A New Keto Acid, 5-Acetoxy-4-ketohexanoic Acid, Formed from Ethanol by Yeast

By Takashi HIRABAYASHI and Tokuya HARADA

*The Institute of Scientific and Industrial Research, Osaka University,
Suita-shi, Osaka, Japan*

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Of five strains of yeast tested, three which produced 5-hydroxy-4-ketohexanoic acid (HKH) were also found to form a new keto acid, when grown on medium containing ethanol as the sole carbon source. The new product was isolated in pure form and the structure was investigated by elementary analysis, infrared spectral analysis, NMR analysis and determination of its degradation product.

The compound was shown to be 5-acetoxy-4-ketohexanoic acid. 5-Acetoxy-4-ketohexanoic acid (AKH) was synthesized and found to be identical with the isolated product.

In our previous papers,^{1,2)} certain strains of yeasts were demonstrated to produce a large amounts of cells and 5-hydroxy-4-ketohexanoic acid (HKH) from ethanol.

These strains have also been found to form a new keto acid from ethanol. The present paper describes the production, isolation, chemical properties and structure of this compound and its identification as 5-acetoxy-4-ketohexanoic acid.

MATERIALS AND METHODS

Microorganism. The following organisms were used in this work: *Hansenula miso* IFO 0146, *Pichia mogii* IFO 0193, *Candida albicans* IFO 0583 and *Candida tropicalis* IFO 0006 (obtained from the Institute of Fermentation, Osaka); *Saccharomyces oviformis* WH 92 (obtained from the Institute of Suntory Co. Ltd.).

Cultures. The organism was maintained on a glucose (1%)-Bacto-peptone (1%)-yeast extract (0.5%) agar medium. Volumes of 95 ml of a medium in 500 ml shake-flasks were inoculated with 5 ml of seed culture grown in the same medium. Medium (a)

contained 150 mg of $(\text{NH}_4)_2\text{HPO}_4$, 100 mg of KH_2PO_4 , 50 mg of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1 mg each of NaCl , $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, and $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in 100 ml of distilled water and ethanol was added as the sole carbon source at the concentrations given in the results. The medium was adjusted to pH 7.2. Ethanol was filtered through a Seitz-filter and then added to the autoclaved medium. Medium (b) supplements medium (a) with 0.02 g of yeast extract. Medium (a) was used for culture of *Hansenula miso* IFO 0146 and *Pichia mogii* IFO 0193 and medium (b) for culture of *Candida albicans* IFO 0583, *Candida tropicalis* IFO 0006 and *Saccharomyces oviformis* WH 92. The culture was shaken reciprocally at 120 strokes per min at an amplitude of 7 cm at 30°C.

Paper chromatography. Paper chromatography was carried out by the descending technique on Toyo-Roshi No. 52 paper. For development the following solvent systems were used; *n*-butanol:acetic acid: water (4:1:5 v/v) (solvent A); *n*-butanol:pyridine: water (6:4:3 v/v) (solvent B). Bromo-cresol-green and ammoniacal silver nitrate reagents were used for detection of acidic and reducing matters, respectively.

Infrared analysis. Infrared analysis of the substances as liquid film was done in an S-type infrared spectrophotometer of Japan Spectroscopic Co. Ltd.

Nuclear magnetic resonance. NMR analysis of the substance as 10% solution in CDCl_3 with tetramethylsilane as the internal standard was done at a sweep

1) T. Harada and T. Hirabayashi, *Agr. Biol. Chem.*, **32**, 1175 (1968).

2) T. Hirabayashi and T. Harada, *ibid.*, **33**, 276 (1969).

width of 500 cps at 60 mega cycles.

RESULTS AND DISCUSSION

Formation of a new keto acid from ethanol

The organisms were cultured for 6 days adding 4% ethanol initially and raising the concentration to 6% after 48 hr. When the yeasts (*Hansenula miso* IFO 0146, *Candida albicans* IFO 0583 and *Saccharomyces oviformis* WH 92) were used, HKH and an unidentified keto acid were detected by paper chromatography in the culture fluid. The R_f values of the unidentified keto acid were 0.92 and 0.83 with solvents A and B, respectively, while those of HKH were 0.82 and 0.65, respectively, as shown previously.²¹ The amount (ca. 20 mg/liter) of this keto acid formed was rather smaller than that (ca. 200 mg/liter) of HKH. Strains of yeasts which did not form HKH (*Pichia mogii* IFO 0193 and *Candida tropicalis* IFO 0006) did not form this keto acid.

Isolation of the keto acid

Hansenula miso IFO 0146 was used to isolate the keto acid. After four days incubation of culture medium adding 4% and 2% ethanol initially and after 48 hr, respectively, the culture fluid was centrifuged at 8000 rpm for 10 min. The supernatant (ca. 4 liters) was evaporated to 1/10 volume *in vacuo* and adjusted to pH 2.0 with sulphuric acid.

The solution was then continuously extracted with ether for 40 hr. The ether extract was evaporated to dryness *in vacuo* to remove ether. The residue was dissolved in 1 ml of water. The solution was placed on a cellulose column (3×30 cm) and the material was eluted with the solvent; *n*-butanol : formic acid : water (19 : 1 : 10 v/v). Fractions of eluate of 10 ml were collected with a fraction collector. The fractions which contained the material with a motility corresponding to that of the keto acid were evaporated to dryness. The residue was mixed with 1 ml

of chloroform and centrifuged. The resulting supernatant was chromatographed on a celite column (1×40 cm). The column was plugged with cotton and the material was eluted with 100 ml portions of 1, 5, 10 and 15% *n*-butanol in chloroform, both solvents being saturated with water after mixing. Appropriate fractions were collected in a fraction collector and evaporated to dryness (yield 80 mg). This material is a viscous oil, soluble in water, ethanol, chloroform and ether and insoluble in petroleum ether (bp 30~60°C).

The keto acid, $[\alpha]_D^{20} -18^\circ$ ($c=2$, water)

Anal. Found: C, 51.01; H, 6.30. Calcd. for $C_8H_{12}O_5$: C, 51.06; H, 6.38%.

Analysis of the infrared absorption spectrum and the nuclear magnetic resonance spectrum of the keto acid

The infrared absorption spectrum of the keto acid (liquid film) is shown in Fig. 1. The strong absorption band at 1250 cm^{-1} is typical for a disturbed stretching vibration of an ester group (CO-O-C). The spectrum showed absorption bands at 2990 and 2950 cm^{-1} for CH_2 groups, CH and CH_3 and at 1740 cm^{-1} for a free acid carbonyl group. The absence of absorption bands near 3400 cm^{-1} indicates the absence of a hydroxy radical.

The position of the protons on the carbon atoms of the keto acid was ascertained from analysis of NMR spectral data. The spectrum taken using 40 mg of the keto acid in CDCl_3 with 2% tetramethylsilane as standard is shown in Fig. 2. The doublet at $\delta=1.45\text{ ppm}$ indicates that a $-\text{CH}_3$ group is adjacent to a $-\text{CH}-$ group. The singlet at 2.18 ppm indicates that there is a $-\text{CH}_3$ group adjacent to a $-\text{CO}-$ group. The triplet at 2.8 ppm indicates that a $-\text{CH}_2\text{CH}_2-$ group is adjacent to carbons without hydrogen atoms. The quartet at 5.2 ppm indicates that a $-\text{CH}-$ group is adjacent to a $-\text{CH}_3$ group. There is a broad absorption near 8.0 ppm for the

FIG. 1. Infrared Spectra (in liquid film) of the Yeast Product and 5-Acetoxy-4-ketohexanoic Acid.

FIG. 2. NMR Spectrum of the Keto Acid Formed from Ethanol.

acidic H. The integral ratios are 3 : 3 : 4 : 1 : 1. On the basis of these results, the most reasonable structure of the keto acid is as follows:

Decomposition products of the keto acid

Twenty mg of the keto acid were dissolved in 0.5 ml of dry methanol and 0.15 ml of a freshly prepared solution of sodium methoxide (0.05 g of sodium dissolved in 10 ml of methanol) was added with stirring at a room temperature. When the reaction was complete

(20 min), a test drop of the suspension gave an alkaline reaction with litmus. The sodium was removed by stirring with a slight excess of Amberlite IR-120 (H^+) until the solution was neutral and the resin was filtered off. Half of the filtrate was acidified to pH 2.0 with sulphuric acid and steam distilled. The distillate was titrated with NaOH and concentrated to 1 ml. A *p*-bromophenacyl ester derivative which was prepared in the usual way from the concentrated matter and recrystallized from ethanol, showed mp 85°C (unchanged on addition of the derivative of authentic acetic acid). The distillate contained 41 μ moles of acetic acid, as shown by titration.

The other half of the filtrate was evaporated to a syrup and examined by paper chromatography. A spot developed, which gave an *R_f* value of 0.82 with solvent A and of 0.65 with solvent B. The *R_f* values were identical with those of HKH. HKH was determined by the method of Bloom *et al.*³⁾ and the filtrate was found to contain 39 μ moles. Positive identification of the acid as HKH was accomplished by preparing the *p*-phenylphenacyl ester. The crystals obtained melted at 131~132°C. There was no depression of

3) R. J. Bloom, P. G. Fuller, J. G. Westerfeld and W. W. Westerfeld, *Biochemistry*, **5**, 3211 (1966).

the mp on mixing them with the derivative of authentic HKH (yeast product).²⁾ Degradation studies showed that the keto acid was composed of equimolar quantities of HKH and acetic acid.

Synthesis of 5-acetoxy-4-ketohexanoic acid

The 5-hydroxy-4-ketohexanoic acid (100 mg) isolated from the broth was refluxed with acetic anhydride (6 ml) in the presence of sodium acetate (120 mg) for 2.5 hr. After cooling, the reaction mixture was poured into ice-cold water (50 ml) and the acetate was extracted with chloroform (50 ml $\times$ 3). The chloroform layer was washed with water, dried over sodium sulfate and then evaporated to dryness, yielding 85 mg of oil. The crude product was dissolved in chloroform and the solution was passed through a celite column as previously described for the isolated product. Fractions containing AKH were combined and evaporated to dryness (yield 30 mg).

This material was arbitrarily assumed to be the purest fraction.

5-Acetoxy-4-ketohexanoic acid $[\alpha]_D^{20} -18^\circ$ ($c=2$, water)

Anal. Found: C, 51.32; H, 6.53. Calcd. for $C_8H_{12}O_5$; C, 51.06; H, 6.38%.

The infrared spectrum of the synthetic AKH was identical with that of the isolated product, as shown in Fig. 1. NMR spectral analysis also indicated that the product was AKH. The melting point of its *p*-phenylphenacyl ester was 108°C and this was not depressed when the material was mixed with the ester of the isolated material. From these results the keto acid was identified as 5-acetoxy-4-ketohexanoic acid.

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