Chemical and biochemical properties of 2-hydroxypentadienoic acid, a homologue of enolpyruvic acid

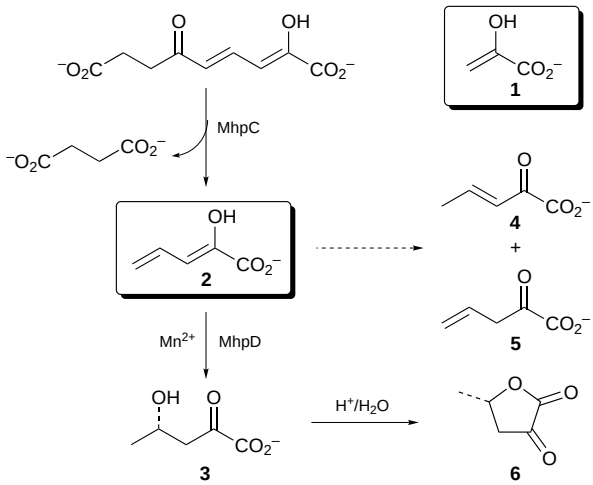
John R. Pollard,† Ian M. J. Henderson and Timothy D. H. Bugg*

Department of Chemistry, University of Southampton, Highfield, Southampton, UK SO17 1BJ

2-Hydroxypentadienoic acid is shown to be a biochemical intermediate on the phenylpropionate catabolic pathway of *Escherichia coli*; its stability and ketonisation behaviour are compared with those of its homologue, enolpyruvate.

The enol tautomer of pyruvic acid, enolpyruvate **1**, has been implicated as an intermediate in several enzymatic reactions.[1,2] The buffer- and enzyme-catalysed ketonisation of **1** have been studied in detail.[1–4] Its extended homologue 2-hydroxypentadienoic acid **2** has been proposed as an intermediate in a number of bacterial *meta*-cleavage pathways,[5–8] however very little is known about its chemical and biochemical properties. We have previously verified using ^{1}H NMR spectroscopy that **2** is the product of the reaction catalysed by C–C hydrolase MhpC, on the phenylpropionate catabolic pathway in *Escherichia coli* (see Scheme 1).[6] However, there is uncertainty over whether the dienol tautomer of **2** or one of two possible keto tautomers **4** or **5** is the substrate for the subsequent enzymatic hydration reaction to give 4-hydroxy-2-oxopentanoic acid **3**.[8–10] Here we analyse some chemical properties of 2-hydroxypentadienoic acid **2**, compare them with those of its homologue enolpyruvate, and confirm the biochemical intermediacy of **2**.

2-Hydroxypentadienoic acid **2** was generated by the reaction of C–C hydrolase MhpC, as previously described.[6] The appearance of **2** was monitored by UV spectroscopy (λ_{max} 270 nm) and organic acids HPLC.‡ Production of **2** was followed by a first-order decay in A_{270} over a 1–10 min time period (see Fig. 1), correlating with the disappearance of the HPLC peak, thus corresponding to the decomposition of **2**. The appearance and disappearance of **2** could be fitted accurately to a double exponential curve, allowing determination of the rate constants for production and decomposition of **2**. By extrapolation of the first-order decay of **2** to zero time, starting from a known concentration of the MhpC substrate (ε_{394} 15 600 M^{-1} cm^{-1}), the extinction coefficient for **2** was determined as ε_{270} = 19 200 M^{-1} cm^{-1} at pH 5.0 in 50 mM NaOAc. This value was found to remain fairly constant over the pH range 5–10, and is somewhat higher than the value of 9600 M^{-1} cm^{-1} reported for enolpyruvate,[2] as expected for a more conjugated system.



Scheme 1 The substrate for MhpC is the extradiol ring fission product of 2,3-dihydroxyphenylpropionic acid, whose structure has been verified previously (ref. 6).

The observed decomposition of **2** was thought to correspond to the ketonisation of its dienol functional group, which could take place either at C-3 (α-protonation) or C-5 (γ-protonation). A sample of **2** generated by MhpC was allowed to decompose, and after lyophilisation was analysed by ^{1}H NMR spectroscopy. The product contained 90% 2-oxopent-3-enoic acid **4**, 10% 2-oxopent-4-enoic acid **5** and a small amount (< 5%) of dienol **2**,§ verifying that decomposition was due to ketonisation, and indicating a 9 : 1 ratio of γ-protonation to α-protonation.

By carrying out the MhpC conversion in various buffers, it was possible to determine the rate of ketonisation (k_{obs}) *versus* pH and buffer type. Compound **2** was found to be more than five-fold more stable in phosphate or acetate buffers than in citrate, Mes or Tris buffers. k_{obs} was found to increase with pH [see Figs. 1 and 2(*a*)], as observed for enolpyruvate,[4] implying that the ketonisation of **2** is base-catalysed. In 50 mM NaOAc pH 5.0 a k_{obs} value of 0.026 min^{-1} was measured, corresponding to a half-life of 27 min. The half-life of enolpyruvate in H_2O at pH 5.0 is approximately 30 s,[3] thus **2** is more than 50-fold more stable than enolpyruvate in aqueous solution. Generation of **2** in 50 mM NaOAc (pH 5.0) in the presence of 5 mM $MnCl_2$ gave a 2.4-fold increased k_{obs}, indicating that the ketonisation of **2** is catalysed by divalent metal ions, as found for enolpyruvate.[3]

It was also found that k_{obs} increased when larger amounts of MhpC were used for its generation [see Fig. 2(*b*)], thus the ketonisation of **2** is also catalysed by MhpC. At normal assay concentrations of MhpC (0.05–0.1 u ml^{-1}), < 20% of the observed decomposition is enzyme-catalysed, but at concentrations of 1 u ml^{-1} approximately 75% of k_{obs} is due to enzyme-catalysed ketonisation. This behaviour can be rationalised mechanistically, since the first step of the MhpC-catalysed reaction is thought to be the ketonisation of a structurally similar dienol precursor at C-5.[6] From the gradient

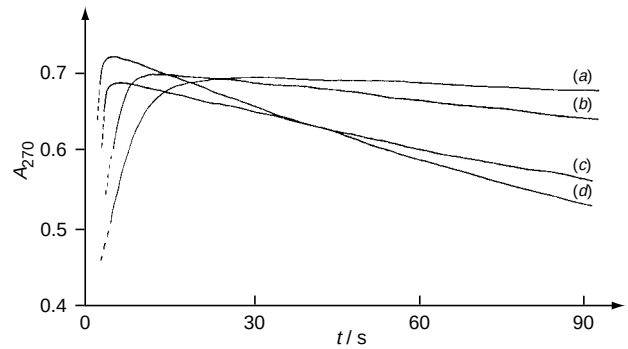


Fig. 1 Production of **2** by the reaction catalysed by hydrolase MhpC, and the subsequent decomposition of **2** at (*a*) pH 5, (*b*) pH 6, (*c*) pH 7 and (*d*) pH 8, as observed by UV spectroscopy. Assays contained 40 μM ring fission product, 0.1 u ml^{-1} MhpC and 50 mM buffer (NaOAc at pH 5, KH_2PO_4 at pH 6, 7 and 8), and were carried out at 20 °C.

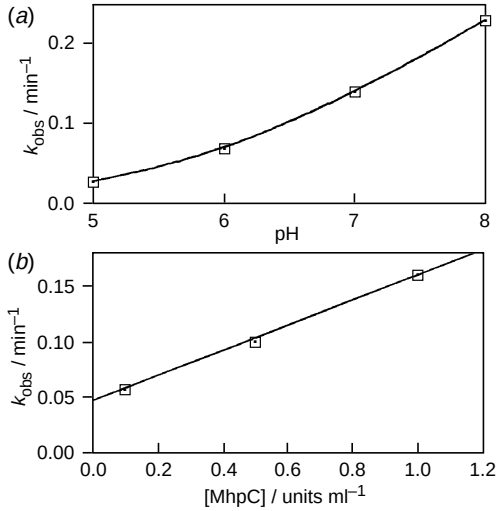


Fig. 2 Deduced first-order rate constants (k_{obs}) for ketonisation of **2**. (*a*) Variation of k_{obs} *versus* pH. Assays contained either 50 mM sodium acetate (pH 5) or 50 mM potassium phosphate (pH 6, 7 and 8). (*b*) Variation of k_{obs} *versus* concentration of the producing enzyme MhpC (measured in units MhpC per ml assay mixture). Specific activity of MhpC is 75 u mg^{-1}, M_r 29 kDa, substrate concentration, 58 μM.

of the plot in Fig. 2(*b*) the rate of MhpC-catalysed ketonisation of **2** can be calculated at 18 min^{-1}, 1.5% of k_{cat} for the MhpC-catalysed production of **2**. The ketonisation of enolpyruvate is also catalysed by an enzyme, pyruvate kinase (see Table 1).[2]

Is the dienol tautomer of **2** the substrate for the ensuing hydratase enzyme MhpD? Addition of *E. coli* extract containing MhpD enzyme activity to a solution of freshly-generated **2** gave rise to a rapid decrease in A_{270}. The rate of the enzyme-dependent decomposition of **2** was proportional to enzyme concentration, and samples of protein containing no MhpD showed no effect on **2**. This assay has been used to purify hydratase enzyme MhpD, as described elsewhere, and the pure enzyme shows the same effect by UV spectroscopy.[11] The conversion of **2** to 4-hydroxy-2-oxopentanoic acid **3** by MhpD has been confirmed by the lactonisation of **3** under acidic conditions, to give a sample of 2-oxo-4-methyl-γ-butyrolactone **6** which shows identical analytical data to synthetic samples of **6**.[12] Enzymatic processing of dienol **2** at rates considerably in excess of k_{obs} for ketonisation of **2** implies that the dienol tautomer is used as a substrate for MhpD-catalysed hydration. Studies to elucidate the mechanism of the MhpD-catalysed hydration are discussed elsewhere.[11] The Mn^{2+}- and MhpC-catalysed decomposition of **2** lead to low yields for large scale substrate conversions of **2** by hydratase MhpD, which requires Mn^{2+} for activity.[11]

Table 1 Comparison of the properties of **2** with its homologue enolpyruvate **1**

	Enolpyruvate **1**	2-Hydroxypentadienoic acid **2**
λ_{max}/nm	225	270
ε_{max}/M^{-1} cm^{-1}	9600[a]	19 200
k_{obs} (pH 5)/min^{-1}	1.32[b]	0.026
$t_{1/2}$ (pH 5)/min	0.5	27
Non-enzymatic catalysis	OH^-, M^{2+} [b]	OH^-, M^{2+}
Enzymatic catalysis (k_{cat}/min^{-1})	pyruvate kinase (50)[a]	MhpC (18)

[a] Data from ref. 2. [b] Data from ref. 3. Measured in 20mM NaOAc (pH 5), 0.2 mM $MnCl_2$.

On the catechol *meta*-cleavage pathway of *Pseudomonas putida*, **2** is generated by a decarboxylase enzyme which forms a 1 : 1 complex with the ensuing hydratase enzyme.[10] It has been proposed that the formation of such a complex favours the processing of the unstable intermediate **2**.[10] We have no evidence of any association between hydrolase MhpC and hydratase MhpD, however it is noteworthy that the catalytic efficiency of purified MhpD is very high (k_{cat}/K_m = 1.1 × 10^7 M^{-1} s^{-1}).[11] In conclusion, 2-hydroxypentadienoic acid shows similar chemical properties but enhanced kinetic stability with respect to its homologue enolpyruvate, and represents a further example of a biologically important enol.

This work was supported by an Earmarked studentship (J. R. P.) and a research grant (B04835) from the BBSRC. We thank Sarah Fleming for assistance with NMR spectroscopy, and Dr Jonathan Percy (University of Birmingham) for helpful comments.

Footnotes and References

* E-mail: tdb@soton.ac.uk
† Present address: Department of Chemistry, University of St. Andrews, Fife, Scotland, UK KY16 9ST.

‡ HPLC analysis of **2**: retention time, 42 min (λ_{max} 270 nm) on Bio-Rad HPX-87H Organic acids column, eluent 0.005 M H_2SO_4, flow rate 0.6 ml min^{-1}.

§ *Selected data* for **4**: δ_H (270 MHz, D_2O) 1.95 (3 H, dd, *J* 1.8, 7.2, H-5), 6.16 (1 H, dq, *J* 16.2, 1.5, H-3), 7.04 (1 H, dq, *J* 16.2, 7.0, H-4). For **5**: δ_H 3.47 (2 H, dt, *J* 7.0, 1.4, H-3), 5.1–5.2 (2 H, m, H-5), 5.85 (1 H, m, H-4).

1 I. A. Rose, *Methods Enzymol.*, 1982, **87**, 84.
2 D. J. Kuo, E. L. O'Connell and I. A. Rose, *J. Am. Chem. Soc.*, 1979, **101**, 5025.
3 B. A. Miller and D. L. Leussing, *J. Am. Chem. Soc.*, 1985, **107**, 7146.
4 J. A. Peliska and M. H. O'Leary, *J. Am. Chem. Soc.*, 1991, **113**, 1841.
5 S. Dagley, *Essays Biochem.*, 1975, **11**, 81.
6 W. W. Y. Lam and T. D. H. Bugg, *J. Chem. Soc., Chem. Commun.*, 1994, 1163.
7 F. Junker, J. A. Field, F. Bangerter, K. Ramsteiner, H. P. Kohler, C. L. Joannou, J. R. Mason, T. Leisinger and A. M. Cook, *Biochem. J.*, 1994, **300**, 429.
8 H. Lian and C. P. Whitman, *J. Am. Chem. Soc.*, 1994, **116**, 10403.
9 W. L. Collinsworth, P. J. Chapman and S. Dagley, *J. Bacteriol.*, 1973, **113**, 922.
10 S. Harayama, M. Rekik, K. Ngai and L. N. Ornston, *J. Bacteriol.*, 1989, **171**, 6251.
11 J. R. Pollard and T. D. H. Bugg, unpublished work.
12 A. Rossini and H. Schinz, *Helv. Chim. Acta*, 1948, **31**, 473. HPLC retention time for lactone **6**: 35 min (see footnote ‡ for conditions).

Received in Glasgow, UK, 1st July 1997; 7/04635I