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(+)-γ-Carboxymethyl-γ-methyl-Δ^{α}-butenolide

A 1,2-RING-FISSION PRODUCT OF 4-METHYLCATECHOL BY *PSEUDOMONAS DESMOLYTICUM*

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1. (+)-γ-Carboxymethyl-γ-methyl-Δ^{α}-butenolide was isolated from resting-cell cultures of *Pseudomonas desmolyticum* incubated in the presence of 5-methylsalicylic acid. 2. The structure of this metabolite was deduced from physical and chemical evidence. 3. The isolated compound must be formed in an enzymic reaction since it shows optical activity. 4. The degradative pathway of 4-methylcatechol by *Ps. desmolyticum* is discussed.

Pseudomonas desmolyticum oxidizes naphthalene to 3-oxoadipic acid through the intermediary formation of salicylic acid and catechol (Treccani, Walker & Wiltshire, 1954), whereas 1- and 2-methylnaphthalenes are metabolized to 2-hydroxy-6-oxohepta-2,4-dienoic acid and 5-formyl-2-hydroxyhexa-2,4-dienoic acid (compound I) respectively, through the corresponding methylsalicylic acids and methylcatechols (Canonica, Fiecchi & Treccani, 1957; Treccani & Baggi, 1962; Treccani, Fiecchi, Baggi & Galli, 1965; Canonica, Fiecchi, Galli Kienle, Scala & Treccani, 1966; Catelani, Fiecchi & Galli, 1968).

In the experiments on the isolation of compound (I), performed with resting cells of *Ps. desmolyticum* incubated with 5-methylsalicylic acid, it was observed that compound (I) accumulated in smaller amounts than expected with respect to the supplied substrate. In addition, the aqueous solution remaining after the ethereal extraction of compound (I) showed a strongly positive lactone test.

The present paper is concerned with the isolation and the identification of the compound responsible for the lactone test. The degradative pathway of 4-methylcatechol in *Ps. desmolyticum* is discussed on the basis of these results and those of our previous work on the induction of diphenol oxygenases (Treccani, Galli, Catelani & Sorlini, 1968).

MATERIALS AND METHODS

Maintenance and growth of organism. A *Pseudomonas* strain isolated by Treccani *et al.* (1954) from elective cultures on naphthalene, similar to the *Ps. desmolyticum* of Gray & Thornton (1928), was used. The conditions of maintenance and growth were as described by Treccani & Fiecchi (1958).

Chemical syntheses. 5-Methylsalicylic acid was prepared as described by Treccani & Fiecchi (1958). (±)-γ-Carboxymethyl-β-methyl-Δ^{α}-butenolide (compound III) was synthesized from *o*-nitro-*p*-cresol, as described by Pauly, Gilmour & Will (1914) and Pauly & Will (1918).

Biological synthesis. 5-Formyl-2-hydroxyhexa-2,4-dienoic acid (compound I) was prepared by oxidation of 5-methylsalicylic acid with resting cells of *Ps. desmolyticum* (Treccani *et al.* 1965).

Chemical determinations. Mono- and di-lactones were detected as described by Cain (1961). The u.v.-spectrophotometric determinations were carried out in a Zeiss PMQ II spectrophotometer. The n.m.r. spectra were recorded on a Perkin–Elmer R10 spectrograph (with tetramethylsilane as internal reference and [D_6]acetone as solvent). An Infracord model 137 spectrophotometer (Perkin–Elmer) was used to obtain i.r. absorption spectra (in Nujol). Mass spectra were recorded on a LKB 9000 spectrometer.

Chromatography. T.l.c. of compound (II) was carried out on Kieselgel G (E. Merck A.-G., Darmstadt, Germany). Glass plates were coated to a thickness of 0.2mm and activated at 100°C for 30min. The solvent system was diethyl ether–benzene (1:1, v/v) and spots were revealed by spraying with 0.5% (w/v) $KMnO_4$.

Melting points. All melting points are uncorrected.

RESULTS

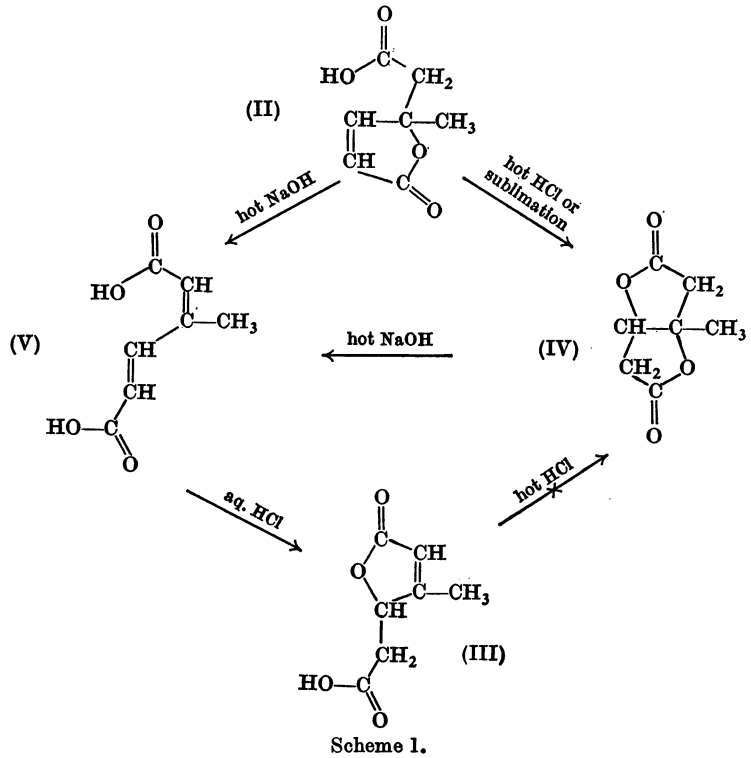
The extraction of compound (I), obtained by oxidation of 5-methylsalicylic acid with a resting-cell suspension of *Ps. desmolyticum*, was performed as reported by Treccani *et al.* (1965). The residual aqueous solution was continuously extracted with diethyl ether for 48h. The organic layer was dried over anhydrous magnesium sulphate and evaporated to dryness. The slightly coloured oily residue was dissolved in a minimum amount of water and the solution was treated with decolorizing charcoal and freeze-dried. The residual oil was shown to be a single compound by t.l.c.; it was acidic and gave

a positive test for lactones, but negative for dilactones. This compound (II) (Found: C, 52.9; H, 5.1; $C_7H_8O_4$ requires: C, 53.8; H, 5.2%) had $[\alpha]_D^{20}$ $+32\pm2°$ (*c* 10mg/ml in water), $\lambda_{max.}$ in ethanol 212nm (ϵ 12000). The i.r. spectrum showed strong bands at 3500 and 1700cm^{-1} (carboxyl) and 1750 and 1655cm^{-1} ($\alpha\beta$-unsaturated γ-lactone). The n.m.r. spectrum of compound (II) included the following signals: two doublets (1H each) whose weighted-mean position fell at 7.92 and 3.15 δ, with coupling constant J_{AB} 5.4Hz. The first signal is due to the vinyl proton β to an $\alpha\beta$-unsaturated lactone group (Bhacca & Williams, 1964), whereas the second one belongs to the vinyl α-proton. A broad signal (1H) was found at 6.6δ due to a carboxylic proton. At 2.94 and 2.82δ there were the weighted-mean positions of two doublets (1H each) attributable to an AB system, with a geminal coupling constant J_{AB} 16.2Hz. The signal belongs to a methylene group adjacent to a carboxyl group and bound to an asymmetric centre. A singlet (3H) was centred at 1.57δ, due to an angular methyl group bound to an oxygen-bearing carbon atom.

Compound (II), treated with an ethereal solution of diazomethane, gave the corresponding methyl ester, b.p. 86°C/1mmHg (Found: C, 56.0; H, 6.0; $C_8H_{10}O_4$ requires C, 56.4; H, 5.8%). Its mass spectrum showed the molecular ion at *m/e* 170. The n.m.r. spectrum was similar to the spectrum of compound (II), except for the absence of the broad singlet at 6.6 δ ($-CO_2H$) and the presence of the singlet centred at 3.70δ due to the 3 protons of the ester ($-CO_2CH_3$).

The results for compound (II) are consistent with the structure of (+)-γ-carboxymethyl-γ-methyl-Δ^{α}-butenolide. In addition, this compound must be different from (±)-γ-carboxymethyl-β-methyl-Δ^{α}-butenolide (compound III) (m.p. 130°C). The u.v. spectrum of compound (III) in ethanol showed $\lambda_{max.}$ 216nm (ϵ 11.000). In the i.r. spectrum there were strong bands at 3470 and 1690 (carboxyl), 1730 and 1640cm^{-1} ($\alpha\beta$-unsaturated γ-lactone); the absorption in the 1400–800cm^{-1} region was different from that of compound (II). The n.m.r. spectrum exhibited the following signals: a broad singlet (1H) was centred at 10.3δ, due to the acidic proton of the carboxyl group. At 5.86δ there was a singlet (1H) for a vinyl proton; this signal showed an allylic coupling (J_{all} 1Hz) with the protons of the methyl group absorbing at 2.18δ. A system was centred at 5.3δ (1H), made up of four lines of approximately equal intensities: it represents the X part of an ABX system (J_{AX} 5.1Hz; J_{BX} 7.4Hz). This proton must be bound to an oxygen-bearing carbon atom. The AB portion was split into eight lines (2H), forming two superimposed quartets, having theoretical intensities of 1:3:3:1. The weighted-mean position of the quartet due to the H_A was at 3.03δ (J_{AB} 16.2Hz and J_{AX} 5.1Hz), whereas the second quartet was centred at 2.55δ (J_{AB} 16.2Hz and J_{BX} 7.4Hz). The AB system was attributed to a methylene group. The singlet at 2.18δ (3H) is due to a methyl group on a double bond, and is affected by allylic coupling (J_{all} 1Hz) with the proton absorbing at 5.86δ.

Chemical evidence for distinct structures for compounds (II) and (III) came from their different behaviour towards dilactonization. When compound (II) was eluted with diethyl ether from an alumina column a crystalline compound (IV) resulted (m.p. 108°C, prisms from diethyl ether). This compound had $[\alpha]_D^{20}-131\pm2°$ (*c* 10mg/ml in water) (Found C, 53.9; H 5.3; $C_7H_8O_4$ requires C, 53.8; H, 5.2%). Compound (IV) gave a positive test for dilactones and had an equivalent weight of 156 with M-sodium hydroxide at 20°C (calc. 156.1) and 78 (calc. 78.05) with M-sodium hydroxide at 80°C. The same compound was also obtained by the following procedures. (*a*) Distillation of crude compound (II), b.p. 130°C/1mmHg; (*b*) boiling compound (II) with concentrated hydrochloric acid followed by ether extraction; (*c*) leaving the crude compound (II) for several weeks. Compound (III) was unaffected by each of these treatments. Proof of the dilactone structure for compound (IV) came from the following physicochemical features. The i.r. spectrum showed neither bands of acidic hydroxyl groups nor those of double bonds. There was a very intense band at 1780cm^{-1}, due to the two saturated γ-lactone groups. The mass spectrum showed the molecular ion at *m/e* 156. The n.m.r. spectrum of compound (IV) showed the following signals: A doublet, absorbing at 5.19δ (1H), due to a proton bound to an oxygen-bearing carbon atom; this signal represents the X part of an ABX system $CO-C(H_A)(H_B)-C(H_X)-O-$ (J_{AX} 5.1Hz and J_{BX} 0.0Hz). The AB portion consisted of six lines: at 3.28δ a quartet was centred due to H_A. This portion was coupled with H_B (J_{AB} 19.2Hz) and H_X (J_{AX} 5.1Hz). The chemical shift of H_B was 2.82δ. The signal was split into a doublet because of the interaction with H_A (J_{AB} 19.2Hz). No interaction occurred between H_B and H_X. From the Karplus function (Bhacca & Williams, 1964) it may be accepted that the dihedral angle between H_B and H_X is 80°, whereas the coupling constant between H_A and H_B accounts for a geminal coupling with a dihedral angle of about 107°. At 3.0δ (2H) a singlet is due to the hydrogen atoms of a methylene group adjacent to a carbonyl group. It must be an AB system having $\delta_{AB} \ll J_{AB}$ so that the two central lines are superimposed and the satellites have very low intensities. A singlet at 1.7δ (3H) is attributable to a methyl group on an oxygen-bearing carbon atom. All these results are consistent with the



Scheme 1.

structure of (−)-1-methyl-3,7-dioxo-2,6-dioxabicyclo-(3,3,0)-octane (β-methylbutanolido-βγ-γ′β′-butanolide) (compound IV).

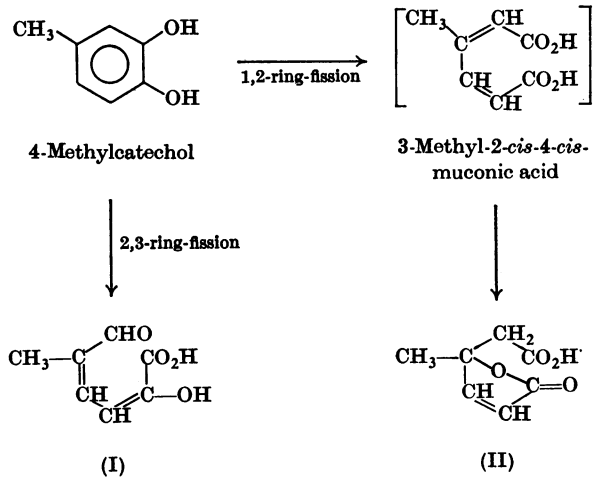
In separate experiments compounds (II), (III) and (IV) were treated for 1 h at 80°C with excess of 2 M-sodium hydroxide. The alkaline solution was made acid to Congo Red with M-sulphuric acid and repeatedly extracted with ether. In all cases 3-methyl-2-*cis*-4-*trans*-muconic acid (compound V) was obtained (m.p. and mixed m.p. 175°C, as reported by Elvidge, Linstead & Sims, 1951).

DISCUSSION

All the physicochemical properties of compound (II), isolated from the incubation media of resting cells of *Ps. desmolyticum* incubated with 5-methylsalicylic acid, are in agreement with the proposed structure of (+)-γ-carboxymethyl-γ-methyl-Δ^{α}-butenolide. Its chemical reactivity is summarized in Scheme 1. Further, compound (II) must be an enzymically formed product of 4-methylcatechol degradation because of its optical activity. This compound can be formed, in principle, from either 3-methyl-2-*cis*-4-*cis*- or 3-methyl-2-*trans*-4-*cis*-muconic acid, since in compound (II) the less-substituted double bond is still present. 3-Methyl-2-*cis*-4-*trans*-muconic acid can be ruled out since its lactonization product is compound (III) (Elvidge *et al.* 1951). From these facts and by analogy with the metabolic pathway of catechol and protocatechuic acid degradation (Dagley, Evans & Ribbons, 1960), we can assume that the precursor of compound (II) is the *cis–cis*-isomer.

Nakagawa, Inoue & Takeda (1963) isolated a methylmuconic acid from the incubation medium of a highly purified pyrocatechase of *Brevibacterium fuscum* incubated with 4-methylcatechol. They assumed that the oxidation product had at least one double bond in the *cis*-configuration, since the extinction maximum decreased with time in M-hydrochloric acid; however, they found a melting point of 235°C, which is characteristic of 3-methyl-2-*trans*-4-*trans*-muconic acid (Elvidge *et al.* 1951).

We never found the dilactone (compound IV) in the ethereal extracts of the acidified medium; thus it can be considered an artifact, despite its optical activity, the second chiral centre arising under the influence of the first present in compound (II). The same conclusion was reached by Landa & Eliasek (1956), who isolated a product identical, but for optical activity, with the synthetic 3,7-dioxo-2,6-dioxabicyclo-(3,3,0)-octane (butanolido-βγ-γ′β′-butanolide) from *Oospora* cultures incubated in the presence of catechol.



4-Methylcatechol

3-Methyl-2-*cis*-4-*cis*-muconic acid

(I)

(II)

Scheme 2. Metabolic pathway of 4-methylcatechol degradation by *Pseudomonas desmolyticum*.

The possible metabolic pathway for 4-methylcatechol degradation is summarized in Scheme 2. These assumptions are also supported by the evidence presented by Treccani *et al.* (1968) that the growth of *Ps. desmolyticum* on different aromatic compounds induces the synthesis of both pyrocatechase and metapyrocatechase. These oxygenases, separated by ammonium sulphate fractionation at 40% and 70% saturation respectively, seem to be specific for the different catechols; in fact, the partially purified metapyrocatechase oxidizes catechol to muconic semialdehyde in poor yield (0.03 mol/mol of catechol), whereas 4-methylcatechol is quantitatively converted into compound (I) under the same conditions. The fraction separated at 40% saturation with ammonium sulphate oxidizes catechol with an oxygen uptake of 1 mol/mol of substrate, leading to the production of 3-oxoadipic acid, whereas 4-methylcatechol is metabolized with the same oxygen uptake, but in this case there is no evidence either of keto acid (Galli, 1968) or of semialdehyde formation.

From the present findings it can be assumed that resting cells of *Ps. desmolyticum* can perform both *ortho-* and *meta-* cleavage of 4-methylcatechol. There is no evidence at present that the lactonic acid (compound II) may be further metabolized by *Ps. desmolyticum*.

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