Studies on the Chemical Constituents of *Xanthoxylum nitidum* (ROXB.) D. C. (*Fagara nitida* ROXB.). II.[1] Examination of the Chemical Constituents by Membrane Filtration: Identification of Magnoflorine, a Water-Soluble Quaternary Aporphine Alkaloid

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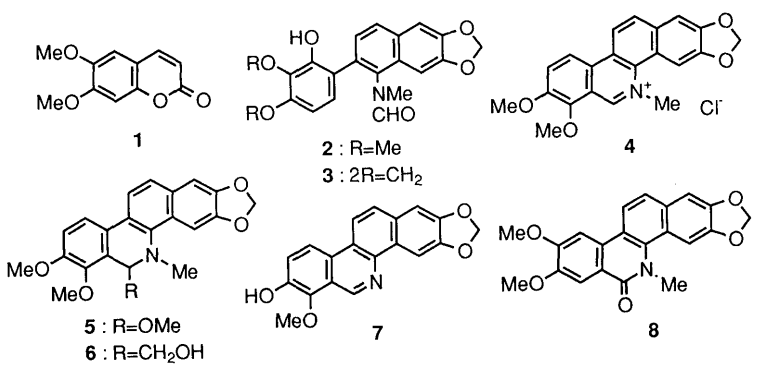
A membrane filtration, the rejection effectivity of which mainly depends on the pore size of the membrane used, was applied to examination of the chemical constituents of *Xanthoxylum nitidum* (ROXB.) D. C. (*Fagara nitida* ROXB.), Rutaceae. The methanol extracts of the wood and bark were subjected to gradient filtrations. Each filtrate given by the separation was semi-quantitatively analyzed by HPLC. Magnoflorine (9), a phenolic quaternary aporphine alkaloid, was dramatically rejected by the ultrafiltration using a membrane with the pore size of 1000 in spite of its expected permeability through the membrane due to its smaller molecular weight than the pore size of the membrane used. Such clear rejection may be attributable to the phenolic functions in 9 because chelerythrine chloride (4), a quaternary benzo[*c*]phenanthridine alkaloid, with a close molecular weight to that of 9, passed through the membrane.

Keywords membrane filtration; magnoflorine; *Xanthoxylum nitidum* (*Fagara nitida*); rejection ratio; HPLC analysis; Rutaceae

Membrane filtration can be widely used for a variety of purposes and has been particularly practically used in bioindustry[2] and the food industry.[3] Filtration effectivity generally depends upon the pore size of the membrane used. However, no application of membrane filtration to the separation of relatively small molecules, including chemical constituents from natural sources, has yet been reported as far as we know.

We previously reported the isolation of eighteen chemical components from the alkaloid fraction of the bark of *Xanthoxylum nitidum* (ROXB.) D. C. (*Fagara nitida* ROXB.), which was fractionated by liquid–liquid partition method.[1] In the course of our continuing studies on Rutaceous plants we applied membrane filtration to separation of the chemical constituents. The methanol extracts obtained from the wood and bark were gradiently filtered using ultrafiltration (UF) and reverse osmosis (RO) membranes with different pore sizes. Semi-quantitative analysis of each fraction given by the separation using high performance liquid chromatography (HPLC) indicated the high rejection ratio of magnoflorine (**9**), a phenolic quaternary aporphine alkaloid, by a UF membrane with a pore size of 1000 (UF 1K). We report here the results of the separation and identification of magnoflorine (**9**).

On the Wood The suspension of the methanol extract of the wood in 25% aqueous methanol was subjected to sequential membrane filtrations as shown in Chart 1 after removal of insoluble materials. Each fraction (W-I—W-IV) was analyzed by HPLC using ultraviolet (UV) at 365 nm as a detector (for other conditions, see Experimental). The HPLC profiles (Fig. 1) showed a dramatic change between W-II and W-III. Peak w-1, the relative concentration of which was 50% in W-II, was diluted to 23% in W-III. In contrast, peak w-2 in W-II was concentrated three fold in W-III and then six fold in W-IV. This indicated that peak w-1 was remarkably rejected by a membrane of UF 1K while peak w-2 freely passed through not only the membrane of UF 1K but also the reverse osmosis membrane with a pore size of 400 (RO 400). Though the compound showing peak w-2 was identified with aesculetin dimethyl ether (**1**) by comparison



with an authentic sample, the component showing peak w-1 remained undefined in this stage.

On the Bark The methanol extract of the bark was examined in the same way as that of the wood (Chart 1 and Fig. 2). In this case detection with UV at 254 nm was more diagnostic than with UV at 365 nm. Comparison using the previously isolated products[1] from the bark of this plant allowed us to identify four peaks [peaks b-2 to b-5]. Among them, arnottianamide (**2**) and/or integriamide (**3**)[4] corresponding to peak b-2 and chelerythrine chloride (**4**) and/or 6-methoxy-5,6-dihydrochelerythrine (**5**)[4] corresponding to peak b-5 were found to be rejected by a membrane of RO 400. The molecular weights (365—384) of these alkaloids might be responsible for their behavior on the membrane filtration. However, interestingly, peak b-4 due to bocconoline (**6**) and/or decarine (**7**)[4] with molecular weight closely similar to them was concentrated in the final fraction (B-IV). The unexpectedly high rejection of peak b-3, attributable to oxynitidine (**8**) (M.W., 363), by a membrane of UF 50K was also observed. In this case it would be reasonable that the concentration of **8** in B-I probably depends on its low solubility to the solvent used for the separation but not to its permeability to the membrane.

The retention time of peak b-1 rejected by a membrane of UF 1K suggested that peak b-1 could be assigned to be peak w-1 in the wood. Thus we tried to separate the compound corresponding to peak w-1 (b-1) from the wood because a simpler peak pattern in HPLC was observed in the wood than in the bark.

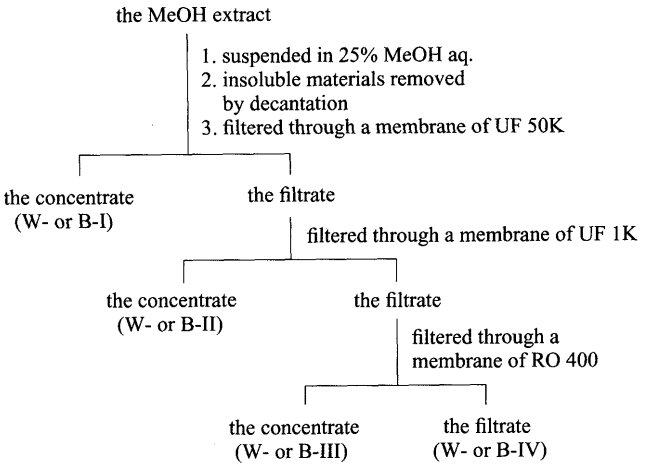


Chart 1

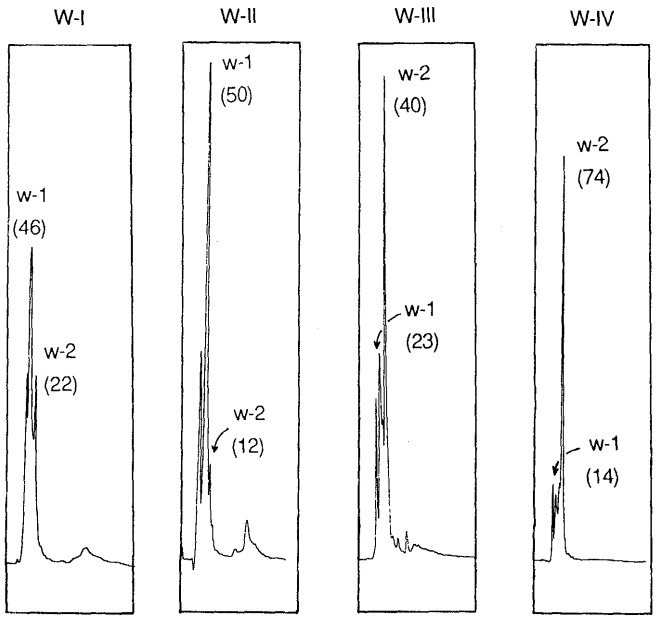


Fig. 1. HPLC Profile of Each Fraction Given by Membrane Filtration of the Methanol Extract of the Wood[a]

a) Number in parenthesis is the relative concentration ratio (%) of the peak to all peaks in each fraction. The peaks were detected by UV (365 nm).

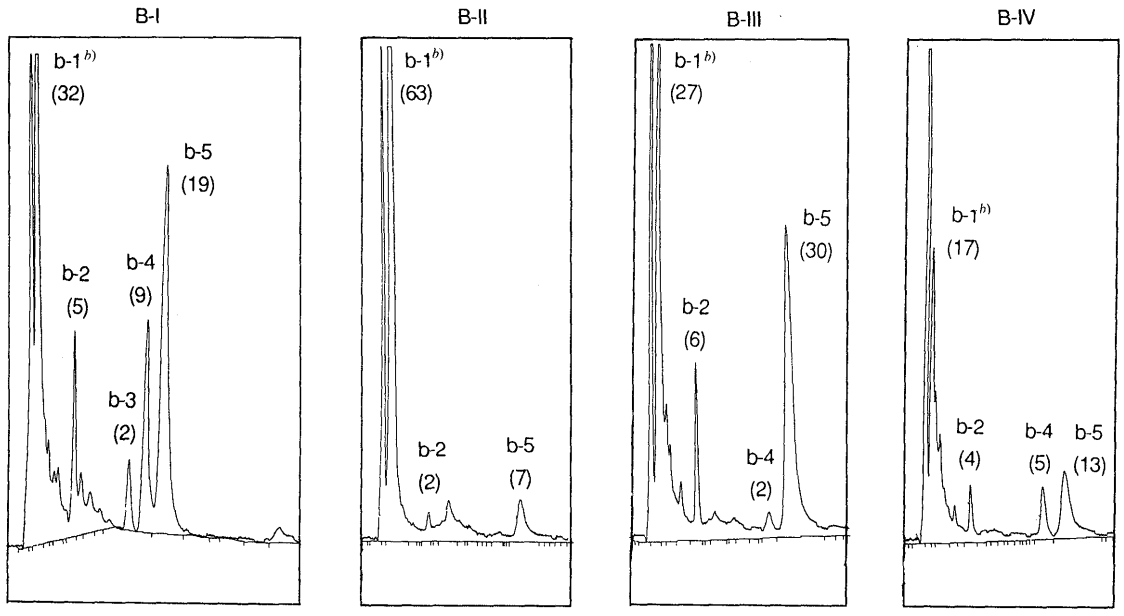


Fig. 2. HPLC Profile of Each Fraction Given by Membrane Filtration of the Methanol Extract of the Bark[a]

a) Number in parenthesis is the relative concentration ratio (%) of the peak to all peaks in each fraction. The peaks were detected by UV (254 nm). *b*) Syringaresinol[1] may be contained in this peak because it shows the same retention time as peak b-1 in HPLC under the conditions used.

TABLE I. NMR Data[a] of Magnoflorine (**9**) and Its Diacetate (**10**)

Carbon	δ_H		δ_C	
	9	**10**	**9**	**10**
1	—	—	150.94	138.42
2	—	—	153.92	154.09
3	6.62 (s)	7.15 (s)	112.02	115.52
4	2.61 (dif. d, J=16.0 Hz) 3.07 (dif. d, J=16.0 Hz)	3.16 (dd, J=14.3, 4.7 Hz) 3.75 (dt, J=12.5, 4.7 Hz)	25.98	26.15
5	2.84 (dif. d, J=11.0 Hz) 3.44 (dd, J=11.0, 4.6 Hz)	3.44 (dt, J=14.3, 5.8 Hz) 3.82 (dd, J=12.5, 5.8 Hz)	63.85	63.91
6a	3.66 (dif. d, J=13.0 Hz)	4.46 (dif. d, J=13.0 Hz)	71.97	71.34
7	2.34 (t, J=13.0 Hz) 2.91 (dif. d, J=13.0 Hz)	2.91 (t, J=13.0 Hz) 3.36 (dd, J=13.0, 3.2 Hz)	32.61	32.33
8	6.53 (d, J=8.0 Hz)	7.21 (d, J=8.5 Hz)	119.38	129.65
9	6.76 (d, J=8.0 Hz)	7.40 (d, J=8.5 Hz)	113.32	116.71
10	—	—	152.42	153.59
11	—	—	149.92	138.83
OMe	3.77 (s)	3.87 (s)	58.34	59.30
OMe	3.84 (s)	3.89 (s)	58.57	59.30
N^+Me	2.74 (s)	3.06 (s)	45.44	46.02
N^+Me	3.17 (s)	3.34 (s)	56.12	56.60
3a	—	—	118.84	130.98
6b	—	—	122.75	124.54
7a	—	—	127.99	129.34
11a	—	—	124.32	126.44
11b	—	—	124.59	127.26
CMe	—	2.28 (s)	—	22.81 22.85
CO	—	—	—	175.12 175.17

a) ^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) in D_2O are reported downfield from external TSP at 0.00 ppm. The assignments are based on DEPT, H-H COSY, HMQC, and ^{1}H-detected heteronuclear multiple bond connectivity (HMBC) experiments.

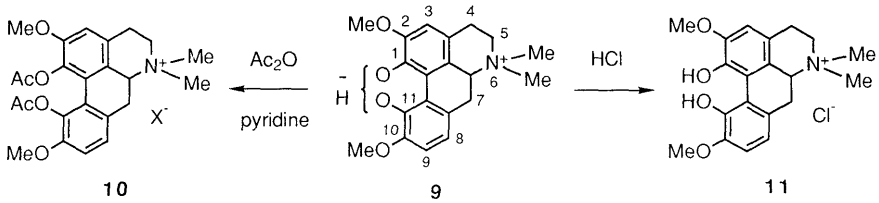


Chart 2

Identification of Magnoflorine (9) and Its Permeability through Membrane The methanol extract of the wood was directly chromatographed on silica gel using chloroform, methanol and 50% aqueous methanol as gradient solvents. Trace of the fractions by HPLC suggested that a compound corresponding to the expected peak would be contained in the fraction eluted with 50% aqueous methanol. Further purification of this fraction by preparative TLC (PLC) gave a water-soluble compound as a pale yellow amorphous powder.

Positive first atom bombardment high resolution mass (FABHRMS) spectroscopy indicated the molecular formula of the compound as $C_{20}H_{23}NO_4$ through the appearance of a positive ion peak at m/z 342.1706 due to a protonated molecular ion (Calcd for $C_{20}H_{24}NO_4$: 342.1722). The absorption band due to a hydroxy group was observed at 3350 cm^{-1} in the infrared (IR) spectrum. The absorption maxima at 229.9, 278.1, and 318.4 nm in the UV spectrum suggested the presence of a conjugated system in its molecule. Data on the proton (^{1}H) and the carbon (^{13}C) nuclear magnetic resonance (NMR) spectroscopy are given in Table I. Distortionless enhancement by polarization transfer (DEPT), proton-proton shift correlation spectroscopy (H–H COSY), and ^{1}H-detected heteronuclear multiple quantum coherence (HMQC) experiments led to the assignment of three methylene groups (two sequential and one isolated), a benzylic methine, a quaternary *N*,*N*-dimethyl, and a methoxyl group. Additionally, three aromatic protons were observed as a singlet and complementary coupled doublets (J=8 Hz) respectively, suggesting the presence of five and four sequentially substituted benzene rings in its molecule. Acetylation with acetic anhydride and pyridine at room temperature gave the diacetate (**10**) [m/z: 426.1916. Calcd for $C_{24}H_{28}NO_6$: 426.1919; ν_{max}^{Nujol} cm^{-1}: 1759] as a pale yellow oil (Chart 2 and Table I). These facts allowed us to safely deduce the parent compound as magnoflorine[5] (**9**), a phenolic quaternary aporphine alkaloid, distributed in *Xanthoxylum* plants but not previously found in this plant. It was finally characterized as a crystalline styphnate, mp 226—234 °C (dec.) [lit.[5] mp 232°C (dec.)]. Thus a compound corresponding to the

TABLE II. Comparison of the Rejection Ratios of **9**, **11**, and **4** by Membranes

Substrate (M.W.)	Membranes	
	UF 3K	UF 1K
9 (341)	5.7—7.3	76.9
11 (377)	1.7	76.0
4 (384)	—	36.5

peak w-1 (b-1) was identified with magnoflorine.

Magnoflorine chloride (**11**) was also prepared from the isolated magnoflorine (**9**) by treatment with hydrochloric acid (Chart 2). Though the chloride (**11**) showed a bathochromic shift in the UV spectrum after addition of alkali, no shift was observed in the case of the isolated magnoflorine (**9**) from a natural source, indicating that **9** was in a phenobetaine (a zwitter ion) form.

Since the molecular weight of magnoflorine was 341 in the phenobetaine form, it should basically pass through a membrane of UF 1K if the pore size of the membrane effectively allows filtration. However, the membrane unexpectedly rejected magnoflorine. The permeability of a substrate in a plant extract may be assumed to depend on the substrate's structural features and/or on possible interaction with other components coexisting in the extract. Thus pure samples of the isolated magnoflorine (**9**) (M.W., 341), magnoflorine chloride (**11**) (M.W., 377), and chelerythorinechloride (**4**) (M.W., 384), a quaternary benzo[*c*]phenanthridine alkaloid with a larger molecular weight than that of magnoflorine, were independently subjected to filtrations using membranes of UF 3K and/or UF 1K. The filtration effectivities of the membranes were estimated by rejection ratios[6] based on HPLC analysis (Table II).

High rejection of both pure forms (**9** and **11**) of magnoflorine by a membrane of UF 1K in nearly the same ratio showed not only no influence of a counter ion on the permeability of magnoflorine through the membrane but also no significant interaction of magnoflorine with other components in the plant extract. On the other hand, the higher permeability of chelerythrine chloride (**4**) suggested that a phenolic function rather than a quaternary nitrogen in its molecule could be responsible for the membrane filtration. The membrane of UF 1K we used was made from cellulose polymer. Therefore, some interaction(s) between the substrate and the membrane itself may partly account for the higher rejection of magnoflorine. Further examination is now in progress.

Experimental

All melting points were measured on a micro melting-point hot stage (Yanagimoto) and are uncorrected. IR spectra were recorded for Nujol mulls on a Hitachi 260-10 or JASCO IR-700 spectrophotometer. NMR spectra were recorded with a JEOL GSX-500 spectrometer with sodium 3-(trimethylsilyl)propionate (TSP) as an external reference. Peak multiplicities are quoted in Hz as singlet (s), doublet (d), triplet (t), quartet (q), double doublet (dd), double triplet (dt), and multiplet (m). Diffused splitting pattern is abbreviated as dif. Mass spectra were measured with a Hitachi M-60 spectrometer using a direct inlet system. For column silica gel 60 (70—230 mesh ASTM, Merck) was used, while for TLC and PLC, DC-Fertigplatten SIL-G 25 UV254 (Macherey–Nagel) and Silica gel GF_{254} (Merck) were used. Ultrafiltration was carried out by a carbosepmicromodule using a ceramic inorganic membrane of 50K (Sumitomojukikai Environment) under a pressure of 1 kg/cm^2 (flow rate, 73.2 l/m^2 h) or a spinflow system using cellulose polymers of 1K (Millipore) under a pressure of 5 kg/cm^2 (flow rate, 16.6 l/m^2 h). Reverse osmosis filtration was carried out by a spinflow system using a polyacrylamide membrane of 400 (Millipore) under a pressure of 30 kg/cm^2 (the flow rate, 30.8 l/m^2 h). For the HPLC system JASCO 880-PU was used as a pump unit, JASCO UVIDEC-100-V as a detector, and JASCO RC-250 as a recorder.

Preparation of the Methanol Extracts Each suspension of the finely chipped wood and bark of *X. nitidum* (ROXB.) D. C. (*F. nitida* ROXB,), which had been collected in Taiwan (Republic of China) in 1981, in methanol was heated under reflux for 25 h. The methanol solutions were evaporated to dryness *in vacuo* to give the extracts.

Membrane Filtration (a) Wood: The extract (0.752 g) obtained from the wood (132 g) was suspended in 25% aqueous methanol (155 ml), insoluble materials (0.209 g) removed by decantation, and then filtered through a membrane of 50 K to give two portions: the concentrate [W-I (0.120 g)] and the filtrate. The filtrate was further subjected to sequential membrane filtration as shown in Chart 1 to give three fractions [W-II (0.158 g), W-III (0.082 g), and W-IV (0.050 g)].

(b) Bark: The same treatment of the suspension of the extract (1.708 g) in 25% aqueous methanol (341 ml) gave four fractions [B-I (0.327 g), B-II (0.419 g), B-III (0.114 g), and B-IV (0.020 g)] after removal of the insoluble materials.

(c) Magnoflorine (**9**), Magnoflorine Chloride (**11**), and Chelerythrine Chloride (**4**): **9** (0.012 g) was dissolved in 25% aqueous methanol (23 ml), **11** (0.023 g) in 25% aqueous methanol (34 ml), and **4** (0.023 g) in 25% aqueous methanol (34 ml). Each solution was subjected to ultrafiltration using membranes of UF 3K and/or UF 1K under the same condition as the extracts.

HPLC Analyses of the Fractions For a column cosmosil 5 C18 (4.6 × 150 mm: Nacalai) was used. An aqueous methanol [MeOH : H_2O (3 : 1, v/v)] was passed through the column at a flow rate of 0.60 ml/min. The peaks were detected using a wavelength of 365 nm. In the case of the fraction obtained from the bark a wavelength of 254 nm was also used.

Magnoflorine (9) The methanol extract (85 g) obtained from the wood (1.26 kg) was chromatographed over SiO_2. After removal of the fractions (total weight, 63.43 g) eluted with $CHCl_3$ and MeOH, successive elution with a mixed solvent of MeOH : H_2O (1 : 1, v/v) gave two fractions [fr. A (6.8 g) and fr. B (2.0 g)]. One part (0.020 g) of fr. B was further purified using PLC (MeOH, Rf=0.24) to give a pale yellow amorphous powder [0.011 g (0.087% from the wood)]. IR ν_{max}: 3350 (OH) cm^{-1}. UV λ_{max} nm: 229.9, 278.1, 318.4. NMR: see Table I. FABHRMS *m/z*: 342.1706. Calcd for $C_{20}H_{24}NO_4$: 342.1722.

Acetylation of Magnoflorine (9) A solution of magnoflorine (**9**) (0.019 g) in Ac_2O (0.35 ml) and pyridine (0.35 ml) was stirred at room temperature for 1 d. After usual work-up purification of the crude product with PLC (MeOH, Rf=0.13) gave a pale yellow amorphous powder (0.012 g). IR ν_{max}: 3350 (OH) cm^{-1}. UV λ_{max} nm: 218.6, 263.5, 297.0. NMR: see Table I. FABHRMS *m/z*: 426.1916. Calcd for $C_{24}H_{28}NO_6$: 426.1919.

Derivatives of Magnoflorine (9) (a) The Chloride (**11**): Magnoflorine (**9**) (0.015 g) was dissolved in 10% HCl (2 ml) and the resultant solution was evaporated to dryness *in vacuo* under 30 °C to give the chloride (0.017 g) as a light brown oil. IR ν_{max}: 3350 (OH) cm^{-1}. UV λ_{max} nm: 224.0, 268.6, 302.1. UV $\lambda_{max}^{MeOH+1\%\ NaOH}$ nm: 229.0, 277.8, 322.2.

(b) The Styphnate: According to the reported method[5] the styphnate (0.009 g), mp 224—234 °C (lit.[5] mp 232 °C), was prepared from the chloride (**11**) (0.017 g).

References and Notes

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4) Unfortunately, these compounds showed the same retention times under the conditions used.
5) H. Ishii, M. Tomita, *Yakugaku Zasshi*, **65**, 123 (1953).
6) The rejection ratio was calculated as $(C_0-C_1)/C_0 \times 100$ (C_0: concentration of the original solution; C_1: concentration of the filtrate solution).