# Chemotaxonomical Studies of the Leaf Oils of *L. umbellata* Thunb.

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Chemotaxonomy, monoterpenes

*L. umbellata* Thunb. (Kuromoji in Japanese) which belongs to the *Lauraceae* family, is deciduous shrub and grows on the mountainous distinct all over Japan. The twigs have been used for the tooth picks since they have a fragrant odor. Before World War II, the essential oil (Kuromoji-yu) had been used for the perfume of the soap because it contains large amounts of linalool. The oil, however, has not been isolated at present.

Kuromoji is sometime divided into several varieties on the basis of morphological differences. According to the expert opinion of an authoritative, Kuromoji divided into several species and their subspecies.

Kuromoji
1. Kuromoji- *L. umbellata* Thunb.,
   Himekuromoji- *var. membraceae* (Maxim.) Momiyama,
   Obakuromoji- *var. lancea* Momiyama.
2. Kekuromoji- *L. sericea* (Sieb. et Zucc.) Blume,
   Usugekuromoji- *var. glabrata* Blume.

The morphological distinction of these five species, however is not clear. In the previous communication[1], we reported the chemotaxonomy of Kuromoji, Kekuromoji, and Usugekuromoji. In addition to the paper, the leaf oils of Himekuromoji and Obakuromoji belonging to the subspecies of Kuromoji were examined from the view point of chemotaxonomy.

The leaf oils were isolated from the fresh leaves by steam distillation. The main individual terpene constituents were isolated by column chromatography followed by preparative gas chromatography and identified by IR spectrum and by gas chromatography by comparison with authentic specimens.

The results of the investigation are shown in Table I. The percentage of the constituents was calculated from the areas of the peaks of gas chromatograms. The main constituents of the plants are following: Kuromoji 1,8-cineole and limonene 37.3 %, linalool 28.6%; Himekuromoji 1,8-cineole and limonene 29.2%, linalool 18.2%, carvone 23.7%; Obakuromoji 1,8-cineole and limonene 35.9 %, linalool 22.9%, caryophyllene 7.8%. We named those three Kuromojis Linalool-kuromoji, Carvone-kuromoji, and Caryophyllene-kuromoji respectively on the basis of the characteristic constituents. It seems reasonable that Carvone-kuromoji derived from Linaloolkuromoji by the biogenetic oxidation of limonene to carvone.

Table I. The compositions of the essential oils of *L. umbellata* Thunb. A: Kuromoji; B: Himekuromoji; C: Obakuromoji D: Kekuromoji; E: Usugekuromoji.

| Compound | $t_R$ [min] | Samples A | B | C [%] | D | E |
|---|---|---|---|---|---|---|
| α-pinene | 2.5 | 7.1 | 5.1 | 6.0 | 11.2 | 11.1 |
| camphene | 2.7 | 4.4 | 3.3 | 3.0 | 5.1 | 10.8 |
| β-pinene | 3.1 | 1.3 | 0.8 | 1.0 | 3.6 | 5.8 |
| $\Delta^3$-carene | 3.3 | 0.7 | 1.0 | 1.0 | 1.6 | 1.2 |
| myrcene | 3.5 | 1.6 | 1.4 | 1.1 | 2.0 | — |
| limonene 1,8-cineole | 3.9 | 37.3 | 29.2 | 35.9 | 50.3 | 3.3 |
| γ-terpinene | 4.4 | 4.9 | 2.2 | 4.0 | 2.6 | 1.4 |
| linalool | 5.3 | 28.6 | 18.2 | 22.9 | 7.1 | 4.2 |
| unknown | 5.9 | 0.7 | 0.3 | — | — | — |
| borneol | 6.5 | 0.5 | 0.4 | 0.8 | 0.6 | — |
| unknown | 6.8 | 2.6 | 1.9 | 3.6 | 1.8 | — |
| unknown | 7.1 | 3.2 | 3.3 | 3.7 | 6.1 | 0.5 |
| carvone | 8.1 | — | 23.7 | 0.5 | 0.6 | 0.2 |
| unknown | 8.3 | 1.6 | — | — | — | — |
| geraniol | 8.6 | 1.3 | 3.2 | 0.8 | 0.6 | 0.3 |
| bornylacetate | 9.3 | 0.6 | 0.4 | 0.8 | 1.0 | 6.7 |
| unknown | 10.1 | 1.6 | — | — | — | 1.8 |
| unknown | 10.3 | — | 0.1 | — | — | — |
| unknown | 10.7 | — | — | 1.5 | — | 1.2 |
| geranylacetate | 11.4 | 1.3 | 1.1 | 0.5 | 3.2 | 2.0 |
| unknown | 11.5 | — | — | — | — | 6.2 |
| unknown | 11.8 | — | 0.4 | 0.5 | — | 2.6 |
| caryophyllene | 12.4 | 0.2 | 1.1 | 7.8 | 1.2 | 13.3 |
| unknown | 13.2 | — | 0.3 | 0.8 | — | — |
| unknown | 13.8 | — | 0.9 | — | — | — |
| unknown | 14.0 | 0.2 | 1.1 | 2.5 | 1.0 | 1.9 |
| unknown | 15.1 | — | — | — | — | 0.6 |
| cadinene | 15.3 | 0.5 | 0.6 | 0.8 | 0.4 | 5.9 |
| unknown | 17.0 | — | 0.3 | 0.5 | — | 0.2 |
| unknown | 19.5 | — | — | — | — | 4.9 |
| unknown | 19.8 | — | — | — | — | 5.5 |
| unknown | 22.0 | — | — | — | — | 4.5 |
| unkownn | 23.0 | — | — | — | — | 2.0 |
| unknown | 23.5 | — | — | — | — | 2.5 |

As seen in Table I, the five species can be easily identified by means of chemical methods.

*Isolation of the leaf oils.* The fresh leaves cut in small pieces were subjected to steam distillation. The oils were extracted with ether and dried over anhydrous sodium sulfate. After the distillation of the ether, leaf oils were obtained (Fig. 1).

Sample A (Kuromoji): On September 7 in 1970, at Izunagaoka in Shizuoka Prefecture. 0.2 % yield, $n_D^{25}$ 1.4658, $\alpha_D^{25}$ -17.0°.

Sample B (Himekuromoji): On October 14, 1971, at Shinkhiro in Aichi Prefecture. 0.4 % yield, $n_D^{25}$ 1.4789, $\alpha_D^{25}$ +10.7°.

Sample C (Obakuromoji): On September 1, 1971, at Yonezawa in Yamagata Prefecture. 0.3% yield, $n_D^{25}$ 1.4717, $\alpha_D^{25}$ -20.5°.

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Fig. 1. Map of Investigation Areas (Japan).
● Caryophyllene-kuromoji;
○ Linalool-kuromoji;
▲ Carvone-kuromoji;
△ Kekuromoji;
◑ Usugekuromoji.

Sample D (Kekuromoji): On July 25, 1970, at Kuromoritoge in Ehime Prefecture. 0.23% yield, $n_D^{25}$ 1.4646, $\alpha_D^{25}$ -16.0°.

Sample E (Usugekuromoji): On october 16 in 1971, at Yoshiwa in Hiroshima Prefecture. 0.08% yield, $n_D^{25}$ 1.4873, $\alpha_D^{25}$ +27.4°.

*The analysis of the oils by gas chromatography.* For identification, gas chromatography was carried out with Hitachi Model K 53 gas chromatograph equipped with an ionization detector. The stainless steel column was packed with 5% SE-30 on Chromosorb W, temperature programmed from 70 to 250 °C.

[1] H. KOMAE, N. HAYASHI, S. KOSELA and T. ARATANI, Flavour Ind. **4**, 208 [1972].

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## Acid Hydrolysis of Colchicine and Related Compounds

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Colchicine, tropolones, kinetics

Acid-catalyzed hydrolysis of the methoxytropone moiety of colchicine has been shown to proceed at a greater rate than in the case of isocolchicine[1]. (Structures are shown in Table I.) No interpretation of this rate difference has been offered. And, while a mechanism for the acid-catalyzed hydrolysis of 2-methoxytropone has been proposed[2], no kinetic data on this type of reaction either for simple methoxytropones or on colchicine type compounds have been reported. Thus, the present investigation was undertaken to examine the effect of structural changes on the rate of acid hydrolysis of colchicine and to examine certain kinetic aspects of this reaction.

### Experimental

Colchicine was purified according to ASHLEY and HARRIS[3]. Other compounds were prepared according to literature procedures (Table I).

The analytical procedure for unhydrolyzed methoxytropones was formulated to take advantage of the salt-forming ability of tropolones in alkaline media. A sample (1 ml) of the acidic reaction mixture was diluted with an alkaline buffer (0.1 M NaOH and 0.1 M $NaH_2PO_4$, pH = 11.8, 5 ml) and extracted with three, 3 ml portions of chloroform. The chloroform extract was diluted to a suitable concentration and the absorbance of the solution, corresponding to the concentration of the unhydrolyzed compound, was measured at the appropriate wavelength (Table I). All compounds were run at 75 °C in 0.127 M HCl containing 5% acetonitrile. Ionic strength was kept constant at 0.15 by addition of KCl. All reactions were followed to at least 75% completion.

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### Results

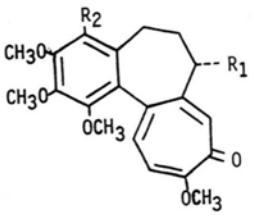
Rate constants determined for the acid hydrolysis of the various compounds are shown in Table I and represent pseudo-first order rate constants obtained from the slopes of log-concentration-versus-time plots.

Hydrolysis rate constants for colchicine, isocolchicine and 2-methoxytropone were determined over a ten-fold range of hydrogen-ion concentration at constant ionic strength and are shown in Table II. The slope of a log $k$ versus log $[H^+]$ plot was unity for each compound.

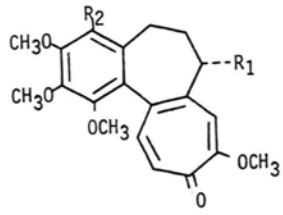
In order to determine the effect of an electron-withdrawing group on the rate of acid hydrolysis of colchicine, the 4-cyano derivative of colchicine was prepared. The cyano derivative was chosen since this substituent would be electron-withdrawing in the 4 position of colchicine which is *meta* to the bond joining the phenyl and tropolone rings. The rate of hydrolysis of 4-cyanocolchicine showed an increase over the rate of hydrolysis of colchicine.

Removal of the acetamido side chain from colchicine and isocolchicine, to give the corresponding desacetamido derivatives, was carried out to deter-

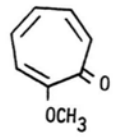
Table I. Summary of data for colchicine and related compounds.



1



2



3

| Compound | $R_1$ | $R_2$ | Hydrolysis rate [hours$^{-1}$][a] constants · $10^2$ | Wavelength used in assay [nm] | Reference for preparation |
|---|---|---|---|---|---|
| Colchicine (**1**) | $NHCOCH_3$ | H | 41.6 | 350.5 | 3 |
| Isocolchicine (**2**) | $NHCOCH_3$ | H | 13.7 | 343 | 4 |
| 4-Cyanocolchicine (**1**) | $NHCOCH_3$ | CN | 52.0 | 339 | 5 |
| Desacetamidocolchicine (**1**) | H | H | 13.0 | 346 | 6 |
| Desacetamidoisocolchicine (**2**) | H | H | 4.64 | 344 | 6 |
| 2-Methoxytropone (**3**) | — | — | 25.4 | 320 | 2 |

[a] In 0.127 M HCl containing 5% acetonitrile, 75 °C.

Table II. Pseudo first-order rate constants at various concentrations of HCl*.

| Concentration of HCl M/L | Rate Constants · $10^2$ [hours$^{-1}$] Colchicine | Isocolchicine | 2-Methoxytropone |
|---|---|---|---|
| 0.134 | 48.6 | 15.0 | 29.1 |
| 0.103 | 34.3 | 10.5 | 19.5 |
| 0.0656 | 24.1 | 6.9 | 14.4 |
| 0.0445 | 17.1 | 5.2 | 9.7 |
| 0.0298 | 11.3 | 3.4 | 6.6 |
| 0.0232 | 8.1 | 2.3 | 4.8 |
| 0.0138 | 6.2 | 1.7 | 3.2 |

* Solutions contained no acetonitrile. Rate constants were determined at 75 °C and at an ionic strength of 0.15.

mine the effect of this side chain on the hydrolysis rate. It can be seen that removal of the acetamido group decreased the hydrolysis rates of both colchicine and isocolchicine to the same extent.

## Discussion

A mechanism for the acid-catalyzed hydrolysis of 2-methoxytropone was suggested several years ago on the basis of product identification and spectral evidence for the existence of a tropylium ion intermediate[2].

The findings in the present investigation that the methoxytropones in Table I were all first order in substrate, and that colchicine, isocolchicine and 2-methoxytropone were first order in hydrogen ion over the range studied are consistent with the proposed mechanism.

The observed increase in hydrolysis rate upon substitution of the electron-withdrawing cyano group in the benzene ring of colchicine indicates that the rate determining step is influenced in a positive manner by an electron deficiency in the substrate. This behaviour would be expected if attack by the water molecule on the protonated tropylium ion were the slow step.

Examination of molecular models reveals that the acetamido group in isocolchicine could interfer with the approach of the attacking water molecule; this is not possible in the case of colchicine. That this type of steric hinderance by the acetamido group in isocolchicine is not a significant factor in the hydrolysis rate difference between colchicine and isocolchicine is shown by the fact that removal of the acetamido side chain of both colchicine and isocolchicine decreases the rates of hydrolysis equally in each case. It is likely, therefore, that electronic effects are more important in determining the hydrolysis rates of colchicine and isocolchicine than steric effects of the acetamido group. Thus, colchicine and isocolchicine may be considered as unsymmetrically substituted derivatives of 2-methoxytropone wherein the unequal effects of the substitutents result in different rates of hydrolysis.

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## Not Freezing Water in the Lamellar System of Chloroplasts

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Chloroplasts, water, PMR-spectroscopy

In the course of small angle X-ray scattering analyses Kreutz and Weber[1] observed that the particles of a soluble protein preparation derived from the lamellar system of chloroplasts by treatment with formic acid, exhibited an unusual high water content of 75 per cent. Viscosimetric investigation by Menke and Ruppel[2] with a preparation obtained by a somewhat modified procedure also pointed to a high hydration of the protein particles. A solution of this protein had a limiting viscosity number $[n]$ of 12 ml/g. With an axis ratio of 1:2.4 of the effective flattened hydrodynamic ellipsoid, also a hydration of 76 per cent was calculated. To our knowledge no investigations have been made on the water content and the state of water in the thylakoid membrane, from which the above mentioned preparations have been obtained. Kuntz, Brassfield, Law, and Purcell[3] observed by means of proton magnetic resonance spectroscopy, that in protein solutions a fraction of the water will not freeze even at —35 °C. The amount of the not freezing water is within the experimental error the same as the amount of hydration water, determined by other methods. It thus appeared obvious to determine with this method the amount of the not freezing water in the lamellar system of chloroplasts.

Stroma-freed chloroplasts of *Antirrhinum majus*, strain 50, were prepared according to an earlier described procedure[4]. The water signals were measured in the HR-mode with external side bands using a Varian HA 100 nuclear resonance spectrometer. For temperature regulation the temperature unit V 6040 was used. The temperature of the sample was determined by temperature depending comparison spectra of methanole. The samples were frozen at —35 °C in the sample holder at the measuring site. The concentration of the stroma-freed chloroplast suspension was 14-19 per cent. The area of the very small water signal, remaining after freezing, was the measure of the amount of not freezing water. These signals were compared with the corresponding signals of bovine serum albumin (Serva cryst., extra pure, > 98 per cent) and of lysozyme (Serva 3 x cryst., extra pure).

The signal area of the chloroplast preparation corresponded at —35 °C to 1.1 ± 0.1 times that of lysozyme and to 1.2 ± 0.1 times the area of bovine serum albumin. For lysozyme Kuntz *et al.*[3] obtained at —35 °C a value of 0.36 g of not freezing water per g protein. The corresponding value for bovine serum albumin was 0.37. Consequently, the stroma-freed chloroplasts contained at —35 °C 0.4 g of not freezing water per g lamellar system or 29 per cent. Hence, the thylakoid membrane and the soluble proteins contain approximately equal amounts of not freezing water. However, the water signal of stroma-freed chloroplasts is broader and lower than the signals of the proteins. From this it should not be necessarily concluded that the not freezing water in the thylakoid membrane is in a different state than in the two proteins. In addition it should be mentioned that not freezing water is not only detectable by proton magnetic resonance but also by means of calorimetry[5,6]. The conclusion from our results is that there is no reason to consider an especially high hydration when building models of the molecular structure of the thylakoid membrane.

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