Isolation and Identification of 4-Hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2H)-furanone, as a Flavor Component in *Shoyu* (Soy Sauce)[†]

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Unstable compounds with intense sweet odor were isolated from *shoyu* and identified as 4-hydroxy-2-ethyl-5-methyl-3(2H)-furanone and 4-hydroxy-5-ethyl-2-methyl-3(2H)-furanone (tautomer ratio, about 3:2) on the basis of spectrometrical investigation. This may be the rare case of isolation of the tautomeric compounds from a natural product.

They were concluded to be major and important flavor components in *shoyu*.

In the previous paper,¹⁾ fifty compounds were presented as flavor components in *shoyu*. Although most of the comparatively large peaks on the chromatogram of a *shoyu* flavor concentrate were identified, some compounds greatly contributing to *shoyu* flavor still remain unknown.

The investigation described in this paper concerns the isolation of one such unknown compound from *shoyu* and the subsequent elucidation of its chemical structure. When a small amount of *shoyu*, for example 10 or 20 liters, was dealt with, the isolation of a sufficient amount of compound was unsuccessful because of its lower stability. Therefore, a fairly great amount of *shoyu* was used in order to get enough material for several kinds of spectra. The compound had a sweet odor similar to that of shortcake.

MATERIALS AND METHODS

Shoyu. Commercial regular *shoyu* was used for the preparation of a flavor concentrate.

Preparation of a flavor concentrate. The outline of the procedure for concentration and isolation is as

follows. *Shoyu*, 780 liters, was distilled at a sample temperature of 36°C under reduced pressure (20 mmHg) in a flash-vacuum-evaporator manufactured by Nichinan Kikai Co., Ltd. The distillate, 413 liters, was extracted with 350 liters of dichloromethane under homogenization. The dichloromethane extract was concentrated to a volume of about 5 liters at a sample temperature of 30°C under reduced pressure (20 mmHg) in a glass flash-vacuum-evaporator. The concentrate (about 5 liters) was dried over anhydrous sodium sulfate and filtered. The final concentration was carried out in a rotary-vacuum-evaporator to a volume of 67 ml at room temperature under reduced pressure (20 mmHg). The flavor concentrate thus obtained had an odor almost the same as that of the original *shoyu*. A typical gas chromatogram of the concentrate is shown in Fig. 1.

Isolation. The flavor concentrate, 67 ml, was fractionated by preparative gas chromatography on an FFAP column (3/8 inches i.d. × 2 m, aluminum) into two fractions (1 and 2). Fraction 2 contained the higher boiling point constituents. It was further fractionated by gas chromatography on an FFAP column (3 mm i.d. × 2 m, stainless) into four fractions (A through D). Fraction 2D consisted of one peak which contained one major compound. The fraction was purified by gas chromatography (FFAP column, 3 mm i.d. × 2 m, stainless). A total of 176 mg of purified fraction 2D was obtained as sticky oil which had a low tendency to crystallize. It exhibited a sweet, shortcake-like odor and this odor seemed to be important for the base of *shoyu* flavor. Immediately before the spectrometrical investigation, it was further purified by the same gas chromatographic method.

[†] Studies on Flavor Components in *Shoyu*. Part II. The main part of this work was presented at the Annual Meeting of the Agricultural Chemical Society of Japan at Sapporo, Japan, on July 24, 1975.

FIG. 1. Gas Chromatogram of *Shoyu* Flavor Concentrate.

Instrument: Shimadzu 4BM-PF (FID).

Column: polyester FF 10%+terephthalic acid 3%, 3 mm i.d. $\times$ 2 m, glass. Oven temp.: 50~180°C (3°C/min). Injector and detector temp.: 240°C. Carrier gas: N₂, 30 ml/min.

Preparative gas chromatography. The preparative gas chromatographic fractionation was carried out on a Varian Model 700 gas chromatograph with a thermal conductivity detector. The following conditions were applied: a 3/8 inches i.d. $\times$ 2 m aluminum column containing 10% FFAP on 60~80 mesh Diasolid L (Nihon Chromato Works, Ltd.), a helium flow rate of 100 ml/min, and an injection temperature of 250°C. The oven temperature was isothermal at 160°C for the effluence of fraction 1, and at 200°C for that of fraction 2. Volumes of 500~700 μ l of the flavor concentrate were injected and a total of 67 ml was fractionated.

Gas chromatography for fractionation and purification. A JEOL Model 750 gas chromatograph with a thermal conductivity detector was used for the second fractionation and purification. The conditions were as follows: a 3 mm i.d. $\times$ 2 m stainless steel column containing 10% FFAP on 60~80 mesh Diasolid L, a helium flow rate of 30 ml/min, and an injection temperature of 230°C. Oven temperature was programmed from 50 to 200°C at a rate of 4°C/min. Each fraction was trapped in a glass tube cooled in a dry ice-ethanol mixture.

High-resolution mass spectrometry (High-MS). High-MS analyses were made on a Hitachi Model RMU-7M double focusing mass spectrometer with a computer, Model HITAC 10. The mass spectrometer was operated under the following conditions: an ionization

electron energy of 70 eV, an ion accelerating voltage of 4.6 kV (m/e max 1000), a total emission of 100 μ A, an ion source temperature of 100°C, and a sample heater temperature of 60°C for direct inlet system. Perfluorokerosene was used as the internal standard, and resolution was set at about 10,000. Elemental compositions of fragment ions and the molecular ion were determined with the high-MS.

Ultraviolet absorption analysis (UV). UV spectra were determined on a Hitachi Model 323 recording spectrophotometer in cells with an optical path length of 1 cm. Samples were dissolved in ethanol and analyzed.

Infrared analysis (IR). IR spectra were recorded on a Hitachi Model EPI-G3 grating infrared spectrophotometer.

Nuclear magnetic resonance spectroscopy (NMR). NMR spectra were obtained with a pulsed-NMR, JEOL Model JNM-PS-100 (100 MHz) with a computer which controlled the pulsing unit, collected the resultant signal, averaged successive pulse results, and finally, performed the Fourier transformation. The signal-to-noise enhancement is theoretically $\sqrt{N}$ with N being the number of sweeps made, thus 25 sweeps yield a fivefold enhancement. Tetramethylsilane was used as an internal standard. Solutions were made in CDCl₃.

Ferric chloride test. The test was conducted by the conventional method.

RESULTS AND DISCUSSION

Fraction 2D which was not identified in the previous paper¹⁾ consisted of one large peak on the gas chromatogram of *shoyu* flavor (Fig. 1). The peak was proved to contain one major compound by combined gas chromatography-mass spectrometry with a 0.25 mm i. d. $\times$ 30 m glass capillary column coated with PEG 20M.

An outstanding feature of this compound was its instability and attempts to carry out chemical transformations were unsuccessful. Thus, thin-layer chromatography on silica gel with various developing solvents indicated many spots and none of the which was major. This instability was one of the major factors which interfered with the various analyses. Accordingly, it was necessary to deal with a large amount of *shoyu*, 500~1000 liters, and purify the compound by gas chromatography immediately before each analysis.

Tentative identification

A tentative identification of this compound rested on the interpretation of one test and four spectra: UV, MS, IR and NMR.

The compound exhibited a UV absorption maximum at 290 nm. The spectrum indicated that the molecule contained a conjugated system (Fig. 2).

FIG. 2. UV Spectrum of Isolated HEMF.

The high-MS revealed a molecular formula $C_7H_{10}O_3$. The high intensity of the parent peak led to the assumption that the structure was cyclic (Fig. 3).

The IR spectrum showed a strong broad hydroxyl band at 3250 cm^{-1} , a strong carbonyl band at 1705 cm^{-1} and a very strong broad band at 1625 cm^{-1} ; the positions and relative intensities of the latter two bands suggested a conjugated carbonyl group, $C=C-C=O$. The absence of bands at $1720\sim 1820\text{ cm}^{-1}$ indicated that the compound did not contain a lactone group (Fig. 4).

FIG. 3. MS Spectrum of Isolated HEMF and Suggested Fragmentation.

The NMR spectrum showed complicated signals which seemed to have resulted from two compounds. The ends, $-CH_3$, of two ethyl groups were respectively recognized on the basis of two triplets with different intensities at 0.98δ (ppm, $J=7\text{ Hz}$) and 1.25δ ($J=7.5$) (Fig. 5).

The ferric chloride test was positive (purple). Functional groups of phenol, enol, oxime and

FIG. 4. IR Spectrum of Isolated HEMF (film).

FIG. 5. NMR Spectrum of Isolated HEMF (in CDCl_3).

hydroxamic acid were proposed. Phenol, oxime and hydroxamic acid groups of them were discarded because of the absence of the corresponding MS fragment ions. It was, hence, indicated that the molecule contained an enol group, $\text{C}=\text{C}-\text{OH}$.

The index of hydrogen deficiency for the molecular formula $\text{C}_7\text{H}_{10}\text{O}_3$ was 3 and it was accounted for by one ring and one conjugated carbonyl group described above. Therefore, the $\text{C}=\text{C}$ bond of the conjugated carbonyl group was identical with that of the enol one.

These results resembled those of a component of pineapple,^{2,3)} beef,⁴⁾ popcorn⁵⁾ and roasted almond⁶⁾ flavors which was identified as 4-hydroxy-2,5-dimethyl-3(2H)-furanone ($\text{C}_6\text{H}_8\text{O}_3$, abbreviated to HDMF). The UV absorption maximum, the prominent features on IR spectrum and the fragment pattern on MS spectrum of the compound in question were very similar to those of HDMF (UV $\lambda_{\text{max}}^{\text{EtOH}}$: 289 nm).

Consequently, the structures of proton tautomers seemed to be reasonable (Fig. 5). When coexistence of tautomers was considered, the low tendency to crystallize was satisfactorily

explained.

Identification and elucidation of structure

The same compounds (A+B) were synthesized from 3-heptyne-2,5-diol by Re *et al.*⁷⁾ who also reported the syntheses of 4-hydroxy-5-methyl-3(2H)-furanone and 4-hydroxy-2,5-dimethyl-3(2H)-furanone. To confirm the identity of the isolated compounds (A+B), the representative values of their spectra were compared with those of the spectra given by Re *et al.* The comparison is presented in Table I. Both compounds were found to be identical. Consequently, the compounds isolated from *shoyu* were confirmed to be tautomers A+B, 4-hydroxy-2-ethyl-5-methyl-3(2H)-furanone and 4-hydroxy-5-ethyl-2-methyl-3(2H)-furanone (abbreviated to HEMF). De Rijke and Boelens⁸⁾ also synthesized HEMF from *tert*-butyl acetoacetate and 2-bromobutanoic acid.

The structures can be rationalized by the following elucidation of spectra. The observed UV maximum value, 290 nm, is in fair agreement with calculated value, 292 nm; simple empirical calculation is as follows: 215 (a base wavelength for the parent chromophore, acyclic unsaturated carbonyl) + 35 ($\alpha\text{-OH}$) + 30 ($\beta\text{-OR}$) + 12 ($\beta\text{-R}$) = 292 nm.

The prominent structural features of the IR spectrum are described in the tentative identification.

Each signal on the NMR spectrum is interpreted in Fig. 5. The tautomerism ratio of A to B was calculated at about 3:2 on the basis of proton intensities of the ethyl group ends.

The principal fragmentation in mass spectrometry is suggested in Fig. 3. It was based on empirical formulas of the fragment ions determined by the high-MS.

Re *et al.*⁷⁾ furthermore described that the synthetic HEMF possessed the striking flavor of cooked fruits. The isolated HEMF also has an intense sweet odor similar to that of shortcake. HEMF is one of the major flavor components in *shoyu* and seems to be one of the important components for *shoyu* flavor.

TABLE I. COMPARISON OF SPECTRAL DATA OF ISOLATED AND SYNTHESIZED HEMF

		Isolated	Synthesized ⁷⁾
UV (in EtOH)		290 nm	293 nm
IR (cm ⁻¹)		3250 (s)	3220 (s)
		1705 (s)	1690 (s)
		1625 (s)	1620 (s)
		1460 (m)	1445 (m)
		980 (m)	968 (m)
		935 (m)	925 (m)
MS (70 eV)	<i>m/e</i>	Relative intensities	
	142 (M ⁺)	94%	66%
	99	28	18
	71	40	42
	57	84	97
	43	100	100
NMR δ ppm (<i>J</i> : Hz)		in CDCl ₃	in CCl ₄
	A	0.98 (3H, t, 7)	0.97 (3H, t, 7)
		1.63~2.13 (2H, m)	1.53~2.10 (2H, m)
		2.26 (3H, d, 1)	2.23 (3H, d, 1)
		4.38 (1H, t, 6)	4.32 (1H, tq, $J_1=5.5$, $J_2 \div 1$)
	B	1.25 (3H, t, 7.5)	1.25 (3H, t, 7.5)
		1.44 (3H, d, 7)	1.42 (3H, d, 7)
		2.63 (2H, q, 7.5)	2.62 (2H, q, 7.5)
		4.47 (1H, q, 7)	4.43 (1H, q, 7)

Isolation of HEMF from a natural product was described here for the first time.

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