pH 2 and extracted with chloroform (2 × $^1/_6$ vol.). The extract was centrifuged to facilitate the removal of the cells and precipitated material and to clarify the chloroform layer. The organic acids were removed from the chloroform extract by washing with 5% aqueous sodium carbonate (2 × $^1/_5$ vol.). The sodium carbonate solution was acidified to pH 2 and extracted with dichloromethane (2 × $^1/_5$ vol.). This extract was dried over anhydrous sodium sulphate and evaporated, leaving the acidic products as a clear brown liquid. The acids were then converted into their methyl esters (1.5 g) using MeOH/HCl.

Analytical gas–liquid chromatography was carried out on a Pye series 104 chromatograph (Model 4) at 150° with a column packing of 10% polyethyleneglycol adipate 1500 on 80–120 mesh acid washed celite. A 15 ft column (10% PEGA 1500 on 60–80 mesh Chromosorb W) at 120° or 150° was used for preparative GLC. Nuclear-magnetic-resonance (NMR) spectra were recorded on Varian HA-100 and Perkin-Elmer R10 instruments for $CDCl_3$ solutions with tetramethylsilane as the internal standard. Mass spectra were measured on an A.E.I. MS9 instrument.

3. Results

Examination of the methylated acid fraction by GLC (fig. 1) showed that it was composed of one major (Peak A) and several minor subfractions. Only those methyl esters giving rise to peaks A, B and C (fig. 1) were obtained in sufficient quantities for further analysis.

North-Holland Publishing Company – Amsterdam

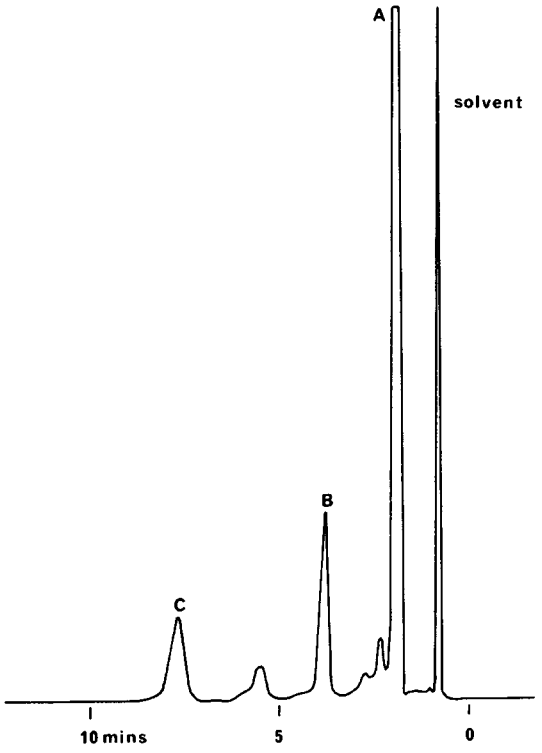

Fig. 1. GLC analysis of the methylated acid fraction resulting from degradation of α-pinene by *Pseudomonas* PX1.

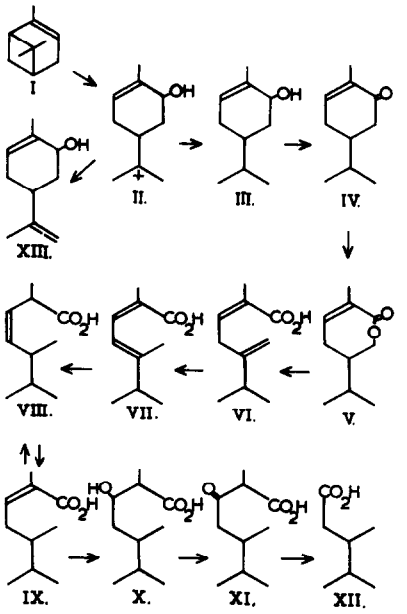

Fig. 2. Proposed pathway of α-pinene (I) degradation by *Pseudomonas* PX1. The compounds identified were VI, VIII, XII and XIII.

Identification of methyl ester A. The pure compound (1.2 g) analysed for $C_8H_{16}O_2$ (Found: C, 66.5; H, 11.3%. Calculated: C, 66.6; H, 11.1%) and showed characteristic I.R. absorption bands for a saturated ester (υ_{max} 1740 cm^{-1}) and *gem*-dimethyl groups (υ_{max} 1375, 1385 cm^{-1}). The mass spectrum did not show a molecular ion (m/e 144) but gave peaks at m/e 129 (3.0), 113 (15.0), 101 (19.5), 87 (40.3), 74 (100), 71 (34.3), 69 (19.4), 59 (21.0), 43 (49.0), 41 (30.0) (numbers in parenthesis refer to ion abundance). The NMR spectrum (60 MHz) showed signals for three methyl groups on methine carbon atoms (coincident doublets; τ 9.08–9.18; 9H; J = 6 cps), two methine protons (complex signal; τ 8.1–8.7), a methylene group α to a carbonyl system (complex signal; τ 7.69–7.9) and a methoxyl group (singlet; τ 6.37; 3 H). On the basis of this information methyl ester A was identified as (−)-methyl 3,4-dimethylvalerate ($[\alpha]_D^{25}$ − 11.0° ± 0.1°) and the free acid was assigned structure XII (fig. 2).

Identification of methyl ester B. The mass spectrum of B (70 mg), $C_{11}H_{20}O_2$, showed peaks at m/e 184 (12.5), 152 (7.5), 109 (25.0), 96 (20.0), 88 (75.0), 86 (100.0), 70 (28.0), 69 (38.1), 55 (62.2), 43 (28.1) and 41 (50.0). The NMR spectrum (100 MHz) revealed an isopropyl group (split doublet; τ 9.16; 6 H; J = 7 cps: septuplet; τ 8.53; 1 H; J = 7 cps), two methyl groups on methine carbons (doublet; τ 9.06; 3 H; J = 7 cps: doublet; τ 8.76; 3 H; J = 7 cps), a methoxyl group (singlet; 3 H; τ 6.34), two vicinal definic protons (doublets; τ 4.55, 4.57; 2 H; J = 4.5 cps) and two methine protons each α to an olefinic proton (multiplet; 1 H; τ 6.9: finely split signal; 1 H; τ 8.06). From this information methyl ester B was identified as methyl 2,5,6-trimethylhept-3-enoate and structure VIII (fig. 2) given to the free acid.

Identification of methyl ester C. The I.R. spectrum of this compound, $C_{11}H_{18}O_2$ (130 mg), showed characteristic absorption bands for an α,β-unsaturated ester (υ_{max} 1710, 1640, 1260 cm^{-1}). The mass spec-

trum showed peaks at 182 (20.0), 167 (10.1), 151 (23.0), 139 (100.0), 123 (72.0), 114 (27.0), 107 (52.0), 83 (54.0), 81 (61.1), 70 (40.0), 69 (53.0), 43 (54.0), 41 (84.4). The NMR spectrum (100 MHz) showed signals for an isopropyl group (doublet; τ 8.9; 6 H; J = 7 cps: multiplet; τ 7.73; 1 H), a vinyl methyl group (finely split singlet; τ 8.15; 3 H), a methylene group sandwiched between two vinylic carbons (doublet; τ 7.11; 2 H; J = 8 cps), a methoxyl group (singlet; τ 6.27; 3 H), two geminal olefinic protons (finely split singlets τ 5.19, 5.31; each 1 H) and an olefinic proton (split triplet, τ 3.19; 1 H; J = 8 cps). On irradiation at the frequency of the isopropyl methine proton (τ 7.73) the only observed effect was the collapse of the fine splitting of the signal at τ 5.19. From this information, methyl ester C was identified as methyl 2-methyl-5-isopropylhexa-2,5-dienoate and the free acid was assigned structure VI (fig. 2).

4. Discussion

The nature of the acidic compounds isolated suggested that the organism used in this study utilized α-pinene in a way different from that exhibited by the PL strain. We were unable to identify any of the organic acids previously reported as being intermediates in α-pinene utilization by the PL strain and although such a finding did not exclude the existence of these pathways in *Pseudomonas* PX1, their presence seemed unlikely. Although growth and adaptation studies using the metabolites isolated have not as yet been attempted with this organism, the isolated organic acids appeared to have a precursor-product relationship and therefore it was tempting to postulate a pathway based only on chemical logic. The pathway we have envisaged for the degradation of α-pinene by *Pseudomonas* PX1 is shown in fig. 2.

An examination of the neutral extracts obtained during this study did not reveal any of the neutral intermediates postulated. However, (+)-*trans*-carveol (XIII) was identified as a fermentation product (I.R. and NMR spectra identical with an authentic sample) and this may have originated from cation II by proton elimination. A satisfactory explanation for the formation of VI would be cleavage of the cyclohexene ring of carvotanacetone (IV) via a lactone intermediate (V). This would be analogous to the cleavage of the bornane nucleus, exhibited during the bacterial oxidation of camphor [3–5].

Acknowledgements

The authors gratefully acknowledge helpful criticisms from Dr. C.W. Bird (Queen Elizabeth College) and a grant from Bush Boake Allen Ltd.

References

[1] O.P. Shukla, M.N. Moholay and P.K. Bhattacharyya, Indian J. Biochem. 5 (1968) 79.
[2] O.P. Shukla and P.K. Bhattacharyya, Indian J. Biochem. 5 (1968) 92.
[3] W.H. Bradshaw, H.E. Conrad, E.J. Corey, I.C. Gunsalus and D. Lednicer, J. Amer. Chem. Soc. 81 (1959) 5507.
[4] J. Hedegaarde, H.E. Conrad and I.C. Gunsalus, Bacteriol. Proc. (1961) 183.
[5] H.E. Conrad, J. Hedegaarde, I.C. Gunsalus, E.J. Corey and H. Uda, Tetrahedron Letters 10 (1965) 561.