Chemistry of Extradiol Aromatic Ring Cleavage: Isolation of a Stable Dienol Ring Fission Intermediate and Stereochemistry of Its Enzymatic Hydrolytic Cleavage

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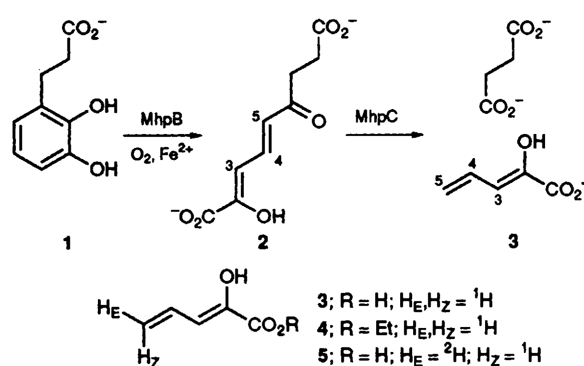
The *meta*-ring fission intermediate on the phenylpropionate catabolic pathway exists as a stable *trans*-dienol; its breakdown by hydrolase enzyme MhpC proceeds with overall replacement of the succinyl group by a proton from water with retention of stereochemistry.

Oxidative cleavage of catechol and other dihydroxyaromatics is a key step in the biodegradation by soil bacteria of naturally occurring aromatic molecules and many aromatic environmental pollutants.[1] Two types of oxidative cleavage are found: *ortho*-, or intradiol, where cleavage is between the two hydroxy groups; and *meta*-, or extradiol, where cleavage is adjacent to the two hydroxy groups. Whilst it has been known for many years that intradiol cleavage gives rise to *cis*-,*cis*-muconic acids,[2] the chemistry of the dienol extradiol cleavage products has not until recently been well characterised, due to their inaccessability and instability. Thus, they have been written variously in the literature with either *cis*- or *trans*-carbon–carbon double bonds, and in either enol or keto forms. Recently the chemistry of 2-hydroxymuconic acids and their intermediacy in the catechol and phenylacetate catabolic pathways has been explored,[3] and a further dienol intermediate on the phenylacetate pathway characterised.[4] Here we report the enzymatic synthesis, isolation and characterisation of the extradiol cleavage product of 2,3-dihydroxyphenylpropionic acid **1**, and the stereochemistry of its enzymatic hydrolysis.

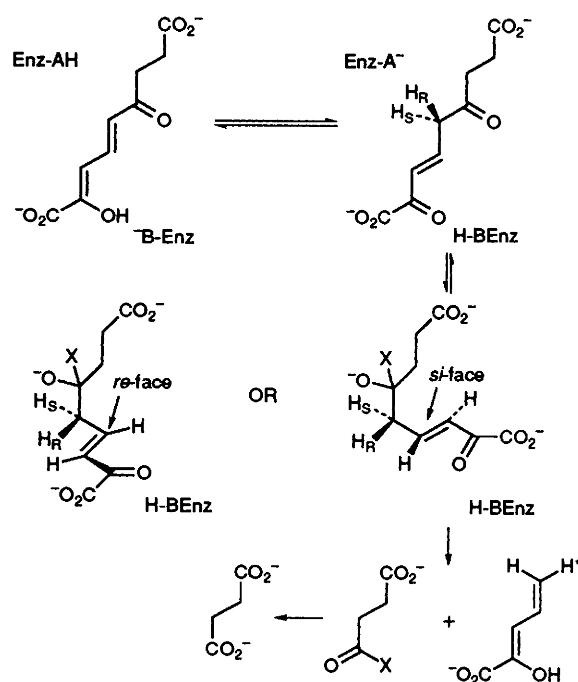
Extradiol cleavage of **1**, an intermediate on the β-phenylpropionate catabolic pathway of *Escherichia coli*,[5] is carried out by 2,3-dihydroxyphenylpropionate 1,2-dioxygenase (MhpB), a non-haem iron(II)-dependent enzyme which has been overproduced and purified to near homogeneity.[6] The ring fission product **2** of the MhpB-catalysed reaction, which at pH 8 or above exists as a bright yellow dienolate anion, had previously been characterised only by UV spectroscopy.[5] **2** is then a substrate for an unusual hydrolytic fragmentation reaction catalysed by 2-hydroxy-6-ketonona-2,4-diene 1,9-dioic acid 5,6-hydrolase (MhpC) yielding succinate and 2-ketopent-4-enoic acid **3**. In order to investigate the chemistry carried out by MhpB and MhpC, isolation and characterisation of **2** was required.

Large amounts of dioxygenase enzyme MhpB were required for effective conversion of **1**, since the holoenzyme is rapidly inactivated by loss of the iron(II) cofactor in dilute solution.[6] However, using 50 units of MhpB in 50 mmol dm^{-3} Tris buffer at pH 8.5, 100 mg of **1** was converted to 90 mg of ring fission product **2**, obtained as a yellow solid after purification by (DEAE) anion exchange chromatography (DEAE = diethylaminoethyl Sephadex). A 270 MHz ^1H NMR spectrum of **2** recorded in [$^2H_6$]acetone showed vinylic signals at δ 7.64 (dd) for 4-H, δ 6.36 (d) and 6.41 (d) for 3-H and 5-H of the dienol, with coupling constants of 11.5 and 15.8 Hz, indicating a *trans* 4,5-double bond, and an *s*-*trans* diene conformation,[7] as drawn in Scheme 1. The regiochemistry of the 2,3-double bond has yet to be determined. ^{13}C NMR and MS data were entirely consistent with the dienol structure shown.† No other isomers were detected, thus if dienol **2** is initially formed as the *cis*-isomer after aromatic ring cleavage, *cis*/*trans* isomerisation must be rapid. ^1H NMR analysis of **2** in ^2H_2O showed that 3-H and 5-H exchange completely with deuterium in 5 min at room temp., but no exchange of 4-H was observed. **2** was stable towards storage either as a solid or a solution in organic solvents.

Hydrolase enzyme MhpC, purified to 50% homogeneity from an overproducing strain of *E. coli*, was used to investigate the hydrolytic fragmentation of **2**. Large scale conversion of **2** by MhpC followed by acidification and extraction into ethyl acetate yielded the hydrolase products **3** and succinic acid, which were analysed by ^1H NMR spectroscopy.‡ Succinic acid was identified by a singlet at δ 2.6, and also using a coupled enzyme assay involving succinyl CoA synthetase, showing ATP- and CoA-dependent P_i production.[8] Analysis of **3** by ^1H NMR revealed that it too existed as a dienol, with vinylic signals at δ 5.19, 5.41, 6.22 and 6.78.§ Identification of **3** was confirmed by synthesis of its ethyl ester



Scheme 1 Proposed mechanism for hydrolase MhpC, showing two stereochemical possibilities for the fragmentation step. The 5_E-H proton of the product derived from enzyme-catalysed protonation is starred. X is either OH or an enzyme active site nucleophile.



Scheme 2

4 by reaction of allyl magnesium bromide with diethyl oxalate in diethyl ether–THF (1 : 1) at −78 °C in 74% yield.[9] The ^{1}H NMR spectrum of **4** was also characteristic of a dienol structure, with chemical shifts for the vinylic protons virtually identical to **3**.§

The clear separation of the 5_Z-H and 5_E-H protons of **3** in the ^{1}H NMR spectrum allowed the elucidation of the overall stereochemistry of the MhpC reaction, by examination of the products of the enzymatic reaction when run in ^2H_2O. Incubation of **2** with MhpC in buffer containing 90% ^2H_2O followed by analysis of the products by ^{1}H NMR spectroscopy revealed that the intensity of the signal for the 5_E-H proton was significantly reduced compared with the 5_Z-H signal, suggesting that the 5_E-H proton is derived from enzyme-catalysed protonation. In order to confirm this result, conversion of **2** was carried out in 100% deuteriated buffer using lyophilised MhpC, and the enzyme-catalysed reaction was monitored directly by 270 MHz ^{1}H NMR spectroscopy. A spectrum of the deuteriated product **5** recorded after 5 min. at room temp. showed signals characteristic of the 3-H, 4-H and 5_Z-H protons, but the 10 Hz doublet at δ 5.2 was completely absent, confirming that it is the 5_E-H proton that is derived from water. The 5_Z-H and 3-H protons were also reduced in intensity by 68 and 39% respectively, due to rapid deuterium exchange of these protons in **2** prior to reaction, as observed above. However, no further deuterium exchange was observed in **5** on further incubation in ^2H_2O for 1 h.

This observation implies that the overall stereochemistry of the MhpC reaction involves replacement of the succinyl moiety of **2** by a proton from water with retention of regiochemistry at C-5. A possible mechanism for the enzyme-catalysed reaction, shown in Scheme 1, involves ketonisation of **2**, followed by nucleophilic attack by either water or an active site nucleophile at C-6, followed by a stereospecific fragmentation. The observed stereochemical course is consistent with either protonation at H_R followed by fragmentation onto the *si*-face of the double bond, or protonation at H_S followed by fragmentation onto the *re*-face of the double bond.

This is the first stereochemical analysis of any member of the family of such hydrolase enzymes found on aromatic degradation pathways.[10] It remains to be determined if other members of this family follow a similar stereochemical course.

Received, 1st March 1994; Com. 4/01256I

Footnotes

† Found for **2**: δ_H (270 MHz, [$^2H_6$] acetone) 7.64 (1 H, dd, *J* 11, 16 Hz), 6.41 (1 H, d, *J* 16 Hz), 6.36 (1 H, d, *J* 11 Hz), 4.65 (1 H, br), 2.94 (2 H, t, *J* 7 Hz), 2.61 (2 H, t, *J* 7 Hz); δ_C (67.9 MHz, [$^2H_6$]acetone) 198.3, 174.0, 165.4, 147.3, 135.7, 130.8, 109.6, 35.6, 28.1

‡ Large scale MhpC conversions were carried out in 50 mmol dm^{-3} potassium phosphate buffer (pH 8.0) at room temp., typically using 10 mg **2** and 15 units MhpC (30 units used for 100% ^2H_2O experiment). Conversions were monitored by decrease in absorbance of **2** at 394 nm, and were usually complete after 5 min.

§ Found for **3**: δ_H (270 MHz, [$^2H_6$]-acetone) 6.78 (1 H, dt, *J* 10, 10, 17 Hz), 6.22 (1 H, d, *J* 10 Hz), 5.41 (1 H, dd, *J* 2, 17 Hz), 5.19 (1 H, dd, *J* 2, 10 Hz). Found for **4**: δ_H (270 MHz, $CDCl_3$) 6.77 (1 H, dt, *J* 10, 10, 17 Hz), 6.20 (1 H, d, *J* 10 Hz), 5.39 (1 H, dd, *J* 1, 17 Hz), 5.23 (1 H, dd, *J* 1, 10 Hz), 4.30 (2 H, q, *J* 7 Hz), 1.33 (3 H, t, *J* 7 Hz); δ_C (67.9 MHz, $CDCl_3$) 165.7, 139.6, 129.9, 120.2, 112.3, 62.4, 14.3.

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