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Biological-Active Triterpenes of Alismatis Rhizoma. I.[1] Isolation of the Alisols

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In order to isolate the hypocholesterolemic principle, the neutral ethyl acetate-soluble fraction of the methanol extract of Alismatis Rhizoma was fractionated in parallel with the biological test. Thus new compounds alisol A (**1**), alisol B (**2**), alisol A monoacetate (**3**), alisol B monoacetate (**4**) and alisol C monoacetate (**5**) were obtained. The compounds **1**, **3**, **4** and **5** were shown to be effective in the test with rats.

A Chinese crude drug "Zexie" (沢瀉),[3] Alismatis Rhizoma, the rhizoma of *Alisma Plantago-aquatica* L. var. *orientale* Samuels. (=*A. orientale* Juzep., Alismataceae) has been used as an important component of several preparations and believed to have a diuretic action. Old literatures stated that the crude drug was remedy for diabetes and swelling.[4] In 1960, Kobayashi found a lipotropic action of the crude drug. It was recognized that both neutral lipid-soluble fraction and water-soluble fraction had a lipotropic action in his biological tests.[5] He isolated choline as one of the active principles from the latter fraction, whereas biological aspects of the former fraction have been the subject of a series of his reports. However, no compound has been isolated from the lipid-soluble fraction.

In our recent study the neutral ethyl acetate-soluble fraction (A-1, see Chart 1) of the crude drug showed a significant hypocholesterolemic action in rats.[6] This prompted us to clarify the active principle. The present paper concerns the isolation of new, hypocholesterolemic substances named alisol A (**1**), alisol A monoacetate (**3**), alisol B monoacetate (**4**) and alisol C monoacetate (**5**) as well as related compound alisol B (**2**) from Alismatis Rhizoma.

The drug material cultivated and prepared at our Company's farm at Fukuchiyama near Kyoto, was powdered and extracted with methanol. After evaporation of the solvent the residue was dissolved in water and extracted with ethyl acetate. The organic layer was separated and washed with aqueous sodium bicarbonate or sodium carbonate solution to remove acidic substances. The ethyl acetate layer (A-1) thus obtained was regarded to be neutral since subsequent extraction of the organic layer with aqueous hydrochloric acid afforded only a trace of basic substance. The fraction A-1 showed a positive hypocholesterolemic action in the biological test with rats; plasma cholesterol level of young male rats fed on

1) A part of this work was reported in *Tetrahedron Letters*, **1968**, 103 and represented at the 11th Symposium on the Chemistry of Natural Products, Kyoto, October **1967**; Biological aspects of alisol A monoacetate (**3**) was also represented at the 4th International Congress on Pharmacology, Basel, July 1969.

2) Location: *Juso-Nishino-cho, Higashiyodogawa-ku, Osaka.*

3) "Zhong Yao Zhi" (中葯志), Vol. I, ed. by Pharmaceutical Institute, Chinese Academy of Medical Science, Peking, 1959, p. 296.

4) J. Sato, "On the Chinese Medicinal Plants" (漢薬の原植物), Japan Society for the Promotion of Science, Tokyo, 1959, p. 329.

5) T. Kobayashi, *Yakugaku Zasshi*, **80**, 1456, 1460, 1465, 1612, 1617 (1960). For the constituents of the crude drug see also R. Combes, Y. Martin and M.R. Brunel, *Compt. rend.*, **234**, 1655 (1952); M. Goto, *Yakugaku Zasshi*, **75**, 1180 (1955).

6) For the biological tests see Y. Aramaki, T. Kobayashi, Y. Imai, S. Kikuchi, T. Matsukawa and K. Kanazawa, *J. Atheroscler. Res.*, **7**, 653 (1967).

a diet containing a large amount of cholesterol was not so heavily elevated when the fraction A-1 was added to the food at the rate of 0.5%.

Preliminary Fractionation and Isolation of the Alisols

Preliminary fractionation of the starting fraction (A-1) was performed as follows (Chart 1). After the fraction was passed through a charcoal column to remove coloring material, the resulting fraction A-2 was subjected to silica gel column chromatography. Elution with chloroform followed by ethyl acetate gave the four fractions A-3-1—A-3-4. The fraction A-3-4, the most effective one was again chromatographed on a silica gel column. Elution with benzene–acetone mixture and with ethyl acetate afforded three fractions designated as A-3-4-1—A-3-4-3; the biological tests clearly revealed that the most polar fraction A-3-4-3 had the strongest activity. Above results demonstrated that hypocholesterolemic constituent was mainly located in the polar fraction.

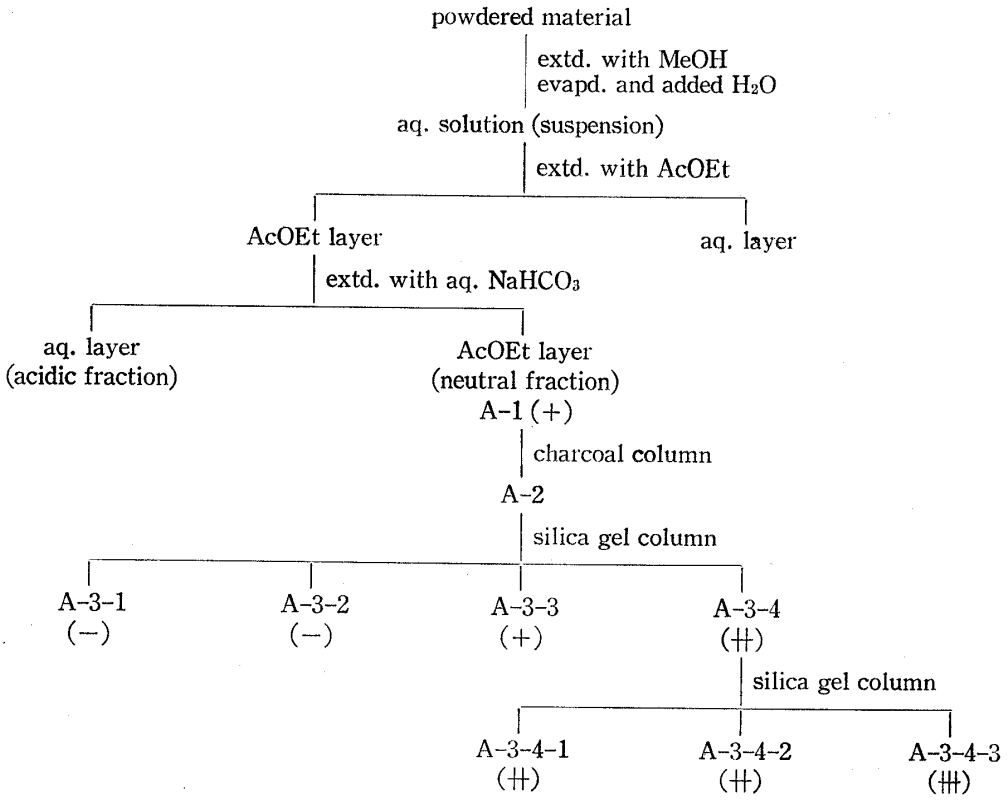

Chart 1. Extraction and Preliminary Fractionation[a)]

a) Hypocholesterolemic activities are given in parentheses.
卅: effective at the dose level of 0.1% in diet
卄: effective at 0.25% in diet
+: effective at 0.5% in diet
−: not effective at 0.25% in diet

In a large scale experiment the starting fraction A-1 was directly chromatographed on a charcoal column. After elution with benzene to obtain the less polar fractions A-2-1—A-2-3, elution was done with ethyl acetate to afford the polar fraction designated as A-2-4, which was then subjected to silica gel column chromatography giving the eleven fractions A-2-4-1—A-2-4-11. Silica gel thin-layer chromatography of these fractions indicated that the fractions A-2-4-6—A-2-4-10 were consisted of the compounds contained in the most effective fraction A-3-4-3 in the preliminary experiment described above. Actually A-2-4-7 and A-2-4-9 showed a strong hypocholesterolemic action. This accentuated the isolation of the active principle.

The fraction A-2-4-9 showed several spots on thin-layer chromatogram. Attempt to isolate the main component of this fraction, named alisol A (**1**), by the use of silica gel chro-

matography failed mainly because the compound did not crystallize. However, acetylation of the fraction followed by silica gel chromatography gave rise to crystalline alisol A triacetate (**6**), and treatment of the latter with potassium carbonate in aqueous methanol regenerated alisol A (**1**). Alisol A thus obtained was effective in the biological test at the dose level of 0.1% in diet.

Treatment of the fraction A-2-4-7 with ethyl acetate afforded a crystalline substance which was later shown to be alisol A monoacetate (**3**), being also effective in the biological test.

Alisol B (**2**), whose structure relates closely to that of **1**, was isolated from the fraction A-2-4-5, showing no hypocholesterolemic action in rats.

The third active principle alisol B monoacetate (**4**) was isolated in a crystalline state together with **2**, from the less polar fractions A-2-2 and A-2-3. Further, the additional active principle alisol C monoacetate (**5**) was obtained by careful silica gel chromatography of the mother liquor of alisol B monoacetate (**4**).

Table I. Physical Properties and Hypocholesterolemic Activities of the Alisols[a)]

Compound	Formula	mp (°C)	$[\alpha]_D$ (°)	Efficacy (%)
Alisol A (**1**)	$C_{30}H_{50}O_5$	amorphous	+ 99	54
Alisol B (**2**)	$C_{30}H_{48}O_4$	166—168	+130	0
Alisol A monoacetate (**3**)	$C_{32}H_{52}O_6$	194—196	+ 86	61
Alisol B monoacetate (**4**)	$C_{32}H_{50}O_5$	162—164	+121	51
Alisol C monoacetate (**5**)	$C_{32}H_{48}O_6$	232—233	+103	55

a) The hypocholesterolemic activities are the ones obtained at the dose level of 0.1%, and are expressed as the mean of five rats in each group.

Physical properties of the alisols (**1**—**5**) are listed in Table I; the molecular formulae being established by mass spectrometry and elementary analyses. Triterpene nature of the alisols was suggested by positive Liebermann–Burchard reaction in the new compounds as well as by their molecular formulae; structural studies are reported in the following papers. The crude drug imported from Korea was also found to contain these new compounds. The yields of the compounds (**1**—**4**) from the Korean drug material were 0.008, 0.01, 0.006 and 0.15%, respectively.

Thin-layer chromatographic investigation showed that the stems and the leaves of *A. Plantago-aquatica* L. var. *orientale* also contain the compounds **1**—**4**; the major components being **2** and **4** as in the case of the crude drug. Thus alisol B (**2**) was isolated from the upper part of the plant in the yield of 0.18% by saponification of the lipid fraction.

Biological Test

Hypocholesterolemic activity was studied in Sprague-Dawley-JCL/T male rats weighing about 100 g, fed on a semi-synthetic diet containing 1% cholesterol, 0.2% sodium cholate and 0.2% choline chloride. The test compound previously dissolved in ethanol or ethyl ether was admixed in the diet. After 10 day-feeding the plasma cholesterol level was determined by Technicon autoanalyser. The plasma cholesterol level of the control group was found to be as high as about 250 mg/100 ml. The biological activity of the compounds was estimated by percentage inhibition of hypercholesterolemia, which was calculated by the following formula:

$$E\,(\%)=\frac{C_C-C_T}{C_C-C_N}\times 100$$

C_C: plasma cholesterol value of control group rats
C_T: value of alisol-treated rats
C_N: value of rats fed on a stock diet

Hypocholesterolemic activities of the alisols are shown in Table I. Subsequent biological studies have shown that these compounds also have lipotropic, diuretic and antiinflammatory activities in rats, thus demonstrating that the new compounds are also responsible for the effects which the crude drug has been believed to possess. However, the present paper deals with the biological aspects no further, because these will appear in the near future.[7]

Experimental

All melting points were measured on Kofler block and were uncorrected. The specific rotations were taken on $CHCl_3$ solutions, $c=1.0\%$. For silica gel thin-layer chromatography (TLC) silica gel G (Merck) was used; detection being done by spraying sulfuric acid followed by heating. For column chromatography silica-gel (Merck, ϕ 0.05—0.2 mm) was used.

Extraction of "Zexie"——The powdered drug material (1 kg) cultivated and prepared at Fukuchiyama, was twice extracted with MeOH (5 liters) at *ca.* 60° for 6 hr. The combined MeOH extracts were evaporated to give a reddish-brown viscous residue (150 g). The residue was diluted with water (200 ml) and the resulting suspension was then extracted with ether (300, 200 ml). The combined ether extracts were washed with aq. $NaHCO_3$ (2×200 ml) and then with water, dried and evaporated to afford 39 g of a brown oil (neutral lipid fraction, A-1). The fraction A-1 (44 g), dissolved in benzene (200 ml) was passed through a charcoal column (Tokusei-shirasagi, Takeda, 45 g) and eluted successively with benzene (3 liters), AcOEt (1.5 liters) and AcOEt–MeOH (1:1) (1.5 liters). The combined eluates were evaporated to give a faint-yellow oil (42.9 g, A-2).

Chromatography-1——The fraction A-2, obtained above was chromatographed on silica gel (400 g). The results are shown in Table II.

Table II. Chromatography-1

Fraction	Eluant	Volume (liter)	Yield (g)
A-3-1	$CHCl_3$	1.0	10.3
A-3-2	$CHCl_3$	2.5	14.1
A-3-3	$CHCl_3$	4.0	8.1
A-3-4	AcOEt	2.0	17.7

Chromatography-2——The fraction A-3-4 (12 g), obtained by chromatography-1, was dissolved in benzene–acetone (10:1) (40 ml) and chromatographed on silica gel (200 g). The results are shown in Table III.

Table III. Chromatography-2

Fraction	Eluant	Volume (liter)	Yield (g)
A-3-4-1	benzene–acetone (10:1)	3.5	5.4
A-3-4-2	benzene–acetone (5:1)	2.5	3.4
A-3-4-3	AcOEt	1.5	2.9

Chromatography-3 and Chromatography-4——The neutral lipid fraction A-1 (330 g) was dissolved in benzene (1 liter) and chromatographed on charcoal (500 g) (Table IV, chromatography-3). The fraction A-2-4 (60 g), obtained by the chromatography, was then subjected to column chromatography using 1 kg of silica gel (Table V, chromatography-4).

Alisol A (1)——The fraction A-2-4-9 (1.0 g), obtained by chromatography-4 (Table V), was dissolved in a mixture of Ac_2O (10 ml) and C_5H_5N (5 ml) and the solution was allowed to stand at room temperature for 16 hr. Work up in the usual manner gave crude acetate (1.2 g). The acetate (1.1 g) was dissolved in

7) Y. Imai, H. Matsumura and Y. Aramaki, *Jap. J. Pharmacol.*, to be published.

Table IV. Chromatography-3

Fraction	Eluant	Volume (liter)	Yield (g)
A-2-1	benzene	6	190
A-2-2	benzene	6	35
A-2-3	benzene	4	10
A-2-4	AcOEt	14	60
A-2-5	AcOEt–MeOH (1:1)	11	15

Table V. Chromatography-4

Fraction	Eluant	Volume (liter)	Yield (g)
A-2-4-1	benzene–acetone (10:1)	2	9
A-2-4-2	benzene–acetone (10:1)	2	7
A-2-4-3	benzene–acetone (10:1)	2	7.5
A-2-4-4	benzene–acetone (5:1)	2	10
A-2-4-5	benzene–acetone (5:1)	3	10
A-2-4-6	benzene–acetone (5:1)	2	2.5
A-2-4-7	benzene–acetone (5:1)	6	6
A-2-4-8	benzene–acetone (3:1)	5	4.5
A-2-4-9	benzene–acetone (3:1)	4	3.5
A-2-4-10	benzene–acetone (3:1)	6	2.5
A-2-4-11	AcOEt	5	0.3

benzene–acetone (7:1) (70 ml), chromatographed on silica gel (120 g) and eluted with benzene–acetone (7:1) to afford 690 mg of alisol A triacetate (**6**), mp 230—231°. Recrystallization from CH_2Cl_2–MeOH gave colorless needles, mp 231—233°, $[\alpha]_D^{21}$ +54.4°. Mass (*m/e*): 556 (M^+-AcOH). *Anal.* Calcd. for $C_{36}H_{56}O_8$: C, 70.10; H, 9.15. Found: C, 70.07; H, 8.92.

A solution of compound **6** (3.5 g) in a mixture of MeOH (250 ml) and 10% aq. K_2CO_3 (100 ml) was refluxed for 6 hr. After concentration, the mixture was extracted with AcOEt (2×100 ml). The combined AcOEt extracts were washed with water, dried and evaporated to dryness. The residue (3.0 g) was chromatographed on silica gel (50 g) and eluted with benzene–acetone (3:1) to give alisol A (**1**) as colorless powder (2.4 g), which showed a single spot on TLC (benzene–acetone (3:2) as a solvent). The compound did not crystallize. $[\alpha]_D^{23}$ +99.6°. Mass (*m/e*): 472 (M^+-H_2O). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1705. *Anal.* Calcd. for $C_{30}H_{50}O_5$: C, 73.43; H, 10.27. Found: C, 73.24; H, 10.38. Reacetylation of alisol A (**1**), thus obtained, gave alisol A triacetate (**6**). The identity was confirmed by admixture and comparison of the infrared (IR) spectra.

Alisol A Monoacetate (**3**)——The fractions A-2-4-6 and A-2-4-7 of chromatography-4 (Table V), were combined and treated with AcOEt. The resulting crystals were collected and washed with AcOEt to give colorless prisms of alisol A monoacetate (**3**) (1.5 g), mp 190—192°. Recrystallization from acetone raised the melting point to 194—196°. $[\alpha]_D^{23}$ +86°. Mass (*m/e*): 514 (M^+-H_2O). IR ν_{max}^{KBr} cm^{-1}: 3450 (OH); 1745 (acetyl); 1705 (ring ketone). *Anal.* Calcd. for $C_{32}H_{52}O_6$: C, 72.14; H, 9.84. Found: C, 72.20; H, 9.96. Acetylation of compound **3** with Ac_2O and C_5H_5N at room temperature gave alisol A triacetate (**6**). Treatment of compound **3** with hot methanolic K_2CO_3 for 1 hr gave alisol A (**1**).

Alisol B (**2**)——The fraction A-2-4-5 (9.5 g) was rechromatographed on silica gel (200 g); the results being given in Table VI. The fraction 3 obtained above was shown to be pure alisol B (**2**) according to

Table VI. Chromatographic Separation of the Fraction A-2-4-5

Ffraction	Eluant	Volume (ml)	Yield (g)	Constituent
1	benzene–acetone (5:1)	700	3.5	
2	benzene–acetone (5:1)	200	1.6	2
3	benzene–acetone (5:1)	600	3.0	2
4	benzene–acetone (5:1)	800	1.2	2

TLC (benzene–acetone (2:1) as a solvent). The fractions 2 and 4 were combined and chromatographed in the same manner to give an additional crop of **2** (1.8 g). Crystallization from AcOEt afforded colorless prisms of **2** (2.0 g), mp 166—168°, $[\alpha]_D^{23}$ +130°. IR ν_{max}^{KBr} cm^{-1}: 3480 (OH); 1705 (ring ketone). *Anal.* Calcd. for $C_{30}H_{48}O_4$: C, 76.22; H, 10.24. Found: C, 75.86; H, 10.25.

Alisol B Monoacetate (4) and β-Sitosterol——a) The fractions A-2-2 and A-2-3 in Table IV (chromatography-3) were combined (45 g), chromatographed on silica gel (700 g) and eluted with benzene–acetone (3:1). The first fraction (5 g), upon standing at room temperature for a week, separated crystals which were collected and washed with EtOH to give β-sitosterol (3 g). Repeated crystallization from EtOH gave colorless leaflets, mp 138—139°, alone or in admixture with an authentic specimen of β-sitosterol.

The first mother liquor of β-sitosterol was evaporated and the residue was chromatographed on silica gel (50 g), using $CHCl_3$ as an eluant to give 1 g of an oil. Rechromatography of the oil using benzene–acetone (6:1) as an eluant, gave 750 mg of compound **4** which according to TLC was pure (for TLC benzene–acetone (3:1) was used as a solvent). Crystallization from AcOEt–*n*-hexane gave colorless prisms, mp 162—164°, $[\alpha]_D^{23}$ +121°. IR ν_{max}^{Nujol} cm^{-1}: 3470 (OH); 1745 (acetyl); 1700 (ring ketone). *Anal.* Calcd. for $C_{32}H_{50}O_5$: C, 74.67; H, 9.79. Found: C, 74.81; H, 9.71.

b) The neutral lipid fraction A-1 (*ca.* 2 kg) obtained from the crude drug material (78 kg) of Korean origin was chromatographed on charcoal (3.0 kg) and eluted successively with benzene (73 liters), AcOEt (70 liters) and AcOEt–MeOH (1:1) (50 liters) to afford 950, 235 and 63 g of an oil, respectively. After the benzene-eluate was allowed to stand in a deep freezer for a week, the resulting crystals were collected and washed with *n*-hexane–AcOEt (8:1) to give 45 g of faint-yellow crystals, which were composed of compound **4** (main component), β-sitosterol and alisol C monoacetate (**5**) according to TLC. The crystals were dissolved in benzene–acetone (10:1) (500 ml) and chromatographed on silica gel (500 g). The results are given in Table VII. The fraction 2 in Table VII was recrystallized from AcOEt–*n*-hexane to give pure compound **4** (22 g).

Table VII. Chromatographic Separation of the Crude Crystals of Compound 4[a)]

Fraction	Eluant	Volume (liter)	Yield (g)	Constituent[a)]
1	benzene–acetone (10:1)	0.5	3	β-sitosterol
2	benzene–acetone (5:1)	3.5	30	**4**(**5**)
3	benzene–acetone (5:1)	1.5	8	**5**(**4**)

a) The minor constituents are given in parentheses.

Table VIII. Chromatographic Separation of Compounds **4** and **5**

Fraction	Eluant	Volume (ml)	Yield (g)	Constituent
1	benzene–acetone (10:1)	500	5.0	**4**
2	benzene–acetone (10:1)	500	4.2	**4**(**5**)
3	benzene–acetone (10:1)	500	3.4	**5**(**4**)
4	benzene–acetone (5:1)	500	2.4	**5**(**4**)

Alisol C Monoacetate (5)——The mother liquor of compound **4** obtained above (*b*) and the fraction 3 in Table VII were combined and chromatographed on silica gel (200 g); the results being shown in Table VIII. The fraction 3 in the Table VIII was treated with benzene and the insoluble white crystals were collected and washed with benzene. The crystals thus obtained were then chromatographed on silica gel (100 g), using $CHCl_3$–AcOEt (3:1) as an eluant, to give an impure portion (1.4 g); subsequent elution afforded a pure sample of compound **5** (1.2 g).

The impure part (1.4 g) obtained above and the fraction 4 in Table VIII were combined and chromatographed three times under a similar condition to give an additional crop of compound **5** (2.5 g). Recrystallization from MeOH gave colorless pillars, mp 232—233°, $[\alpha]_D^{24}$ +102.9°. Mass (*m/e*): 528 (M^+). IR ν_{max}^{KBr} cm^{-1}: 3470 (OH); 1745 (acetyl); 1705 (ring ketone at C_3); 1690 and 1642 (CO–C=C). *Anal.* Calcd. for $C_{32}H_{48}O_6$: C, 72.69; H, 9.15. Found: C, 72.53; H, 8.95. Compound **5** had a *Rf*-value slightly smaller than that of compound **4** on TLC (benzene–acetone (3:1) as a solvent).

Isolation of Alisol B(2)from the Stems and Leaves of *Alisma Plantago-aquatica L.* var. *orientale Samuels* (Saponification Procedure)——The dried stems and leaves (600 g) of *Alisma Plantago-aquatica* L. var. *orientale*,

harvested in October 1967, were chopped up and extracted twice with MeOH (15 liters) at room temperature for 1 week with occasional agitating. The combined extracts were concentrated to a volume of about 500 ml, and the resulting dark green, aqueous suspension was extracted with AcOEt (300, 200, 200 ml). The organic layers were combined, washed with water (200 ml), dried and evaporated to leave a dark green mass (16 g). MeOH (200 ml) and anhydrous K_2CO_3 (9 g) were added to the residue and the mixture was refluxed for 2 hr. After evaporation of MeOH, water was added to the residue and the resulting aqueous suspension was extracted with AcOEt. The AcOEt layer was washed with water, dried and evaporated to give an unsaponifiable lipid fraction (10 g) as a greenish yellow mass. A benzene solution of the unsaponifiable fraction was passed through a column, which was packed with 20 g of chromatographic charcoal (Tokusei-shirasagi), and eluted with benzene (500 ml), followed by AcOEt (700 ml). The combined eluates were evaporated to dryness to give a red oil (9.5 g) which was then submitted to column chromatography on silica gel (50 g); the results being given in Table IX.

Table IX. Chromatographic Separation of the Unsaponifiable Lipid Fraction obtained from the Stems and Leaves

Fraction	Eluant	Volume (ml)	Yield (g)
1	benzene–acetone (10:1)	200	5.60
2	benzene–acetone (10:1)	200	0.90
3	benzene–acetone (10:1)	200	0.38
4	benzene–acetone (5:1)	200	0.34
5	benzene–acetone (5:1)	500	1.30
6	benzene–acetone (3:1)	100	0.19
7	benzene–acetone (3:1)	100	0.11
8	acetone	250	0.25

The fractions 5 and 6 of the Table IX were combined, rechromatographed on silica gel (10 g) and eluted with benzene–acetone (5:1) to afford a viscous oil (1.5 g) which showed a single spot identical with alisol B (**2**) on TLC (for TLC benzene–acetone (2:1) was used as a solvent). Crystallization of the oil from AcOEt gave colorless prisms (420 mg), which were identified with an authentic sample of **2** by IR spectral comparison. The mother liquor of **2** was evaporated to dryness and the residue was acetylated with 5 ml each of Ac_2O and C_5H_5N at room temperature for 5 hr. Column chromatography of the acetylation product was then carried out on silica gel (20 g) using benzene–acetone (20:1) as the eluant to obtain 820 mg of alisol B diacetate.[8]

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8) The compound will appear again in the Part IV of the series; colorless plates, mp 143—145°, $[\alpha]_D^{23}$ +117.5°.