The Phylogenetic Distribution of Sterols in Tracheophytes

WILLIAM R. NES, KENNETH KREVITZ, JOHN JOSEPH, WILLIAM D. NES, BARRY HARRIS, and **GEOFFREY F. GIBBONS,** Department of Biological Sciences, Drexel University, Philadelphia, PA 19104, and **GLENN W. PATTERSON,** Department of Botany, University of Maryland College Park, MD 20742

ABSTRACT

The sterols of nine mature plant species in seven families ranging from the subphylum Lycopsida through the Filicopsida and the classes Gymnospermae and Angiospermae in the Pteropsida were structurally and stereochemically defined. Two plant categories were found. In the first, comprised by *Dryopteris (Thelypteris) noveboracensis, Polystichum acrostichoides, Dennstaedtia punctilobula, Osmunda cinnamomea, Ginkgo biloba, Cucurbita pepo,* and *Kalmia latifolia,* 24α-alkylsterols were dominant and were composed principally of 24α-ethylcholesterol (sitosterol) or (in *Cucurbita pepo*) 24α-ethyllathosterol and its *trans*-22-dehydro derivative (spinasterol). Depending on the species, small amounts of 24α-ethyl-*trans*-22-dehydrocholesterol (stigmasterol), 24α-methylcholesterol (campesterol), 24β-methylcholesterol (dihydrobrassicasterol, always less than campesterol), cholesterol, lathosterol, 24α-ethyllathosterol, 24ξ-methyllathosterol, *trans*-24-ethylidenelathosterol (Δ^7-avenasterol), and (tentatively identified) 24-ethyl-24(25)-dehydrolathosterol were present. *Spinacea oleracea* was also confirmed as belonging to Category I, and, except as described in what follows, Category I represents all other configurationally investigated vascular plants. The second category of plants contained only 24β-ethylsterols. Only one species (*Kalanchoe daigremontiana*) belonging to the family Crassulaceae was found, but one other (the genus *Clerodendrum* in the family Verbenaceae) is already known. *K. daigremontiana* contained 25(27)-dehydroclionasterol (clerosterol) and 25(27)-dehydroporiferasterol. The primitive *Lycopodium complanatum* was intermediate between Categories I and II; the sterols with a 24-C_2-group had only the 24α-configuration (sitosterol with some stigmasterol), but the principal sterols with a 24-C_1-group (ergosterol and dihydrobrassicasterol) possessed the 24β-configuration. *C. pepo* seeds, which are already known to contain principally 24β-ethylsterols, contrast sharply with our finding that tissue (pericarp of the fruit) from the mature plant contains only 24α-ethylsterols. This apparent evolutionary recapitulation (Category II to I) during development coupled with statistical dominance of Category II plants among the algae and fungi and of Category I plants in the Tracheophytes, and the existence of an intermediate type in the species examined from the lower Tracheophyte (Lycopsida) lead logically to the conclusion that the 24α-alkyl structure, especially 24α-ethyl-Δ^5-sterols (sitosterol and the much rarer stigmasterol) constitutes the most highly evolved type of 24-alkylsterol. By inference from our knowledge of biosynthesis, corroborated by the spectrum of sterols found here, the pathway [through $\Delta^{24(25)}$-sterols] leading to 24α-alkylsterols appears to be higher than the pathway [through $\Delta^{25(27)}$-sterols] which leads to 24β-alkylsterols. The sterols of these and other plants were also found amenable to classification according to their nuclear unsaturation (Δ^5, A; Δ^7, B; and $\Delta^{5,7}$, C). Tracheophytes of Category A have been most frequently encountered, but *C. pepo* was shown to be of Category B throughout its ontogeny. While no Tracheophytes of pure Category C have been discovered, *L. complanatum* was shown to be of the mixed A-C-Type. Based on these facts and ideas, some previously suggested lines of botanical evolution are examined. The chemical data fail to verify a line from Magnoliales to the *Cucurbitaceae,* from Magnoliales through Theales to Ericales, nor from Ranales to Saxifragales. However, they are consonant with a relationship between Cucurbitaceae and Theales and between Rosales and Lamiales. Triterpenoids found in various of the families studied included cycloartenol and friedelin. The spectroscopic properties of the latter are described.

INTRODUCTION

With the recent development of reversed

phase chromatographic methods for the separation of homologous sterols (1-3) and of proton magnetic resonance spectroscopy at 220 MHz for configurational analysis at C-25 (4,5), it has become possible to examine the relationship between structure and occurrence of sterols with quantitative and stereochemical precision and thereby to shed light on the biosynthetic pathways operating at various stages of the evolutionary hierarchy. Proton magnetic resonance (PMR) spectroscopy is of special significance. Since we have not seen the same spectrum given by two different sterols, the spectra at 220 MHz constitute a "finger print." In the case of the Phylum Tracheophyta, we recently used this methodology to examine the sterols of representatives drawn from a cross-section of ferns and seed-bearing plants (4). One plant each in the subphylum Filicopsida (the New York fern) and in the Pteropsida class Gymnospermae (a pine) were examined. Among the Angiospermae, we included the two most primitive orders (Magnoliales and Ranales), one more advanced order (Leguminales) of the woody Dicotyledons (Lignosae), and one order (Cruciales) in the herbaceous Dicotyledons. In all cases, 24α-ethylcholesterol was the major component and no 24β-ethylsterol was detectable. However, contrary to earlier suppositions, the 24-methylcholesterol was a mixture of 24α- and 24β-methyl epimers in which the former was dominant. This work made it clear that two pathways must operate simultaneously, both generally as well as in a given plant, at the 24-methyl level. On the other hand, at the 24-ethyl level, a pathway usually exists apparently only for a single configuration (24α), but the problem is complicated by the fact that 24β-ethylsterols do exist in higher Tracheophytes. They have been found in the seeds of several genera in the family Cucurbitaceae of the order Cucurbitales (6-9) and in two species of the genus *Clerodendrum* in the order Verbenales (10,11). In order to ascertain just how common one or other of the configurations is, we have continued our investigation of plants arranged phylogenetically.

In the present study, we chose a representative (*Lycopodium complanatum*) of the subphylum Lycopsida which is less advanced than either the Filicopsida or Pteropsida previously analyzed. We also chose three more ferns [Hay-scented and Christmas ferns in the common family Polypodiaceae and Cinnamon in the family of so-called "flowering ferns" (Osmundaceae)] as well as a representative (the maidenhair tree) of the Gymnospermae which is less advanced than the pine previously studied and a representative (*Kalanchoe daigremontiana*) of the biochemically and morphologically primitive Crassulaceae family in the herbaceous angiosperm order Saxifragales. Furthermore, the New York fern (4) was reexamined for minor components detected by gas liquid chromatography (GLC) but not previously reported on. From the woody Dicotyledons, we also chose a plant (mountain laurel) in the family Ericaceae from the order Ericales which is believed (12) to have arisen from Magnoliales through an order (Theales) different from those (Dilleniales, Coriariales, and Rosales) suggested (12) as intermediates to the previously studied (4) Leguminales. Finally, we examined the fruit and leaves of the pumpkin from the family Cucurbitaceae in the order Cucurbitales, because the seeds are known (6-9) to contain principally 24β-ethylsterols.

The nomenclature used in this paper takes cholesterol, its Δ^7-analog, lathosterol, and certain other sterols with well established trivial names as parents. It is summarized in Table I.

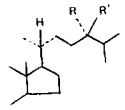
MATERIALS AND METHODS

Cultivated male maidenhair tree leaves (*Ginkgo biloba*), and the leaves and pericarp (fleshy part of the fruit) of cultivated pumpkin plants (*Cucurbita pepo*), the leaves of wild mountain laurel (*Kalmia latifolia*), and fronds of the wild New York fern (*Dryopteris noveboracensis*) (also known as *Thelypteris noveboracensis*) were collected in southeastern Pennsylvania. Specimens of *Kalanchoe daigremontiana* Hamet and Perr., native to S.W. Madagascar, were grown in the Drexel University greenhouse. Whole plants were used for chemical study. One sample (stems and fronds) of *Lycopodium complanatum* and the sample of the Christmas fern (*Polystichum acrostichoides*) (fronds) were collected from wild growths in Massachusetts in the summer. The other sample of *L. complanatum* was collected in Virginia in November. The fronds of the hay-scented [*Dennstaedtia punctilobula* (Michx.) Moore] and Cinnamon (*Osmunda cinnamomea* L.) ferns were collected from wild growths in Maryland. For *Spinacea oleracea*, we used fresh leaves sold commercially as food.

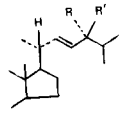
Except as noted, the 4,4-dimethyl- and 4-desmethyl substances were isolated from the specimens as previously described (4) through continuous acetone extraction, saponification, and sequential chromatography on alumina with ether graded into hexane and on lipophilic Sephadex (Lipidex-5000) with 5% hexane in methanol. This procedure yields sterols from both the free and esterified pools. Spectral and gas liquid chromatographic procedures were

TABLE I

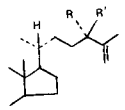
Sterol Nomenclature Used



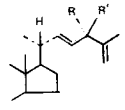
Substitution		Name	
R	R′	Δ^5-Series	Δ^7-Series
H	H	Cholesterol	Lathosterol
H	CH_3	24α-Methylcholesterol (Campesterol)	24α-Methyllathosterol (24-Epifungisterol)
CH_3	H	24β-Methylcholesterol (22-Dihydrobrassicasterol)	24β-Methyllathosterol (Fungisterol)
H	C_2H_5	24α-Ethylcholesterol (Sitosterol)	24α-Ethyllathosterol (22-Dihydrospinasterol)
C_2H_5	H	24β-Ethylcholesterol (Clionasterol)	24β-Ethyllathosterol (22-Dihydrochondrillasterol)



Substitution		Name	
H	H	*trans*-22-Dehydrocholesterol	*trans*-22-Dehydrolathosterol
H	CH_3	*trans*-22-Dehydrocampesterol (24-Epibrassicasterol)	24α-Methyl-*trans*-22-dehydrolathosterol (22-Dehydro-24-epifungisterol)
CH_3	H	Brassicasterol	24β-Methyl-*trans*-22-dehydrolathosterol (*trans*-22-Dehydrofungisterol)
H	C_2H_5	*trans*-22-Dehydrositosterol (Stigmasterol)	24α-Ethyl-*trans*-22-dehydrolathosterol (Spinasterol)
C_2H_5	H	*trans*-22-Dehydroclionasterol (Poriferasterol)	24β-Ethyl-*trans*-22-dehydrolathosterol (Chondrillasterol)



Substitution		Name	
CH_3	H	24β-Methyl-25(27)-dehydrocholesterol (Codisterol)	24β-Methyl-25(27)-dehydrolathosterol
C_2H_5	H	24β-Ethyl-25(27)-dehydrocholesterol (Clerosterol) (25(27)-Dehydroclionasterol)	24β-Ethyl-25(27)-dehydrolathosterol



Substitution		Name	
CH_3	H	24β-Methyl-*trans*-22,25(27)-bisdehydrocholesterol (*trans*-22-Dehydrocodisterol)	24β-Methyl-*trans*-22,25(27)-bisdehydrolathosterol (*trans*-22,25(27)-Bisdehydrofungisterol)
C_2H_5	H	24β-Ethyl-*trans*-22,25(27)-bisdehydrocholesterol (25(27)-Dehydroporiferasterol) (*trans*-22-Dehydroclerosterol)	24β-Ethyl-*trans*-22,25(27)-bisdehydrolathosterol (25(27)-Dehydrochondrillasterol)

also described earlier (4). PMR spectra were performed at 220 MHz in $CDCl_3$ unless otherwise noted. Retention times in GLC are described (RRT) in terms of their values relative to the retention time of cholesterol, and the liquid phase was XE-60 at 235 C. Representative mass and PMR spectra are listed in Tables II–VI for sterols derived from plants examined in the present work. For comparison, especially in the case of epimers at C-24, authentic sterols are included for some of which spectral data have not heretofore been published. The corresponding information in the one case of a mature plant in which 24β-ethylsterols were found to be dominant is given in that part of the Results section dealing with *K. daigremontiana.* Additional data on Δ^7-sterols is given in the Results section under *C. pepo.*

RESULTS

Lycopodium complanatum

The mass spectrum of the 4-desmethylsterol fraction from alumina of the specimen collected in the summer showed the presence of materials with molecular weights of 396, 414, 400, and 412 (in descending order of the intensity of M^+). Three sterol fractions [50 mg combined from 118 g (wet wt) of plant] were then separated on Sephadex. The fastest

TABLE II

Typical Mass Spectra of Δ^5 and Δ^7-Sterols from Tracheophytes

	m/e, %						
	Cholesterol series				Lathosterol series		
Side chain	24–H	24–CH_3	24–C_2H_5	24–C_2H_5-Δ^{22}	24–H	24–C_2H_5	24–C_2H_5–Δ^{22}
Source	Christmas Fern	Christmas Fern	Christmas Fern	Soybeans	NY Fern	NY Fern	*C. pepo* Pericarp
Fragmentation							
M^+	386, 100	400, 100	414, 100	412, 66	386, 100	414, 100	412, 28
M^+-CH_3	371, 32	385, 29	399, 29	397, 9	371, 29	399, 26	397, 10
M^+-H_2O	368, 47	382, 42	396, 44	394, 11	368, 2	369, 2	394, 1
$M^+-CH_3-H_2O$	353, 33	367, 30	381, 28	379, 13	353, 5	381, 4	379, 1
$M^+-C_3H_7$	-	-	-	369, 22	-	-	369, 13
M^+-85	301, 43	315, 41	329, 39	327, 5	-	-	-
$M^+-C_7H_{11}$ or $_{12}O$	275, 56	289, 47	303, 44	300, 42	-	-	-
M^+–(SC = Side Chain)	273, 26	273, 241	273, 25	273, 23	273, 21	273, 21	273, 31
M^+–SC–2H	271, 15	271, 2	271, 2	271, 59	-	-	271, 100
M^+–SC–H_2O	255, 36	255, 29	255, 28	255, 100	255, 75	255, 67	255, 40
M^+–SC–H_2O–2H	253, 6	253, 1	-	-	-	-	-
M^+–SC–C_3H_6	231, 30	231, 27	231, 24	231, 18	231, 31	231, 25	231, 18
M^+–SC–C_3H_8	229, 18	229, 12	229, 11	229, 21	229, 24	229, 20	229, 21
M^+–SC–C_3H_6–H_2O	213, 43	213, 39	213, 39	213, 45	213, 33	213, 24	213, 20
M^+–SC–C_3H_8–H_2O	211, 9	211, 2	211, 1	-	211, 2	211, 1	211, 3

TABLE III

Mass Spectra of Tracheophyte Ergosterol and Related Standards

Side chain	m/e, % in 7-Dehydrocholesterol series					
	24–H[a]	24α–CH_3[b]	24α–CH_3–Δ^{22}[c]	24β–CH_3–Δ^{22}[d]	24β–CH_3–Δ^{22}[e]	24β–CH_3–Δ^{22}[f]
Fragmentation						
M^+	-	-	-	-	396, 86	396, 99
M^+-CH_3	-	-	-	-	381, 3	381, 4
M^+–HOX (X = H or Ac)	366, 100	380, 100	378, 100	375, 100	378, 5	378, 7
M^+–HOX–CH_3	351, 13	365, 10	363, 5	363, 8	363, 100	363, 100
M^+–HOX–C_3H_5	325, 1	-	-	-	337, 45	337, 41
M^+–HOX–C_3H_7	-	337, 1	335, 2	335, 2	-	-
M^+–SC (SC = Side Chain)	-	-	-	-	271, 23	271, 30
M^+–HOX–SC	253, 20	253, 18	253, 29	253, 45	253, 59	253, 52
M^+–HOX–SC–C_3H_6	211, 13	211, 30	211, 11	211, 14	211, 36	211, 34
M^+–HOX–SC–C_3H_8	209, 2	209, 1	209, 5	209, 5	209, 4	209, 8

[a]7-Dehydrocholesteryl acetate synthesized from cholesterol.

[b]7-Dehydrocampesteryl acetate biosynthesized (*Tetrahymena pyriformis*) from 24α-methylcholesterol. We thank Dr. R.L. Conner and Mrs. J.R. Landrey for the incubations, a full report of which will be made elsewhere.

[c]24-Epiergosteryl acetate derived as described under footnote-b.

[d]Ergosteryl acetate from yeast.

[e]Ergosterol from yeast.

[f]Ergosterol from *Lycopodium complanatum*.

TABLE IV

Representative Proton Magnetic Resonance (PMR) Spectra of Tracheophyte Sterols in the Cholesterol Series[a]

Group at C–24 and side chain double bond	Chemical shift in ppm from TMS at 220 MHz								
	H[b]	α–CH_3[c]	β–CH_3[d]	α–C_2H_5[e]	β–C_2H_5[f]	α–CH_3–Δ^{22}[g]	β–CH_3–Δ^{22}[h]	α–C_2H_5–Δ^{22}[i]	β–C_2H_5–Δ^{22}[j]
Proton position									
C–18 (s)	0.68	0.68	0.68	0.68	0.68	0.69	0.70	0.70	0.70
C–19 (s)	1.01	1.01	1.01	1.01	1.01	1.02	1.01	1.01	1.01
C–21 (d) (J = 6)	0.91	0.91	0.92	0.92	0.93	1.00	1.01	1.02	1.03
C–26,27 (d) (J = 6)	0.87	0.77 0.85	0.77 0.86	0.81 0.84	0.81 0.83	0.82 0.83	0.82 0.83	0.80 0.85	0.79 0.85
C–28 (d) (J = 6)	-	0.80	0.78	-	-	0.91	0.91	-	-
C–29 (t) (J = 7-8)	-	-	-	0.85	0.86	-	-	0.80	0.81

[a]Abbreviations: s = singlet; d = doublet; t = triplet; J is given in Hz.

[b]New York fern.

[c]Main 24-methyl component of Christmas fern.

[d]Main 24-methyl-Δ^5-sterol from *Lycopodium complanatum.*

[e]*Kalmia latifolia.*

[f]*Chlorella ellipsoidea.*

[g]Diatoms, ref. 5.

[h]*Brassica rapa* seeds.

[i]Soybeans.

[j]*Chlorella ellipsoidea.*

TABLE V

Representative Proton Magnetic Resonance (PMR) Spectra of Tracheophyte Sterols and Standards in the Lathosterol Series[a]

Group at C−24 and side chain double bond	Chemical shift in ppm from TMS at 220 MHz								
	H[b]	α−CH_3[c]	β−CH_3[d]	α−C_2H_5[e]	β−C_2H_5[f]	α−CH_3−Δ^{22}[g]	β−CH_3−Δ^{22}[h]	α−C_2H_5−Δ^{22}[i]	β−C_2H_5−Δ^{22}[j]
Proton position									
C−18 (s)	0.53	-	0.54	0.54	0.54	-	0.54	0.55	0.55
C−19 (s)	0.80	-	0.80	0.80	0.80	-	0.80	0.80	0.80
C−21 (d) (J = 6)	0.92	-	0.92	0.93	0.93	-	1.01	1.02	1.03
C−26,27 (d) (J = 6)	0.87 0.86	- -	0.85 0.77	0.84 0.82	0.84 0.82	- -	0.83 0.82	0.85 0.80	0.85 0.79
C−28 (d) (J = 6)	-	-	0.78	-	-	-	0.91	-	-
C−29 (t) (J = 7)	-	-	-	0.85	0.86	-	-	0.80	0.82

[a]Abbreviations: s = singlet; t = triplet; J is given in Hz.

[b]From New York fern.

[c]Probably (MS) present in New York fern and *C. pepo* but insufficient amount for PMR.

[d]From *Chlorella emersonii* or signifies doublet.

[e]From *C. pepo* pericarp, or implies doublet.

[f]From *Chlorella emersonii.*

[g]Unknown.

[h]Synthetic; gift of H. Kircher.

[i]From *Spinacea oleracea.*

[j]From *Chlorella emersonii.*

TABLE VI

Proton Magnetic Resonance (PMR) Spectra of Sterols in the 7-Dehydrocholesterol Series[a]

Side chain	Chemical shift in ppm from TMS at 220 MHz				
	24–H[b]	24α–CH_3[c]	24α–CH_3–Δ^{22}[c]	24β–CH_3–Δ^{22}[d]	24β–CH_3–Δ^{22}[e]
C–18 (s)	0.61	0.62	0.63	0.63	0.63
C–19 (s)	0.94	0.95	0.95	0.95	0.95
C–21 (d) (J = 6)	0.94	0.93	1.03	1.04	1.04 (1.03 sh)
C–26,27 (d) (J = 6)	0.87	0.80 0.78	0.84 0.83	0.84 0.83	0.84 0.83
C–28 (d) (J = 6)	-	0.81	0.92	0.92	0.92 (0.91 sh)

[a]Abbreviations: s = singlet; t = triplet; J is given in Hz.

[b]Prepared synthetically from cholesterol.

[c]Prepared biosynthetically (*Tetrahymena pyriformis*) from 24α-methylcholesterol. We thank Dr. R.L. Conner and Mrs. J.R. Landrey for the incubations, a full report of which will appear elsewhere.

[d]Isolated from aerobically grown yeast or signifies doublet.

[e]Isolated from *Lycopodium complanatum*, or implies doublet in left-hand column.

moving component (15 mg) as described in a preliminary communication (13) was primarily ergosterol. This was demonstrated by its movement in GLC (RRT 1.30) and by its UV spectrum (λ_{max} 271, 282, and 293 nm) and mass and PMR spectra (Tables III and VI) when compared to that of ergosterol and 24-epiergosterol which was prepared biochemically from 24α-methylcholesterol (13). The PMR spectra of the epimers differ in the position of the signal for C-21 which is 2 Hz downfield in ergosterol. An upfield shoulder (intensity ca. 0.5) on the doublet for C-21 indicated the presence of 24-epiergosterol as a minor component. Only one other report (14) exists of ergosterol in a Tracheophyte and the configuration at C-24 was not determined. Not described in our preliminary paper was the next fraction (12 mg) which was a mixture of stigmasterol and 24-methylcholesterol in a ratio of 2:1, respectively. The ratio is based on the intensities of the signals for C-18 in the PMR spectrum. In the mass spectrum, the peaks for m/e 400 and 412 were equal, but stigmasterol, a $\Delta^{5,22}$-sterol, has a weaker peak for the molecular ion than do Δ^{5}-sterols due to enhanced allylic cleavage at the 17(20)-bond (Table II). The mass and PMR spectra were exact composites throughout of stigmasterol and 24-methylcholesterol. The 24α-configuration of the stigmasterol was demonstrated by comparison of the C-21 doublet with that of authentic samples of stigmasterol from soy beans and poriferasterol from *Chlorella ellipsoidea* (15) (Table IV). The doublet for C-21 and the triplet for C-29 were displaced downfield (1-2 Hz) in the latter (4,5). The triplet was interferred with by the 24-methylcholesterol in the *Lycopodium* sterol mixture, but the C-21 signal, being shifted due to the Δ^{22}-bond, was clearly visible as the downfield branch. It was exactly at the position found in stigmasterol. The configuration of the 24-methylcholesterol was also demonstrated by PMR spectroscopy. The diagnostic doublets (4) for C-21 and C-28 clearly showed the major epimer was 24β-methylcholesterol (Table IV). Indeed, there was only a suggestion (weak shoulders) of the presence of 24α-methylcholesterol. Also not reported in our preliminary paper (13) was the slowest moving component (19 mg) on Sephadex. It was very pure, identical with sitosterol in all respects (GLC, MS, and PMR), and different from its 24-epimer, clionasterol, in the PMR. Clionasterol was isolated from *Chlorella ellipsoidea*. Thus, the *Lycopodium* sterols were in descending order of their amounts, ergosterol, sitosterol, stigmasterol, and 22-dihydrobrassicasterol with small amounts of materials tentatively identified (PMR) as 24-epiergosterol and campesterol. The UV spectrum of the sterol mixture from the plant collected in the fall showed the typical absorption for the $\Delta^{5,7}$-system but less than from the summer sample.

The Ferns

In all four cases, the major sterol was sitosterol (GLC, MS, PMR) unaccompanied by stigmasterol (no m/e 412 peak occurring in the 24-methyl fraction with which it moves on Sephadex). The epimeric 24-methylcholesterols were always present as minor components (GLC, MS, and PMR for each fraction from each plant). The α/β-ratio for the latter deter-

mined by PMR in a manner described earlier (4) varied slightly, but campesterol was always present to a greater extent (ca. twice as much) than dihydrobrassicasterol. In the case of the Christmas fern, cholesterol (GLC, MS, and PMR) was also present. The New York fern similarly yielded cholesterol (GLC, MS, PMR). Thus, two of the ferns possessed the homologous Δ^5-series: 24–H, 24–CH_3, and 24–C_2H_5. The New York fern also contained by GLC the analogous Δ^7-homologs. Enriched fractions of the latter were obtained from the more polar half of the sterol band from alumina chromatography. They were obtained pure by sequential chromatography on thin layers of silica gel (removing Δ^5-sterols which moved faster) and a column of Sephadex (separating the homologs). Lathosterol moved the fastest. It was identified by GLC, MS, and PMR. 24α-Ethyllathosterol, moving the slowest, was similarly identified. The configuration at C-24 was demonstrated by comparison with authentic samples of 24α-ethyllathosterol from *Spinacea oleracea* (16) and 24β-ethyllathosterol from *Chlorella emersonii* (17). The PMR spectra of the epimeric standards (Table V) showed the expected (4,5) downfield shifts in the singals for C-21 and C-29 yielding clearly different absorption patterns which, incidentally, spectroscopically confirms that *S. oleracea* has sterols with the opposite configuration from that in *C. emersonii*. The spectrum of the fern sterol was identical with that of the 24α-epimer from *S. oleracea*. While there was a suggestion of 24-methyllathosterol in the GLC of the appropriate chromatographic fractions, there was too little to identify.

The ferns, in summary, contained 24α-ethylcholesterol, no stigmasterol, lesser amounts of 24α-methylcholesterol which was in greater quantity than 24β-methylcholesterol, and, in certain cases, still smaller amounts of cholesterol. Δ^7-Sterols appearing only in the New York fern were in lesser amount than Δ^5-sterols, but, as in the latter case, the 24α-ethyl component was in greater amount than the component with a 24-H-atom. No seasonal variant was observed (GLC) in the relative constituents with the New York fern which was examined both in early summer and the fall. From a summer sample, 58 mg of sterol from 100 g of wet wt of tissue was obtained. The sterols were in the following ratio: 24–C_2–Δ^5 to 24–C_1–Δ^5 to 24–H–Δ^5 to 24–C_2–Δ^7 to 24–H–Δ^7: 1.0 to 0.18 to 0.11 to 0.08 to 0.07. The Christmas fern yielded 29 mg of sterol per 100 g wet wt of tissue. The 24–C_2–Δ^5–, 24–C_1–Δ^5–, and 24–H–Δ^5– components were present in a ratio of 1.0 to 0.18 to 0.10. An unidentified component (RRT 1.81) which probably was 24α-ethyllathosterol was also present at about the level of cholesterol.

Ginkgo biloba

The sterol of the maidenhair tree has been reported (18) to be 24-ethylcholesterol (beleived but unproven to be the 24α-epimer, sitosterol, identified by m.p., GLC, and IR) containing a trace of its 22-dehydroderivative (thought to be stigmasterol identified only by GLC). We separated the two components by chromatography on lipophilic Sephadex for further study. The major component, moving the slower, had the same rate of movement in GLC as sitosterol. It possessed a PMR spectrum identical with that of sitosterol (4,5) and distinctly different from that of the epimeric clionasterol (4,5) proving the suspected 24α-ethyl configuration to be correct. The component moving faster on Sephadex had a retention time in GLC the same as 24-methylcholesterol which is similar to that of stigmasterol. Its mass spectrum, however, was identical to that of 24-methylcholesterol rather than the suspected stigmasterol. Based on the molecular ions at m/e 398, 400, and 412, the sample was 83% 24-methylcholesterol, 10% "stigmasterol", and 5% "brassicasterol." Minor components also were evident from enhanced fragmentations at m/e 314, 299, 172, and 229. It is not obvious what they were due to, although they are found with 25(27)-dehydroclionasterol. The PMR spectrum was identical with that of an authentic 60:40 mixture (4) of 24α- and 24β-methylcholesterol and different from either alone (4,5). A weak "doublet" (J = 7) from a minor component appeared at 0.98 ppm which, however, does not agree with expectation for stigmasterol, brassicasterol, or 25(27)-dehydroclionasterol. Cholesterol was also apparent in the mixed sterols by GLC. The major sterols of the maidenhair tree are, thus, in descending order of amount, 24α-ethyl-, 24α-methyl, and 24β-methylcholesterol and probably cholesterol itself with trace amounts of unidentified materials of molecular weights 412, 398, and perhaps others. The leaves yielded 55 mg of sterol per 100 g wet wt. The 24–C_2–Δ^5–, 24–C_2–$\Delta^{5,22}$–, 24–C_1–Δ^5–, and 24–H–Δ^5–sterols were present in a ratio of 1.0 to trace to 0.10 to 0.10.

Kalanchoe daigremontiana

The 4-desmethylsterol mixture was composed of two sterols (RRT of 1.42 and 1.54). They were separated on Sephadex. The faster moving (RRT 1.42) exhibited the mass spectrum [m/e 410 (M^+, 19%), 395 (M^+–CH_3, 5%),

392 (M^+-H_2O, 4%), 381 ($M^+-C_2H_5$, 14%), 377 ($M^+-CH_3-H_2O$, 4%), 363 ($M^+-C_2H_5-H_2O$, 11%), 325 (M^+–85, 7%), 314 ($M^+-C_7H_{12}$, 9%), 309 (M^+–101, 11%), 300 (M^+–110, 41%), 271 (M^+–side chain–2H, 100%), 255 (M^+–side chain–H_2O, 58%), 253 (M^+-H_2O–side chain–2H), 239 (8%), 215 (17%), and 213 (M^+-H_2O-side chain–C_3H_6, 26%]; PMR spectrum [0.70 (s, C-18), 0,83 (t, J = 7.5 Hz, C–29], 1.03 (d, J = 6.5 Hz, C–21), 1.01 (s, C-19), and 1.65 ("d", J = 1 Hz, C–26) ppm; m.p. (151 C); and IR spectra (ν_{max} 965 and 890 cm^{-1}) expected of 25(27)-dehydroporiferasterol [previously isolated from *Clerodendrum campbelli* from which its structure and configuration were demonstrated (11)]. The slower moving component (RRT 1.55, m.p. 135-136 C, ν_{max} 890 cm^{-1}) in mass spectroscopy showed m/e 412 (M^+, 100%), 397 (M^+-CH_3, 25%), 394 (M^+-H_2O, 17%), 381 ($M^+-C_2H_7$, 5%), 379 ($M^+-CH_3-H_2O$, 28%), 328 (M^+–84, 18%), 314 ($M^+-C_7H_{14}$, 45%), 299 ($M^+-C_8H_{17}$, 50%), 281 (15%), 271 (M^+–side chain–2H, 75%), 255 (M^+–side chain–H_2O, 33%), 253 (M^+–side chain–H_2O–2H, 20%), 231 (M^+–side chain–C_3H_6, 38%), 229 (M^+-side chain–C_3H_8, 33%), and 213 (M^+-H_2O–side chain–C_3H_6, 67%). In the PMR spectrum, signals appeared at 0.68 (s, C–18), 0,80 (t, J = 7.5 Hz, C–29), 0.91 (d, J = 6 Hz, C–21), 1.01 (s, C–19), and 1.57 ("d", J = 1 Hz, C–26) ppm. These data prove the compound to be either the 22-dihydro derivative [25(27)-dehydroclionasterol] of the faster moving component or the analogous derivative [25(27)-dehydrositosterol] of the latter's 24-epimer. The 24β-configuration of both fast and slow moving components was demonstrated as follows. The mixed 4-desmethylsterols from alumina chromatography of the neutral lipid were converted to their corresponding 3,5-cyclo-6β-yl methyl ethers by treatment with fused KOAc in methanol at reflux for 2 hr. The chromatographically (Al_2O_3) purified but unseparated pair of methyl ethers (RRT 0.46 and 0.50) was hydrogenated (PtO_2, dioxane/HOAc) to give a single 3,5-cyclosteryl methyl ether (RRT 0.50). Retrorearrangement (fused ZnOAc, HOAc, 6 hr at reflux) gave a single Δ^5-sterol which had a m.p. (136-137 C) and PMR spectrum identical with clionasteryl acetate (from *Chlorella ellipsoidea*). Clionasteryl and sitosteryl acetates have distinctly different melting points and PMR spectra (4,5). Our *Kalanchoe* sterols must, therefore, both have had the 24β-ethyl configuration and been 25(27)-dehydroclionasterol [clerosterol previously isolated from *Clerodendrum infortunatum* (10) but without proof for the configuration at C–24] and 25(27)-dehydroporiferasterol (*trans*-22-dehydroclerosterol). Other $\Delta^{25(27)}$-sterols, viz. cyclolaudenol and the 24β-methyl analog (codisterol) of 25(27)-dehydroclionasterol, occur in algae (19,20), and the former in Tracheophytes, e.g., ferns (21). The 25(27)-designation rather than 25(26) is made by the following analogy. When 25(27)-dehydroporiferasterol was labelled from 2-^{14}C-MVA, the methylene carbon was found to be unlabelled (11). If we designate C–26 as the labelled carbon derived from 2-^{14}C-MVA, then cyclolaudenol, codisterol, clerosterol, and the latter's 22-dehydro derivative become $\Delta^{25(27)}$-sterols on the assumption that the same biosynthetic stereospecificity occurs in both algal and Tracheophyte systems and is the same for the first as well as second C_1-transfer. To our knowledge, this work represents the first proof of configuration at C–24 for clerosterol, the first time both clerosterol and its 22-dehydro derivative have been found in the same plant, and only the second time either has been reported. From the very watery *K. daigremontiana* plants was isolated 5.4 mg of sterol per 100 g of wet wt. Each of the two components comprised about one-half of the total amount.

It is also interesting that we observed a 1 Hz split in the "singlet" for C–26 in both $\Delta^{25(27)}$-sterols. Tentatively, we interpret this to result from a coupling with the H-atom on C–24 which implies C–26 lies between the H-atom and C–28 (as shown in Table I) in the preferred conformation. In turn, since a higher multiplet was not observed, the two H-atoms on C–28 must lie on the side opposite to C–26 with C–29 projecting away ("up") from the remainder of the side chain.

Kalmia latifolia

The only sterol found was sitosterol (m.p., GLC, MS, PMR) at a level of 59 mg/100 g wet wt. Since a ketone in even larger amount (78 mg/100 g) was present (moving on alumina just after the hydrocarbons), we also examined it to determine whether it was steroidal. While it was not, it is an interesting enough compound to report on.

The ketone possessed an RRT of 2.81, a.m.p. greater than 230 C, and a carbonyl peak (ν_{max} 1790 cm^{-1}) in the IR indicative of the pentacyclic triterpenoid friedelin already known (21-23) to be present in the genus *Rhododendron* of the family Ericaceae to which *Kalmia* belongs. The spectral and other properties described in what follows agree with this assignment of structure. The mass spectrum of the ketone indicated a molecular weight (M^+

97%) of 426 for $C_{30}H_{50}O$. In addition to loss of CH_3 (m/e 411, 31%), fragmentations occurred at and near the ring junctions yielding C_5H_9O (m/e 341, 17%), $M^+-C_9H_{16}$ (302, 62%), $M^+-C_{10}H_{19}$ (287, 24%), $M^+-C_{11}H_{21}$ (273, 98%), $M^+-C_{13}H_{24}$ (246, 62%), $M^+-C_{14}H_{26}$ (232, 60%), $M^+-C_{15}H_{28}$ (218, 81%), and a combination of $M^+-C_{16}H_{29}$ and $C_{14}H_{21}O$ (205, 100%) resulting from clevage through the center of ring C. Upon reduction with $LiAlH_4$, the carbonyl group disappeared (IR), and the product fragmented (MS) in nearly the same way as the ketone except that the fragments occurred two mass units higher, the cleavage in ring A was insignificant, and a peak at m/e 205 remained with one at 207. The reduction product also showed M^+-H_2O and $M^+-H_2O-CH_3$. In the PMR spectrum of the ketone, two multiplets near 2.26 and 2.36 ppm for the H-atoms on C–2 and C–4, five singlets for three protons at 0.71, 0.85, 0.94, 1.04, and 1.16 ppm, and one singlet for six protons at 0.99 ppm were observed. A doublet also appeared at 0.87 ppm (J = 6.5 Hz). These signals account for theoretical expectation for the eight methyl groups in friedelin. The doublet is clearly from the methyl group on C–4. Partial assignment of the remaining signals can be made from the spectrum of the corresponding alcohol. While the peaks at 0.85, 0.94, 0.99, and 1.16 remained unchanged, the doublet moved to 0.93 (J - 6.5 Hz) in the alcohol and the other two singlets (0.71 and 1.04 ppm) moved to 0.96 and 1.00 ppm. They are, therefore, probably the closest ones to C–3 and represent the CH_3 groups at C–5 and C–9, respectively. The two peaks at 0.99 ppm are probably the *gem*-dimethyl group and the one at 1.16 ppm (which does not appear in the spectra of lanosterol or cycloartenol) is probably the CH_3-group at the *cis*-juncture between rings D and E. The spectral properties confirm the structural assignment as friedelin. To our knowledge, these spectra have not previously been described for this unique cyclization product of squalene oxide. While friedelin occurs in a variety of botanical families (24), we have not observed it in any of the other families examined in this or our earlier work (4).

Cucurbita pepo

In view of the presence of 24α- and 24β-alkylsterols and the unusual absence of Δ^5-sterols in pumpkin seeds (6-9), we made an exceptionally careful study of the mature plant. The pericarp (after removal of seeds and associated structures) and the leaves of plants collected in early autumn were examined separately. In addition to separations on Sephadex, the 4-desmethylsterol fraction (Al_2O_3-chromatography) from the pericarp was acetylated and further separated by chromatography on a thick layer of silica gel impregnated with 10% of $AgNO_3$ in the solvent system hexane-chloroform-acetic acid (75:25:0.6, v/v). The sterol fraction from the leaves was chromatographed directly as the free alcohol on silica gel impregnated with 10% $AgNO_3$ in the solvent system chloroform-ligroin-acetone (75:23:4, v/v). A monoene fraction (Fraction 1, with the same rate of movement as sitosterol) and two diene fractions [with the rates of movement, respectively, of stigmasterol (Fraction 2) and fucosterol (Fraction 3)] were eluted for further study. The analytical data are recorded as follows. R_f (SiO_2) refers to the rate of movement of the alcohol in thin layer chromatography (TLC) on silica gel in chloroform. R_s (RP) refers to the rate of movement of the alcohol relative to cholesterol in reversed phase TLC (25). R_f ($AgNO_3$) is the rate of movement of the acetate after one development (unless otherwise noted) on a thin layer of silica gel impregnated with 10% of $AgNO_3$ in the solvent system hexane-chloroform-acetic acid (75:25:0.6, v/v). All compounds showed only "end absorption" in the ultraviolet spectrum. The value quoted is at 220 nm in ethanol. The Liebermann-Burchard test (LB) was performed in acetic anhydride-sulfuric acid (19:1, v/v) at room temperature. A color reaching maximum intensity in a minute or less is described as "fast," and one requiring 30 min as "slow." The following data were obtained with authentic steroids. Sitosterol possessed R_f (SiO_2) 0.18; R_s (RP) 0.79; R_f ($AgNO_3$) 0.23; RRT 1.54; LB slow, blue, and its Δ^7-isomer (24α-ethyllathosterol) showed R_f (SiO_2) 0.18; R_f ($AgNO_3$) 0.23; RRT 1.80; LB fast, violet. Spinasterol possessed R_f (SiO_2) 0.18; R_s (RP) 0.94; R_f ($AgNO_3$) 0.19; RRT 1.53; LB fast, violet. Fucosterol exhibited R_f (SiO_2) 0.18; R_s (RB) 1.01; R_f ($AgNO_3$) 0.14; RRT 1.63, LB slow, blue. Isofucosterol possessed R_f (SiO_2) 0.18; R_f ($AgNO_3$) 0.10; RRT 1.68, LB slow, blue. Stigmasterol showed R_f (SiO_2) 0.18; R_s (RP) 0.29; RRT 1.34.

Fraction 1 (24α-ethyllathosterol) from the pericarp possessed R_f (SiO_2) 0.18; R_f ($AgNO_3$) 0.23; RRT 1.80; LB fast, violet; ϵ 1,500; m.p. 142-144 C (152-155 C as acetate); ν_{max} 800, 830 cm^{-1}; m/e for the acetate 456 (M^+), 441 (M^+-CH_3), 396 (M^+-HOAc), 381 (M^+-CH_3-HOAc), 255 (M^+-HOAc–side chain), 213 (M^+–60–side chain–42).

Fraction 2 (24α-ethyl-22-dehydrolathosterol, spinasterol) from the pericarp possessed

R_f (SiO_2) 0.18; R_f ($AgNO_3$) 0.18; RRT 1.54; LB fast, violet; ϵ 1,400; m.p. 159-161 C (172-177 C as acetate); ν_{max} 800, 830, 970 cm^{-1}; m/e for the acetate 454 (M^+), 439 (M^+-CH_3), 411 ($M^+-C_3H_7$), 394 (M^+–HOAc), 379 (M^+-CH_3–HOAc), 351 ($M^+-C_3H_7$–HOAc), 315 (M^+–side chain), 273 (M^+–side chain–42).

Fraction 3 from the pericarp was not investigated except by gas liquid chromatography (GLC) which showed two major (93%) components (ratio of 1:1), RRT 1.79 and 1.95 agreeing with the values for peposterol and Δ^7-avenasterol as discussed below.

Fractions 1 and 2 from the leaves were not well separated perhaps due to different relative amounts compared to the pericarp fractions. They were combined and submitted to preparative GLC. One fast and one slow moving component were observed equivalent, respectively, to fractions 2 and 1. The latter 24ξ-ethylsterol possessed R_f (SiO_2) 0.18; R_s (RP) 0.81; R_f ($AgNO_3$) 0.23; RRT 1.80; LB fast, violet; ϵ 3,000; m.p. 141-143 C; m/e for the alcohol 414 (M^+), 399 (M^+-CH_3), 396 (M^+-H_2O), 381 ($M^+-CH_3-H_2O$), 273 (M^+–side chain), 255 (M^+-H_2O–side chain) 213 (M^+-H_2O–side chain) 213 (M^+-H_2O–side chain–42); δ 0.53 (s, C–18), 0.79 (s, C–19), 0.92 (d, C–21), 0.85 (poorly resolved multiplet, C–26,27,29) ppm at 100 MHz. The fast moving component in GLC (24ξ-ethyl-22-dehydrolathosterol, spinasterol) possessed R_f (SiO_2) 0.18; R_s (RP) 0.94; R_f ($AgNO_3$) 0.18; RRT 1.55; LB fast, violet; ϵ 1,900; m.p. 158-161 C; ν_{max} 800, 830, 970 cm^{-1}, m/e for the alcohol 412 (M^+), 397 (M^+-CH_3), 369 ($M^+-C_3H_7$), 351 ($M^+-C_3H_7-H_2O$), 273, 272 and 271 (M^+–side chain–0, 1, and 2 H–atoms), 255 (M^+–side chain–H_2O), 213 (M^+–side chain–H_2O–42).

Fraction 3 from the leaves showed a peak with a shoulder on the leading side in GLC. The two components were separated as the acetates on preparative chromatoplates of silica gel containing 15% of $AgNO_3$ in the solvent system hexane-chloroform-acetic acid (75:25:0.6) by two or more developments. The one moving slower on the chromatoplate (*trans*-24-ethylidenelathosterol, Δ^7-avenasterol) possessed R_f (SiO_2) 0.18, R_f (15% $AgNO_3$ 2 passes of solvent) 0.11; RRT 1.98 (2.11 as acetate); LB fast, violet; m.p. 139-141 C (acetate); ν_{max} 800, 830 cm^{-1}; m/e; for the acetate 454 (M^+), 439 (M^+-CH_3), 394 (M^+–HOAc), 379 (M^+-CH_3–HOAc), 356 ($M^+-C_7H_{14}$), 315 (M^+–side chain), 314 (M^+–side chain–1), 313 (M^+–side chain–2), 273 (M^+–side chain–42), 255 (M^+–side chain–HOAc), 253 (M^+–side chain–HOAc–2); δ for the acetate 0.53 (s, C–18), 0.80 (s, C–19), 0.97 doublet (C–21, C–26, C–27), 1.59 (d, J = 6 Hz, C–29), 2.01 (CH_3 of acetyl), 5.15 (m, H on C–7 and C–28) ppm at 100 MHz. The faster moving component (which we shall call peposterol) showed R_f (SiO_2) 0.18; R_f (15% $AgNO_3$, 2 passes) 0.15; RRT 1.75 (1.89 as acetate); LB fast, violet; m.p. 135-137 C (acetate); ν_{max} 800, 930 cm^{-1}; m/e for acetate 454 (M^+), 439 (M^+-CH_3), 394 (M^+–HOAc), 379 (M^+-CH_3–HOAc), 356 ($M^+-C_7H_{14}$), 315 (M^+–side chain), 314 (M^+–side chain–1) 313 (M^+–side chain-HOAc–2), 273 (M^+–side chain–42), 255 (M^+–side chain–HAOc), 253 (M^+–side chain–HOAc–2); δ 0.51 (s, C–18), 0.80 (s, C–19), 0.90 (d, J = 6 Hz, C–21), 0.93 (t, J = 6-7 Hz, C–29), 1.56 (sh 1.59, C–26 and C–27), 2.01 (CH_3 on acetyl) ppm at 100 MHz.

The α-configuration at C–24 was demonstrated by PMR at 220 MHz in a later set of experiments in which the pericarp 4-desmethylsterols derived from the free and esterified pools were chromatographically separated on Sephadex. The two main components had m.p.'s (142-143 C and 157-159 C), RRTs (1.79 and 1.53) and mass and PMR spectra which were identical with 24α-ethyllathosterol and its *trans*-22-dehydro derivative, spinasterol, respectively, isolated from spinach, and the PMR spectra differed from the 24β-epimers isolated from *Chlorella emersonii*. The PMR spectrum of the leaf 24ξ-ethyllathosterol isolated by argentation chromatography was taken earlier at 100 MHz. Unfortunately, the resolution, while confirming the Δ^7-assignment (C–18 signal at 0.53 ppm), was insufficient for configurational determination and too little sample was left for examination at 220 MHz. Presumably the configuration is the same as in the pericarp specimens. Similarly the PMR spectrum of peposterol (one of the minor components from leaf "Fraction 3") was taken not only at 100 MHz but on a very small sample. The resolution of the multiplets left something to be desired. Only six methyl peaks (other than for acetyl at 2.01 ppm) were seen. The ones at 0.51 and 0.80 ppm were clearly singlets for C–18 and C–19, in the Δ^7-series. The other four experimental peaks (0.86, 0.93, and 1.00 and 1.56 ppm) agree with the interpretation of singlets and multiplets given in the paragraph describing "Fraction 3" indicating the sterol is probably 24-ethyl-24(25)-dehydrolathosterol. However, until a larger sample is available for further study we prefer to make the structural assignment only tentatively. A sterol assigned this structure has already been obtained (26) from sunflower but was mixed with Δ^7-avena-

sterol. The interesting signal from C–29 in the PMR spectrum is not recorded, and the doublet from C–29 in the Δ^7-avenasterol confuses the interpretation of the peaks in the 1.6 ppm region where C–26 and C–27 should give signals. However, it is claimed (26) that two singlets appeared at 1.57 and 1.66 ppm for the $\Delta^{25(27)}$-sterol. In our case, only the former was seen as a strong peak (1.56 ppm), although a weak multiplet did appear from 1.70 to 1.74 ppm. Thus, unfortunately in neither the present nor previous (26) case is a completely adequate structural analysis possible.

In addition, the following compounds were present based on weak extra peaks for M^+ in the mass spectra of the samples of 24α-ethyllathosterol isolated by argentation chromatography: lathosterol, m/e 386 ca. 0.5 of m/e 414 (leaf); 24ξ-methyllathosterol, m/e 400 2.0% of m/e 414 (leaf) and 5.0% of m/e 414 (pericarp). The pericarp 24ξ-methyllathosterol (presumably of the α-configuration) was further identified by the presence of an 8% component showing a peak at RRT 1.45 contaminating the 24α-ethyllathosterol (RRT 1.80). Had the minor component been the Δ^5-isomer, the RRT would have been 1.29. From the pericarp fractions appearing in the alumina column prior to the appearance of 4-desmethylsterols, two triterpenes were isolated, one had the same mass spectrum as authentic cycloartenol (as the acetate, m/e 468, 453, 408, 393, 365, 357, 339, 297, 286, 271, and 175). The other (as the acetate, m/e 468, 453, 408, 393, 301, 289, 241, 229, 218, 205, 191, and 189) was not further studied. The ester and free alcohol fractions of the pericarp chromatographically separated prior to saponification were also investigated separately in a similar manner. The same 4-desmethylsterols were obtained. Moreover, no Δ^5-sterols could be detected either in the ester or the free sterol pools by MS, GLC, PMR, or Liebermann-Burchard Test.

C. pepo thus contains 24α-ethyl-*trans*-22-dehydrolathosterol and 24α-ethyl-lathosterol in descending order of amount with much smaller quantities of lathosterol, 24ξ-methyllathosterol (presumably in the 24α-series), *trans*-24-ethylidenelathosterol, and (tentatively) 24-ethyl-24(25)-dehydrolathosterol. The two principal components were present at levels (mg/100 g of wet tissue) of 8.0 and 7.0, respectively, in the leaf and of 1.8 and 1.7 in the much more watery pericarp. In *S. oleracea* leaf, the values were 4.3 and 3.5, respectively. *trans*-24-Ethylidenelathosterol amounted to about 3 in *C. pepo* leaves which also contained about 1 of the "$\Delta^{24(25)}$"-isomer, 0.1 of the 24-methyl component, and 0.04 of lathosterol.

DISCUSSION

In our previous (4) and present work, we have examined thirteen families in the Phylum Tracheophyta. Using derived hydrocarbons, Mulheirn (27), has also examined *Zea mays* bringing to fourteen the number of families studied with PMR at 220 MHz. The plants range from the subphylum Lycopsida characterized by simple vascular tissue, poorly developed roots, and sporangia through the Filicopsida with a more complex vascular system, but simple roots and no flower, fruit, or seed to the true seed bearing plants (Pteropsida) culminating in the class Angiospermae with flowers, fruit, and the most complex of plant vascular tissue extending through large and well developed roots, stems, and leaves. While the number of families examined represents only a few of those existing, the evolutionary range gives validity to some taxonomic and phylogenetic considerations. They are particularly interesting with sterols as a tool, because all algae and all Tracheophytes, so far as is known, biosynthesize sterols, and because there are cogent reasons (28) for believing sterols perform a vital function as architectural components in the lipid leaflet of membranes.

It is clear from our work that there are two extreme types of plant in terms of the configuration at C–24. In Category I are plants containing exclusively or primarily 24α-alkylsterols, while in Category II are those containing exclusively or primarily 24β-alkylsterols (Table VII). Furthermore, this categorization depends on ontogeny. Seeds of the family Cucurbitaceae contain 24β-ethylsterols (6-8) together with, as recently demonstrated with *C. pepo* (9), 24α-ethylsterols in smaller amount (ca 23%). However, we have shown that in mature tissue of this plant, only 24α-ethylsterols are present in consequential amount. This appears to be an evolutionary recapitulation, since sterols of the great majority of the investigated nonvascular plants (algae and fungi) contain only 24β-alkylsterols. Our work also shows that an intermediate type exists in *Lycopodium complanatum* in which the 24-ethylsterol possesses the α-configuration exclusively as do plants of Category I, but, in the 24-methylsterols, the β-configuration is dominant as in plants of Category II. This chemical intermediacy correlates with primitive vascularization in the Lycopsida and suggests that increasing proportions of 24β-alkylsterols may exist in the liverworts and mosses which are "higher" than algae in being Embryophytes but "lower" than Tracheophytes in not being vascularized. Configurational data are not yet

TABLE VII

Classification of Tracheophyte Families by Side Chain[a]

Category I (Primarily 24α-Alkyl)[b]	Intermediate[c]	Category II (Primarily 24β-Alkyl)[b]
Pteropsida		
Angiospermae		
Leguminosae		
Pisum sativum (s);		
Glycine max (s)		
Ericaceae		
Kalmia latifolia (ℓ)		
Brassicaceae		
Brassica oleracea (ℓ)		
Chenopodiaceae		
Spinacea oleracea (ℓ)		
Cucurbitaceae		Cucurbitaceae
Cucurbita pepo (p,ℓ)		*Cucurbita pepo* (s) and others
Magnoliaceae		Verbenaceae
Liriodendron tulipifera (ℓ)		*Clerodendrum infortunatum* *Clerodendrum campbellii*
Podophyllaceae		Crassulaceae
Podophyllum peltatum (ℓ)		*Kalanchoe daigremontiana* (ℓ)
Gymnospermae		
Pinaceae		
Pinus pinea (s, en, e)		
Ginkgoaceae		
Ginkgo biloba (ℓ)		
Filicopsida		
Polypodiaceae		
Polystichum acrostichoides (f)		
Dennstaedtia pucti lobula (f)		
Dryopteris (Thelypteris) *noveboracensis* (f)		
Osmundaceae		
Osmunda cinnamomea (f)		
	Lycopsida *Lycopodium complanatum* (ℓ)	

[a]Abbreviations used: e = embryo of seed; en = endosperm of seed; f = fronds; ℓ = leaves; p = pericarp of fruit; s = whole seeds.

[b]The plants listed are those examined in our present and previous (4) work. Included are also the only other two families known (10,11) to contain 24β-ethylsterols. In all of the plants in Category I the tissues examined have only 24-ethylsterols in the 24α-series, and the 24-methylsterols, when present, were always a minor fraction and principally, but not exclusively, of the α-configuration. In the one plant (*K. daigremontiana*) with the 24β-ethylsterols which we examined, except for a very small amount (ca. 2%) of unidentified sterol, all of the sterol had the 24β-ethyl structure. In *Cucurbita pepo* seeds 77% of the sterol has the 24β-ethyl structure (6). Reports (10,11) on the two species of Clerodendrum do not make clear the percentage of epimers, but we presume the absence of mention of 24α-sterols implies the 24β-ethylsterols to be dominant.

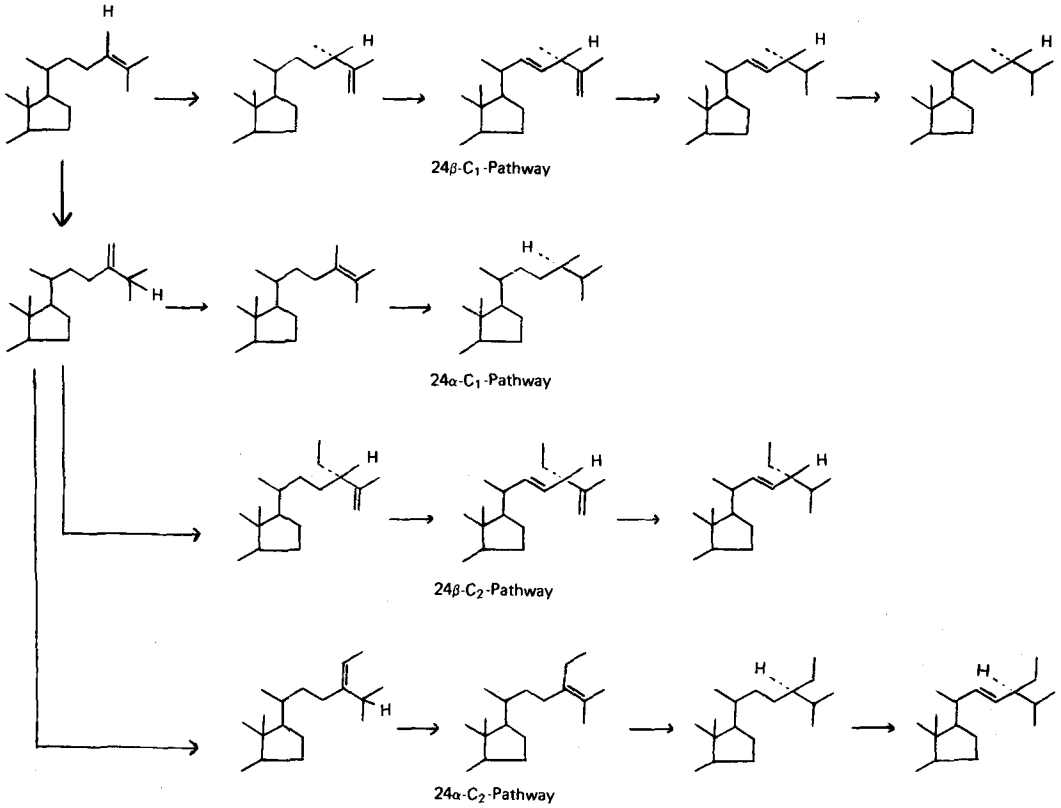
[c]In *L. complanatum* the only 24-ethylsterol possessed the α-configuration but most of the 24β-methylsterol possessed the 24β-configuration. The ratio of 24α-alkylsterol to 24β-alkylsterol was ca. 1.6 to 1.0. Thus the plant was closer to Category I than to II.

available on these interesting organisms, but at least, in the case of the mosses, 24ξ-methyl- and 24ξ-ethylcholesterol are reported to be present (29-31).

In addition to variations in the side chain, the type and degree of unsaturation in the nucleus function as taxonomic and phylogenetic markers. Again, there are two extreme types of Tracheophyte: Category A containing primarily Δ^5-sterols and Category B with Δ^7-sterols. The Cucurbitaceae are the most well studied of Category B. The seeds of several genera in this family have already been found (6-9) to possess only Δ^7-sterols, and we have now found that the fruit and leaves of *C. pepo* also contain only Δ^7-sterols. *C. pepo* has, therefore, been examined at all stages of ontogeny and is unquestionably in Category B throughout its development. *S. oleracea* leaves are similarly known to contain Δ^7-sterols (16). We have confirmed this and find further that there are no detectable Δ^5-sterols. *S. oleracea* is, therefore, another plant in Category B at least in the mature stage. Based on the sterols, in

Scheme I

Probable Pathways to Tracheophyte Sterols



leaves or in the oils of seeds of e.g., *Camellia japonica* and *Thea sinensis* (32,33), these plants similarly belong to Category B as probably do *Acacia* species (34). Most other plants studied, such as the others examined by us in the present and previous work (4), are of Category A. It is interesting that *Ginkgo* nuts were recently examined (40) and found, as were the male leaves in our work, to contain only Δ^5-sterols in the homologous series (in increasing amount: 24–H, 24–CH_3, and 24–C_2H_5 with some 24-ethyl-Δ^{22}-sterol).

From the foregoing discussion, it is apparent statistically that most plants are of Categories I and A, i.e., containing principally 24α-alkyl-Δ^5-sterols. For this reason, we believe the 24α-alkyl-Δ^5-structure, and especially the one with the 24α-ethyl group commonly represented by sitosterol, constitutes the most highly evolved of the alternative structures. This is given further credence by the apparent evolutionary recapitulation in *C. pepo* from Category II to I, by the intermediate status of *L. complanatum*, and by the sterols of algae (Category II).

The biosynthetic pathway in plants of Category II is thought to proceed through the introduction of a $\Delta^{25(27)}$-bond at the time of C_1-transfer to C–24 or C–28 (11). Our finding that *Kalanchoe daigremontiana* contains 24β-ethyl-$\Delta^{25(27)}$-sterols is further evidence for such a pathway (Scheme I), and it is presumably more primitive than the one to 24α-alkyl-sterols. The latter probably occurs through $\Delta^{24(25)}$-sterols (35-37). Our tentative detection of 24-ethyl-24(25)-dehydrolathosterol and its association in mature *C. pepo* plants with the $\Delta^{24(28)}$-isomer (Δ^7-avenasterol) and with 24α-ethyllathosterol and its 22-dehydro derivative (spinasterol) is consistent with a route (Scheme I) in which the second C_1-transfer yields a $\Delta^{24(28)}$-sterol which is isomerized to the $\Delta^{24(25)}$-isomer followed by reduction to the 24α-ethyl end-product. The existence of the $\Delta^{24(25)}$- and $\Delta^{25(27)}$-routes to the epimeric sterols are given further substantiation by the finding (M.L. McKean and W.R. Nes, unpublished observations) that 24-tritiolanosterol leads in *Pinus pinea* to labelled 24-methylcholesterol (presumably the 24β-component) but to unlabelled 24α-ethylcholesterol, while

2-^{14}C-MVA produces more label in the latter than in the former.

If we extend our categorization to include plants with dominant sterols bearing the $\Delta^{5,7}$-diene structure (Category C), it becomes possible to include the algae under the system. Thus, *Chlorella ellipsoidea* (15) is Category II-A, *Chlorella emersonii* (17) is II-B, and *Chlorella simplex* (38) is II-C. It is interesting to note that the blue-green *Phormidium luridium*, which is much more primitive then the *Chlorella* species each of which is of a single category, is of a mixed category (A,B, and C) (39). Similarly, *Lycopodium complanatum* is mixed (A and C), and, relative to the Pteropsida and most of the Filicopsida examined where single double bond categories exist, *L. complanatum* is more primitive. The full significance of these mixed types remains to be demonstrated, but they would appear to represent plants in which the sterol pathway is not kinetically evolved to the point found in others of a single double bond category. By analogy to animals, the pathway Δ^7 to $\Delta^{5,7}$ to Δ^5 presumably operates in plants. In most plants, as in animals, this pathway is kinetically completed with little or no steady-state concentration of intermediates, i.e., the plants are of single category (A,B, or C). In view of the data presented here and elsewhere, we belive it plausible to believe, if tentatively, that plants in Category B, *C. pepo* for instance, represent an evolutionary line which did not develop the gene (or its expression) for the Δ^5-dehydrogenase, that plants in Category C are in a line lacking the Δ^7-reductase, and that plants of a mixed category (or ones bordering on it, e.g., the New York fern with some Δ^7-sterols in a larger pool of Δ^5-sterols) reflect the quite different condition of having the enzymes, and therefore the genetics, but not having the fully developed regulatory genes for perfect kinetic control. An alternative explanation for Category B amd C plants is retroevolution in which the genetics for the appropriate enzyme has been lost. Unfortunately, no obvious way exists to discriminate between these possibilities at the present time, but on the assumption that retroevolution has not taken place it is possible to assess the phylogenetics of the plants examined. This means that in the order of evolution of the unsaturation in ring B, we expect Categories C, B, and A, while for the configuration we expect II and I. A plant of Category II-C would therefore be the most primitive with which, for instance, *Chlorella simplex* agrees in its morphology relative to, say, *Pisum sativum*, an angiosperm of Category I-A, and we would place *C. simplex*, *C. emersonii*, and *C. ellipsoidea* in the relative (direct or parallel) evolutionary order in which they are mentioned.

Two quite different taxonomic and phylogenetic relationships have been suggested in the literature for the angiosperms by Hutchinson (12) and Cronquist (41). Our chemotaxonomy based on the sterols offers a way of examining them. From morphologic, geographic, and other parameters, both Hutchinson and Cronquist place Magnoliales (including *Liriodendron tulipifera*) as the most primitive order of flowering plant closely associated with Ranales of the Hutchinson system which Cronquist [at least in so far as the species (*Podophyllum peltatum*) we examined is concerned] regards as Ranunculales. Hutchinson considers Magnoliales to have given rise to woody dicots and Ranales to herbaceous dicots and perhaps also to monocots, while in the Cronquist system the woody and herbaceous divisions are not recognized. Since the family *Cucurbitaceae* and the examined species of Theales are of Category B and both Magnoliales and Ranales (Ranunculales) are of Category A, the sterol data correlate with neither system in the sense of a direct progenitor role of Magnoliales for these Category B plants. It seems to us that the sterols may indicate a parallel evolution with rather than a direct evolution from Magnoliales. Similarly, Hutchinson's suggestion of the evolution of Saxifragales from Ranales is not verified, since *Kalanchoe daigremontiana* (Saxifragales) is of Category II and *Podophyllum peltatum* (Ranales) is of Category I, nor is his suggestion of the line to Ericales through Theales from Magnoliales verified, since *Kalmia latifolia* (Ericales) is of Category I-A while representatives of Theales, e.g., the genus *Camellia*, are of Category I–B (32,33). The Theales to Ericales (and Capparales including *Brassica*) line of Cronquist similarly is not consonant with our data. On the other hand, there are aspects of the Cronquist system with which our data either do agree or with a slight change in his system would agree. He places *Cucurbita* in Violales emanating from Theales and both are, in fact, of Category I-B in the mature stage. He also places *Crassulaceae* (which includes *Kalanchoe*) in the Rosales with Leguminosae (which includes *Pisum* and *Glycine*), and the former is supposed to have given rise to the latter with which the change from Category II (*Kalanchoe*) to Category I (*Pisum* and *Glycine*) would agree. *Clerodendrum* (Category II-A) in Lamiales is placed above Rosales. Again the sterol data agree, but only if the evolutionary line to *Clerodendrum*

bifurcates (at or before *Crassulaceae* of Category II-A) before the formation of Leguminosae of Category I-A. Unfortunately, despite the coincidence of the data in and above Rosales with the previous classifications, the sterol data do not support the origin of Rosales itself (in the Cronquist system) from Magnoliales (Category I-A) in view of his placing Crassulaceae (Category II-A) in the order. In Hutchinson's system, *Crassulaceae* is supposed to have emanated from Ranales which agrees no better.

The detailed disagreements delineated between sterol structure and previously suggested phylogenetics should not, however, obscure more general agreements. Thus, the line of Cronquist from Magnoliales to Ranunculales to *Spinacea* in Carophyllales is verified (all Category I-A). Furthermore, the sterol structures are consistent with, while not proving, that many angiosperms are higher and could have arisen from the gymnosperms, since the two species of the latter studied were of Category I-A as are most of the angiosperms studied. Similarly, the Filicopsida are of Category I-A. This tells us that evolution of the pathway to 24α-ethylsterols in the Δ^5-series was accomplished quite early chronologically and is not associated with the presence of characters such as flowering. In summary, if retroevolution did not occur in the sterol pathway which at the present is not amenable to experimental verification, some of the angiosperms e.g., Cucurbitaceae, could not have arisen from a line comprised by Filicopsida, Gymnospermae, and Magnoliales and must have diverged very early, but the sterol data do not conflict with the thesis that many flowering plants could have arisen from Magnoliales.

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