A Spectroscopic Study on *p*-Hydroxyphenylpyruvic Acid. Keto-Enol Tautomerism and Stability of Its Complex with Fe^{+3} Ions*

L. Cassidei, A. Dell'Atti, and O. Sciacovelli

Istituto di Chimica fisica dell'Universita, Via Amendola 173, 70126-Bari, Italy

Z. Naturforsch. **35 c**, 1–5 (1980); received July 20/October 15, 1979

p-Hydroxyphenylpyruvic Acid, Tautomerism, Complex Stability of pHPPA-$FeCl_3$

^1H-, ^{13}C-NMR, IR, UV-Vis, and MS spectra of *p*-hydroxyphenylpyruvic acid (pHPPA) have been recorded and fully interpreted.

pHPPA exists in solution as a mixture of interconverting forms: keto, hydrated keto and only one enol tautomer to which the Z configuration has been assigned according to the value (3.7 Hz) of the vicinal $^1H-C=C-^{13}COOH$ coupling constant.

In organic solvents the enol isomer is far more stable whereas in aqueous solutions the keto form predominates. The tautomeric equilibrium is pH-dependent and the anion is present as keto form not only in aqueous solution but also in H_2O-DMSO mixtures with a high content of DMSO.

The Z enol tautomer does form a coloured complex ($\lambda_{max} = 680$ nm) with Fe^{+3} ions. The complex decomposes rapidly in all considered solvents except DMSO.

In view of a possible use of H_2O-DMSO mixtures for clinical analysis purpose, the enol fraction of pHPPA and the stability of the pHPPA-$FeCl_3$ complex in H_2O-DMSO mixtures have been examined. Our results suggest that a solvent composition containing at least 80 vol.% in DMSO could be an appropriate solvent for pHPPA determination by $FeCl_3$ method.

p-Hydroxyphenylpyruvic acid (pHPPA) is a significant product of tyrosine metabolism in transitory and hereditary tyrosinemia [1]. Its determination in biological fluids contributes to obtain a precocius and valuable picture of the amino acid metabolic diseases. Actually, the colorimetric detection of pHPPA by the ferric chloride method is practically impossible: only a green-blue flash of colour can be observed [2]. Because pHPPA exhibits keto-enol tautomerism [3–5], this fact could be attributed to low amount in solution of the tautomer responsable for the coloured complex formation and to low chemical stability of this complex. It is, therefore, evident that a detailed investigation (i) of the keto-enol tautomerism of this substance and (ii) of the stability of its complex with Fe^{+3}, in different organic solvents and in organic solvent-aqueous mixtures, imposes in order to identify and control the factors affecting the determination of pHPPA by $FeCl_3$. For this purpose, NMR spectroscopy in concurrence with UV-Vis, IR, and MS spectroscopic techniques has been proved to be very suitable.

* This work was supported financially by C.N.R.

Abbreviations: pHPPA, *p*-hydroxyphenylpyruvic acid; DMSO, dimethylsulphoxide; TMS, tetramethylsilane; DSS, sodium 2,2-dimethyl-2-silapentane-5-sulfonate; PPA, phenylpyruvic acid; CW continuous wave.

Reprint requests to Dr. O. Sciacovelli.

0341-0382/80/0100-0001 $ 01.00/0

Moreover, because pHPPA is a substrate or product in many enzyme reactions which are believed to involve either the keto or the enol form [4, 6] a better knowledge of its tautomeric properties may be useful to elucidate the mechanism of reactions in which it is involved.

Experimental Section

Instruments and techniques

The ^1H-NMR spectra were recorded at 100 MHz on a Varian HA 100 spectrometer. Usually 0.5 M solutions were employed and a small amount of TMS was added to generate the lock and/or internal reference signal. For the spectra recorded in H_2O-DMSO mixtures it was more convenient to lock the spectrometer on the DMSO signal. The solubility of pHPPA in water was too low to obtain CW ^1H NMR spectra. The increase of solubility in buffered solutions (pH = 6) was enough for a satisfactory signal-to-noise ratio in a single scanned CW spectrum. In this case sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) was used as internal reference and lock substance.

The FT ^{13}C-NMR spectra, ^1H noise decoupled and undecoupled, were recorded at 25.2 MHz on a Varian XL 100 spectrometer (1 M in DMSO-d_6, 0.48 Hz per data point, number of transient: 1000 for

^1H noise decoupled and 28000 for ^1H undecoupled spectra).

The infrared spectra of pHPPA and its sodium salt in KBr pellets were recorded on a Perkin-Elmer 257. The UV-Vis measurements were carried out using a Zeiss PMQ II spectrophotometer fitted up with a thermostatted cell-holder. Mass spectra were obtained with a Perkin-Elmer 270 MS under the following experimental conditions: source pressure, 10^{-7} mm Hg; electron beam energy, 70 eV; ion-accelerating voltage, 2 kV.

The mole fraction of the H_2O-DMSO mixtures was determined from the data of Cowie and Toporowski [7] by measuring the refractive index at 25 °C on an Abbè refractometer using the sodium D line. The volume percentages were calculated from the mole fraction data.

The keto-enol equilibrium in pure solvents and H_2O-DMSO mixtures was investigated by UV spectrophotometry and ^1H NMR spectroscopy. From optical measurements the enol fraction was obtained using the expression $C_E = A/(\varepsilon \cdot 1)$. The absorbance, A, was measured in corrispondence to the maximum absorption of the enol on assuming that absorption of the keto form was negligible [3, 5, 10].

For the ^1H-NMR spectra of pHPPA recorded in pure solvents the enol fraction was obtained from the integrals of the signals of the $-CH_2-$ keto protons and of the $-CH=$ enol proton. In H_2O-DMSO solvent mixtures the integrals of the aromatic protons signals were used to calculate the enol fraction, because the intense signal due to H_2O protons did not permit accurate integration of $-CH_2$-signal. In order to obtain reliable values of the enol fraction at least ten integrals were averaged. All measurements concerning the keto-enol tautomerism were made 24 h after preparing the solutions so as to reach equilibrium.

The stability of the pHPPA-$FeCl_3$ complex was studied at 25 °C by recording the decrease of the absorbance ($\lambda_{max} = 680$ nm) of solutions, with different pHPPA concentrations and $FeCl_3$/pHPPA mole ratios, in H_2O-DMSO mixtures covering the range 20 – 100 vol.% DMSO.

Materials

Commercially available solvents were used. pHPPA was purchased from Fluka AG, Buchs SG, Switzerland.

Experimental Results

^1H NMR spectra of pHPPA in organic deuterated solvents (DMSO, methanol, acetone and dioxane) display an intense singlet in the olefinic region ($\delta = 6.3_5$), whereas either no signal (in methanol and dioxane solutions) or a small one at $\delta = 3.9_6$ (in DMSO and acetone solutions) is observed in the absorption range of methylene protons. On the contrary, in the spectrum recorded in aqueous solution buffered at pH = 6 the methylene resonance ($\delta = 4.1$) is very strong and the olefinic signal ($\delta = 6.3$) small. Moreover, an additional small signal is found at $\delta = 3.1$ which can be assigned to the hydrated keto tautomer: $-CH_2-C(OH)_2-$. In the aromatic region the chemical shifts (δ) of the AA'BB' systems can be attributed by considering the substituent effects on the chemical shift of benzene protons [8] (H – C(6): 6.8_5 (enol), 6.7_7 (keto); H – C(5): 7.6_4 (enol), 7.0_5 (keto)). The effects for $-C=C(OH)-COOH$ group (*o:* + 0.45; *m:* ~ 0; the plus sign refers to downfield shift) are derived from the PPA [9].

The percentages of the enol and keto forms in organic solvents have been calculated from the integrals of the olefinic and methylene proton signals (see Table I). In aqueous solution the proximity of the very intense H_2O peak prevents integration of the methylene resonance. The use of deuterated buffer solutions allow us to only estimate the percentages of the tautomers because of the progressive deuteration of the $-CH=$ and $-CH_2-$ signals. Exact values (Table I) of the tautomeric population in water have been obtained, at low pH values, from UV spectra by assuming that the UV spectrum of the enol form undergoes no significant variations in different solvents. UV data for pure enol form have been derived from methanol and dioxane solutions because ^1H NMR spectra show no presence of the keto tautomer in these solvents. The following molar extinction coefficients, at 25 °C, are calculated: $\varepsilon_{303\,nm}$ (methanol) = 25400 and $\varepsilon_{307\,nm}$ (dioxane) =

Table I. Equilibrium percentages of the enol form in different media at 30 °C.

Solvent	%
Acetone	~98
DMSO	96.5
H_2O(HCl 1 M)	16.4
H_2O(HCl; pH = 1)	14.3
H_2O(HCl; pH = 2)	10.7

Table II. Thermodynamic quantities relative to enol-keto equilibrium for undissociated pHPPA in H_2O(HCl 1 M) and DMSO.

Solvent	$\Delta F°_{20\,°C}$ [cal/mol]	ΔH [cal/mol]	ΔS [cal/°Kmol]
H_2O(HCl 1 M)	−900	−2000	−4.0
DMSO	2000	7200	17.2

Table III. ^{13}C NMR parameters of pHPPA in DMSO-d_6 (1 M).

Carbon atom	Chemical [a] shift (δ)	Coupling [b] constant [Hz]
C (1)	167.1	$^3J(C_1H_3)=3.7$
C (2)	139.2	$^2J(C_2H_3)=2.44$
C (3)	111.1	$^1J(C_3H_3)=157.2$; $^3J(C_3H_5)=4.9$
C (4)	126.3	$^3J(C_4H_6)=7.8$
C (5)	131.4	$^1J(C_5H_5)=160$
C (6)	115.6	$^1J(C_6H_6)=159$
C (7)	156.7	$^2J(C_7H_6)=2$; $^3J(C_7H_5)=9$

[a] Referred to TMS as internal standard.
[b] Absolute values.

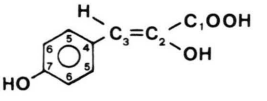
25 300, on the assumption that at these wave-lengths absorption of the keto form is negligible [3, 5, 10].

The tautomeric equilibrium in aqueous solutions cannot be investigated accurately at pH higher than 2, because of the overlap of the enol anion absorption band with a medium intensity band originated from the keto tautomer [5]. This absorption band which is not present in the UV spectra of aqueous solutions of PPA is probably the B-band of the benzene chromophore shifted to longer wave-length by the presence of an auxochromic group such as OH.

Table I shows the different behaviour of pHPPA in aqueous and organic solvents. In aqueous solutions the keto form predominates and the tautomeric equilibrium is pH-dependent, as reported by other Authors, too [3]. In organic solvent pHPPA is far more stable in its enol form [11]. However, ^1H NMR spectra of *p*-hydroxyphenylpyruvate in H_2O-DMSO solutions with a high content of DMSO (80 vol.%) show that the anion is largely present as keto isomer. These results indicate that DMSO and probably also the other organic solvents stabilize the undissociated enol form, exclusively.

Thermodynamic quantities have been calculated for the undissociated acid in DMSO (30−78 °C) and H_2O (HCl 1 M) (30−60 °C) assuming ideal behaviour (see Table II). The data display a dominant role of solvation in stabilizing both tautomers. The decrease of entropy in DMSO may be attributed to the formation of strong hydrogen bond between hydroxyl groups of pHPPA and DMSO molecules [9].

The possibility that the spontaneous tautomerism produces both Z and E tautomers must be considered. The ^1H-, ^{13}C-NMR and UV spectra indicate the presence of only one enol tautomer in water and various organic solvents. The configuration can be derived from ^{13}C NMR spectra, ^1H undecoupled. ^{13}C-NMR parameters of pHPPA in DMSO-d_6 are reported in Table III. The Z configuration [5, 11] can be assigned on the basis of the value of the vicinal $^1H-C=C-^{13}COOH$ coupling constant, which is typical for a cis arrangement of the coupled nuclei in fragments $^1C-C=C-^{13}C$ with similar sums of substituents electronegativities [11, 12]. Presumably, the stability of the Z enol form is due to the conjugation of the phenyl ring, the olefinic and carboxylic groups.



IR spectra analysis

The infrared spectra of pHPPA and its sodium salt in the solid state show some remarkable differences as a consequence of the unusual behaviour of phenylpyruvic acids which are stable in the enol form as undissociated acid and in the keto one as anion.

In the IR spectrum of pHPPA two absorption band are present in the OH stretching vibration region: a band between 3500 and 3360 cm^{-1} attributable to the overlap of the signals arising from the enol and the associated phenol hydroxyl groups, whereas a broad band between 3300 and 2500 cm^{-1} originates from the OH-stretching of the bonded carboxylic group. A strong band at 1695 cm^{-1} can be assigned to the $\nu_{C=O}$ of the carboxylic group. The decrease in frequency compared to aliphatic α keto acids could be ascribed to the decrease of the C=O bond force constant due to the conjugative effect of an α, β double bond. The $\nu_{C=C}$ vibration occurs at 1600 cm^{-1}. A band at 1200 cm^{-1} could be assigned to the stretching vibration of the =C−OH group on the basis of the calculated value of 1205 cm^{-1} for ν_{C-OH} for vinyl alcohols and the lack of this

strong absorption in the spectrum of the sodium salt of pHPPA according to the presence of only the keto form [13]. In the spectrum of sodium *p*-hydroxyphenylpyruvate a strong band between 3550 – 3200 cm^{-1} is assigned to the OH stretching vibration of the phenolic group. The carbonyl group gives rise to the characteristic band at 1720 cm^{-1} due to the stretching vibration of the C=O bond. A very strong band at 1635 cm^{-1} originates from the asymmetric stretching of the COO^- group whereas a low intense band at 1400 cm^{-1}, absent in the IR spectrum of pHPPA can be assigned to the symmetric stretching of the carboxylate anion and/or to the scissoring band of the methylene group in the fragment $-CH_2-\overset{\overset{O}{\|}}{C}-$.

Mass spectra of pHPPA

Tabular presentation of the spectrum is given in Table IV. The two main fragmentation sequences confirmed by metastable peaks are similar to those observed for PPA [11]. The presence of a *p*-hydroxyl group does not seem to influence the fragmentation process at least in the main cleavage and rearrangement modes. The circumstance that for the second fragmentation path only two fragmentation steps corresponding to those of PPA are present and the very prominent peak at $m/e = 107$ (an intense base peak is absent in the mass spectrum of PPA) indicate the presence of a stabilized $|C_7H_7O|^+$ ion which probably corresponds to the peak observed for cresols as a result of benzylic C – H cleavage.

Properties of $FeCl_3$-pHPPA complexes

Addition of $FeCl_3$ to pHPPA solutions in the considered organic solvents and water yields a "flash" of a green-blue colour in all the solvents except DMSO. As ^1H NMR spectra show, the disappearing of the colour is due to decomposition of pHPPA. In DMSO the colour appears to be stable in time and ^1H NMR spectra show that only the Z enol tautomer does combine with the ferric cation to form a coloured complex ($\lambda_{max} = 680$ nm). From these findings it can be argued that a study on the stability of the complex in DMSO-H_2O mixtures would give useful information about the possibility to detect pHPPA in biological fluids simply by adding a solution of $FeCl_3$ in DMSO. Moreover, since the colour development depends on the enol content of H_2O-DMSO mixtures and on the enolization rate of the keto form, the keto-enol equilibrium of pHPPA has been studied throughout the composition range at 25 °C.

Fig. 1 shows that the enol fraction increases regularly with the volume percentage in DMSO. The trend of the plot is not linear and shows that it would be favourable to operate with solutions richer in DMSO.

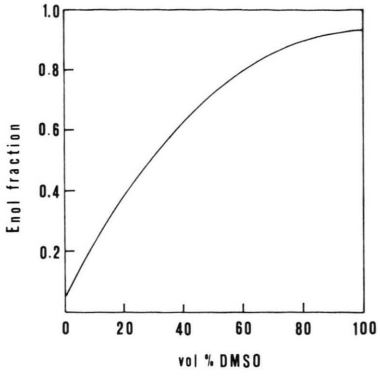


Fig. 1. Enol fraction versus volume percentage in DMSO at 25 °C.

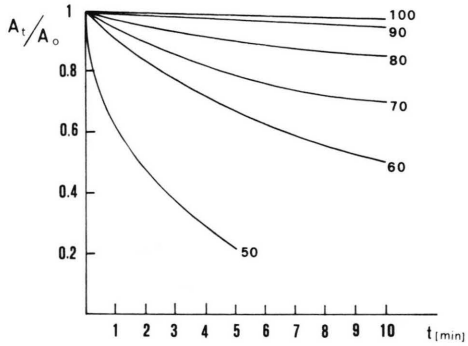


Fig. 2. Decrease in time of the absorbance ratio A_t/A_0 (680 nm, 25 °C) of pHPPA-$FeCl_3$ complex in H_2O-DMSO mixtures with different compositions. A_t and A_0 are the absorbance values of the sample at the time t and $t = 0$, respectively. For solutions containing less than 50 vol.% DMSO the fading of the colour is too fast to allow reliable absorbance measurements.

Table IV. Salient fragment ions from pHPPA.

m/e (intensity referred to base peak)
Path I 180 (31) $\xrightarrow{-CO,H_2O}$ 134 (31) $\xrightarrow{-CO}$ 106 (24) $\xrightarrow{-H}$ 105 (10) $\xrightarrow{-CO}$ 77 (23) $\xrightarrow{-C_2H_2}$ 51 (10)
Path II 180 (31) $\xrightarrow{-COOH}$ 135 (5) $\xrightarrow{-CO}$ 107 (100)

The curves in Fig. 2 display the decrease in time of the absorbance ratio A_t/A_0 at 650 nm, of pHPPA-$FeCl_3$ complex in H_2O-DMSO mixtures. A_t and A_0 are the absorbance of the sample at the time t and $t = 0$, respectively. The quantity A_t/A_0 has been reported instead of A_t, because it allows to compare samples with different concentrations of pHPPA and $FeCl_3$. The decomposition products of the complex show no absorption at 680 nm; therefore, the A_t/A_0 ratio is proportional to the amount of the complex present in solution and its decrease has to be related to the chemical stability of the complex. The time dependence of A_t/A_0 indicates that the complex stability increases with the content of DMSO in the solvent mixtures. Solutions containing more than 80 vol.% in DMSO show a satisfactory colour stability which appears to be independent of the initial concentration of pHPPA ($0.3 \div 6.3 \times 10^{-3}$ M) and of $FeCl_3$/pHPPA mole ratio ($4 \div 1$) in the limits of experimental error. Moreover, at this solvent composition the enol fraction falls between 0.90 and 0.94, thus allowing an instantaneous development of the green-blue colour and making the colorimetric analysis practically independent of the rather low enolization rate of pHPPA [3, 5]. Our results suggest the use of such solvent compositions for pHPPA determination by the $FeCl_3$ method. Improvement in the colour stability can also be achieved by keeping the temperature as low as possible. Indeed, the decomposition rate of the complex appears to depend firstly on the solvent composition and secondly on the temperature.

[1] H. Bickel, F. P. Hudson, and L. J. Woolf, Phenylketonuria, G. Thieme Verlag, Stuttgart 1971; J. B. Stanburg, J. B. Wyngaarden, and D. S. Fredrickson, The Metabolic Basis of Inherited Disease, McGraw-Hill Book Company, 1972.
[2] G. H. Thomas and R. Rodney Howell, Selected Screening Tests for Genetic Metabolic Diseases, Year Book Medical Publishers Inc., Chicago 1973.
[3] T. Bücher and E. Kirberger, Biochim. Biophys. Acta **8,** 401 (1952).
[4] W. E. Knox and B. M. Pitt, J. Biol. Chem. **225,** 675 (1957).
[5] P. O. Larsen and E. Wieczorkowska, Acta Chem. Scand. B **28,** 92 (1974).
[6] I. Saito, Y. Chujo, H. Shimazu, M. Yamane, T. Matsuuza, and H. J. Cahnmann, J. Am. Chem. Soc. **97,** 5272 (1975); E. C. C. Lin, B. M. Pitt, M. Civen, and W. E. Knox, J. Biol. Chem. **233,** 668 (1968).
[7] J. M. G. Cowie and P. M. Toporowski, Can. J. Chem. **39,** 2240 (1961).
[8] L. M. Jackman and S. Sternhell, Applications of NMR Spectroscopy in Organic Chemistry, Pergamon Press, 1969.
[9] L. Cassidei, A. Dell'Atti, and O. Sciacovelli, Z. Naturforsch. **31 c,** 641 (1976).
[10] S. S. Tate, A. K. Grzybowski, and S. P. Datta, J. Chem. Soc. **1964,** 1372.
[11] O. Sciacovelli, A. Dell'Atti, A. De Giglio, and L. Cassidei, Z. Naturforsch. **31 c,** 5 (1976).
[12] J. A. Stobbe and G. L. Kenyon, Biochemistry **10,** 2669 (1971); U. Vögeli and W. Von Philipsborn, Org. Magn. Res. **7,** 617 (1975).
[13] M. Avram and G. Mateescu, Infrared Spectroscopy, Wiley-Interscience 1972.