

STUDIES ON THE ISOMERS OF PHENYLHYDRAZONES OF SOME ALIPHATIC α -KETO ACIDS

BY

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ABSTRACT

Studies were done on the isomers of phenylhydrazones of some aliphatic α -keto acids by thin layer chromatography, spectrographic analysis, and other methods. It was found that each α -keto acid gives two different isomers of phenylhydrazones, *syn*- and *anti*-isomers, with some exceptions. In visible and ultraviolet regions, absorption maximum of the *syn*-isomer appeared at a longer wavelength than that of the *anti*-isomer in ethyl acetate and in aqueous 0.1 M NaHCO₃ solution. In infrared region, N-H and C=O stretching bands of the *syn*-isomer existed at longer wavelengths than the corresponding bands of the *anti*-isomer, respectively, as in the case of isomers of their 2,4-dinitrophenylhydrazones. This difference in absorption bands of the two isomers was presumed to be attributable to the formation of a hydrogen bond between C=O and N-H groups in the *syn*-isomer.

Introduction

For the identification of α -keto acids, which are important metabolites in living organisms, 2,4-dinitrophenylhydrazine method has most widely been used for the following reasons: The reagent easily and quantitatively reacts with α -keto acid to form a scarcely soluble hydrazone derivative, which is most stable among other hydrazone derivatives and can be detected easily on chromatography because of its yellow color. However, there exists some inconvenience in the 2,4-dinitrophenylhydrazone method because the absorbance due to the reagent at the wavelength appropriate for the determination is not negligible.

In contrast, phenylhydrazine is colorless and has a negligible absorbance at longer wavelengths, though it has some disadvantages as a reagent. Therefore, it may be possible to use phenylhydrazine for the determination of some kinds of carbonyl compounds which have a considerable absorbance at longer wavelengths. In fact, for the determination of pyridoxal phosphate, phenylhydrazine has successfully been used (1).

So far as reported, phenylhydrazones of most α -keto acids are known colorless. However, on the isomers of pyruvic acid phenylhydrazone (PA-PHZ), Hayashi (2) reported the existence of two different isomers, yellow and colorless. From the above-mentioned observations, it is presumed that the color of phenylhydrazone is closely related to the

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configuration of the isomer. However, the isomers of phenylhydrazones of α -keto acids seem not to have been studied except for the studies by Hayashi. On the other hand, studies on the isomers of 2,4-dinitrophenylhydrazones of α -keto acids have been performed in considerable detail (3-8). For example, it was established by Katsuki *et al.* (7,8), that 2,4-dinitrophenylhydrazone of α -keto acid, such as glyoxylic acid (GA), pyruvic acid (PA), 2-oxobutyric acid (OBA), 2-oxovaleric acid (OVA) or 2-oxocaproic acid (OCA) has two different isomers, *syn*- and *anti*-isomers. They were reported to have differences in their behaviors on chromatography, visible and infrared spectra and in some other properties.

The present study is related to separation of isomers of phenylhydrazones of some aliphatic α -keto acids, examination of their properties, and to the assignment of their isomers. These studies were started as a fundamental investigation for the determination of some carbonyl compounds by the use of phenylhydrazine reagent.

EXPERIMENTAL

Studies were performed on phenylhydrazones of the following nine α -keto acids: GA, PA, OBA, OVA, OCA, 2-oxo-3-methylvaleric acid (OMVA), 2-oxoisovaleric acid (OIVA), 2-oxo-3,3-dimethylbutyric acid (ODMBA) and mesoxalic acid (MOA).

Keto Acid

GA was prepared by the oxidation of tartaric acid with periodic acid (9). PA was purchased from Tokyo Kasei Chemical Company. OBA and OIVA were purchased from Sigma Chemical Company. OVA, OCA and OMVA were synthesized by the condensation of corresponding fatty acid esters with diethyl oxalate in the presence of sodium ethoxide, followed by hydrolysis with hydrochloric acid according to Adicks and Andersen's method (10). ODMBA was prepared by the oxidation of pinacolone with KMnO_4 followed by extraction with diethyl ether (11). MOA was prepared by hydrolysis of mesoxalic acid diethylester with hydrochloric acid. Mesoxalic acid diethylester was a generous gift of Prof. I. Hayashi.

Preparation of Phenylhydrazone

Phenylhydrazine hydrochloride solution (10 g in 1 liter of 2 N hydrochloric acid) was added drop by drop with shaking to a water (or alcohol) solution of carbonyl compound. The mixture was allowed to stand for few hours. The precipitate formed was filtered and washed with 0.5 N hydrochloric acid and then with a small amount of distilled water. After the crystals were dried in a desiccator under reduced pressure, it was recrystallized from ethyl acetate, benzene or ether.

Separation of Phenylhydrazone Isomer

When phenylhydrazine is allowed to react with α -keto acid according to the usual method (12), only one isomer, usually with no color (W-form), is obtained. However, the other isomer was obtained by treating the above hydrazone as follows: Phenylhydrazone, 300 mg, is dissolved into 50 ml of ethyl acetate saturated with 6 N HCl, and the solution is allowed to stand for 30 min at room temperature. The fraction of the other isomer increased rapidly during this treatment (this isomer is expressed as Y-form). Then, the solution was shaken with excess amounts of 1 M NaHCO_3 solution, and the precipitate formed was filtered and washed twice with distilled water. This operation was carried out as quickly as possible. It was dried on sulfuric acid in a vacuum desiccator and recrystallized from benzene or

ethyl acetate immediately. All the crystals obtained by isomerization were yellow.

Thin Layer Chromatography

Wakogel B5 (Wako Pure Chemical Industries Co., Ltd.) was used as the adsorbents. The chromatographic solvent system used was: ethyl acetate saturated with 0.1 M NaHCO_3 /methanol (5:1). The phenylhydrazones were detected by ultraviolet light of short wavelengths (F1-3L, type, Tokyo Shibaura electric Co., Ltd.).

Absorption Spectra

The measurement of absorption spectra in visible and ultraviolet regions of the phenylhydrazones were performed with a Hitachi Perkin-Elmer 139 UV-Spectrophotometer and a Hitachi Double Beam Spectrophotometer, Model 124, respectively. The infrared absorption spectra were taken by the KBr pellet method with a Perkin-Elmer Model 521 Grating IR Spectrophotometer.

RESULTS AND DISCUSSION

Determination of Purity of the Two Isomers of the Hydrazones

The purity of the two isomers of phenylhydrazones prepared by the method described above was confirmed by thin layer chromatography. The results are shown in Fig. 1. The spot of the W-isomer was always located at a position lower than that of the Y-isomer. With the increase in the number of carbon atoms of α -keto acids, R_s values of the spots increased (methyl red (MR) was used as an internal standard). Since Y-forms of GA-PHZ and OBA-PHZ were unstable, the conversion of the Y-form to the W-form occurred due to the isomerization while these were kept in a desiccator for a long time. Therefore, the Y-form was apt to be contaminated by small amounts of the W-form. As for OMVA-PHZ, OIVA-PHZ and ODMBA-PHZ, only one form was obtained.

As evidently can be seen from the results of chromatography (Fig. 1), the phenylhydrazones were found free from detectable impurities.

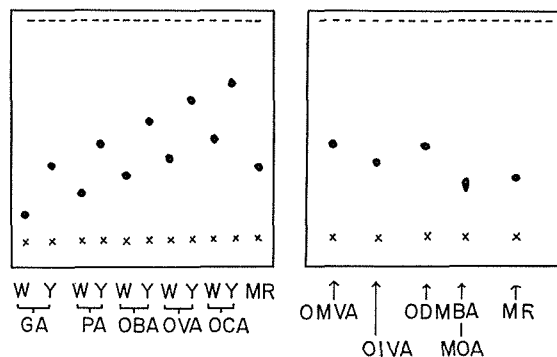


Fig. 1. Thin layer chromatogram of the phenylhydrazones.

The results of elemental analysis of the hydrazones are shown in Table 1. The experimental values well agreed with the calculated values. From these results, it is evident that these pairs of hydrazones were pure and different from each other.

Table 1. Elemental Analysis of the Phenylhydrazones

Phenylhydrazones of keto acids		C		H		N	
(form)		Calcd.	Found	Calcd.	Found	Calcd.	Found
GA	Y*		58.38		4.92		16.98
		58.53		4.91		17.06	
	W		58.38		4.83		17.06
PA	Y		60.92		5.69		15.52
		60.67		5.62		15.73	
	W		60.90		5.81		15.84
OBA	Y		62.73		6.08		14.55
		62.48		6.29		14.57	
	W		62.23		6.35		14.39
OVA	Y		63.96		6.92		13.28
		64.06		6.84		13.58	
	W		64.30		6.96		13.59
OCA	Y		65.71		7.08		12.76
		65.44		7.32		12.72	
	W		65.79		7.45		12.82
OMVA		65.44	65.32	7.32	7.38	12.72	12.47
OIVA		64.06	64.25	6.84	6.90	13.58	13.35
ODMBA		65.44	65.16	7.32	7.40	12.72	12.73
MOA		51.93	52.04	3.87	3.84	13.32	13.46

* Contaminated by some amounts of the W-form.

Table 2 shows melting points of both isomers of the phenylhydrazones. With the increase in carbon number of α -keto acid, melting point of the hydrazone decreased, except for the case of GA-PHZ. It can also be seen that melting point of the Y-form is higher than that of the W-form in the case of the lower keto acids and *vice versa* in the case of higher keto acids. This fact is quite similar to the behaviors of the two isomers of 2,4-dinitrophenylhydrazones of α -keto acids (8). Throughout this experiment, the measurement of melting point was carried out by the following method: The sample held in a capillary was immersed in a bath kept at a constant temperature and was examined whether it melts in a moment or not. At this measurement, the temperature of the bath with the sample was not raised. This procedure was repeated by raising temperature of the bath by few

Table 2. Melting points of the Phenylhydrazones

Phenylhydrazones of keto acids	Y-form (°C)	W-form (°C)
GA	125*	135
PA	155**	187**
OBA	144.5*	154
OVA	117.5	104
OCA	128	90
OMVA	136.5	—
OIVA	143	—
ODMBA	168	—
MOA	162	—

* Contaminated by some amounts of the W-form.

** These melting points accorded with the literature values (2).

degrees each time until true melting point was found. This method was used because isomerization often occurred while the measurement of the melting point was carried out. For example, melting point of Y-form of PA-PHZ was 155°C but the yellow color of the compound disappeared when the sample was allowed to stand in a bath at 140°C. When melting point of the heated sample was measured again after cooling to the room temperature, it showed 187°C. This melting point accorded with that of the W-form. Inspection of the product by thin layer chromatography revealed that this colorless crystal did not contain any impure substance, and accorded well with R_s value of the W-form.

Ultraviolet Absorption Spectra

Figure 2 shows absorption spectra of Y-forms of PA-PHZ dissolved in ethyl acetate and in aqueous 0.1 M NaHCO_3 . The wavelength of absorption maximum of the Y-form was longer than that of the W-form.

Table 3 shows absorption maxima of the other phenylhydrazones. On all the hydrazones, the wavelengths showing absorption maxima of the Y-forms were longer than those of the W-forms without exception. All of OMVA-PHZ, OIVA-PHZ, ODMBA-PHZ and MOA-PHZ showed absorption spectra of the Y-form-type.

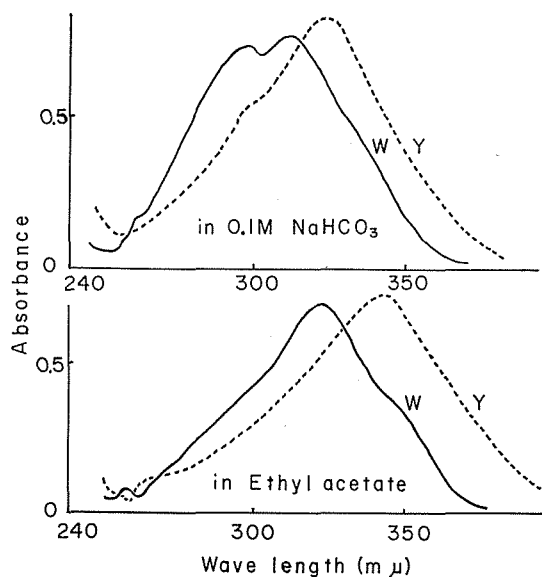


Fig. 2. Absorption spectra of Y- and W-forms of PA phenylhydrazone in 0.1 M NaHCO_3 and in ethyl acetate.

Table 3. Absorption spectra of the Phenylhydrazones in Ultraviolet and Visible regions

Phenylhydrazones of keto acids	(form)	λ_{\max} in Ethyl acetate	λ_{\max} in 0.1M NaHCO ₃
GA	Y	—	—
	W	336	324.5
PA	Y	342	325.4
	W	317	311.5, 290.5
OBA	Y*	345	324.8
	W	318	312.8 291.0
OVA	Y	345	322.3
	W	318	312.8 290.2
OCA	Y	345	323
	W	318	312, 290.2
OMVA		345	318.2, 286.8
OIVA		345	317, 283
ODMBA		340	275
MOA		357	326

* Contaminated by some amounts of the W-form.

Infrared Absorption Spectra

Figures 3, 4 and 5 show infrared absorption spectra of the Y- and W-forms of the hydrazones in the region of 4,000 to 300 cm^{-1} .

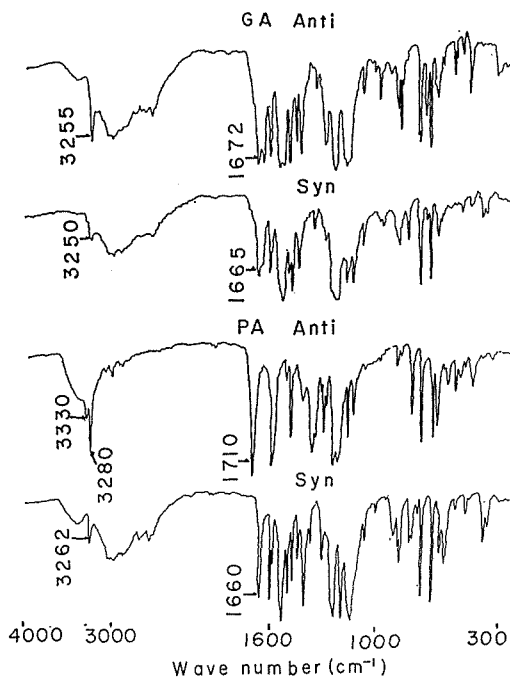


Fig. 3. Infrared spectra of the isomers of the phenylhydrazones (I).

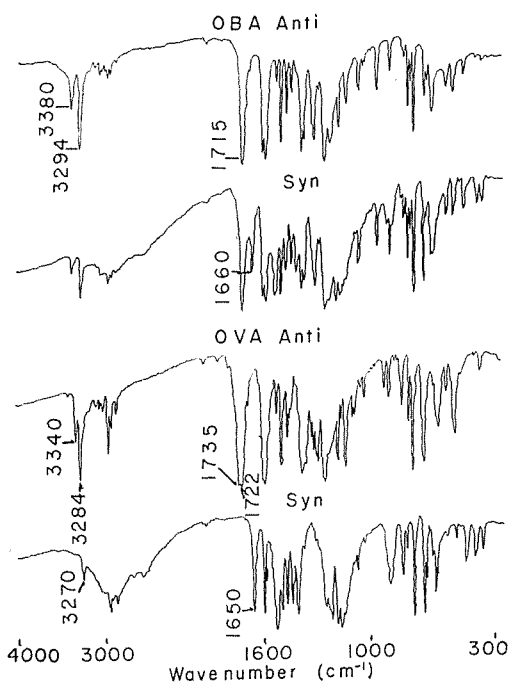


Fig. 4. Infrared spectra of the isomers of the phenylhydrazones (II). *Syn*-isomer of OBA-PHZ was contaminated by the *anti*-isomer.

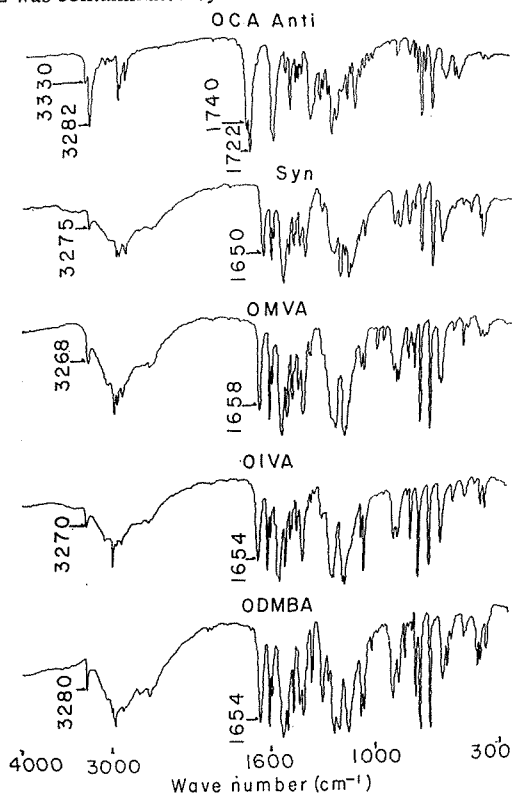
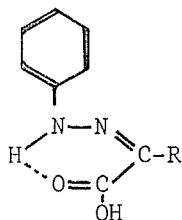


Fig. 5. Infrared spectra of the isomers of the phenylhydrazones (III).

As N-H stretching band, each W-isomer of PA-PHZ, OBA-PHZ, OVA-PHZ and OCA-PHZ showed two absorption bands in the region of 3,500 to 3,200 cm^{-1} , whereas each Y-isomer of these hydrazones except that of OBA-PHZ*¹) showed a single band which was at a longer wavelength than the corresponding band of the W-isomer. Each of W-isomers of PA-PHZ and OBA-PHZ showed a single C=O stretching band and each of W-isomers of GA-PHZ, OVA-PHZ and OCA-PHZ showed two absorption bands as C=O stretching band in the region of 1,750 to 1,640 cm^{-1} . On the other hand, each of all the Y-isomers of these hydrazones except that of OBA-PHZ*¹) showed a single C=O stretching band, which was at a longer wavelength than the corresponding band of the W-isomer.

From these results, it can be summarized that each absorption band of C=O and N-H groups of the Y-form is at a longer wavelength than the corresponding bands of the W-isomer. This difference, judging from the analogy to their 2,4-dinitrophenylhydrazone derivatives (7), is attributable to the formation of a hydrogen bond between C=O and N-H groups of the Y-isomer as follows:



OIVA-PHZ, OMVA-PHZ and ODBA-PHZ showed characteristic absorption bands of the Y-form as the stretching bands for C=O and N-H groups.

Assignment of Y- and W-forms

From the results of spectrographic analysis of 2,4-dinitrophenylhydrazones of 2-methoxy carbonyl compounds (for example, 2-methoxypropiophenone), Ramirez *et al.* (13) showed that N-H absorption band of the *syn*-form appears at 3,200 cm^{-1} due to the formation of an intramolecular hydrogen bond of N-HO type between N-H and $\text{CH}_3\text{-O}$ groups on the same side of C=N bond.

Similar observations were made on *syn*-isomers of 2,4-dinitrophenylhydrazones of 2-oxopantolactone (5), phenylglyoxylic acid (6) and some aliphatic α -keto acids (3) or substituted hydrazone derivatives of ethyl-4-cyano-2-oxobutyrate (14).

It was concluded by these studies that a hydrogen bond is formed between C=O and N-H groups in them just like the case of the Y-isomers of α -keto acid phenylhydrazones as described above.

From the visible-, ultraviolet- and infrared spectra and other behaviors described above, it is evident that Y-form of phenylhydrazones of α -keto acids can be assigned to the *syn*-isomer and W-form to the *anti*-isomer.

In addition, it was observed from the experiments shown above that the isomerization from the *anti*-form to the *syn*-form occurred in organic solvent in the presence of hydrogen ion and that the isomerization from the *syn*-form to the *anti*-form occurred during heat treatment similarly to the case of 2,4-dinitrophenylhydrazone derivatives.

* Y-Isomer of OBA-PHZ showed two respective bands as N-H and C=O stretching bands but not one band each, but they are presumably due to the contamination by the W-isomer.

These properties of both isomers of phenylhydrazones of α -keto acids were similar to those of both isomers of 2,4-dinitrophenylhydrazones, respectively, except that the phenylhydrazone derivatives were more unstable than the 2,4-dinitrophenylhydrazones.

As described before, phenylhydrazine might be used more advantageously than 2,4-dinitrophenylhydrazine for the determination of special kinds of carbonyl compounds. Studies are now in progress based on this idea.

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