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Metabolism of Biphenyl

STRUCTURE AND PHYSICOCHEMICAL PROPERTIES OF 2-HYDROXY-6-OXO-6-PHENYLHEXA-2,4-DIENOIC ACID, THE *META*-CLEAVAGE PRODUCT FROM 2,3-DIHYDROXYBIPHENYL BY *PSEUDOMONAS PUTIDA*

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The structure of 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid for the *meta*-cleavage product of 2,3-dihydroxybiphenyl by a *Pseudomonas putida* strain was demonstrated on the basis of its chemical and physicochemical properties and those of its derivatives.

Previous work (Catelani *et al.*, 1971; Catelani *et al.*, 1973) suggested that the metabolism of biphenyl by *Pseudomonas putida* proceeds to benzoic acid via 2,3-dihydro-2,3-dihydroxybiphenyl, 2,3-dihydroxybiphenyl and a *meta*-cleavage product whose structure was proposed as 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid. This hypothesis was advanced mainly from the evidence provided by analogy with other similar metabolic pathways (e.g. Kojima *et al.*, 1961; Dagley *et al.*, 1965; Catelani *et al.*, 1968; Baggi *et al.*, 1972) and by mass-spectrometric features of the isolated compound.

In this work the structure of 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid for the *meta*-cleavage product of 2,3-dihydroxybiphenyl by *Ps. putida* has been unequivocally demonstrated on the basis of other chemical and physicochemical properties. These results are also very useful as material for spectroscopic correlations.

Materials and Methods

Biological synthesis

2-Hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid was prepared as described by Catelani *et al.* (1973) from 2,3-dihydroxybiphenyl; washed suspensions of the *Ps. putida* strain isolated by Catelani *et al.* (1970) were used.

Spectral analyses

Mass spectra were recorded on an LKB model 9000 spectrometer at 70eV. U.v. spectra were recorded in a Zeiss PMQII spectrophotometer; an Infracord model 137 (Perkin–Elmer Ltd., Beaconsfield, Bucks., U.K.) was used to obtain i.r. spectra (in Nujol). N.m.r. spectra were obtained with an HA model 100D (Varian Ass., Palo Alto, California, U.S.A.), with tetramethylsilane as internal reference and fully-labelled [U-^{2}H]acetone as solvent.

Chemical determinations

The potentiometric titrations were performed using a model 333 digital pH/mV meter (A.M.El. Ltd., Milan, Italy). Enols were detected by the $FeCl_3$ test. The presence of a free aldehyde group was assayed by the test of Dickinson & Jacobsen (1970).

Chemical syntheses

2,3-Dihydroxybiphenyl was prepared as described by Catelani *et al.* (1973). 4-Benzoylbutyric acid was synthesized as reported by Sommerville & Allen (1933). Both synthesized compounds were checked for identity and purity by elemental analyses and the g.l.c.-mass spectrometry technique; their melting point values were in complete agreement with literature values.

All melting points are uncorrected.

Preparation of derivatives

Picolinate derivatives were prepared as described by Canonica *et al.* (1966); methyl esters were prepared with diazomethane in diethyl ether. Hydrogenation experiments were performed in the standard apparatus, as described by Canonica *et al.* (1966).

Chromatography

T.l.c. was done on silica-gel plates (Baker-flex IB-F no. 5002; J. T. Baker Chemical Co., Deventer, Holland), activated by heating at 110°C for 30min. The developing solvent system was acetic acid–hexane–chloroform (1:8:2, by vol.); spots were revealed by a u.v. lamp (at 254nm).

G.l.c. of phenolic substances was performed by using a C.Erba model G.T. 200 gas chromatograph with a flame-ionization detector. A stainless-steel column (2m×2mm internal diam.) packed with 1%

SE30 on silanized Chromosorb G (60–80 mesh) was used at the following temperatures: column 190°C, injector 230°C, detector 220°C. The carrier gas was N_2 at 35ml/min. The flame was fed with H_2 at 70.7kPa and air at 120kPa. For g.l.c. of methyl esters a stainless-steel column of the same dimensions was used, packed with 1% ethylene glycol succinate (C.Erba LAC886) on silanized Chromosorb G (60–80 mesh), at the following temperatures: column 150°C, injector 210°C, detector 200°C. The N_2, H_2 and air were supplied as described above. When the gas chromatograph was combined with the LKB model 9000 mass spectrometer, He was used as carrier gas; columns and temperatures were the same as reported above.

Results

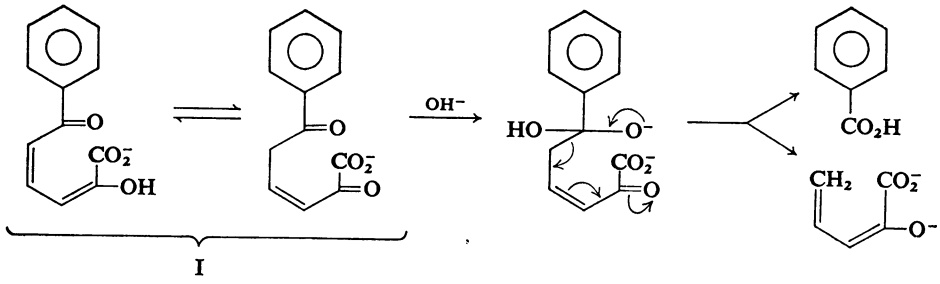
Properties of the meta-cleavage product from 2,3-dihydroxybiphenyl

The substance, isolated from washed suspensions of *Pseudomonas putida* incubated with 2,3-dihydroxybiphenyl, appeared as a light brown microcrystalline powder. After repeated crystallizations from diethyl ether–light petroleum (b.p. 40–60°C) (1:1, v/v) it showed m.p. 112°C (decomp.). Elemental analysis was as follows. Found: C, 65.8; H, 4.5; $C_{12}H_{10}O_4$ requires C, 66.0; H, 4.6%. The mass spectrum gave the molecular weight (218), also confirmed by potentiometric titration. From the mass spectrum the presence in the molecule of a C_6H_5CO group (parent peak at *m/e* 105) could be deduced. Still unexplained was the presence of an intense peak at *m/e* 202, which increased its intensity when the mass spectrum was run at 15eV. A carboxyl group was also present (peaks at $201 = M^+-17$; $173 = M^+-45$); a peak at 157 showed that the compound with molecular weight 202 also had a carboxyl group.

In the titration curve two end points could be observed: pK_1 and pK_2 values of the diprotic acid were 3.55 and 7.70 respectively, accounting for the presence of a carboxyl and an enolic hydroxyl group. The presence of a keto–enol tautomerism was supported both by a positive $FeCl_3$ test and by the characteristic u.v. spectrum (at pH12, $\lambda_{max.}$ 435nm, log ε 4.5 and $\lambda_{max.}$ 257nm, log ε 3.7; at pH3, $\lambda_{max.}$ 336nm, log ε 4.3 and $\lambda_{max.}$ 263nm, log ε 3.9).

In the i.r. spectrum (in Nujol) the following bands appeared: 3380 (very intense, for a strongly associated hydroxyl group), 3200–2600 (broad, for a carboxylic hydroxyl group), 1715 (very intense, for an α,β-unsaturated oxo group), 1700 (very intense, for an α,β-unsaturated carboxyl group), 1648 and 1599 (two intense bands, for a diene group conjugated to carboxyl and oxo groups), 1585 and 1565 (two intense bands, for a phenyl group), 1260 (very intense, probably for a C–O–H bending), 1220–1160 (a multiplet for the different C=O stretchings), 1100–1023 (another multiplet for the different C–OH stretchings), 983 (for a double bond in the *trans* configuration), 895, 871, 780 (double bond), 710–680cm^{-1} (monosubstituted benzene ring with an electron-attractive substituent). This spectrum appeared very similar to that of α-hydroxymuconic semialdehyde and its homologues (Kojima *et al.*, 1961; Treccani *et al.*, 1965; Catelani *et al.*, 1968; Baggi *et al.*, 1972).

The n.m.r. spectrum (in fully-labelled [U-^{2}H]-acetone) showed the following signals: a broad singlet (2H) was centred at about 6.7δ, owing to the acidic protons of the enolic and carboxylic groups. The chemical shift was greatly dependent on concentration, and the signal disappeared on addition of ^2H_2O. At 6.52δ a doublet was centred (1H), owing to the proton on C-3; the signal was resolved into the double doublet ($J_{3,5} = 0.5$Hz; $J_{3,4} = 12$Hz) because this proton was coupled to the allylic proton on C-5 and the vicinal proton on C-4 (transoid configuration). The proton on C-5 absorbed at 7.34δ as another double doublet, showing an allylic coupling ($J_{3,5} = 0.5$Hz) to the proton on C-3, and *trans*-olefinic coupling ($J_{4,5} = 15.5$Hz) to the proton on C-4. A system of three lines was centred at 7.91δ (1H). A fourth line must presumably be hidden by the multiplet in the range 7.91–8.10δ. This completed a quartet of signals of approximately the same intensities; this quartet must be due to the proton on C-4, showing a *trans*-olefinic coupling ($J_{4,5} = 15.5$Hz) to the proton on C-5 and the transoid coupling to the proton on C-3 ($J_{3,4} = 12$Hz). The two multiplets that appeared in the range 7.50–7.70δ (3H) and 7.95–8.10δ (2H) were attributable to the protons of the aromatic ring; their shapes were identical to those appearing in the spectra of other monosubstituted benzenes carrying electron-attractive substituents. Double-irradiation experiments confirmed all these hypotheses; irradiation at 6.52δ induced partial decoupling of the quartet at 7.91δ, which became a doublet with one band falling under the multiplet at 7.95–8.10δ. Irradiation at 7.34δ led to similar results. Irradiation in the range 7.50–7.70 caused loss of multiplicity in the band at 7.95–8.10δ, which became a sharp singlet. As in the case of 2-hydroxy-6-oxo-2,*trans*-4,*trans*-heptadienoic acid (Catelani *et al.*, 1968) the *trans* configuration of the 4,5-double bond must be due to the acidic treatment during the extraction of the *cis* isomer from the medium. However, in this case the signals for protons on C-3 and C-5 did not disappear upon addition of ^2H_2O to the acetone solution: this implied that the enol form of this compound was very stable in these conditions. No signal appeared attributable to an aldehydic proton; on the other hand, the test for aldehydes (see the Materials and Methods section) was negative.



Scheme 1. *Proposed mechanism of benzoic acid formation from 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid in alkaline medium (pH 12)*

I, 2-Hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid.

Picolinate derivative

The *meta*-cleavage compound gave a picolinate derivative as shown by a characteristic u.v. spectrum of the mixture after reaction ($\lambda_{max.}$ 282 and 249nm).

Hydrogenation products

The *meta*-cleavage compound was hydrogenated in 0.01M-NaOH solution with Pd [5% (w/w) on $CaCO_3$] as catalyst. When the yellow colour disappeared (H_2 uptake, 2mol/mol) the reaction mixture was filtered, acidified to pH2 and repeatedly extracted with 30ml portions of diethyl ether. The ethereal extracts were combined, dried over anhydrous $MgSO_4$ and evaporated under reduced pressure in the cold. The crude residue was dissolved in boiling pentane and filtered. The solution was concentrated and cooled to −20°C. A white microcrystalline powder was obtained, uncorr. m.p. 132°C (Found: C, 64.5; H, 6.1; $C_{12}H_{14}O_4$ requires C, 64.8; H, 6.3%). The i.r. spectrum (in Nujol) showed bands at 3380 (very intense, for a strongly associated hydroxyl group), 3200–2500 (broad, for a carboxyl group), 1725 (saturated carboxyl group), 1685 (aromatic oxo group), 1600 and 1580 (two bands for the phenyl group), 1410 (α-oxo methylene), 1360, 1330 and 1310 (a multiplet due to C–O–H bendings), 1250 and 1215 (C=O stretchings), 695cm^{-1} (for monosubstituted benzene ring with an electron-attractive substituent).

The u.v. spectrum of the tetrahydro derivative showed $\lambda_{max.}$ 240 (log ε approx. 3.5), showing the loss of conjugation. In the mass spectrum the molecular-ion peak ($M^+ = 222$) was absent; the peak with highest *m/e* value was present at 204, corresponding to M^+-H_2O; other representative peaks appeared at 160 (M^+-H_2CO_3), 120 ($C_6H_5COCH_3^+$), 105 ($C_6H_5CO^+$) and 77 ($C_6H_5^+$). Methylation of the tetrahydro derivative with CH_2N_2 resulted in a compound whose mass spectrum showed no molecular-ion peak ($M^+ = 236$); the peak with the highest *m/e* value was present at 218 (M^+-H_2O) and other representative peaks appeared at 177 (M^+-$COOCH_3$), 120 ($C_6H_5COCH_3^+$), 105 ($C_6H_5CO^+$) and 77 ($C_6H_5^+$).

From the same hydrogenation reaction benzoic acid was also isolated. Its formation was due to the alkaline medium as was demonstrated by separate experiments. The mechanism of the formation of benzoic acid in alkaline solution may be that shown in Scheme 1; this mechanism is similar to that of a retro-Claisen reaction. Other hydrogenation experiments, carried out at pH7 (in 0.05M-phosphate buffer), did not lead to the formation of benzoic acid. The main constituent in the reaction mixture was a compound whose mass spectrum was identical with that of authentic 4-benzoylbutyric acid. Its formation is yet unexplained. However, it is clear that pH values, by influencing the distribution of the different tautometric forms of the reacting compound, greatly affect both the qualitative and the quantitative composition of the hydrogenation mixture.

Discussion

All the physicochemical properties of the *meta*-cleavage product of 2,3-dihydroxybiphenyl metabolism by *Pseudomonas putida*, as well as the physicochemical properties of its derivatives, are consistent with the proposed structure of 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid. Correlation with u.v.-spectroscopic properties of 2-hydroxymuconic semialdehyde and its homologues points out the presence in the molecule of a chromophore with a high degree of conjugation. The pK values are consistent with the presence of an enol group and a carboxyl group. Mass spectra clearly indicate the presence in the molecule of a benzoyl group, and together with the n.m.r. spectrum exclude the presence of an aldehyde group. The position of the enolic hydroxyl group and the proof that the molecule has a straight chain can be obtained from the n.m.r. spectrum.

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