Enzymology and regulation of the atropine metabolism in pseudomonas putida

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CHAPTER 3

SYNTHESIS AND PROPERTIES
2- PHENYLMALONIC SEMI-ALDEHYDE

3.1 INTRODUCTION

During the study of the metabolism of atropine, indications were obtained for a role of 2-phenylmalonic semi-aldehyde (pma, 3-oxo-2-phenyl propanoic acid) (see fig. 3.1 V) as intermediate in this breakdown. It appeared highly desirable to have the disposal of this compound. However, in the large handbooks one will search in vain for references with information about the properties and synthesis of this compound in spite of its rather simple structure. The methyl and ethyl ester of pma have been synthesized but the acid in free form would be not stabile and was therefore considered to be unknown (Rodd 1956).

An attempt to synthesize the pma by oxidation of tropic acid using alkaline potassium permanganate resulted in a product which was able to induce the tropic acid enzymes. Further analysis revealed that not pma but phenylglyoxylic acid was synthesized. By this lucky coincidence, a gratuitous induction of the tropic acid enzymes was discovered, that will be discussed in chapter 10.

Later in this research, one single reference was found in the literature which describes the synthesis of pma (Strukov 1952). The paper provides only limited information on the nature of this product. It mentions the melting point and the spontaneous decomposition in water in phenylacetaldehyde and carbon dioxide.

Synthesis of pma has been carried out according to this recipe. The reaction product was identified as pma in the enol form (enol-pma).

The keto-enol tautomerism of pma appeared to have a major role in studies of the effect of this compound on the enzymatic dehydrogenation of tropic acid. Therefore, the tautomeric properties of pma have been studied more in detail. A quantitative colorimetric assay of enol-pma was designed. It was possible to demonstrate that the pma disappears in ethanol at 0^0 C. whereas infrared analysis showed the simultaneous formation of the keto form of pma (keto-pma). The tautomeric rearrangement has been studied in aqueous conditions as well. These results have been very relevant to explain the kinetics of the enzymes TDH and PDC.

3.2 THE SYNTHESIS OF THE 2-PHENYLMALONIC SEMI-ALDEHYDE

The pma (V) is synthesized starting from phenylacetic acid according to the reaction scheme in fig 3.1. Properties of intermediate compounds and of the final product are listed in Table 3.2

Fig.3.1
Synthesis of 2-phenylmalonic semi-aldehyde (pma)

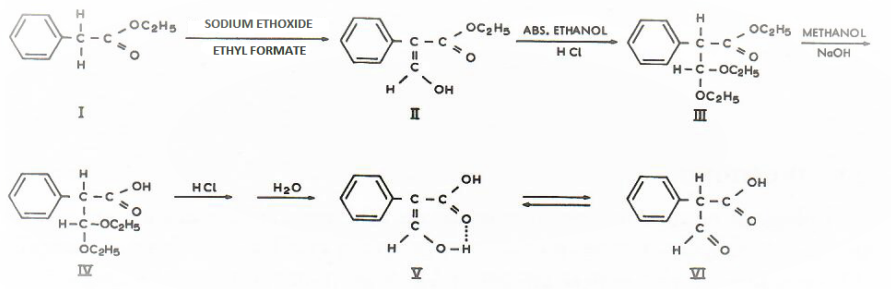


Table 3.2
Properties of the products in the synthesis of pma
b.p. = boiling point m.p = melting point

Compound	Experimental	Literature or calculated value
II ethyl ester of pma	b.p. 124-126^0 / 12 mm n_D^{23} 1.5312	b.p. 139-140^0 / 16 mm n_D 1.5322 (Beilstein)
III diethyl acetal of ethyl ester of pma	b.p 142-144^0 / 6 mm n_D^{24} 1.5318	b.p. 166-168^0 / 20mm (Strukov, 1952)
IV diethyl acetal of pma	m.p, 127.5-128 ^{0} C-H-O analysis 65.50% C 7.58% H 27.21% O	m.p. 121-123^0 (Strukov 1952) C-H-O analysis 65.53% C 7.61% H 26.86% O
V pma	m.p. 85-94^0 (decomposition) C-H analysis 66.02% C 5.02% H	m.p. 101-103^0 (decomposition) (Strukov 1952) C-H analysis 65.85% C 4.91% H

Phenylacetic acid was converted in its ethyl ester (I); the ethyl ester of pma (II) was made by condensation with ethyl formate in sodium ethoxide, according to the method of Friedman and Gladych (1956). The product was purified by extraction and distillation.

Compound II mixed with ferric chloride dissolved in methanol reacted with a violet color that disappeared after the addition of bromine water. This indicated that compound II was present in enol-form either totally or partially.

Further synthesis was according to Strukov (1952). Compound II dissolved in dry ethanol was converted in the diacetal of the ethyl ester of pma by perfusion using dry hydrochloric vapor. After neutralization with alcoholic lye, compound III was isolated by distillation under reduced pressure. Next the ester bond was hydrolyzed using 2 N NaOH in 80% methanol. The diethyl acetal of pma (IV) was sedimented after acidification, recrystallized from toluene and identified based on the melting point and elementary analysis.

The pma was obtained by stirring compound IV in concentrated HCl at 0^0, followed by addition of an equal volume distilled water. The product was collected on a filter, thoroughly washed with ice water, washed with petroleum ether (boiling point 40-60^0) and dried in vacuum.

3.3 IDENTIFICATION OF 2-PHENYLMALONIC SEMI-ALDEHYDE

The elementary analysis of the synthesized product (table 3.2) correlates well with the theoretically expected values. The compound reacts with $FeCl_3$ in ethanol forming a violet complex. This is an indication that the compound is completely or partially in the enol-form. In water decomposition occurs under the formation of the characteristic odor of phenylacetaldehyde.

The final identification of the pma was based on the analysis of the infrared and nuclear magnetic resonance spectra, on the gas chromatografic identification of the decomposition product as phenylacetaldehyde and biochemical evidence (this thesis chapter 6 and 7) The melting point, the only property described by Strukov was not suitable for characterization (3.3.1).

3.3.1 The decomposition range

The crude reaction product decomposes at the melting point (101-103^0) into phenylacetaldehyde and carbon dioxide (Strukov 1952). The product synthesized in this thesis project appeared to decompose at 85-94^0. After three recrystallizations using carbon tetrachloride as solvent, nice needle-form crystals were obtained, but the decomposition range remained 10-15^0 below the literature value. Small crystals appeared to decompose much earlier compared to the larger ones. It seemed that the crystal size could have a significant effect on the range of the decomposition. This was confirmed: a part of a large crystal was pulverized to

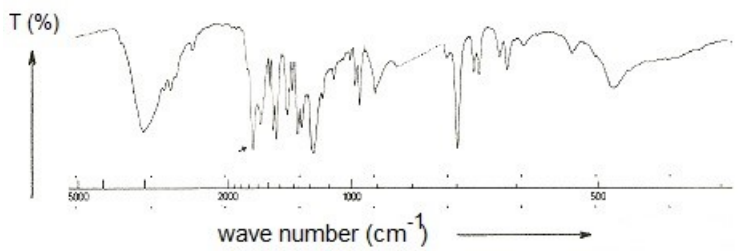
powder. The decomposition range observed for the crystal and the powder was 92-93^0 and 58-67^0 respectively.

It is clear that the melting or decomposition range of pma is not suitable as a parameter for its characterization; the discrepancy between the experimental values and those found in literature does not exclude that the pma compound synthesized by Strukov and the one made in this study are identical. However, Strukov's crystals must have been of respectable dimensions.

3.3.2 Infra-Red and Nuclear Magnetic Resonance spectroscopy

The results of the infra-red and nuclear magnetic resonance spectroscopy have been interpreted by Dr H. Meppelder. He concluded that the product synthesized is indeed the enol form of 2-phenylmalonic semi-aldehyde. His conclusion is based on the following observations. Fig 3.3 shows the infra-red spectrum of the recrystallized product dissolved in carbon tetrachloride. The spectrum of the solid compound is identical.

Fig 3.3
Infra-red spectrum of pma in carbon tetrachloride
X-axis: wavenumber (cm^{-1}); Y axis: transmission T (%)



If pma would have been synthesized as the keto form one would expect the characteristic absorption of the stretch vibration of the aldehyde C=O at 1740-1720 cm^{-1} and for the C=O of the carboxyl group at 1725-1700 cm^{-1}. Both absorptions are totally absent in the spectrum.

For pma in the enol-structure one expects to find the stretch vibration of the carbonyl C=O at an absorption of about 1648 cm^{-1}, which has been found by Friedman and Gladych (1956) for the C=O of the tropine ester of pma. The spectrum of pma shows a strong band at 1653 cm^{-1} and is nicely in agreement with the literature data mentioned. This frequency is abnormally low, because the normal value for an $\alpha-\beta$ non-saturated acid is about ± 1700 cm^{-1}: (the structurally related atropic acid C_6H_5-C(=CH_2)-COOH has a value of 1694 cm^{-1}).

The bathochromic shift could have been caused by the formation of an intramolecular hydrogen bridge (see fig 3.1 V), for which the hydroxyl group of the enol form is available.

The C=C in the side chain of atropic acid absorbs at 1620 cm^{-1}; for an enol structure of pma in which the hydroxyl group is involved in a hydrogen bridge, one may expect a lower value for the absorption of the C=C as well. So, the absorption at 1574 cm^{-1} can be attributed to the C=C bond. Therefore, the infra-red spectrum is not compatible with the keto form of the pma, but can be interpreted for that compound in the enol form.

The nuclear magnetic resonance spectrum lacks the characteristic absorption of the aldehyde group. It shows absorptions corresponding with the presence of an ethylene- and a hydroxyl- proton. A full confirmation of the enol structure of pma was obtained by comparison of its NMR spectrum with that of atropic acid and by a study of the effect of trifluoracetic acid.

3.3.3 Gas Liquid Chromatography

More proof of the identity of pma was obtained by the observation that During thermic decomposition phenylacetaldehyde is formed. This was investigated by gas liquid chromatography. A solution of pma in ether was injected in the gas chromatograph with the injection block at a temperature of 200^0. Only one product was found under condition as described in chapter 2.12; it had a retention time of 8.8 min, identical to that of the reference phenylacetaldehyde.

3.3.4 Biochemical indications

The enzyme tropic acid dehydrogenase is able to accelerate dehydrogenation of tropic acid as well as hydrogenation of pma. After enzymatic hydrogenation of pma, tropic acid was detected as product by thin layer chromatography (see chapter 6).

3.4 TAUTOMERIC REARRANGEMENT OF 2-PHENYLMALONIC SEMI-ALDEHYDE

3.4.1 Quantitative assay of the enol form

A quantitative assay for enol-pma was developed to explain the role of the tautomeric rearrangement of pma in the interpretation of the kinetics of the tropic acid dehydrogenase TDH. This is based on the quantitative enol assay, reported by Kaufman and Richter (1925) making use of the color reaction of an

enol-compound with $FeCl_3$ in methanol. The experimental conditions have to be controlled very well to make the method usable for quantitative purposes. The assay could be disturbed by a shift in the keto-enol rearrangement by the formation of acetal or by oxidation of the enol by $FeCL_3$. This can be corrected as described in chapter 2.13 by registration absorption of the $FeCl_3$-enol complex at 600 nm during a short time period and by extrapolation to time = 0.

3.4.2. Shift of the keto-enol equilibrium in ethanol at 0^0.

The stability of enol pma in various solvents has been investigated using the $FeCl_3$-enol assay. The enol concentration remains constant at last during several hours if the pma is dissolved in dry diethyl ether. In ethanol a decrease of the enol concentration is observed. The decrease proceeds at 0^0 by a first order reaction with a rate constant of 0.2 - 0.3 $hour^{-1}$;about half amount of the enol-pma is converted in about 3 hours. The most logical explanation for this decrease is the shift of the keto-enol equilibrium in favor of the keto-form but a decomposition of enol- or keto-pma into phenylacetaldehyde might occur as well. The decrease might also be explained by the formation of the diethyl acetal of pma, by which keto-pma would be withdrawn from the keto-enol equilibrium.

In chapter 6 a quantitative enzymatic assay is reported for the total amount of pma (keto + enol); this assay does not react on the diethyl acetal or on phenylacetaldehyde. Using this assay, it was concluded that the total amount of pma in ethanol at 0^0 did not decrease more than 5% during 3 hours. Therefore, the decrease of enol-pma must have been accompanied by the formation of keto-pma.

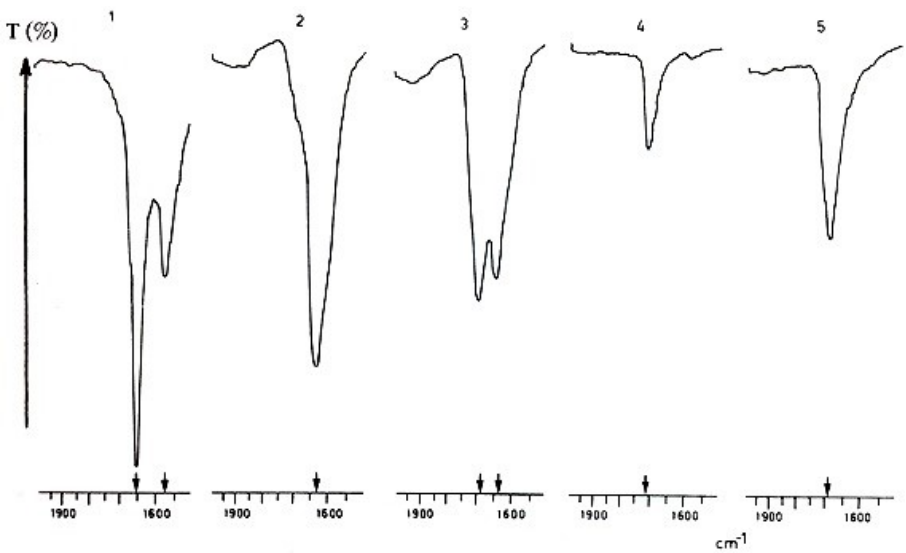
Direct indications for the formation of keto-pma have been obtained using infra-red spectroscopy. Spectra of pma were recorded in the range of 2000-1500 cm^{-1} with carbon tetrachloride or ethanol as solvent in a refrigerated infra-red cell. Usually stretching vibrations of carbonyl groups in an individual aldehyde or carboxylic groups are accompanied by absorptions at 1740 and 1700 cm^{-1}. The enol form does not show absorption at these frequencies (see this chapter 3.2.2) but for the keto-form absorption maxima are expected in this region of the spectrum.

Fig 3.4.1 shows a part of the spectrum of enol-pma dissolved in carbon tetrachloride with absorption maxima at 1653 and 1574 cm^{-1}. Fig. 3.4.2 is the spectrum of pma dissolved in ethanol at 0^0 and measured immediately. This spectrum shows the well known broadening of the absorption maxima and a shift to a lower wavelength as the result of interaction with the solvent. The absorption maximum was found at 1635 cm^{-1}. Fig 3.4.1 shows the spectrum of in ethanol and stored at 0^o for 4 hours. A considerable peak absorption is observed at 1690 cm^{-1}.

The absorption at 1635 cm $^{-1}$ has decreased. In fig. 3.4 numbered 4 and 5 show the spectra of phenylacetaldehyde and of the diacetal of pma respectively in amounts that can be formed maximally from pma. The position and the size of these absorption maxima in the spectra in fig 3.4.3 do not suggest the conversion in one of these two compounds to some extent. In addition, the total amount of pma in the cuvette was hardly decreased as it was shown by the enzymatic assay mentioned. One may conclude that the decrease of the concentration of enol-pma, as observed with the $FeCL_3$ assay is accompanied with the formation of a compound with a maximum absorption at 1690 cm^{-1}. Since an absorption in that area is expected for the carbonyl group van keto-pma in ethanol, this provides direct evidence for the shift of the keto-enol equilibrium in a pma solution in ethanol at 0^0 in favor of the keto form.

Fig 3.4

Spectral data indicating the formation of keto-pma



The infra-red spectrum with 100% transmission T on the Y axis is recorded for 2000-1500 cm^{-1} (5.0-6.6 μ) at 0^0 for the following samples (concentration 0.2 M).

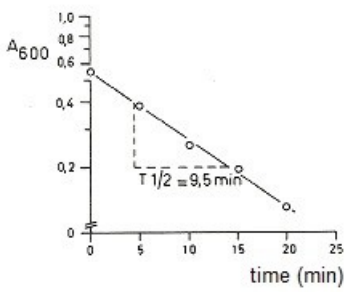
pma in ether
pma in ethanol at 0^0 (measured directly)
same (measured after 4 hours)
phenylacetaldehyde in ethanol
diethyl acetal of pma in ethanol

3.4.3 Rate of the tautomeric rearrangement in aqueous solvent

The speed of the conversion of enol-pma into keto-pma has been studied in aqueous solvent under conditions of the assay of the TDH and PDC enzymatic activity, using the quantitative enol assay. The buffer was tris-HCl because phosphate buffer reacts with the $FeCl_3$ in the assay. During fixed periods of time, tubes containing 0.40 ml 0.2 M tris-HCl buffer (pH7.5) and 20 µL enol-pma in ether (10 mg/ml) were incubated at 25^0 C. Then 2.55 ml ferric chloride regent (2.13) was added. The amount of enol-pma was calculated from the absorption at 600 nm. Fig. 3.5 shows the enol concentration plotted semi-logarithmically against the time. The enol-pma is converted according to a first order reaction with a reaction constant k = 0.073 min^{-1}. Half-life T1/2 = 9.5 min. The data presented in chapter 6 will show that also in this solvent the disappearance of enol-pma is accompanied with the formation of an equal amount of keto-pma. Therefore, the reaction constant mentioned regards the rate of the tautomeric rearrangement in aqueous condition.

Fig. 3.5

Effect of incubation in buffer on the enol content of pma



Enol-pma was incubated in 0.4 ml (0.2M) tris-HCl pH 7.5 during various periods of time. Thereafter 2.55 ml $FeCl_3$ was added and the enol content assayed according to chapter 2.13.