Biotransformation of 3-Methylphthalate by *Micrococcus* sp. Strain 12B

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When *Micrococcus* strain 12B grown on *o*-phthalate was incubated with 3-methylphthalate, three compounds accumulated. These were shown to be 2-pyrone-3-methyl-4,6-dicarboxylic acid, 3,4-dihydroxy-6-methylphthalic acid, and 5-hydroxy-3-methylphthalic acid, all previously undescribed. A pathway for the formation of these compounds is proposed.

INTRODUCTION

Micrococcus sp. strain 12B, which was isolated from compost by enrichment for growth with dibutylphthalate (Eaton & Ribbons, 1982*b*), is able to grow with several other phthalate esters as well as the phthalate anion. Washed suspensions of cells grown in the presence of phthalate are capable of oxidizing several phthalate analogues including 3-methylphthalate (Eaton & Ribbons, 1982*a*; Ribbons *et al.*, 1984). 3-Methylphthalate did not support growth of *Micrococcus* sp. strain 12B but it was transformed to several UV-absorbing products. This is believed to be the first report of the metabolism of 3-methylphthalate.

METHODS

Transformation of 3-methylphthalate. A 1 l overnight culture of *Micrococcus* sp. strain 12B in mineral salts medium (R medium) supplemented with phthalate (0.2%, w/v) and yeast extract (0.02%, w/v) was used to inoculate a 14 l Microferm fermenter (New Brunswick) containing 9 l of the same medium (Eaton & Ribbons, 1982*b*). After overnight incubation at 30 °C with stirring and forced aeration, cells were harvested as described by Eaton & Ribbons (1982*a*) and subsequently resuspended in 6 l 50 mM-KH₂PO₄/NaOH buffer, pH 6.8, in the Microferm fermenter. After the addition of 1.2 g 3-methylphthalic anhydride (Eastman Kodak) cells were incubated at 30 °C with stirring and forced aeration. Progress of the transformation was monitored spectrophotometrically, and when it appeared complete after 20 h, the cells were removed by centrifugation. The supernatant was concentrated to 200 ml at 50 °C in a rotary evaporator, acidified to pH 2 with HCl, and extracted three times with equal volumes of diethylether. After the ether extract was dried over Na₂SO₄, the ether was evaporated; the residue was dissolved in 3 ml ethanol/water (1:1, v/v) and applied to a column (41 × 2.5 cm) of Sephadex LH-20 (Pharmacia) and eluted with the same solvent. Fractions were assayed by diluting them in 50 mM-KH₂PO₄/NaOH buffer, pH 6.8, and recording their UV-visible spectra. Peak fractions were pooled and, after removal of the solvent at 50 °C under reduced pressure in a rotary evaporator, the compounds were identified.

Spectrometric identification of transformation products. UV and visible spectra were recorded with a Cary model 118C dual beam spectrophotometer. Proton magnetic resonance spectra were obtained by R. Block of the Papanicolaou Cancer Research Institute (Miami, Florida, USA) with a Varian HA 100-D15 spectrometer at 100 MHz. Samples were dissolved in deuterated dimethylsulphoxide with tetramethylsilane as internal standard. Mass spectra of 3-methylphthalate transformation products B and C were obtained by K. Pfaffenberger of the Department of Epidemiology and Public Health, University of Miami (Florida, USA) with a Finnigan model 4000 GC/MS operating in the electron impact mode at 70 eV. The mass spectrum of 3-methylphthalate transformation product A was obtained by R. Bottcher of the Department of Chemistry, Florida State University (Tallahassee, Florida, USA) with an AEI MS 902 spectrometer operating in the electron impact mode.

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RESULTS

Washed cells of *Micrococcus* sp. strain 12B grown on phthalate transformed 3-methylphthalate (added as 3-methylphthalic anhydride) to products having an increased UV absorbance and a shift in the peak absorbance (Fig. 1). Three products of this transformation were separated by chromatography on Sephadex LH-20 (Fig. 2) and identified.

Product A. (75 mg.) This was identified as 2-pyrone-3-methyl-4,6-dicarboxylic acid (Fig. 3, compound VII), the lactone of 4-carboxy-2-hydroxy-5-methylmuconic acid (Fig. 3, compound VI). The compound melted at 236–238 °C, and did not give a colour with the phenol reagents FeCl_3 and 2,6-dibromo-*N*-chloro-*p*-benzoquinoneimine or with the Evans (1947) catechol reagent.

The proton magnetic resonance spectrum of product A showed three methyl protons and a vinylic proton: δ_{H} p.p.m. 2.49 (3H, s), 7.49 (1H, s). The mass spectrum showed a parent ion (M^+) at m/e 198 with major fragmentation products at m/e 180 ($M^+ - \text{H}_2\text{O}$), 153 ($M^+ - \text{COOH}$) and 125 ($M^+ - \text{COOH} - \text{CO}$). Similar fragmentation products ($M^+ - \text{COOH}$) and ($M^+ - \text{COOH} - \text{CO}$) are present in the mass spectra of the related lactones 2-pyrone-6-carboxylic acid (Eaton & Ribbons, 1982*b*) and 2-pyrone-4,6-dicarboxylic acid (Eaton & Ribbons, 1982*b*; Maruyama, 1979). The UV spectra of product A have, at pH 2, a maximum at 306 nm (ϵ 8320) (ϵ is the molar absorption coefficient, units $\text{l mol}^{-1} \text{cm}^{-1}$) with a shoulder at 229 nm (ϵ 6030); at pH 7, a maximum at 304 nm (ϵ 8510) with a shoulder at 218 nm (ϵ 8510); and at pH 12, a maximum at 303 nm (ϵ 8320).

Product B. (120 mg.) This was identified as 3,4-dihydroxy-6-methyl-phthalic acid (Fig. 3, compound III). It gave an olive green colour with FeCl_3 , a purple colour with 2,6-dibromo-*N*-chloro-*p*-benzoquinoneimine, and a positive Evans (1947) test for catechols. In aqueous solution the compound was a fluorescent green under UV light as would be expected for an aromatic compound having a hydroxyl group *ortho* to a carboxyl group. The compound melted at 221–224 °C. The proton magnetic resonance spectrum showed δ_{H} 2.64 (3H, s) and 7.26 (1H, s) consistent with a methyl group and an aromatic proton. The mass spectrum showed no parent ion at m/e 212 but there were major mass peaks at m/e 194 ($M^+ - \text{H}_2\text{O}$), 150 ($M^+ - \text{CO}_2 - \text{H}_2\text{O}$) and 122 ($M^+ - \text{CO}_2 - \text{CO} - \text{H}_2\text{O}$). The UV-visible spectra of product B had at pH 2, a maximum at 252 nm (ϵ 5940); at pH 7, maxima at 399 nm (ϵ 7940), 306 nm (ϵ 2190), and 226 nm (ϵ 12300); and at pH 12, maxima at 429 nm (ϵ 10000) and 289 nm (ϵ 6920) with a shoulder at 330 nm (ϵ 2340).

Product C. (23 mg.) This was identified as 5-hydroxy-3-methyl-phthalic acid (Fig. 3, compound VIII). It gave a rose colour with 2,6-dibromo-*N*-chloro-*p*-benzoquinoneimine and a negative Evans test. The proton magnetic resonance spectrum in deuterated dimethylsulphoxide was consistent with three benzylic protons and two non-adjacent aromatic protons: δ_{H} 2.62 (3H, s), 6.89 (1H, s), 7.62 (1H, s). The mass spectrum did not show a parent ion at m/e 196 but there were major mass peaks at m/e 178 ($M^+ - \text{H}_2\text{O}$), 134 ($M^+ - \text{H}_2 - \text{CO}_2$) and 106 ($M^+ - \text{H}_2\text{O} - \text{CO}_2 - \text{CO}$).

DISCUSSION

Some of the early enzymes of phthalate catabolism in *Micrococcus* sp. strain 12B are relatively non-specific and they catalyse the transformation of several ring-substituted analogs (Eaton & Ribbons, 1982*a*; Ribbons *et al.*, 1984) and ester derivatives (Eaton & Ribbons, 1982*c*). Thus, phthalaldehyde is converted to 3,4-dihydroxyphthalaldehyde and dimethylphthalate gives 3,4-dihydroxyphthalate-2-methyl ester after incubation with induced cells. Both products lack a carboxyl group at the C-2 position and are not substrates for the next enzyme of the pathway, 3,4-dihydroxyphthalate-2-decarboxylase. 3-Methylphthalate is a substrate analogue without this restriction and can be metabolized further. In the present study, this was documented by isolation of an oxidation derivative of the 4,5-ring cleavage product.

Fig. 1

Fig. 2

Fig. 1. Transformation of 3-methylphthalate by *Micrococcus* sp. strain 12B. Washed suspensions of cells grown on phthalate were incubated with 3-methylphthalate as described in Methods. Samples were taken at: 0 h (a), 1.25 h (b), 3.5 h (c), 6.75 h (d), 8.75 h (e), and 17.5 h (f). Cells were removed by centrifugation and the UV spectra of the supernatants were recorded after 6-fold dilution in 50 mM- $\text{KH}_2\text{PO}_4/\text{NaOH}$ buffer, pH 6.8.

Fig. 2. Separation of the products of the transformation of 3-methylphthalate by chromatography on Sephadex LH-20. Peak A had an absorbance maximum at 304 nm ($\circ$), peak B had an absorbance maximum at 314 nm ($\square$), and peak C had absorbance maxima at 244 nm (∇) and 288 nm ($\triangle$). The solvent was ethanol/water (1:1, v/v); the arrow indicates one column volume eluted.

Fig. 3. Proposed pathway for the transformation of 3-methylphthalate by *Micrococcus* sp. strain 12B grown with phthalate. I, 3-Methylphthalate; II, 3,5-cyclohexadiene-3,4-dicarboxylate-5-methyl-1,2-diol; III, 3,4-dihydroxy-6-methylphthalate; IV, 6-methylprotocatechuate; V, 4-carboxy-2-hydroxy-5-methylmuconic semialdehyde; VI, 3-carboxy-5-hydroxy-2-methylxalocrotonate; VII, 4,6-dicarboxy-3-methyl-2-pyrone.

The assignment of structures VII, III and VIII (Fig. 3) to the 3-methylphthalate transformation products A, B, and C, respectively, and the proposed reaction pathways for their formation were based on UV, mass and NMR spectra, on various colour reactions, and on the pathway shown to be used for phthalate catabolism in *Micrococcus* sp. strain 12B (Eaton & Ribbons, 1982*a, b, c*). Spectra obtained for product B indicated that it was 3,4-dihydroxy-6-methylphthalate or 4,5-dihydroxy-6-methylphthalate. Structure III was assigned to product B since this compound was fluorescent, like 3,4-dihydroxyphthalate, the analogous metabolite from phthalate in *Micrococcus* sp. strain 12B; 4,5-dihydroxyphthalate, the *Pseudomonas* sp. intermediate in phthalate metabolism is not fluorescent.

The spectra obtained from product A were consistent with it being structure VII (Fig. 3). Compound VII could be formed during isolation by the spontaneous lactonization of 3-carboxy-5-hydroxy-2-methylmuconate (Fig. 3, VI) or, alternatively, as the product of the enzyme-catalysed dehydrogenation of compound V or its hemiacetal as shown for the protocatechuate 4,5-dioxygenase pathway in *Pseudomonas testosteroni* (Kersten *et al.*, 1982). The former is consistent with the pathway proposed for phthalate metabolism in *Micrococcus* strain 12B (Eaton & Ribbons, 1982*b*) in which compound VI would be the expected intermediate of 3-methylphthalate metabolism while the latter possibility is suggested by the work of Maruyama (1979, 1983) who showed that in *Pseudomonas ochraceae* 2-pyrone-4,6-dicarboxylic acid, which is analogous to compound VII, is the product of an enzyme-catalysed dehydrogenation of the ring cleavage product, 4-carboxy-2-hydroxy-muconic semialdehyde, and an intermediate in phthalate metabolism in that organism.

Product C was identified as 4-hydroxy-6-methylphthalate (Fig. 3, compound VIII). By analogy with the transformations of phthalaldehyde and dimethylphthalate by dioxygenative hydroxylation (Eaton & Ribbons, 1982*a, c*) in this strain, it is proposed that compound VIII arose as a non-enzymic dehydration product of the 3,4-dihydro-3,4-diol (Fig. 3, compound II) during acidification and extraction.

The overall yield of these products represents only 15% of the 3-methylphthalate provided to the culture. Other non-UV-absorbing intermediates may account for some of the balance. There was no attempt to optimize or quantify the accumulation and isolation of the three compounds produced by the wild-type *Micrococcus* sp. strain 12B.

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