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Further Study on Mutagenic Furoquinoline Alkaloids of Dictamni Radicis Cortex: Isolation of Skimmianine and High-Performance Liquid Chromatographic Analysis

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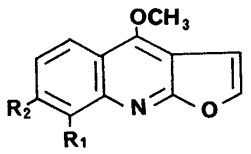
A mutagenic furoquinoline alkaloid, skimmianine, was isolated from Dictamni Radicis Cortex in addition to dictamnine and γ-fagarine. The clear-cut separation of these mutagenic furoquinoline alkaloids from this drug was accomplished by high-performance liquid chromatography (HPLC) on a reversed-phase column and the homogeneity of each peak was confirmed by means of gas chromatography-mass spectrometry. The conditions for the quantitative analysis of these furoquinoline alkaloids by HPLC, and application of this HPLC method to the analysis of the constituents of the commercial Dictamni Radicis Cortex are reported. The relationship between furoquinoline content and mutagenicity is also discussed.

Keywords——Dictamni Radicis Cortex; *Dictamnus dasycarpus*; furoquinoline alkaloid; dictamnine; γ-fagarine; skimmianine; mutagenicity; HPLC; quantitative analysis

Dictamni Radicis Cortex (the root of *Dictamnus albus* subsp. *dasycarpus*; Japanese name: Hakusen-pi; Rutaceae) is an active ingredient of Chinese medicines and has been used for the treatment of jaundice and skin diseases.

Previously, we reported that the mutagenic principles of this plant are represented by two furoquinoline alkaloids, dictamnine (**1**) and γ-fagarine (**2**).[1] Dictamnine has been isolated from this plant produced in Korea and China,[2] and from *Dictamnus albus* L. produced in Southern Europe.[3] As a continuation of this series of studies, the present paper reports the isolation of an additional mutagenic furoquinoline alkaloid, skimmianine (**3**). The separation of the furoquinoline alkaloids **1**—**3** by high-performance liquid chromatography (HPLC) and its application to the quantitative analysis of the mutagenic furoquinolines in this crude drug are also described.



	R_1	R_2
1	H	H
2	OCH_3	H
3	OCH_3	OCH_3

Chart 1

Experimental

General Procedure——Proton nuclear magnetic resonance (^{1}H-NMR) spectra were taken in $CDCl_3$ on a JNM GX-270 spectrometer (internal standard: tetramethylsilane (TMS)) at 270 MHz. The melting point of **3** was taken on a micro hot stage and is uncorrected. Infrared (IR), ultraviolet (UV) and mass spectra (MS) were measured with JASCO A 202, Hitachi 557 and JEOL JMS D-300 spectrometers, respectively.

Extraction and Separation of 3——Commercial Dictamni Radicis Cortex (1 kg) was repeatedly extracted with hot MeOH and the MeOH solution was concentrated to dryness. The MeOH extract was extracted with 5% HCl. The HCl layer was made alkaline with aqueous NH_4OH and extracted with $CHCl_3$. The $CHCl_3$ extract was

chromatographed on Sephadex LH-20 (5 cm i.d. × 30 cm) with EtOAc to give the mutagenic fraction. On silica gel column chromatography with cyclohexane–EtOAc (3 : 1) followed by preparative thin-layer chromatography (TLC) (on a plate of Kiesel gel 60 F_{254} S, 20 × 20 cm, Merck; solvent, cyclohexane–EtOAc (4 : 3)), this fraction afforded **3** in a yield of 0.0005%. **3**: colorless prisms (from C_6H_6–hexane), mp 174—175 °C (lit.[4] mp 176—177 °C). MS *m/z*: 259 (M^+). UV λ_{max}^{MeOH} nm: 210, 247. IR ν_{max}^{KBr} cm^{-1}: 1570, 1360, 1075. ^{1}H-NMR ($CDCl_3$) δ: 4.04 (3H, s), 4.12 (3H, s), 4.45 (3H, s), 7.06 (1H, d, J=3.0 Hz), 7.25 (1H, d, J=9 Hz), 7.60 (1H, d, J=3.0 Hz), 8.03 (1H, d, J=9 Hz).

Plant Materials Used for HPLC Analysis——Commercial Dictamni Radicis Cortex were purchased in Osaka (sample Nos. 1—3) and Tokyo (sample Nos. 4—6) markets in 1984—1985.

Authentic Samples——Authentic samples of **1** and **2** were extracted and purified according to the previous paper.[1] Authentic skimmianine was a generous gift from Dr. Ishii of Chiba University.

Extraction and Separation for Analysis——Dictamni Radicis Cortex (2 g) was extracted with hot MeOH (100 ml) for 5 min three times to ensure complete extraction of furoquinoline alkaloids. The combined MeOH extracts were concentrated to dryness *in vacuo*. A suspension of the residue in H_2O (20 ml) was extracted with EtOAc (15 ml) three times and the combined EtOAc layers were evaporated *in vacuo* to 5.0 ml.

For quantitative analysis, 1.0 ml of the EtOAc fraction was concentrated to dryness. The residue was dissolved in MeOH containing *o*-phenylphenol as an internal standard (0.4 mg/ml) (10 ml) and subjected to HPLC.

HPLC——A model M-6000A pump (Waters), a model U6K sample injection valve (Waters), a variable-wavelength UV detector (JASCO) and a variable-wavelength fluorescence detector (JASCO) were used. Column: TSK GEL ODS 120T (4.6 mm i.d. × 25 cm), prepacked. Injection volume: 5 μl. Mobile phase and flow rate: CH_3CN–CH_3OH–H_2O (30 : 17 : 53) at 1.0 ml/min. Detection: 244 nm (UV); excitative wavelength 333 nm and emissive wavelength 438 nm (fluorescence). The combined eluates of each peak (five times) were concentrated to dryness and the residue was subjected to gas chromatography-mass spectrometry (GC-MS).

GC-MS——A JEOL D-300 mass spectrometer coupled with a Yanaco G-2800 gas chromatograph was used. Column: 3% OV-17 on Chromosorb W AW DMCS 80—100 mesh (3 mm i.d. × 1 m). Column temperature: 190—240 °C at 5 °C/min. Carrier gas and flow rate: He at 20 ml/min. Ionization voltage and current: 70 eV, 300 μA. Ion source temperature: 210 °C. Scanning interval: 5 s. Retention times: **1**, 5.2 min; **2**, 10.3 min; **3**, 13.2 min.

Mutagenicity Assay——See the previous paper.[1]

Results and Discussion

A methanolic extract of Dictamni Radicis Cortex was extracted with 5% HCl. From this fraction, a furoquinoline alkaloid (**3**), in addition to **1** and **2**, was isolated by Sephadex LH-20 and silica gel column chromatography followed by preparative TLC. On the bases of UV, IR,

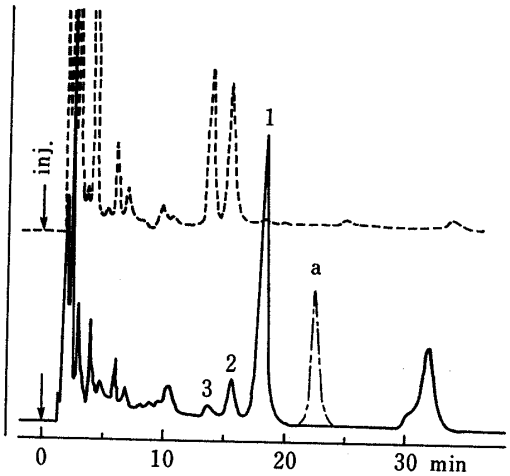


Fig. 1. High-Performance Liquid Chromatogram of the Ethyl Acetate Fraction of Dictamni Radicis Cortex

Column: TSK GEL ODS-120T (4.6 mm i.d. × 25 cm). Mobile phase: CH_3CN–CH_3OH–H_2O (30 : 17 : 53). Flow rate: 1 ml/min. Detector: ——, UV 244 nm (range 0.16); – – – –, fluorescence Ex. 333 nm, Em. 438 nm (range 16). 1, dictamnine (**1**); 2, γ-fagarine (**2**); 3, skimmianine (**3**). a: *o*-phenylphenol (internal standard).

Table I. Mutagenicities of Furoquinoline Alkaloids

	His$^+$ revertant colonies/μg	
	TA 100+S9	TA 98+S9
1	77	52
2	82	44
3	77	27

TABLE II. Contents (%) of Furoquinoline Alkaloids

Sample No.	n	1	2	3
1	3	0.037±0.001	0.006±0.000	0.002±0.000
2	3	0.035±0.001	0.005±0.000	0.001±0.000
3	3	0.044±0.001	0.006±0.000	0.002±0.000
4	3	0.032±0.001	0.005±0.000	0.001±0.000
5	3	0.063±0.001	0.009±0.000	0.002±0.000
6	3	0.090±0.001	0.009±0.000	0.002±0.000

electron impact mass (EI-MS) and NMR spectra, as well as comparison of other physical constants, **3** was identified as skimmianine, which is widely distributed in rutaceous plants.[4,5]

The mutagenicity of **3** in the presence of S9 mix was as strong as that of **1** or **2** towards *Salmonella typhimurium* TA 100 (77 revertant colonies per μg), but about half as strong towards TA 98 (27 revertant colonies per μg), while **3** showed only a weak activity without S9 mix, as in the case of **1** or **2** (Table I). It seems likely that the number of methoxyl groups on the furoquinoline skeleton is less important for the manifestation of base change-type mutagenicity, but has definite significance in relation to frame shift-type mutagenicity.

In order to determine the relationship between the content of these furoquinolines and the mutagenicity of this drug, quantitative analysis of **1**—**3** was conducted. A suspension of the methanolic extract of this crude drug was extracted with ethyl acetate. HPLC of the ethyl acetate fraction on a reversed-phase column of octadecylsilylated silica gel using CH_3CN–CH_3OH–H_2O (30 : 17 : 53) as a mobile phase gave excellent separation of the three furoquinoline alkaloids, as shown in Fig. 1.

The identity and purity of each HPLC peak in the present study were confirmed as follows. The spectra and retention times in GC-MS of the three HPLC peaks were found to be identical with those of corresponding authentic samples. Because **2** and **3** are fluorescent, the ratios of the peak height of UV absorbance to that of fluorescence intensity were compared with those of authentic samples, and the results indicated the purity of each peak.

For quantitative analysis of the mutagenic furoquinoline alkaloids **1**—**3** in Dictamni Radicis Cortex by HPLC, *o*-phenylphenol was found to be appropriate as an internal standard. Highly sensitive detection by fluorescence measurement was useful for **2** and **3** because of their low contents. Calibration plots of peak height ratio *vs.* concentration were found to be linear up to 0.5 μg/injection (UV absorption) or 0.05 μg/injection (fluorescence). Calibration curves of **1**—**3** could be extrapolated through the origin. Quantitative analysis of **1**—**3** in commercial Dictamni Radicis Cortex was conducted by means of the present procedure, and the results are summarized in Table II.

We previously reported that the mutagenic principles in Swertiae Herba were represented by seven tetraoxygenated xanthones.[6] The mutagenicities of the furoquinoline alkaloids in the present study are higher than those of the xanthone derivatives in Swertiae Herba. However, it is noteworthy that the total content of these mutagenic furoquinoline alkaloids in this plant was 0.04—0.11%, which is about one-tenth of that of the mutagenic xanthone derivatives in Swertiae Herba.[7] The mutagenicity of this plant was about one-tenth of that of the same weight of Swertiae Herba.[1,6]

The quantitative analysis of these furoquinoline alkaloids in other rutaceous plants is under study.

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