Journal of the Pharmaceutical Socicty of Korea
17, 152—160 (1973)

Sterols and Terpenoids from Phytolacca esculenta

Won Sick Woo and Sam Sik Kang*

(Received July 5, 1973)

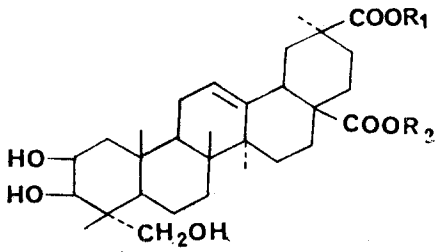
Abstract-Phytolaccagenin, as a minor free terpenoid, was isolated from the roots of *phytolacca esculenta* VAN HOUTTE. A mixture of sterols was also isolated and identified as α-spinasterol and Δ^7-stigmastenol, which never seemed to have been found in Phytolaccaceae.

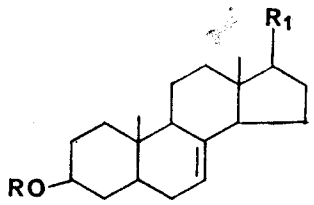
Phytolacca esculenta VAN HOUTTE is a perennial weed, roots of which have been used an indigenous medicine against edema and rheumatism in the Far East, including China, Japan, and Korea. In our laboratory, an anti-inflammatory triterpenoid, named jaligonic acid has recently been isolated[1] and its structure was fully defined as 2β,3β,23-trihydroxy-olean-12-ene-28,30-dicarboxylic acid (**I**)[2].

In the process of isolation of **I**, phytolaccagenin (**II**) was obtained as a minor component. Moreover sterols were isolated from the unsaponifiable fraction.

Triterpenoids—Precipitate obtained by acidification of NaOH soluble fraction followed by chromatography on silica gel yielded a microcrystalline substance, $C_{31}H_{48}O_7$, mp 317-319°, $[\alpha]_D^{20}=+114.7°$. This compound was Liebermann-Burchard (pink) positive and its ir spectrum showed a hydroxyl peak at 3430cm^{-1}, ester at 1732cm^{-1}, and free carboxyl at 1698cm^{-1}. It underwent easy saponification with methanolic KOH and the product was identical with jaligonic acid (**I**). Methylation with diazomethane gave a substance which was identical with jaligonic acid dimethylester (**III**). Since carbomethoxyl group at C-17 can be hydrolysed only under relatively drastic conditions,[3] this compound should be 30-monomethylester of jaligonic acid (**II**). This was confirmed by comparison with authentic specimen prepared from **I**. The mass spectrum (Fig. 1) of **II** exhibits typical retro-Diels-Alder fragmentation involving the 12:13-double bond.[4] The principal ion fragments are summarized in scheme I. The peak arising out of such a fragmentation is observed at *m/e* 292 (a species) which can then lead to the ion fragments appearing at *m/e* 246, 232, and 187 due to the loss of carboxyl, carbomethoxyl, or both groups, respectively. Loss of COOH+H from **II** gives rise to a peak at *m/e* 486 (M-46), which is hardly recognizable in the mass spectrum of jaligonic acid 28-monomethylester (**IV**). The spectrum of **IV**,

*Natural Products Research Institute, Seoul National University, Seoul, Korea.



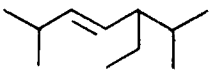


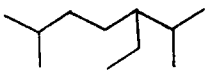
I $R_1=R_2=H$

II $R_1=Me$ $R_2=H$

III $R_1=R_2=Me$

IV $R_1=H$ $R_2=Me$

V $R_1=$  a R=H b R=Ac c R=Bz

VI $R_1=$  a R=H b R=Ac c R=Bz

however, shows a relatively intense peak at *m/e* 472[M-(COOCH$_3$+H)], thus these peaks could be used as a tool for distingshing from each other.

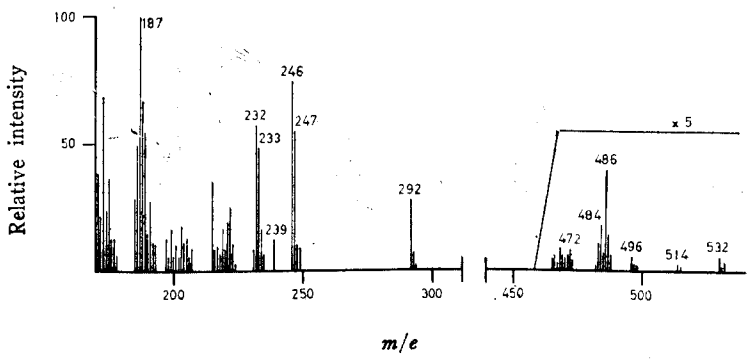
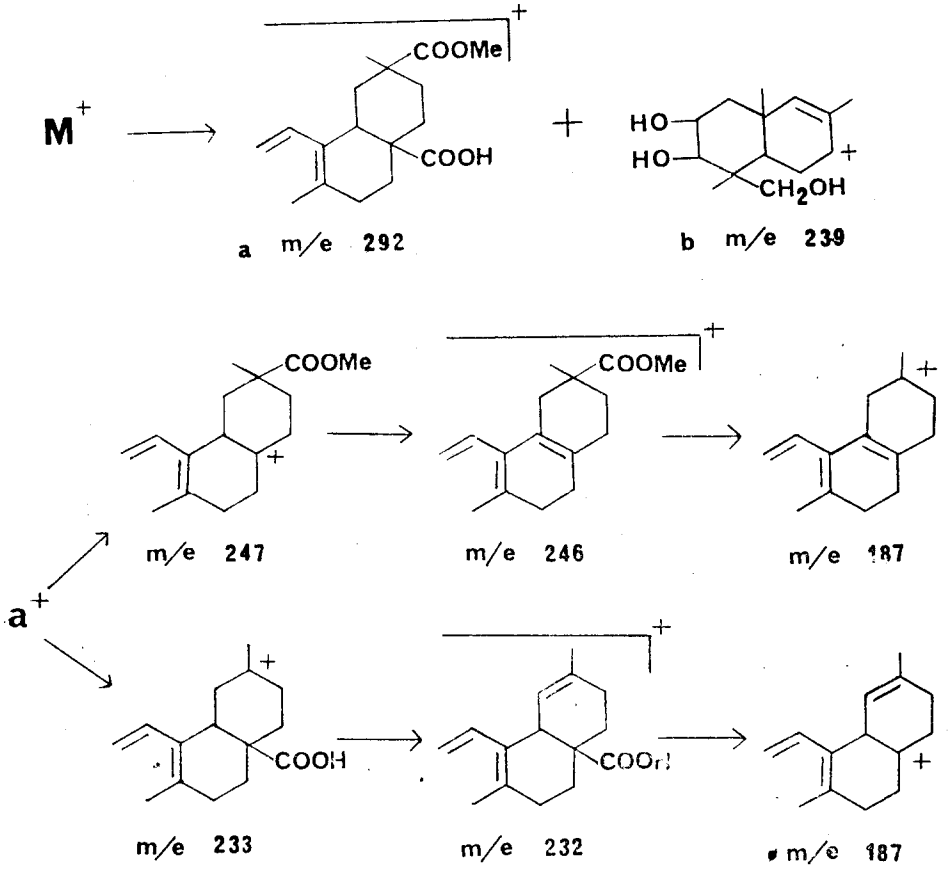


Fig. 1—Mass spectrum of 30-monomethyl jaligonate.

Sterols—Crude phytosterol mixture from Et_2O soluble unsaponifiable fraction was subjected to repeated recrystallizations from MeOH by which a steady rise of the melting point was brought about until finally fraction of colorless plates, $C_{29}H_{48}O \cdot 1/2\,H_2O$, mp 166-169°, $[\alpha]_D^{16}=-4.9°$ was obtained. This fraction was homogenous by tlc on silica gel plates with or without $AgNO_3$ and the melting point and specific rotation of this fraction was not altered substantially by further recrystallizations. The mass spectrum, however, shows that



Scheme 1—Mass spectral fragmentation of 30-monomethyljaligonate.

this fraction is still contaminated with a minor amount of related compound. The mass spectrum of this mixture has parent ions at *m/e* 412 and 414 and fragmentation peaks at *m/e* 397 and 399(M—CH_3) 394(M—H_2O), 383(M—C_2H_5), 379(397—H_2O), 369(M—*i*-C_3H_7), 351(369—H_2O), 300(M—part of side chain), 273(M—side chain), 271(273—2H), 255(273—H_2O), 253(271—H_2O), 246[273—(C16 and 17)], and 231(273—ring D), 229(246—HO), 213(231—H_2O). These fragmentations indicate that a major component is the Δ^7-sterol with a 22 : 23-unsaturated side chain.[5-11] The double bond between C-7 and C-8 could also be recognized from the 18-methyl and 19-methyl singlets at unusually high field (δ=0.54 and 0.79).[11-14] On treatment of this sterol mixture with the modified Liebermann-Burchard reagent the change of absorption at 620nm with the reaction time was quite simlar to that of the Δ^7-sterol type (Fig. 2) and the maximal molar *L* value was 1.84 in good agreement with that (1.87) for α-spinasterol reported by Idler, *et al.*[15]

The ir spectrum shows a peak at 965cm^{-1} characteristic of a 22 : 23-*trans* double bond[16-20] in addition to three peaks in the 790—850cm^{-1} region indicating a trisubstituted

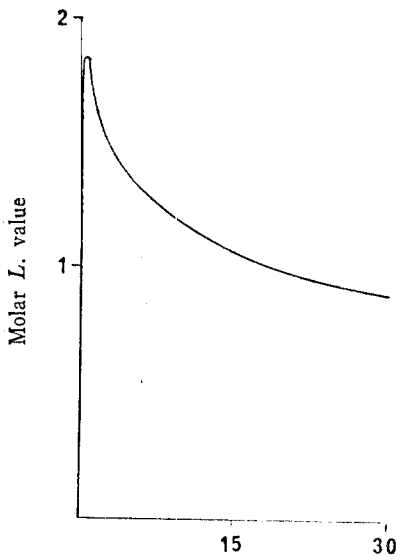


Fig. 2-Reactivity with the modified Liebermann-Burchard reagent of sterol isolated from *P. esculenta*.

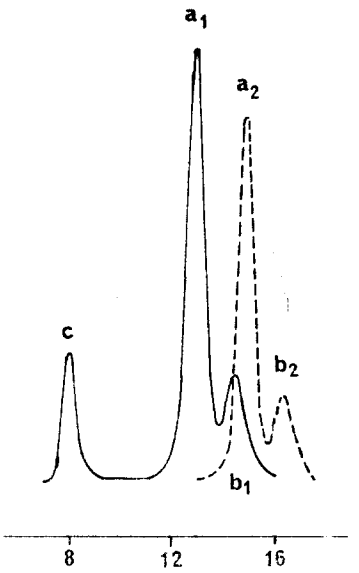


Fig. 3-Gas-liquid chromatogram of the separation of sterol mixture (—) and of acetate mixture(···). Peaks correspond to: a_1, α-spinasterol; b_1, Δ^7-stigmastenol; a_2, α-spinasteryl acetate; b_2, Δ^7-stigmastenyl acetate; c, cholesterol(internal standard).

double bond between C-7 and C-8.[20-23] Moreover, evidence for the presence of 22 : 23-unsaturation was provided by the 21-methyl doublet at farther low field, 57.8 and 64.5 Hz (J=6.7Hz) than those of the saturated side chain sterols.[13,24]

These data are consistent with the presence of α-spinasterol (**Va**) and Δ^7-stigmastenol (**VIa**).

This sterol mixture was readily acetylated and benzoylated. The fractional crystallization of these products led to the mixtures containing a higher proportion of derivatives of **V** but did not lead to complete separation of the components with the limited quantities available. The separation of two acetates or benzoates could be achieved by tlc on $AgNO_3$ impregnated silica gel plates.

Analysis of sterol mixture and its acetate by glc on 2% OV-17 column gave the result presented in Fig. 3. The major component has retention time identical to authentic α-spinasterol (**Va**) (peak a_1); a second component corresponds to Δ^7-stigmastenol (**VIa**) (peak b_1). Peaks a_2 and b_2 correspond to α-spinasteryl acetate (**Vb**) and Δ^7-stigmastenyl acetate (**VIb**), respectively.

Fig. 4 shows the mass spectra of **Va, Vb, VIa,** and **VIb** obtained by combined gas chromatography-mass spectrometry. The fragmentation patterns are identical to those of

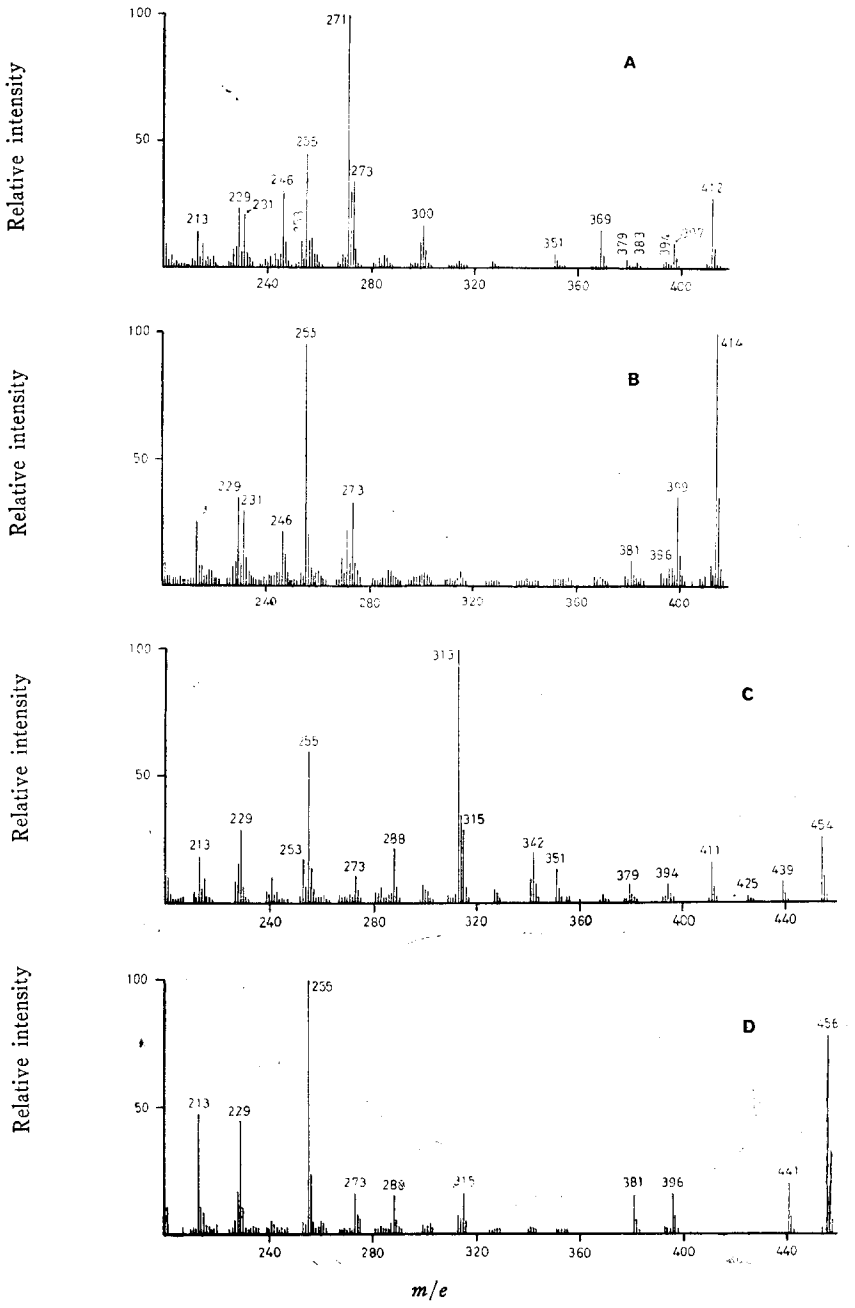


Fig. 4—Mass spectra of α-spinasterol (A). Δ^7-stigmastenol (B), α-spinasteryl acetate (C), and Δ^7-stigmastenyl acetate (D).

authentic samples, respectively. The acetate mixture was chromatographed on silica gel with $AgNO_3$ to give **Vb** in pure state. This is the first reported occurrence of these Δ^7-sterols in Phytolaccaceae, although the isolation of an unidentified phytosterol $C_{31}H_{56}O \cdot H_2O$, mp 166° (its acetate, mp 175—175.5°) from berries of *P. americana* L. has been reported.[25]

EXPERIMENTAL

The melting points were taken on a Mitamura-Riken apparatus and are uncorrected. The ir spectra were determined in KBr discs with a JASCO Model IR-S spectrophotometer and the nmr spectra were recorded in deuteriochloroform solution with a JEOL Model JEM-3H-60 instrument; peak positions are reported in ppm or Hz downfield from tetramethylsilane as an internal standard.

Extraction and Separation of Phytolaccagenin (II)—The ether extracts of roots were prepared as described earlier.[2] When this extracts were partitioned between Et_2O and 10% $NaHCO_3$ soln., jaligonic acid (**I**) was concentrated in aqueous layer. Et_2O layer was extracted with 5% NaOH soln. The precipitate formed on acidification of the alkaline solution with HCl, which consisted mainly of **II** and a trace amount of **I**, was chromatographed on silica gel. Elution of the column with $CHCl_3$-MeOH-7%HOAc(5 : 1 : 1) yielded in the earlier fractions phytolaccagenin(**II**), which on recrystallization from MeOH gave colorless needles, mp 317-319°, $[\alpha]_D^{20}$=+114.7° (c=0.83, MeOH), ir 3430cm^{-1}(OH), 1732cm^{-1} (ester), 1698cm^{-1}(COOH), 1660 and 824 cm^{-1} (double bond). It was identified with an authentic sample synthesized as below.

Anal. Calcd for $C_{31}H_{48}O_7$: C, 69.90; H, 9.08. Found: C, 69.98; H, 9.03.

Synthesis of Phytolaccagenin(II)—Jaligonic acid (**I**) was methylated with 3% MeOH-HCl as discribed earlier[2] to give **II**, mp 317-318°.

Hydrolysis of Phytolaccagenin(II)—A sample of **II** (20mg) was heated under reflux in 10% methanolic KOH soln. (20ml) for 5hr. Much H_2O was added and acidified with HCl, extrated with Et_2O, washed, dried, evaporated, and crystallized from MeOH to furnish prisms of **I**, mp 319—320°, undepressed on admixture with an authentic sample and having same tlc behavior and ir spectrum.

Dimethylester (III)—A sample of **II** (100mg) was esterified when dissolved in MeOH and treated with ethereal diazomethane. The crude product was chromatographed on silica gel (eluent, $CHCl_3$-MeOH-NH_4OH-H_2O=20 : 4 : 1 : 3) and crystallized from MeOH as needles, mp 213—215°, identical with an authentic sample by mixed mp, ir, and co-tlc.

28-Monomethyljaligonate(IV)—This compound was obtained by saponification of **III** under the same conditions as described earlier.[2] mp 321-324°; ir 1732 cm^{-1} (ester), 1710 cm^{-1} (acid); mass m/e 532(M^+, 0.5%), 514(M-H_2O, 0.5%), 496(M-2H_2O, 0.6%), 486[M-(COOH+H), 1%], 484[M-(H_2O+HCHO), 1.5%], 472[M-(COOMe+H), 2.8%], 292(a species, 28.5%), 247(a-COOH, 20%), 246[a-(COOH+H), 52.2%], 239(b species, 12.2%), 233(a-COOMe, 48.1%), 232[a-(COOH+H), 57%], 187[a-

(COOMe+COOH+H), 100%].

Tlc behavior (Table I) and spectra of ir and mass were very different from those of **II**.

Isolation of Sterol Mixture(Va and VIa)—The neutral Et_2O soluble fraction was saponified with 5% methanolic KOH for 2 hr. After filtering, filtrate was diluted with H_2O

Table I-Tlc behavior of terpenoids.*

Solven t system	Rf for compd.			
	I	II	III	IV
$CHCl_3$-MeOH-7%HOAc(5 : 1 : 1)	0.14	0.44	0.78	0.40
$CHCl_3$-MeOH-NH_4OH-H_2O(60 : 30 : 3 : 4)	0.29	0.76	0.94	0.71
i-PrOH-toluene-NH_4OH-H_2O(16 : 4 : 1 : 3)	0.23	0.59	0.85	0.54

*Compounds on the plates were detected by heating after treatment with 50% H_2SO_4.

and extracted with Et_2O. The repeated recrystallization of the unsaponifiable matter from MeOH gave mixture of **Va** and **VIa** as colorless plates, mp 166-169°, $[\alpha]_D^{16}=-4.9°$ (c=1.12, $CHCl_3$), ir 3450 cm^{-1} (OH), 965 cm^{-1} (disubstituted double bond), 840, 825 and 790 cm^{-1} (trisubstituted double bond), nmr δ 0.54 (s, 18-CH_3), 0.79 (s, 19-CH_3), 0.96 and 1.08 (d, 21-CH_3), 3.50 (b, H-C-OH), and 5.0-5.3 (m,-C=CH-and -CH=CH-). Glc data (Fig. 3) and mass spectrum showed that it contained mainly α-spinasterol (**Va**) and a trace amount of Δ^7-stigmastenol (**VIa**) which could not be removed by repeated recrystallization from various solvents.

Anal Calcd for $C_{29}H_{48}O\cdot 1/2H_2O$; C, 82.60; H, 11.71, Found; C, 82.55; H, 11.56.

Acetylation of Sterol Mixture—The sterol mixture(300mg) was heated with Ac_2O (8ml) and pyridine (5ml) for 2hr. Puring onto ice gave solids which were crystallized from MeOH to give the mixture of **Vb** and **VIb**, as needles, mp 178-181°, $[\alpha]_D^{14}=-2.0°$ (c=1.23, $CHCl_3$), ir 1742 and 1245 cm^{-1} (acetate), R*f* (silica gel with $AgNO_3$, solvent; cyclohexane-*i*-propyl ether=49 : 1, 4 times) 0.52 (**Vb**) and 0.61 (**VIb**). Mass spectrum showed M^+ at *m/e* 454 and a small speak at *m/e* 456. Other peaks are at *m/e* 439 and 441(M-CH_3), 425(M-C_2H_5), 411(M-*i*-C_3H_7), 394 and 396(M-HOAC), 379 and 381 [M-(CH_3+HOAc)], 351 (411-HOAc), 342 (M-part of side chain), 315 (M-side chain), 313 (315-2H), 288[315-(C16 and 17)], 273(315-ring D), 255(315-HOAc), 253(313-HOAc), 229(288-OAc), and 213(273-HOAc). Glc data (Fig. 3) showed that **Vb** predominated.

Benzoylation of Sterol Mixture—The sterol mixture (150mg) was heated with benzoyl chloride (0.2ml) and pyridine (10ml) for 2hr. Working up in the usual way afforded a benzoate mixture (**Vc** and **VIc**), which was crystallized from $CHCl_3$-MeOH mixture as plates, mp 196—198°, $[\alpha]_J^{15}=+7.2°$ (c=0.55, $CHCl_3$), ir 1725 and 1275 cm^{-1}, (benzoate), R*f* (same conditions as above) 0.70(**Vc**) and 0.77 (**VIc**).

Gas-Liquid Chromatography of Sterols and Derivatives—A Yanagimoto Model GLG-5DH gas chromatograph equipped with a hydrogen flame ionization detector was used for

most of this work. The chromatographic column was 150cm×3mm glass U-tube contained Chromosorb W (60-80 mesh) coated with 3% SE-30. In all cases, on-column injection was employed and temperatures were: column, 200-280° (6°/min); injector, 240-310°; detector, 230°. The nitrogen flow was 12.5 ml/min. Cholesterol was always run with the samples. For the analysis on 2% OV-17, a Shimadzu Model GC-4B fitted with FID was used. The column was 150cm×4mm glass and contained 2% OV-17 on chromosorb W (60-80 mesh). Column temp., 265°; injection temp., 240°; detector temp., 320° carrier gas, N_2(40ml/min).

Separation of Acetate Mixture by Column Chromatography—Silica gel (240 g), $AgNO_3$ (60 g), and distilled H_2O (400 ml) were mixed and dried under occasional stirring for 5hr at 110°. The mixture was suspended in petroleum ether-Et_2O (98 : 2) and packed in the column, on which the mixture of sterol acetates (200mg) was subjected. The column was eluted with same solvent and the fractions were monitored by glc. From the earlier fractions, a mixture containing varied proportions of two components was yielded. The latter fractions having only **Vb**, on recrystallization from MeOH, gave colorless needles, mp 180—181°. The ir and mass spectra and the retention time of gas chromatography of this compound were completely identified with those of authentic sample.

Anal Calcd for $C_{31}H_{50}O_2$:C, 81.88; H, 11.08. Found:C, 81.81; H, 11.03.

The Liebermann-Burchard Reaction—This reaction was carried out by treatment of the sample in 2ml of glacial acetic acid with 4.2ml of a previously chilled 20 : 1 mixture of acetic anhydride:H_2SO_4. The absorbance was measured at a wavelength of 620nm in a recording spectrophotometer (Shimadzu Model MPS-50L).

In order to compare with other data, molar L values calculated from the following formula:

$$\text{Molar } L = 1.9 \times A \times \frac{W}{w}$$

where A=absorbance; W=molecular weight of sample; w=g of sample taken.

Acknowledgments—We are indebted to Dr. O. Terauchi, Dokushima University, for the specimens of α-spinasteryl acetate and Δ^7-stigmastenyl acetate. We are gratefull to Mr. Yamakuchi, Tokyo University, for the measurements of the mass spectra of terpenoids, and to Mr. Ishijima, Japan Electron Optics Laboratory, for the combined gas chromatography-mass spectrometry analysis of steroids.

REFERENCES

1. W.S. Woo, *This Journal*, **16**, 99(1971)
2. W.S. Woo, *Lloydia*, **36**, 326(1973)
3. C. Djerassi, G.H. Thomas, and H. Monsimer, *J. Am. Chem. Soc.*, **77**, 3579(1955)
4. H. Budzikiewicz, J.M. Wilson, and C. Djerassi, *J. Am. Chem. Soc.*, **85**, 3688(1963)

5. M. Lenfant, E. Zissmann, and E. Lederer, *Tetrahedron Lett.*, **1967**, p-1049
6. B.A. Knight, *J. Gas Chromat.*, **5**, 273(1967)
7. M.C. Gershengorn, A.R.H. Smith, G. Goulston, L.J. Goad, T.W. Goodwin, and T.H. Haines, *Biochemistry*, **7**, 1698(1968)
8. M. Lenfant, R. Ellous, B.D. Das, E. Zissmann, and E. Lederer, *Eur. J. Biochem.*, **7**, 159 (1969)
9. M.A. Abdul-Alin, A.F. Aboulezz, M.B.E. Fayez, and A.E. Seedhom, *Z. Anal. Chem.*, **217**, 268 (1966)
10. S.G. Wyllie and C. Djerassi, *J. Org. Chem.*, **33**, 305(1968)
11. J.W. Clark-Lewis and I. Dainis, *J. Aust. Chem.*, **20**, 1961(1967)
12. J.N. Shooley and M.T. Rogers, *J. Am. Chem. Soc.*, **80**, 5121(1958)
13. M.J. Thompson, S.R. Dutky, G.W. Patterson, and E.L. Gooden, *Phytochemistry*, **11**, 1781 (1972)
14. W. Sucrow, *Chem. Ber.*, **99**, 3559(1966)
15. D.R. Idler and C.A. Baumann, *J. Biol. Chem.*, **203**, 389(1953)
16. J.J. Johnson, M.F. Grostic, and A.O. Jensen, *Anal. Chem.*, **29**, 468(1951)
17. K. Takeda, T. Kubota, and Y. Matsui, *Chem. Pharm. Bull.*, **6**, 437(1958)
18. R.N. Jones, *J. Am. Chem. Soc.*, **72**, 5322(1950)
19. H. Heftmann, B.E. Wright, and G.W. Liddel, *Arch. Biochem. Biophys.*, **91**, 266(1960)
20. H. Rosenkrantz, A.T. Milhorat, and M. Farber, *J. Biol. Chem.*, **195**, 503(1952)
21. D.R. Idler, A.A. Kandutsch, and C.A. Baumann, *J. Am. Chem. Soc.*, **75**, 4326(1953)
22. P. Bladon, I.M. Fabian, H.B. Henbest, H.P. Koch, and G.W. Wood, *J. Chem. Soc.*, **1951**, p-2401
23. H. Hirschmann, *J. Am. Chem. Soc.*, **73**, 5357(1952)
24. G. Slomp and F.A. Mackellar, *J. Am. Chem. Soc.*, **84**, 204(1962)
25. L.D. Jack and C.H. Rogers, *J. Am. Pharm. Assoc.*, **31**, 81(1942)