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Studies on the Constituents of Orchidaceous Plants. V.[1] Isolation, Structure, and C-13 Signal Assignments of Novel Methylsterols from *Nervilia purpurea* SCHLECHTER[2]

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Four new methylsterols, cyclonervilasterol, 24-epicyclonervilasterol, dihydrocyclonervilasterol, and 24-epidihydrocyclonervilasterol, were isolated from the methylsterol fraction of *Nervilia purpurea* SCHLECHTER by reversed-phase high-performance liquid chromatography. The structures **2a**, **3a**, **5a**, and **6a** were proposed for these compounds, respectively, based on chemical evidence and two-dimensional nuclear magnetic resonance spectroscopy including INADEQUATE (Incredible Natural Abandance Double Quantum Transfer Experiment).

Keywords——*Nervilia purpurea*; methylsterol; reversed-phase HPLC; 2-D NMR; 2-D INADEQUATE; cyclonervilasterol; 24-epicyclonervilasterol; dihydrocyclonervilasterol; 24-epidihydrocyclonervilasterol; ^{13}C-NMR

In previous papers,[1,3] we reported the characterization of chemical constituents of *Nervilia purpurea* SCHLECHTER and *N. aragoana* GAUD (Orchidaceae), which are used as a folk medicine "I-tiam-hong" in Taiwan,[3] and the proton and carbon-13 signal assignments of cycloeucalenol-type triterpenes from *N. purpurea* by two-dimensional nuclear magnetic resonance (2-D NMR) spectroscopy.[1] This paper deals with the isolation, structure elucidation, and ^{13}C signal assignments of novel methylsterols named cyclonervilasterol (**2a**), 24-epicyclonervilasterol (**3a**), dihydrocyclonervilasterol (**5a**), and 24-epidihydrocyclonervilasterol (**6a**) from substance MB (a methylsterol mixture) obtained from *N. purpurea*.[2,4]

Substance MB[4] obtained from the dichloromethane extract of dried herbs of *N. purpurea* was shown to be a complex mixture by gas chromatography (GC) and mass spectrometry combined with gas chromatography (GC-MS). The mass chromatogram obtained by the GC-MS method is reproduced in Fig. 1, and proved the substance to consist of five components, corresponding to the molecular formulae $C_{29}H_{46}O$ (**1a**: M^+ *m/z* 410), $C_{29}H_{48}O$ (**4a**: M^+ *m/z* 412), $C_{30}H_{48}O$ (M^+ *m/z* 424), $C_{30}H_{52}O$ (M^+ *m/z* 428), and $C_{31}H_{50}O$ (M^+ *m/z* 438).

The methylsterol mixture was acetylated as usual and the acetate was separated by preparative thin-layer chromatography (TLC)[6] on silver nitrate-impregnated silica gel to give two substances, corresponding to the molecular formulae $C_{31}H_{48}O_2$ (**1b**: M^+ *m/z* 452) and $C_{31}H_{50}O_2$ (**4b**: M^+ *m/z* 454). However, both of these substances (**1b** and **4b**) were considered to be mixtures of isomers, since in the ^{1}H- and ^{13}C-NMR spectra some of the signals appeared as double lines, although they showed only a single peak in GC analysis.

Unexpectedly, substance MB revealed six peaks on high-performance liquid chromatography (HPLC) with a reversed-phase column (TSK-GEL LS-410A ODS-120A column) using hexane–isopropanol–acetonitrile (5 : 15 : 80) as the eluent (Fig. 2). Thus, preparative HPLC of this mixture led to the isolation of four compounds: cyclonervilasterol (**2a**), 24-epicyclonervilasterol (**3a**), dihydrocyclonervilasterol (**5a**), and 24-epidihydrocyclonervilasterol (**6a**), together with two minor components (peaks 3 and 4).[7]

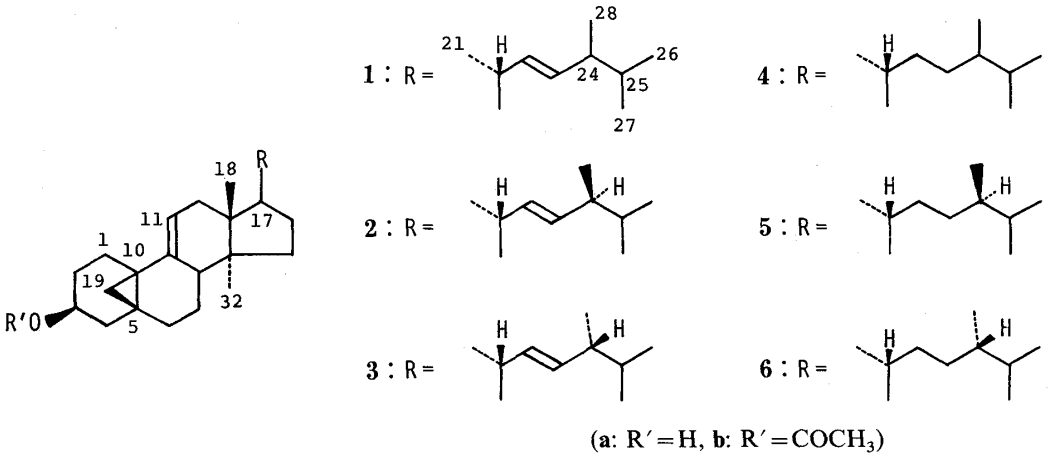

Chart 1[5]

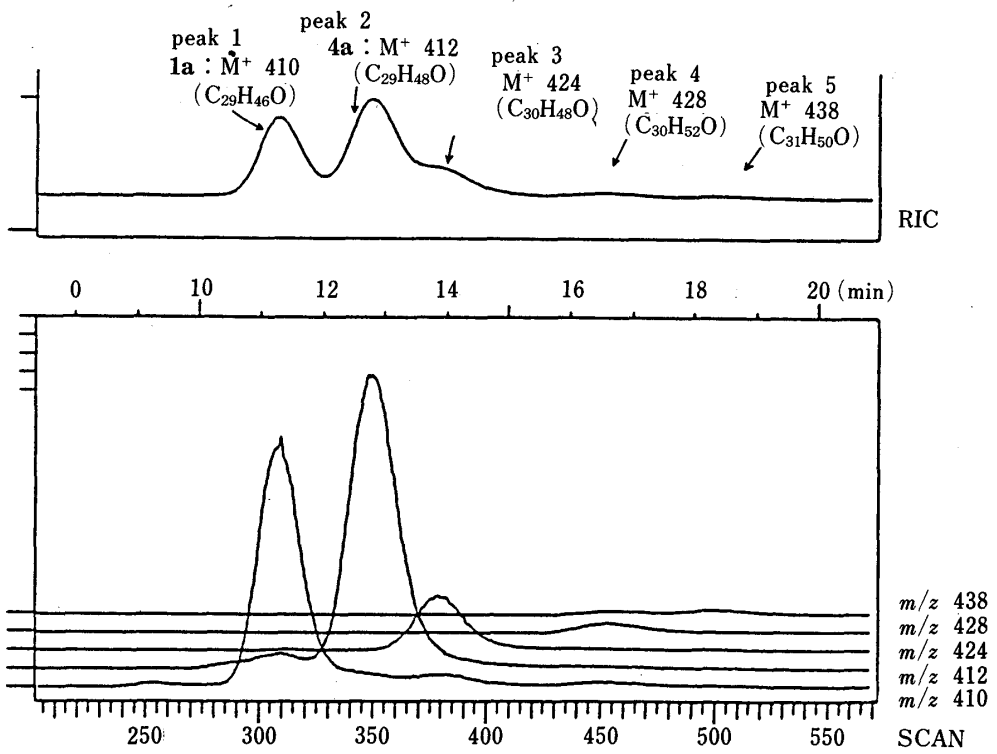

Fig. 1. Gas Chromatogram and Mass Chromatogram of the Methylsterol Mixture Obtained from *N. purpurea*

Cyclonervilasterol (**2a**), mp 151—152 °C, $[\alpha]_D$ +15.2 °, and 24-epicyclonervilasterol (**3a**), mp 153—154 °C, $[\alpha]_D$ −10.8 °, were determined to have the same molecular formula $C_{29}H_{46}O$ by high-resolution MS measurements. The ^{1}H-NMR spectrum of **2a** showed a multiplet due to an olefinic proton at δ 5.43, two quartets due to *trans*-oriented olefinic protons at δ 5.14 and 5.20 (J=7, 15 Hz), a multiplet due to a hydroxy-bearing methine proton at δ 3.60, and a pair of doublets due to cyclopropyl methylene protons at δ 0.63 and 0.67 (J=4.0 Hz), along with signals due to four secondary methyls and two tertiary methyls (Table I). The ^{1}H-NMR spectrum of **3a** was almost identical with that of **2a** except for the 21-methyl signal (Table I). The MS of **2a** (Fig. 3) showed peaks at m/z 410 (M^+), 395 (*a*), 392 (*b*), 377 (*c*), 367 (*d*), 285 (*e*), and 267 (*f*), which could be reasonably explained by the fragmentations shown in Chart 2. The MS of **3a** is identical with that of **2a**.

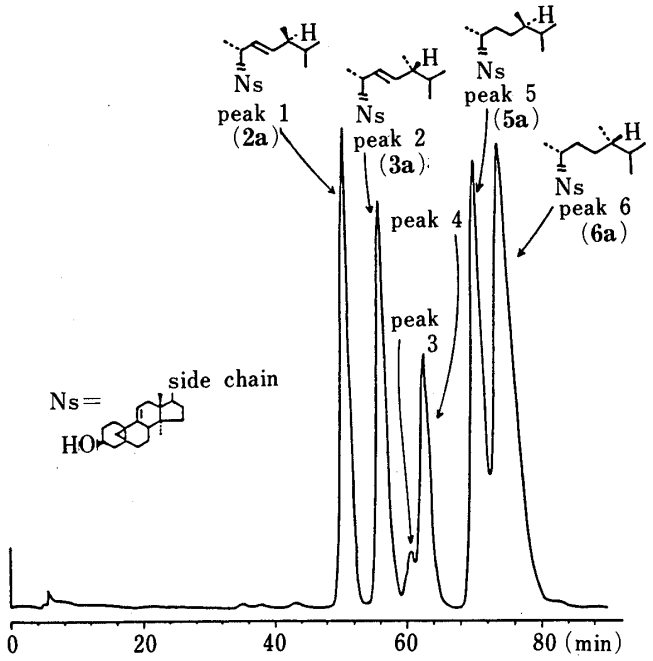

Fig. 2. HPLC Chromatogram of the Methylsterol Mixture Obtained from *N. purpurea*

Conditions: column, TSK-GEL LS-410A ODS (30 cm × 7.5 mm i.d.); solvent, hexane–isopropanol–acetonitrile 5:15:80; flow rate, 2.0 ml/min; detector setting, UV 225 nm; temperature, 20 °C.

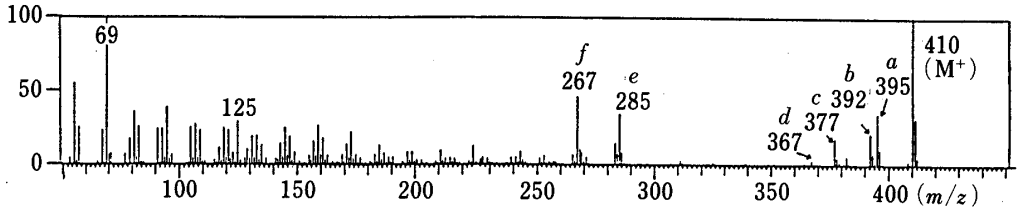

Fig. 3. Mass Spectrum of Cyclonervilasterol (**2a**)

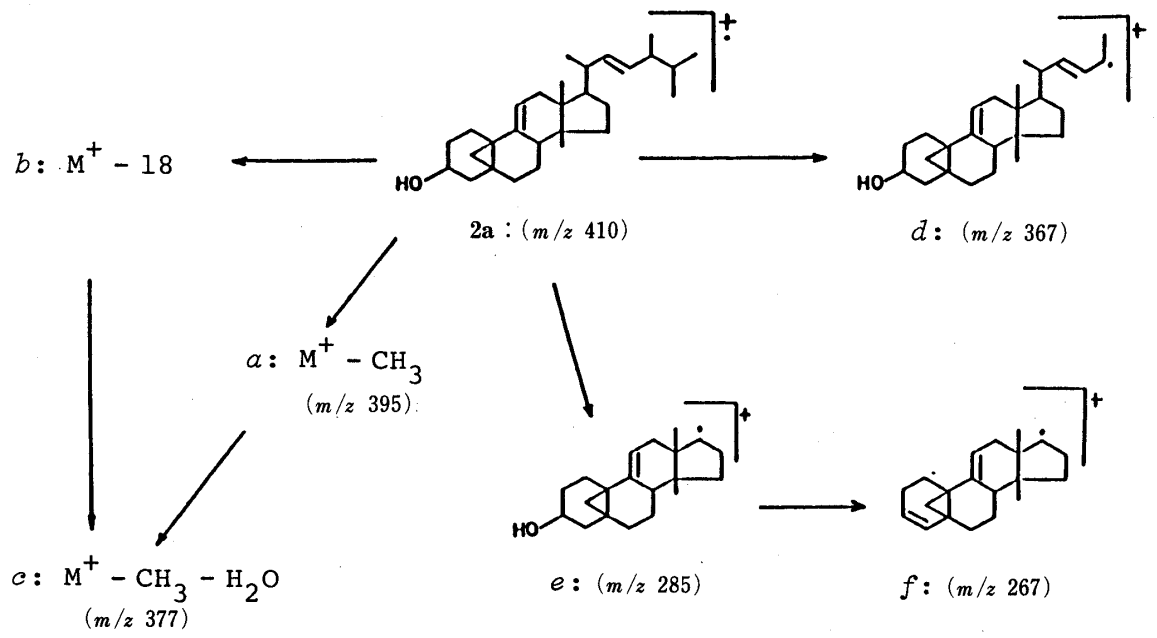

Chart 2

Dihydrocyclonervilasterol (**5a**), mp 128—129.5 °C, $[\alpha]_D$ +24.5° and 24-epidihydrocyclonervilasterol (**6a**), mp 129—130 °C, $[\alpha]_D$ +11.8°, were determined to have the same molecular formula $C_{29}H_{48}O$ by high-resolution MS measurements. The ^{1}H-NMR spectrum

TABLE I. ^{1}H-NMR Spectral Data for

Compounds	3-H	18-Me	19-C$\underline{H}_2$	21-Me
2a (24-*S*/α)	3.60 m ($W_{1/2}=16$)	0.686 s	0.63 d, 0.67 d (4.0) (4.0)	0.966 d (6.51)
3a (24-*R*/β)	3.60 m ($W_{1/2}=16$)	0.686 s	0.63 d, 0.67 d (4.0) (4.0)	0.978 d (6.49)
5a (24-*R*/α)	3.60 m ($W_{1/2}=16$)	0.673 s	0.63 d, 0.67 d (4.0) (4.0)	0.875 d (6.23)
6a (24-*S*/β)	3.60 m ($W_{1/2}=16$)	0.670 s	0.63 d, 0.67 d (4.0) (4.0)	0.887 d (5.8)

δ values in $CDCl_3$ and coupling constants in Hz. *a*) The higher-field signal was arbitrarily assigned to the 27-methyl group.

of **5a** was similar to that of cyclonervilasterol (**2a**), but it was characterized by the absence of two of the olefinic protons at δ 5.14—5.20. The ^{1}H-NMR spectrum of **5a** was almost identical with that of **6a** except for the 27-methyl signal[8] (Table I). The MS of **5a** and **6a** are identical with each other, and they showed the molecular ion peak at *m/z* 412 and significant peaks ascribable to the fragment ions (M^+-15), (M^+-18), ($M^+-15-18$), *e*, and *f* (see Chart 2).

Catalytic hydrogenation of **2a** and **3a** on Adams catalyst gave the corresponding dihydro compounds, each showing the molecular ion peak at *m/z* 412 ($C_{29}H_{48}O$), which were found to be identical with **5a** and **6a**, respectively, by HPLC, MS, and ^{1}H-NMR comparisons. Furthermore, cyclonervilasterol (**2a**) and 24-epicyclonervilasterol (**3a**), and accordingly, dihydrocyclonervilasterol (**5a**) and 24-epidihydrocyclonervilasterol (**6a**) were considered to be epimeric with each other in view of the close similarity of their spectral properties.

The facts that the ^{1}H-NMR spectrum of **2a** exhibited signals due to *trans* olefinic protons, and the MS of **2a** and **5a** showed significant peaks at *m/z* 285 (*e*) and 267 (*f*), suggest that compound **2a** has a disubstituted double bond in the side chain. Thus, we examined the reaction at the side chain of **1b**.

Treatment of cyclonervilasteryl acetate (**1b**) (a mixture of **2b** and **3b**) with osmium tetroxide in pyridine gave a tetraol (**7**), $C_{31}H_{52}O_6$. Subsequent oxidation of this tetraol (**7**) with lead tetraacetate, followed by Jones oxidation and methylation with diazomethane, gave a minute amount of methyl ester (**8**), which showed the molecular ion peak at *m/z* 130 ($C_7H_{14}O_2$) in the GC-MS. Eventually this was identified as methyl 2-methylisovalerate (**8**) by GC and GC-MS comparisons with an authentic sample (**8**) prepared from ergosterol (**9**).[9]

From the above observations coupled with a consideration of the biogenetic relation to the *Nervilia* sterols and triterpenes,[4,10] cyclonervilasterol (**2a**) was presumed to be a methylsterol having a cyclopropane moiety and an ergosterol-type side chain. On the basis of the ultraviolet (UV) absorption band at 210 nm, the other double bond was believed to be conjugated with the cyclopropane ring in a transoid orientation.[11] Then, we examined several chemical reactions in order to obtain more detailed information concerning this conjugated system.

Collins oxidation of **1a** (a mixture of **2a** and **3a**) gave a 3-keto derivative (**10**),[12] mp 140—141 °C, IR ν_{max} 1700 cm^{-1}, whose MS showed the molecular ion peak at *m/z* 408 ($C_{29}H_{44}O$). This compound (**10**) did not isomerize under various alkaline conditions and its ^{1}H-NMR spectrum showed AB quartet signals due to active methylene protons at δ 2.55 and 2.65 ($J=$ 18 Hz), suggesting that the C(5) of **10** may be a quaternary sp^3-carbon rather than an sp^2-carbon. Sodium borohydride ($NaBH_4$) reduction of the ketone (**10**) regenerated cyclonervilasterol (**1a**) (a mixture of 24-epimers).

Oxidation of the tetraol (**7**) with lead tetraacetate afforded mainly an oily keto dialdehyde (**11**), whose MS (Fig. 4a) showed significant peaks at *m/z* 416 (M^+), 398 (*g*), 387 (*h*), 359 (*i*),

Methylsterols from *Nervilia purpurea*

26- and 27-Me[a)]	32-Me	28-Me	11-H	22- and 23-H
0.836 d, 0.818 d (6.76) (6.76)	0.700 s	0.914 d (6.76)	5.43 m	5.14 dd, 5.20 dd (15, 7) (15, 7)
0.835 d, 0.820 d (6.8) (6.8)	0.698 s	0.913 d (6.76)	5.44 m	5.16 dd, 5.25 dd (15, 7) (15, 7)
0.852 d, 0.805 d (6.79) (6.79)	0.698 s	0.774 d (5.46)	5.43 m	
0.856 d, 0.782 d (6.84) (6.84)	0.698 s	0.780 d (5.94)	5.43 m	

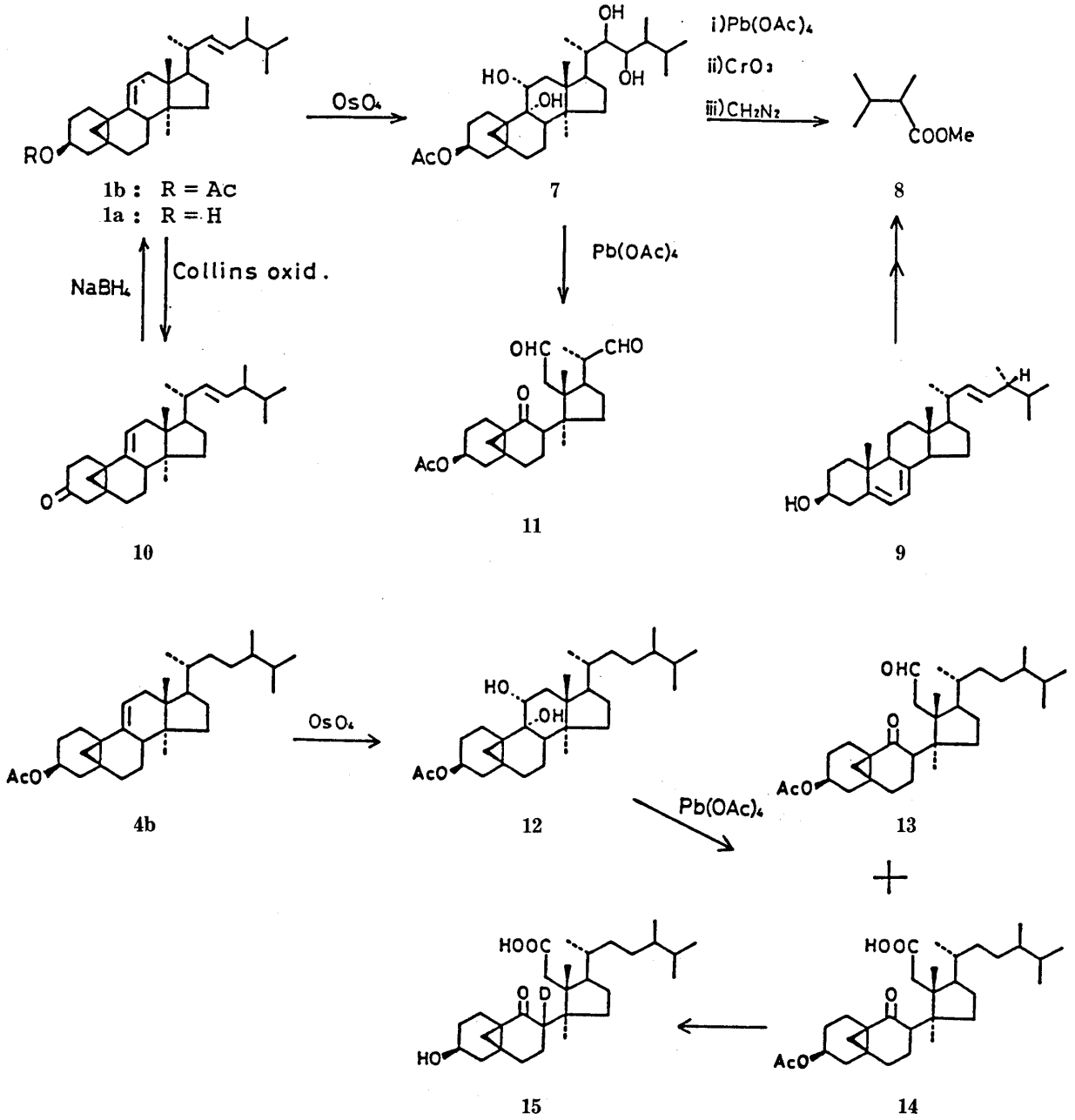

Chart 3

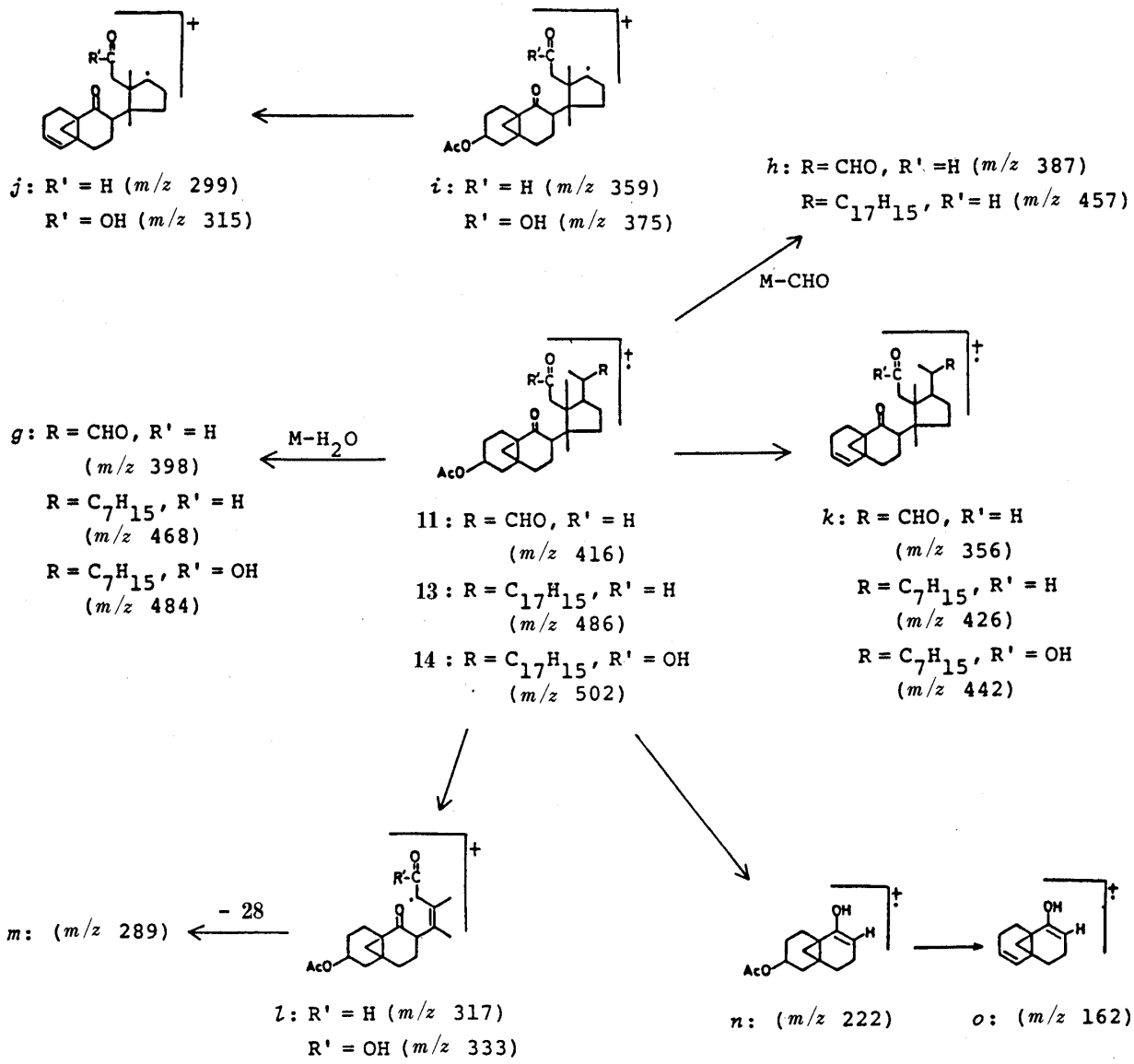

Chart 4

356 (*k*), 317 (*l*), 299 (*j*), 287 (*m*), 222 (*n*), and 162 (*o*), which could be reasonably explained by the fragmentations shown in Chart 4.

Similarly, treatment of dihydrocyclonervilasteryl acetate (**4b**) (a mixture of 24-epimers) with osmium tetroxide in pyridine gave a diol (**12**), which showed the molecular ion peak at *m/z* 488 ($C_{31}H_{52}O_4$) in the MS. Subsequent oxidation of this diol (**12**) with lead tetraacetate led to a keto aldehyde (**13**), $C_{31}H_{50}O_4$, and a small amount of keto acid (**14**), $C_{31}H_{50}O_5$. The MS of **13** (Fig. 4b) showed the molecular ion peak (*m/z* 486) and fragment peaks attributable to *g*, *h*, *k*, *i*, *l*, *j*, *m*, *n*, and *o* (see Chart 4). The MS of **14** (Fig. 4c) also showed significant peaks at *m/z* 502 (M^+), 333 (*l*, R′=OH), 222 (*n*), and 162 (*o*). The characteristic peaks at *m/z* 222 (*n*: $C_{13}H_{18}O_3$) and 162 (*o*: $C_{11}H_{14}O$), observed commonly, can be explained on the basis of the well-known McLafferty fragmentation.

Treatment of **14** with sodium deuteroxide in methanol-d_1 gave a monodeuterated product (**15**). The MS of **15** exhibited the molecular ion peak at *m/z* 461 ($C_{29}H_{47}O_4D$), and as expected, the McLafferty fragments appeared at *m/z* 181 (*n′*: base peak) and 163 (*o′*) (Chart 5).

From the above findings, the locations of the cyclopropane and the double bond must be

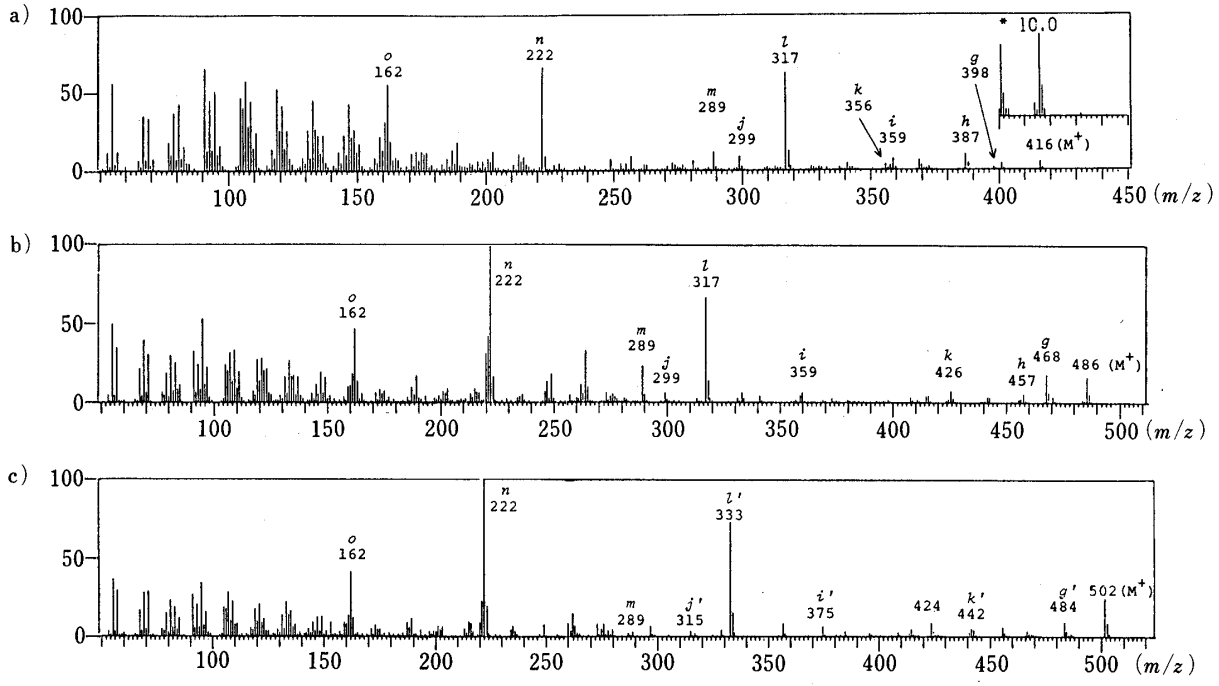

Fig. 4. Mass Spectra of the Keto Dialdehyde (**11**), Keto Aldehyde (**13**), and Keto Acid (**14**)

a) keto dialdehyde (**11**), b) keto aldehyde (**13**), and c) keto acid (**14**).

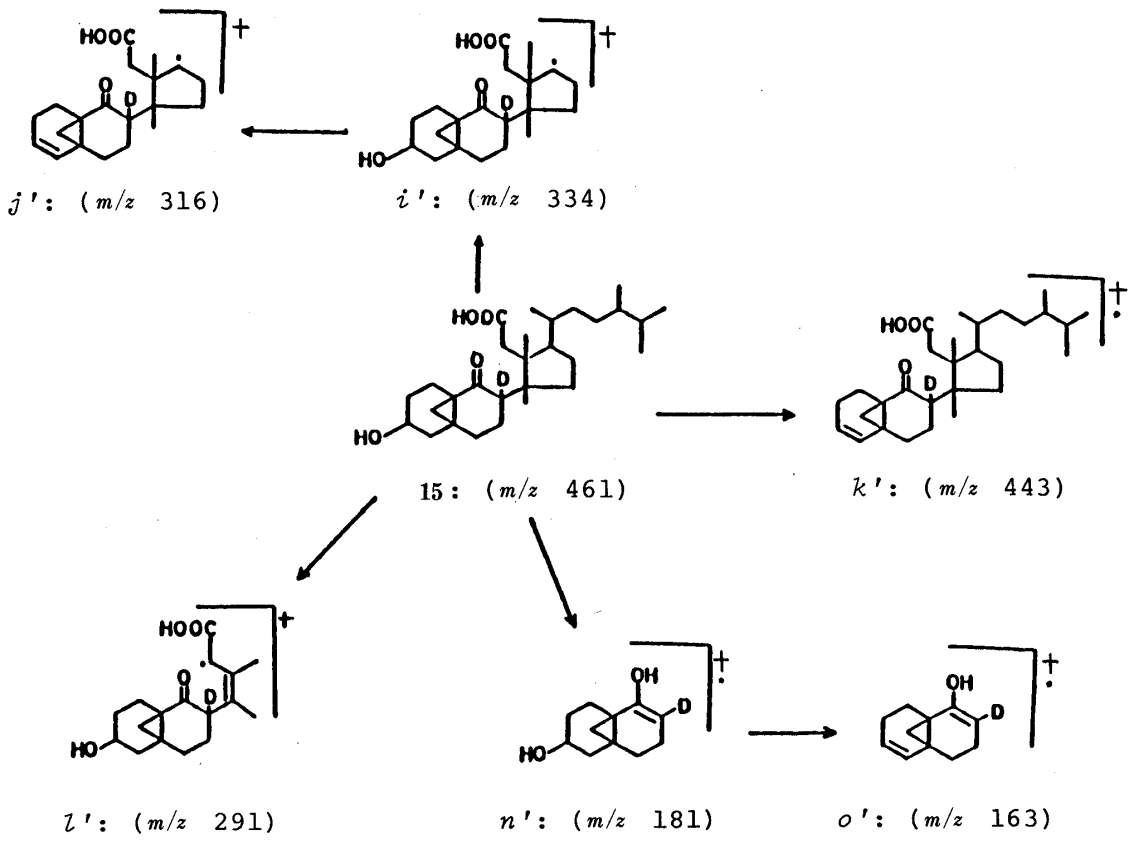

Chart 5

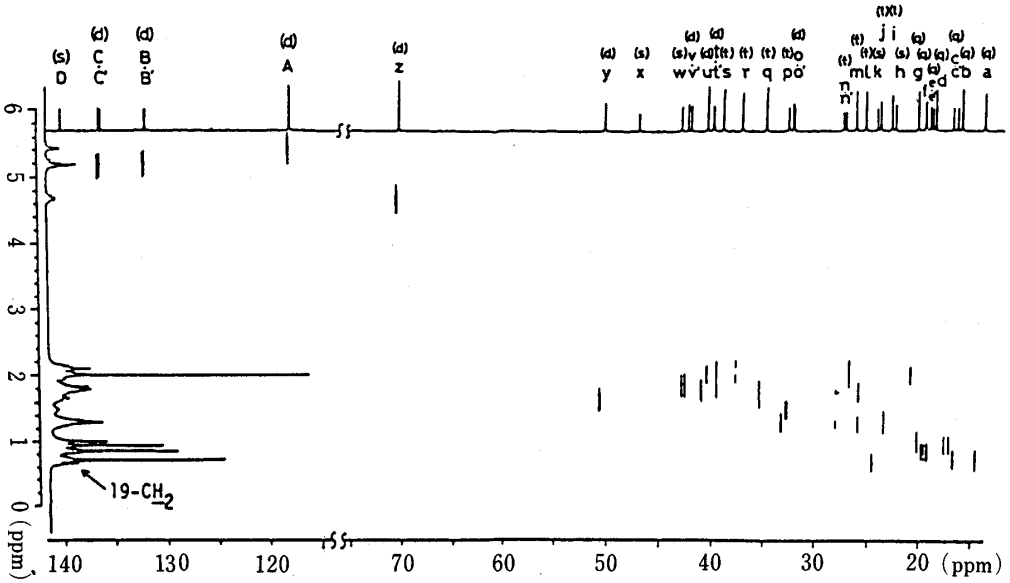

Fig. 5. The ^{1}H–^{13}C Shift Correlated Spectrum of Cyclonervilasteryl Acetate (**1b**: A Roughly 1 : 1 Mixture of **2b** and **3b**)

The ^{1}H shifts are the ordinate and the ^{13}C shifts are the abscissa; sp^2 carbons are marked with capital letters A—E and sp^3 carbons with small letters a—z in the order of increasing δ value. The carbonyl carbon signal (δ 170.5) is outside the spectrum. Some of the signals appear as double lines because the sample is the 24-epimeric mixture. The multiplicities of carbon signals were determined by means of the off-resonance and INEPT methods, and are indicated as (s), (d), (t), and (q).

at C(5)–C(10) and C(9)–C(11), respectively. The positions of two tertiary methyl groups were considered to be at C(13) and C(14), since the MS of **11**, **13**, and **14** showed significant peaks ascribable to the fragment ion *l*. Thus, the gross structures of cyclonervilasterol (and 24-epicyclonervilasterol) and dihydrocyclonervilasterol (and 24-epidihydrocyclonervilasterol) can be represented by the formulae **1a** and **4a**, respectively.

Next, in order to confirm the proposed structures for these compounds, we applied 2-D NMR spectroscopy[13] including 2-D INADEQUATE[14] (Incredible Natural Abandance Double Quantum Transfer Experiment) to them.

First, the ^{1}H–^{13}C shift correlated spectrum of cyclonervilasteryl acetate (**1b**) (roughly 1 : 1 mixture of **2b** and **3b**)[15] was measured. The result is shown in Fig. 5, in which some of the signals, indicated as c·c′, e·e′, n·n′, o·o′, t·t′, v·v′, B·B′, and C·C′, appear as double lines because the sample is the 24-epimeric mixture. The multiplicities of carbon signals were determined by means of the off-resonance and INEPT (Insensitive Nuclei Enhanced by Polarization Transfer) methods, and are indicated as (s), (d), (t), and (q). As shown in Fig. 5, the carbon signals due to the acetyl methyl (peak g), the carbinol methine (peak z), the disubstituted olefin (peaks B and C), and the trisubstituted olefin (peak A and D) were readily assigned. In addition, the carbon j which is correlated with the proton at δ 0.63 (1H, d, J= 4 Hz) was recognized to be the methylene carbon of the cyclopropane ring.

Then the 2-D INADEQUATE spectrum of **1b** was measured to clarify the carbon–carbon connectivities in the molecule. The pulse system employed was the (90°)–τ–(180°)–τ–(90°)–$t_1/2$–(135°)–$t_1/2$–t_2 sequence.[14] The results are reproduced in Figs. 6 and 7, in which the slanting lines on the contour map indicate coupled ^{13}C–^{13}C pairs.

Figure 6 shows that carbon z (d) is directly connected with carbons l (t) and s (t), carbon A (d) with carbons D (s) and r (t), carbon D (s) with carbons A (d), k (s), and u (d), carbon B (d) with carbons C (d) and v (d), carbon C (d) with carbons B (d) and t (d), and carbon E (s) with carbon g (q). Further, in Fig. 7 a correlation was observed between each ^{13}C–^{13}C couple

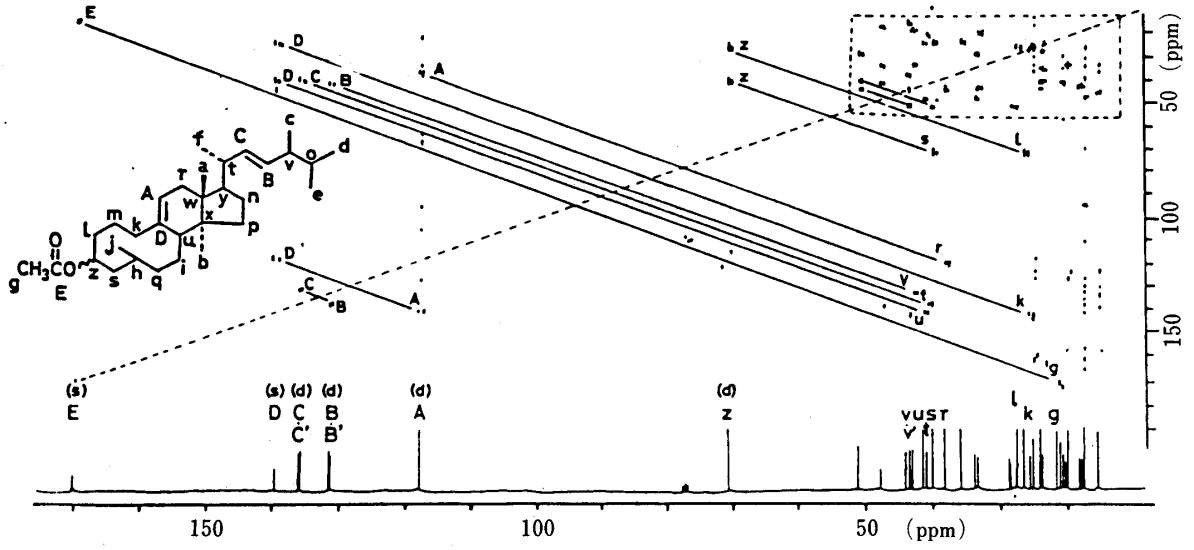

Fig. 6. The 2-D INADEQUATE Spectrum (Contour Map) of Cyclonervilasteryl Acetate (**1b**: A Roughly 1 : 1 Mixture of **2b** and **3b**)

The spectrum was measured on a Nicolet NT-300 spectrometer (65 h run), using 150 mg of the sample in $CDCl_3$. The conventional ^1H-decoupled ^{13}C spectrum runs along the lower axis. Coupled ^{13}C–^{13}C pairs are joined by the slanting lines. The rectangular region at the upper right is enlarged and reproduced in Fig. 7.

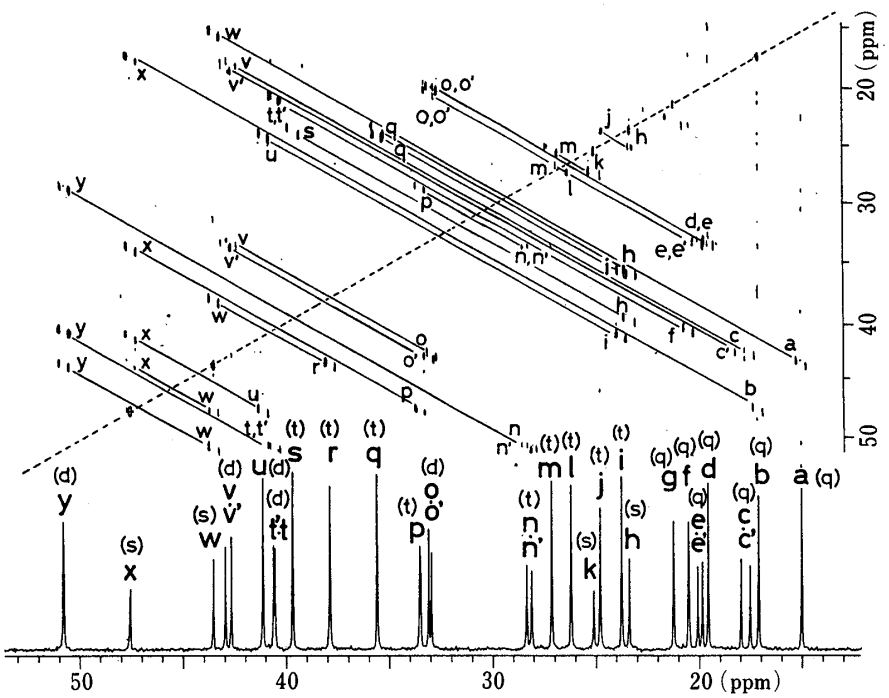

Fig. 7. The Enlarged Contour Map of the Rectangular Region of the Upper Right of Fig. 6

in carbon series l(t)→m(t)→k(s), s(t)→h(s)→q(t)→i(t)→u(d), h(s)→j(t), and so on. It followed that carbons l, m, and k are assigned to C(2), C(1), and C(10) and carbons s, h, q, i, u, and j are assigned to C(4), C(5), C(6), C(7), C(8), and C(19), respectively. Also it is apparent that carbons D(s) and A(d) are assigned to C(9) andC(11), respectively. Further extensive analyses of the ^{13}C–^{13}C correlated peaks in Figs. 6 and 7 led to the structure **16** for cyclonervilasteryl acetate (**1b**). Although correlations could not be detected between the carbons h and k and the

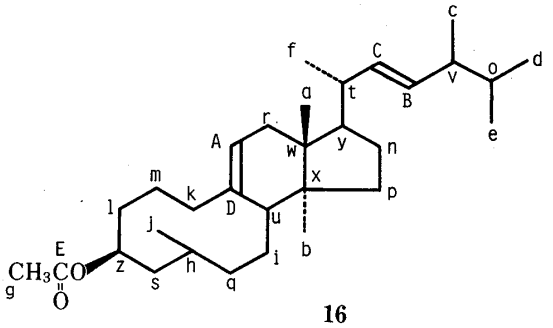

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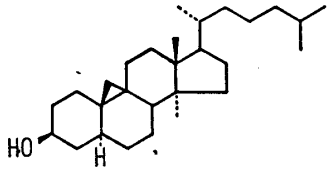
Chart 6



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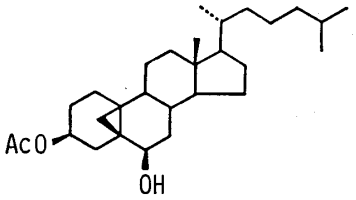

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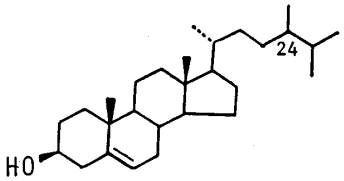

19 : 24-*R*/*α*
20 : 24-*S*/*β*

Chart 7

carbons j and k, there must be chemical bondings between them because the carbons h and k are quaternary and the carbon j is secondary (cyclopropane methylene). Thus, the gross structure of cyclonervilasteryl acetate (**1b**), and hence, the structures of cyclonervilasterol (**2a**), 24-epicyclonervilasterol (**3a**), dihydrocyclonervilasterol (**5a**), and 24-epidihydrocyclonervilasterol (**6a**) were proved.

Turning now to the stereochemistry of the above methylsterols, the configurations at the C(10), C(13), C(14), and C(17) positions were deduced on the basis of the biogenetic analogy with the sterols and triterpenes obtained from this plant[4,10] and with pollinastanol (**17**)[16] which is a well-known methylsterol. The configuration of the C(3)-hydroxy group was believed to be *β*-equatorial in view of the NMR behavior of C(3)–H (δ 3.60, m, $W_{1/2}$ about 16 Hz), which is comparable with that of 3*β*-acetoxy-6*β*-hydroxy-5*β*,19-cyclocholestane (**18**)[17] (δ 4.72, m, $W_{1/2}$ about 16 Hz). Consideration of the stereochemical course of $NaBH_4$ reduction of the ketone (**10**) compared with that of unhindered steroid 3-ketone[18] also supported this conclusion. As to the stereochemistry of the C(24) position, the 24*S* configuration could be assigned to cyclonervilasterol (**2a**) and 24-epidihydrocyclonervilasterol (**6a**), and the 24*R* configuration to 24-epicyclonervilasterol (**3a**) and dihydrocyclonervilasterol (**5a**), based on the shielding values of the 21- and 27-methyl signals compared with those of the 24-epimeric sterols reported by Rubinstein *et al.*,[19] who found that the 27-methyl group in campesterol (**19**: 24-*R*/*α*) and its acetate resonates at slightly lower field than that of dihydrobrassicasterol (**20**: 24-*S*/*β*), and the 21-methyl group in 24-epibrassicasteryl acetate (24-*S*/*α*) resonates at slightly higher field (*ca.* 0.002 ppm) than that of brassicasteryl acetate (24-*R*/*β*). This conclusion was further supported by the HPLC behavior compared with that of analogous sterols,[20] the 24*α* epimers of which are usually eluted faster than the 24*β* epimers.

Our present result provided the first example of naturally occuring methylsterols having a cyclopropane ring at the C(5)–C(10) position and of the effective separation of a 24-epimeric pair with a saturated side chain by reversed-phase HPLC. These compounds are of interest from the viewpoint of the biogenetic relation with natural sterols and triterpenes.

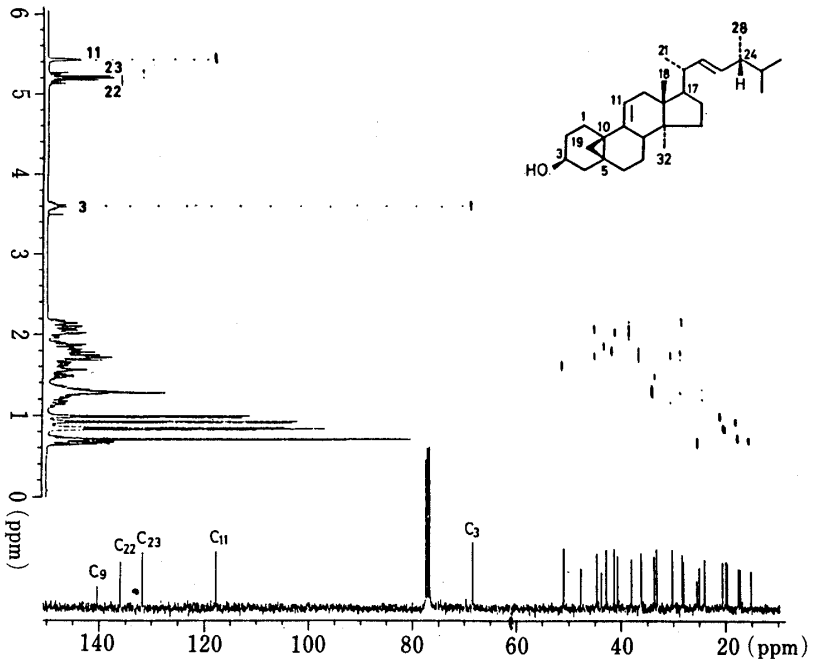

Fig. 8. The ^{1}H–^{13}C Shift Correlated Spectrum of 24-Epicyclonervilasterol (**3a**)

The multiplicities of carbon signals were determined by means of the off-resonance and INEPT methods. The high-field region is enlarged and reproduced in Fig. 9.

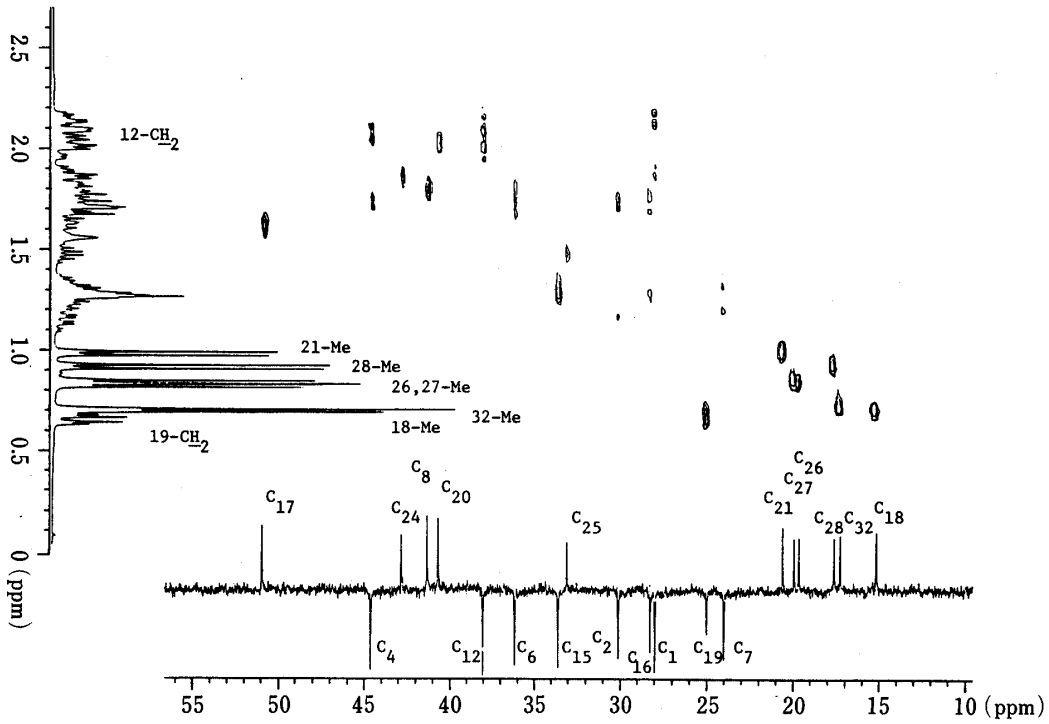

Fig. 9. Enlarged ^{1}H–^{13}C Shift Correlated Spectrum of the High-Field Region in Fig. 8

The multiplicities of carbon signals were determined by means of the DEPT method. Quaternary resonances are suppressed, CH_3 and CH sites give positive intensities, while CH_2 sites give negative intensities.

Next, we examined the ^{13}C-NMR spectra of these methylsterols and their derivatives by application of the INEPT and DEPT[14] (Distortionless Enhancement of Polarization Transfer) methods and 2-D NMR spectroscopy.

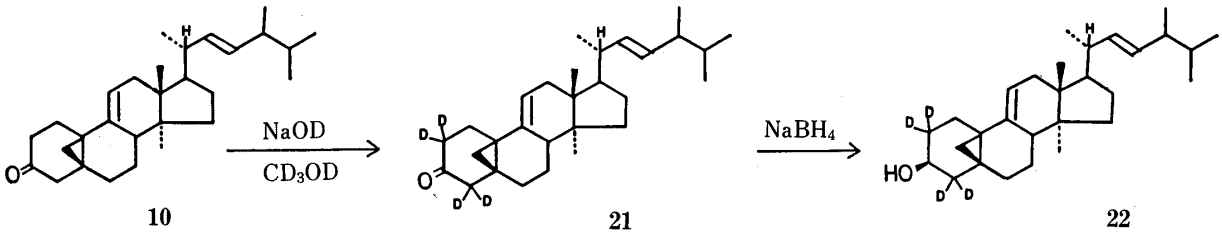

Chart 8

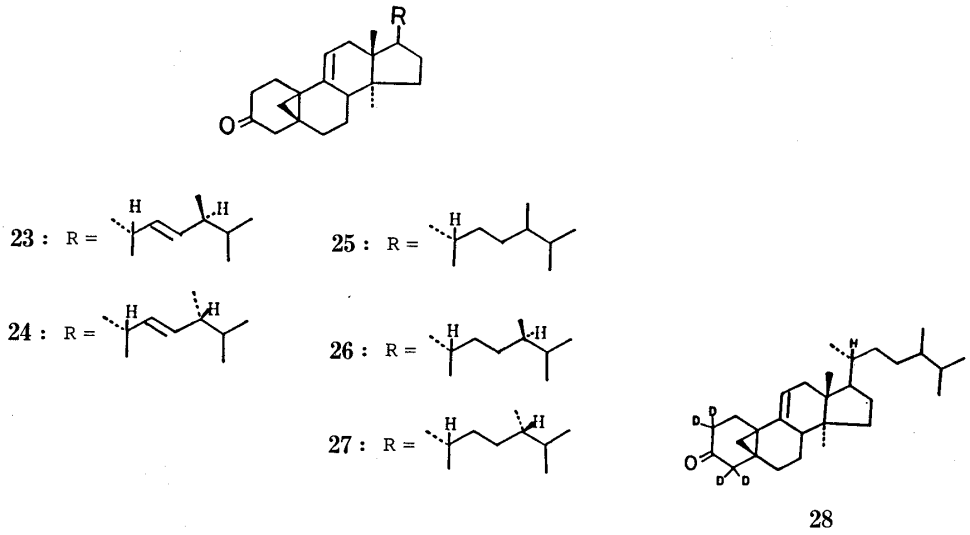

Chart 9

The ^{13}C-signals of the cyclonervilasteryl acetate (**2b**) and 24-epicyclonervilasteryl acetate (**3b**) were determined on the basis of the INADEQUATE data of **1b** shown in Figs. 6 and 7, in which the ^{13}C signals are automatically assigned except for the C(26) and C(27) signals. However, some of the signals appear as double lines because the sample (**1b**) is a 24-epimeric mixture, and therefore, exact assignments of these carbon signals were made on the basis of the comparison of the spectrum of 24-epicyclonervilasteryl acetate (**3b**) with that of **1b**. The results are summarized in Table II.

Subsequently, we carried out the ^{13}C-signal assignments of cyclonervilasterol (**2a**) and 24-epicyclonervilasterol (**3a**) based on the ^{1}H–^{13}C shift correlated NMR measurement (Figs. 8 and 9), which led readily to the assignment of the signals due to C(9), C(22), C(23), C(11), C(3), C(12), C(18), C(19), C(21), C(26), C(27), C(28), and C(32) (Table II). Furthermore, the assignments of the C(2) and C(4) signals were confirmed by comparison of the ^{13}C-NMR spectrum of **3a** with that of the alcohol-2,2,4,4-d_4 (**22**), which was synthesized from the ketone (**10**) through the introduction of deuterium (NaOD and CD_3OD) followed by $NaBH_4$ reduction as shown in Chart 8. Assignments of the other carbon signals were made on the basis of the comparison of the spectrum of **3a** with those of **1a** and the acetates **2b** and **3b**. It should be noted that the chemical shifts for the side chain carbons of the above compounds are consistent with those of brassicasterol (24*R*) and 24-epibrassicasterol (24*S*).[21]

The ^{13}C signals of the 3-keto derivatives (**23**) and (**24**) were then examined in the same manner. The ^{1}H–^{13}C shift correlated NMR measurement of the ketone (**10**: a mixture of **23** and **24**) (Figs. 10 and 11) led readily to assignments of the signals due to C(22), C(23), C(11), C(9), C(4), C(18), C(28), C(32), C(26), C(27), and C(21) (Table II). In this compound, however, the ^{1}H-NMR signals for the 19-methylene protons were obscured by overlapping with the methyl proton signals. A decoupling experiment by irradiation at δ 0.72 gave a new

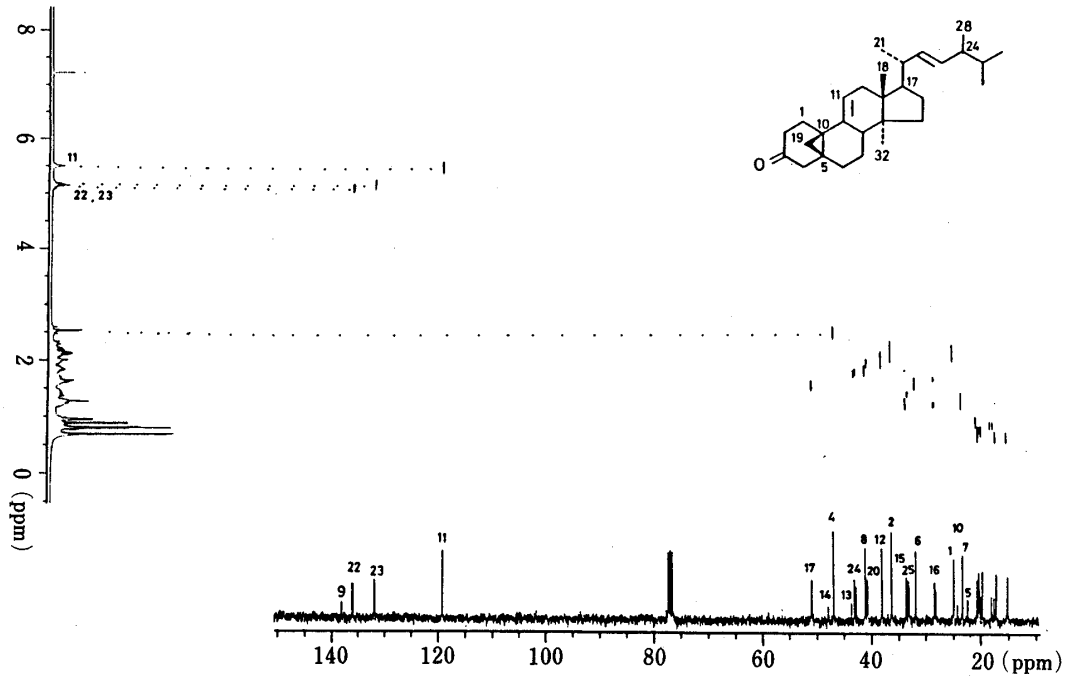

Fig. 10. The ^{1}H–^{13}C Shift Correlated Spectrum of the 3-Keto Compound (**10**: 24-Epimeric Mixture)

The carbonyl carbon signal (δ 215.59) is outside the spectrum. The multiplicities of carbon signals were determined by means of the off-resonance and INEPT methods. Some of the signals appear as double lines because the sample is an epimeric mixture. The high-field region is enlarged and reproduced in Fig. 11.

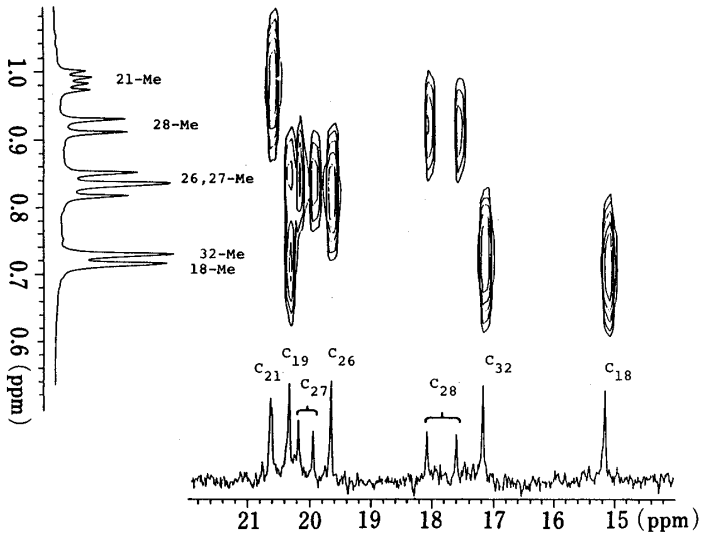

Fig. 11. Enlarged ^{1}H–^{13}C Shift Correlated Spectrum of High-Field Region in Fig. 10

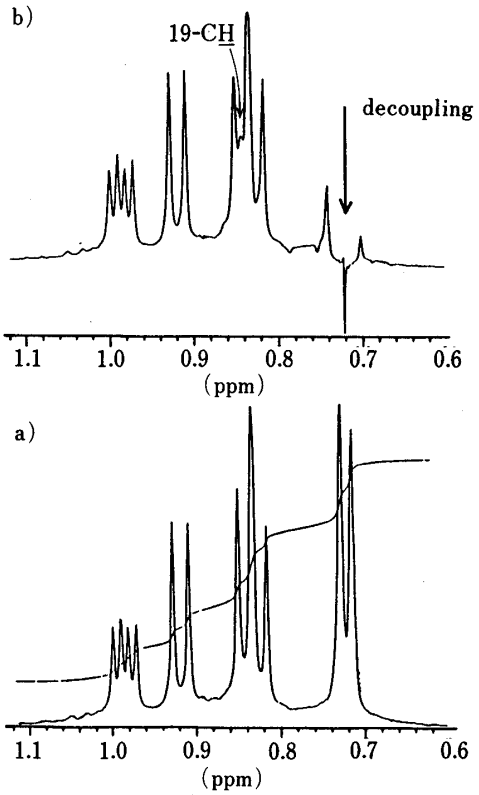

Fig. 12. ^{1}H-NMR Spectrum of the 3-Keto Compound (**10**)

a) normal spectrum (300 MHz).
b) decoupling experiment.

TABLE II. ^{13}C-NMR Data for the Methylsterols and Derivatives

Carbon	**2a** (24-*S*/α)	**3a** (24-*R*/β)	**2b** (24-*S*/α)	**3b** (24-*R*/β)	**23** (24-*S*/α)	**24** (24-*R*/β)	**5a** (24-*R*/α)	**6a** (24-*S*/β)	**5b** (24-*R*/α)	**6b** (24-*S*/β)	**26** (24-*R*/α)	**27** (24-*S*/β)
1	27.99		27.27		25.17[a]		27.96		27.27		25.00[a]	
2	30.18		26.40		36.32		30.18		26.40		36.30	
3	68.40		70.81		215.59		68.39		70.81		211.72	
4	44.64		39.83		47.00		44.62		39.83		46.96	
5	24.02		23.59		22.84[a]		23.96		23.57		22.41[a]	
6	36.17		35.71		32.05[a]		36.18		35.74		31.99[a]	
7	24.02		23.89		23.33		24.02		23.89		23.34	
8	41.39		41.35		41.24		41.37		41.35		41.19	
9	140.54		140.16		139.14		140.43		140.04		138.10	
10	25.48		25.29		24.60[a]		25.44		25.26		24.27[a]	
11	117.84		118.14		119.39		117.92		118.23		119.45	
12	38.09		38.08		38.13		38.19		38.20		38.19	
13	43.79		43.77		43.80		43.88		43.86		43.86	
14	47.73		47.71		47.98		47.57		47.57		47.77	
15	33.68		33.64		33.70		33.64		33.61		33.60	
16	28.53	28.24	28.49	28.24	28.40	28.14	27.84	27.89	27.83		27.78	
17	51.05		51.01		51.06		51.28	51.16	51.25	51.11	51.27	51.14
18	15.18		15.19		15.21		14.97		14.98		15.00	
19	25.07		24.97		20.46		25.05		24.97		20.49	
20	40.62		40.70	40.62	40.60	40.67	36.28	36.63	36.30	36.61	36.62	
21	20.62		20.59		20.64		18.34	18.62	18.34	18.57	18.35	18.58
22	136.31	136.11	136.30	136.10	136.20	136.00	34.08		34.08		34.08	
23	132.19	131.90	131.97	131.86	132.17	132.07	30.69	30.99	30.69	31.00	30.70	31.00
24	43.15	42.86	43.13	42.84	43.11	42.85	38.94	39.19	38.93	39.17	38.94	39.19
25	33.30	33.16	33.28	33.14	33.29	33.16	32.47	31.52	32.47	31.50	32.49	31.53
26	19.67		19.66		19.67		18.39	17.58	18.34	17.59	18.40	17.64
27	20.19	19.95	20.18	19.95	20.07	19.87	20.21	20.52	20.21	20.53	20.21	20.54
28	18.20	17.62	18.06	17.61	17.91	17.48	15.46	15.54	15.45	15.50	15.48	15.53
32	17.28		17.26		17.22		17.26		17.23		17.22	
$OCOCH_3$			21.38						21.38			
$OCOCH_3$			170.52						170.55			

δ values in $CDCl_3$. *a*) Assignments are tentative.

singlet at δ 0.84 as shown in Fig. 12, indicating the location of the 19-methylene signals in this area. Thus, the C(19) signal could be assigned as shown in Fig. 11. Furthermore, the assignments of the C(2) and C(4) signals were confirmed by comparison of the ^{13}C-NMR spectrum of **10** with that of the ketone-2,2,4,4-d_4 (**21**). Here, the assignments of the C(1), C(5), C(6), and C(10) signals were made with the aid of the deuterium isotope effect, reported by Wehrli and Wirthlin,[22] who showed that in the spectrum of 2-methylcyclohexane-2,6,6-d_3 the carbon adjacent to the deuterated carbon shifted upfield by 0.1 ppm and the next adjacent carbon shifted upfield to a smaller extent (0.05 ppm). After Wehrli *et al.*, the signals at δ 25.17, 22.84, 32.05, and 24.60 in the spectrum of **10** were ascribed to C(1) ($\Delta\delta = -0.12$), C(5) ($\Delta\delta = -0.18$), C(6) ($\Delta\delta = -0.03$), and C(10) ($\Delta\delta = -0.06$), respectively. Assignments of other ^{13}C signals were done based on the comparison with those of **1b**. Furthermore, the ^{13}C signals of each 24-epimer (**23** and **24**) were analyzed by comparison with those of **10** (Table II).

Finally, the ^{13}C-NMR spectra of dihydrocyclonervilasteryl acetate (**5b**), 24-epidihydrocyclonervilasteryl acetate (**6b**), dihydrocyclonervilasterol (**5a**), and 24-epidihydrocyclonervilasterol (**6a**) and the 3-keto derivatives (**26** and **27**) were examined in a similar manner and the results are given in Table II. The assignments of the C(2) and C(4) signals of **26** and **27** were confirmed by comparison of the ^{13}C-NMR spectrum of **25** (a mixture of **26** and **27**) with that

of the ketone-2,2,4,4-d_4 (**28**). In this case, the deuterium isotope effect was also observed for the signals due to C(1) (δ 25.03, $\Delta\delta = -0.15$), C(5) (δ 22.43, $\Delta\delta = -0.16$), C(6) (δ 31.95, $\Delta\delta = -0.06$), and C(10) (δ 24.30, $\Delta\delta = -0.02$). It should be mentioned here that the C(2) and C(4) signals of the 3-keto derivatives (**23**, **24**, **26**, and **27**) showed substantial down-field shifts, as expected, while the C(1), C(5), C(6), C(10), and C(19) signals exhibited considerable up-field shifts. This result suggests that the conformation of ring A may be altered by conversion of the methylsterol into the keto derivatives, but this problem is still under investigation.

Experimental

Melting points were determined on a Kofler-type apparatus and are uncorrected. Optical rotations were measured in chloroform solutions on a JASCO DIP-4 automatic polarimeter at 22 °C. UV spectra were taken with a Shimadzu 202 UV spectrometer in EtOH solutions and infrared (IR) spectra with a JASCO IRA-2 spectrometer in KBr discs unless otherwise noted. ^{1}H-NMR spectra were measured on Varian XL-200 and Nicolet NT-300 spectrometers in $CDCl_3$ solutions using tetramethylsilane as an internal standard; chemical shifts are recorded in δ values. MS and high-resolution MS were obtained with a JEOL JMS-D 300 spectrometer (ionization voltage, 70 eV; accelerating voltage, 3 kV) using a direct inlet system or a GC injection system (2% OV-17 2 m × 2 mm i.d. glass column; column temperature 280 °C; injection temperature 300 °C; carrier gas He). GC was done on a Shimadzu GC-6AM instrument with a 2% OV-17 column (2 m × 3 mm i.d. glass tube; injection temperature, 300 °C; column temperature, 280 °C; carrier gas, N_2). HPLC and preparative HPLC were performed on a Waters Associates ALC/GPC 201 D compact-type liquid chromatograph using a TSK-GEL ODS-120A column (column size 25 cm × 4.6 mm i.d.; detector setting, UV 225 nm) or TSK-GEL LS-410 ODS (column size 30 cm × 7.5 mm i.d.; detector setting, UV 225 nm) with hexane–isopropanol–acetonitrile (5 : 15 : 80) as the eluent (flow rate 2.0 ml/min). Column chromatography was done with Mallinkrodt silica gel. Preparative TLC was carried out on Merck Kieselgel GF_{254} with MeOH–$CHCl_3$ mixture (5 : 95 or 3 : 97), or silver nitrate-impregnated silica gel plates prepared from $AgNO_3$ (20%) and Merck Kieselgel $PF_{254+366}$ with ether–hexane mixture, and the plates were examined under UV light. Extraction of substances from silica gel was done with MeOH–$CHCl_3$ (1 : 9) and solutions were concentrated *in vacuo*. For drying organic solutions, anhydrous $MgSO_4$ was used.

Separation and Properties of Methylsterols from Substance MB (Methylsterol Fraction)——Substance MB (methylsterol fraction) (90 mg),[4] obtained previously from the dichloromethane extract of dried herbs of *N. purpurea*, was treated with acetic anhydride (0.5 ml) and pyridine (0.5 ml) overnight at room temperature. Usual work-up and recrystallization from ether–MeOH yielded an acetate mixture (90 mg), which was separated by preparative TLC on 20% $AgNO_3$–silica gel plates with ether–hexane (1 : 20) as the eluent. The less polar fraction was recrystallized from ether–MeOH to give dihydrocyclonervilasteryl acetate (**4b**: 24-epimeric mixture) (23 mg), colorless plates, mp 80—82 °C, $[\alpha]_D$ +19.8° (c = 1.05). MS m/z: 454 (M^+), 394, 379, 366, 313, 285, 267, 225, 173. High-resolution MS: Found 454.3818, Calcd for $C_{31}H_{50}O_2$ (M^+) 454.3811. IR ν cm^{-1}: 1735, 1240 (OAc), 1635, 890 (C=CH). ^{1}H-NMR δ: 0.64 (1H, d, J = 4.0 Hz, 19-CH_2), 0.68, 0.70 (each 3H, s, 18- and 32-CH_3), 0.785 (3H, d, J = 6.6 Hz, 28-CH_3), 0.785 (70%), 0.815 (30%) (3H, d, J = 6.6 Hz, 27-CH_3), 0.86 (3H, d, J = 6.8 Hz, 26-CH_3), 0.88 (30%), 0.89 (70%) (3H, d, J = 6.8 Hz, 21-CH_3), 2.03 (3H, s, OAc), 4.71 (1H, m, C<u>H</u>–OAc), 5.44 (1H, m, C=C<u>H</u>–). The more polar fraction was recrystallized from ether–MeOH to give cyclonervilasteryl acetate (**1b**: 24-epimeric mixture) (40 mg), colorless plates, mp 125—126 °C, $[\alpha]_D$ +5.8° (c = 0.94). MS m/z: 452 (M^+), 392 (base peak), 377, 364, 349, 285, 267, 225, 173. High-resolution MS: Found 452.3664, Calcd for $C_{31}H_{48}O_2$ (M^+) 452.3654. IR ν cm^{-1}: 1735, 1240 (OAc), 1630, 890 (C=CH). ^{1}H-NMR δ: 0.64 (1H, d, J = 4.0 Hz, 19-CH_2), 0.68, 0.69 (each 3H, s, 18- and 32-CH_3), 0.825, 0.84 (each 3H, d, J = 6.5 Hz, 26- and 27-CH_3), 0.91 (3H, d, J = 6.8 Hz, 28-CH_3), 0.974 (50%), 0.984 (50%) (3H, d, J = 6.5 Hz, 21-CH_3), 2.03 (3H, s, OAc), 4.70 (1H, m, C<u>H</u>–OAc), 5.19 (2H, m, –C<u>H</u>=C<u>H</u>–), 5.44 (1H, m, C=C<u>H</u>–).

Cyclonervilasterol (1a: 24-Epimeric Mixture)——The acetate (**1b**: 24-epimeric mixture) (25 mg) was refluxed with 3% KOH–MeOH (1.5 ml) for 30 min and the reaction mixture was worked up as usual. Recrystallization of the product from ether–MeOH gave **1a** (24-epimeric mixture) (19 mg), colorless needles, mp 154—155 °C, $[\alpha]_D$ +6.7° (c = 1.06). MS m/z: 410 (M^+), 395, 392, 377, 285, 267, 225, and 173. High-resolution MS: Found 410.3593, Calcd for $C_{29}H_{46}O$ (M^+) 410.3548. ^{1}H-NMR δ: 0.63 (1H, d, J = 4.0 Hz, 19-CH_2), 0.683, 0.698 (each 3H, s, 18- and 32-CH_3), 0.818, 0.834 (each 3H, d, J = 6.8 Hz, 26- and 27-CH_3), 0.913 (3H, d, J = 6.8 Hz, 28-CH_3), 0.968 (50%), 0.978 (50%) (3H, d, J = 6.4 Hz, 21-CH_3), 3.61 (1H, m, C<u>H</u>–OH), 5.20 (2H, m, –C<u>H</u>=C<u>H</u>–), 5.43 (1H, m, C=C<u>H</u>–).

Dihydrocyclonervilasterol (4a: 24-Epimeric Mixture)——Dihydrocyclonervilasteryl acetate (**4b**: 24-epimeric mixture) (50 mg) was hydrolyzed in the same manner as above and the product was recrystallized from ether–MeOH to give **4a** (24-epimeric mixture) (39 mg), colorless needles, mp 125—127 °C, $[\alpha]_D$ +17.6° (c = 0.61). MS m/z: 412 (M^+), 397, 379, 353, 313, 285, 267, 243, 225, 173. High-resolution MS: Found 412.3702, Calcd for $C_{29}H_{48}O$ (M^+) 412.3705. ^{1}H-NMR δ: 0.64 (1H, d, J = 4.0 Hz, 19-CH_2), 0.670, 0.698 (each 3H, s, 18- and 32-CH_3), 0.781 (3H, d, J = 6.3 Hz, 28-CH_3), 0.781 (70%), 0.808 (30%) (3H, d, J = 6.3 Hz, 27-CH_3), 0.854 (3H, d, J = 6.4 Hz, 26-CH_3), 0.881 (30%), 0.885 (70%) (3H, d, J = 6.1 Hz, 21-CH_3), 3.62 (1H, m, C<u>H</u>–OH), 5.44 (1H, m, C=C<u>H</u>–).

Isolation of Methylsterols by HPLC——Substance MB (mixture) (20 mg) was subjected repeatedly to preparative HPLC on a TSK-GEL LS-410A ODS column with hexane–isopropanol–acetonitrile (5 : 15 : 80) as the eluting solvent at 20 °C to give cyclonervilasterol (peak 1, **2a**) (3 mg), mp 151—152 °C; 24-epicyclonervilasterol (peak 2, **3a**) (1.7 mg), mp 153—154 °C; peak 3 (0.2 mg),[7] mp 121—123 °C; peak 4 (0.5 mg),[7] mp 131—133 °C; dihydrocyclonervilasterol (peak 5, **5a**) (2.3 mg), mp 128—129.5 °C; 24-epidihydrocyclonervilasterol (peak 6, **6a**) (4.1 mg), mp 129—130 °C.

Cyclonervilasterol (**2a**): Colorless needles (ether–MeOH), mp 151—152 °C, $[\alpha]_D$ +15.2° (c=0.46). UV λ_{max}^{EtOH} nm (log ε): 210 (3.72). MS m/z: 410 (M^+), 395, 392, 377, 285, 267, 225, 175. High-resolution MS: Found 410.3571, Calcd for $C_{29}H_{46}O$ (M^+) 410.3548. ^{1}H-NMR: see Table I. ^{13}C-NMR: see Table II.

24-Epicyclonervilasterol (**3a**): Colorless needles (ether–MeOH), mp 153—154 °C, $[\alpha]_D$ −10.8° (c=0.62). UV λ_{max}^{EtOH} nm (log ε): 211 (3.76). MS m/z: 410 (M^+), 395, 392, 377, 285, 267, 225, 173. High-resolution MS: Found 410.3551, Calcd for $C_{29}H_{46}O$ (M^+) 410.3548. ^{1}H-NMR: see Table I. ^{13}C-NMR: see Table II.

Dihydrocyclonervilasterol (**5a**): Colorless needles (MeOH), mp 128—129.5 °C, $[\alpha]_D$ +24.5° (c=0.8). UV λ_{max}^{EtOH} nm (log ε): 211 (3.75). MS m/z: 412 (M^+), 397, 394, 379, 285, 267, 243, 225, 173. High-resolution MS: Found 412.3727, Calcd for $C_{29}H_{48}O$ (M^+) 412.3706. ^{1}H-NMR: see Table I. ^{13}C-NMR: see Table II.

24-Epidihydrocyclonervilasterol (**6a**): Colorless needles (MeOH), mp 129—130 °C, $[\alpha]_D$ +11.8° (c=0.43). UV λ_{max}^{EtOH} nm (log ε): 211 (3.72). MS m/z: 412 (M^+), 397, 394, 379, 285, 267, 243, 225, 173. High-resolution MS: Found 412.3712, Calcd for $C_{29}H_{48}O$ (M^+) 412.3705. ^{1}H-NMR: see Table I. ^{13}C-NMR: see Table II.

Catalytic Hydrogenation of Cyclonervilasterol (2a)——Cyclonervilasterol (**2a**) (0.5 mg) was hydrogenated in ether (0.5 ml) and MeOH (0.5 ml) with PtO_2 (1 mg) for 12 h. After removal of the catalyst by filtration, the reaction mixture was purified by preparative TLC with MeOH–$CHCl_3$ (2 : 98) and then recrystallized from MeOH to give the dihydro compound (**5a**) (0.3 mg), colorless needles, mp 125—127 °C. This compound was proved to be identical with dihydrocyclonervilasterol (**5a**) by MS, ^{1}H-NMR, and HPLC comparisons.

Catalytic Hydrogenation of 24-Epicyclonervilasterol (3a)——24-Epicyclonervilasterol (**3a**) (0.5 mg) was hydrogenated in the same manner as above and the product was recrystallized from MeOH to give the dihydro compound (**6a**) (0.3 mg), needles, mp 125—126 °C. This compound was proved to be identical with 24-epidihydrocyclonervilasterol (**6a**) by MS, ^{1}H-NMR, and HPLC comparisons.

Osmium Tetroxide Oxidation of Cyclonervilasteryl Acetate (1b: 24-Epimeric Mixture)——Osmium tetroxide (10 mg) was added to a solution of **1b** (5 mg) in pyridine (0.8 ml) and the mixture was allowed to stand overnight at room temperature. Thereafter, a solution of sodium bisulfite (2 g) in water (2.5 ml) was added to the reaction mixture and the whole was stirred for 30 min. The mixture was then poured into ice-water, basified with Na_2CO_3, and extracted with CH_2Cl_2. The extract was washed successively with 2% HCl and dil. Na_2CO_3, dried, and concentrated. The residue was purified by preparative TLC to give a tetraol (**7**) (3.7 mg), amorphous, MS m/z: 520 (M^+), 502, 460 (base peak), 442, 424, 341, 323, 285, 265, 249, 189. High-resolution MS: Found 520.3753, Calcd for $C_{31}H_{52}O_6$ (M^+) 520.3761. ^{1}H-NMR δ: 0.62, 0.67 (each 1H, d, J=5.0 Hz, 19-CH_2), 0.70—1.08 (CH_3 × 6), 2.02 (3H, s, OAc), 3.40—3.80 (C<u>H</u>–OH × 2), 3.97 (1H, q, J=11, 6 Hz, C<u>H</u>–OH), 4.74 (1H, m, C<u>H</u>–OAc).

Lead Tetraacetate Oxidation of the Tetraol (7) Followed by Jones Oxidation——Lead tetraacetate (15 mg) was added to a stirred solution of **7** (2.5 mg) in dry benzene (0.5 ml) and dry ether (0.5 ml) and stirring continued for 12 h at room temperature. The reaction mixture was diluted with water, and extracted with ether. The extract was dried and concentrated. The residue was dissolved in acetone (0.5 ml) and then Jones reagent was added under stirring at 0 °C until the solution remained yellowish. After stirring for 5 min, MeOH was added. The reaction mixture was neutralized with aq. Na_2CO_3 and extracted with CH_2Cl_2. The CH_2Cl_2 extract was washed with brine, dried, and concentrated. The residue (1.1 mg) was chromatographed on silica gel. Elution with ether–pentane gave a minute amount of a fatty acid, which was dissolved in ether and treated with excess diazomethane at room temperature. Evaporation of the solvent gave the methyl ester (**8**), which was identified as methyl 2-methylisovalerate (**8**) by GC and GC-MS comparisons with a sample prepared from ergosterol (**9**).

Synthesis of Methyl 2-Methylisovalerate (8)——1) Conversion of Ergosterol (**9**) to Ergost-22-en-3-one: A solution of ergosterol (5 g), cyclohexanone (20 ml), and $Al(OiPr)_3$ (1.7 g) in toluene (110 ml) was refluxed with stirring for 40 min. The reaction mixture was worked up in the usual manner to afford a crystalline mass, which was recrystallized from ether–MeOH to give ergost-4,7,22-trien-3-one (3.2 g), mp 130—132 °C. This was refluxed with conc. HCl (5 ml) in MeOH (210 ml) for 2 h and the reaction mixture was worked up as usual. Recrystallization of the product from MeOH–pentane gave ergost-4,6,22-trien-3-one (2.4 g), colorless needles, mp 103—105 °C. Subsequently, a mixture of ergost-4,6,22-trien-3-one (2.4 g) and 10% Pd/C (140 mg) in 1% KOH–MeOH (130 ml) was hydrogenated at room temperature for 12 h. Usual work-up and recrystallizations from acetone–ether gave ergost-22-en-3-one (2.2 g), colorless plates, mp 105—107 °C.

2) Ozonolysis of Ergost-22-en-3-one Followed by Jones Oxidation: Ozone was passed into a solution of ergost-22-en-3-one (2.2 g) in dry CH_2Cl_2 (40 ml), MeOH (1 ml), and anhydrous pyridine (0.23 ml) at −60 °C for 1.5 h. Then glacial acetic acid (4 ml) and zinc powder (1 g) were added, and the mixture was vigorously stirred for 2 h at room temperature. The reaction mixture, after removal of the zinc powder by filtration, was steam-distilled and the distillate (300 ml) was saturated with NaCl and extracted with CH_2Cl_2. The CH_2Cl_2 solution was washed successively with 5% HCl, water, and dil. Na_2CO_3 and then dried and concentrated to leave an oily residue (40 mg). MS m/z: 101

($M^+ + 1$), 74 (base peak), 56, 43. ^{1}H-NMR δ: 0.83—1.1 ($CH_3 \times 3$), 9.56 (1H, d, J=2.5 Hz, CHO). This aldehyde (40 mg) was dissolved in acetone (0.8 ml), then Jones reagent was added under stirring at 0 °C until the solution remained yellowish. After stirring for 5 min, MeOH was added. The reaction mixture was neutralized with aq. Na_2CO_3 and extracted with CH_2Cl_2. The CH_2Cl_2 extract was washed with brine, and then dried and concentrated to leave an oily residue (32 mg). The residue was distilled *in vacuo* to give 2-methylisovaleric acid (20 mg), colorless liquid, bp 150 °C (26 mmHg). MS *m/z*: 117 ($M^+ + 1$), 101, 82, 74, 73, 56, 43. ^{1}H-NMR δ: 0.85—1.21 ($CH_3 \times 3$), 11.26 (1H, br s, COOH). This product dissolved in ether and treated with excess diazomethane at room temperature. Evaporation of the solvent gave methyl 2-methylisovalerate (**8**). MS *m/z*: 130 (M^+), 115, 90, 88, 71, 57, 43.

Collins Oxidation of Cyclonervilasterol (1a: 24-Epimeric Mixture)——Collins reagent (20 mg) was added to a stirred solution of **1a** (24-epimeric mixture) (9 mg) in dry CH_2Cl_2 (1 ml) and stirring continued for 5 h at room temperature. The reaction mixture was chromatographed on silica gel with CH_2Cl_2 and the eluate was crystallized from MeOH to afford a ketone (**10**: 24-epimeric mixture) (6.5 mg), colorless needles, mp 140—141 °C. IR ν_{max}: 1700 cm^{-1}. MS *m/z*: 408 (M^+), 393, 354, 310, 283, 281, 267, 265, 257, 241. High-resolution MS: Found 408.3344, Calcd for $C_{29}H_{44}O$ (M^+) 408.3390. ^{1}H-NMR δ: 0.715, 0.725 (each 3H, s, 18- and 32-CH_3), 0.825, 0.845 (each 3H, d, J=6.8 Hz, 26- and 27-CH_3), 0.92 (3H, d, J=6.7 Hz, 28-CH_3), 0.985 (50%), 0.991 (50%) (3H, d, J=6.6 Hz, 21-CH_3), 2.54, 2.65 (each 1H, d, J=18 Hz, 4-CH_2), 5.22 (2H, m, –C<u>H</u>=C<u>H</u>–), 5.56 (1H, m, C=C<u>H</u>–).

Sodium Borohydride Reduction of the Ketone (10)——$NaBH_4$ (5 mg) was added to a solution of the ketone (**10**) (2.5 mg) in anhydrous MeOH (0.5 ml), and the mixture was stirred at room temperature for 1 h. After neutralization by addition of 5% HCl and evaporation of MeOH under reduced pressure, the mixture was diluted with water and extracted with CH_2Cl_2. The combined CH_2Cl_2 extracts were washed with brine, dried, and concentrated. The residue was purified by preparative TLC with $CHCl_3$ as the eluent and then crystallized from ether–MeOH to afford an alcohol (**1a**) (1.5 mg), which was identified by GC and ^{1}H-NMR comparisons with cyclonervilasterol (**1a**) (24-epimeric mixture).

Alkaline Treatment of the Ketone (10: 24-Epimeric Mixture)——A solution of the ketone (**10**) (3 mg) and NaOMe (6 mg) in anhydrous MeOH (1 ml) was refluxed for 1 h. After neutralization by addition of 5% HCl and evaporation of MeOH under reduced pressure, the mixture was diluted with water and extracted with CH_2Cl_2. The combined CH_2Cl_2 extracts were washed with brine, dried, and concentrated. The residue was purified by preparative TLC with $CHCl_3$ and then recrystallized from MeOH to afford the starting material (**10**) (1.3 mg), whose identity was confirmed by the ^{1}H-NMR spectrum.

Lead Tetraacetate Oxidation of the Tetraol (7)——Lead tetraacetate (4 mg) was added to a stirred solution of **7** (3.7 mg) in dry ether (0.1 ml) and dry benzene (0.1 ml) and stirring continued for 20 min at room temperature. The reaction mixture was diluted with water, and extracted with ether. The extract was dried and concentrated. The residue (2 mg) was purified by preparative TLC to give a keto dialdehyde (**11**) (0.8 mg), oil, MS *m/z*: 416 (M^+), 401, 398, 387, 359, 356, 317, 299, 289, 222, 162. ^{1}H-NMR δ: 0.94, 1.65 (each 3H, s, 18- and 32-CH_3), 1.135 (40%), 1.138 (60%) (3H, d, J=7.2 Hz, 21-CH_3), 2.85 (1H, ddd, J=16, 3, 2 Hz, 12-H), 3.11 (1H, dd, J=16, 2 Hz, 12-H), 4.85 (1H, m, C<u>H</u>–OAc), 9.77 (1H, d, J=3 Hz, –C<u>H</u>O), 10.0 (1H, br s, –C<u>H</u>O).

Osmium Tetroxide Oxidation of Dihydrocyclonervilasteryl Acetate (4b: 24-Epimeric Mixture)——Osmium tetroxide (10 mg) was added to a solution of **4b** (24-epimeric mixture) (7 mg) in pyridine (0.4 ml) and the mixture was allowed to stand overnight at room temperature. Thereafter, a solution of sodium bisulfite (2 g) in water (2.5 ml) was added to the reaction mixture and the whole was stirred for 30 min. The reaction mixture was then poured into ice-water, basified with Na_2CO_3, and extracted with CH_2Cl_2. The extract was washed successively with 2% HCl and dil. Na_2CO_3, dried, and concentrated. The residue was purified by preparative TLC (developed with MeOH–$CHCl_3$ (5:95)) to give a diol (**12**) (4 mg), amorphous, MS *m/z*: 488 (M^+), 470, 428, 410, 392, 320, 304, 291, 265, 261, 189. High-resolution MS: Found 488.3892, Calcd for $C_{31}H_{52}O_4$ (M^+) 488.3863. ^{1}H-NMR δ: 0.62, 0.67 (each 1H, d, J=5.0 Hz, 19-CH_2), 0.785 (70%), 0.815 (30%) (3H, d, J=6.8 Hz, 26- and 27-CH_3), 0.81, 0.93 (each 3H, s, 18- and 32-CH_3), 0.865 (3H, d, J=6.7 Hz, 26- and 27-CH_3), 0.915 (30%), 0.925 (70%) (3H, d, J=6.6 Hz, 21-CH_3), 0.93 (3H, s, 32-CH_3), 2.0 (3H, s, OAc), 3.97 (1H, q, J=11, 6 Hz, C<u>H</u>–OH), 4.75 (1H, m, C<u>H</u>–OAc).

Lead Tetraacetate Oxidation of the Diol (12: 24-Epimeric Mixture)——Lead tetraacetate (3 mg) was added to a stirred solution of **12** (4 mg) in dry benzene (1 ml) and stirring continued for 10 min at room temperature. The reaction mixture was worked up in the usual manner to afford an oily residue (4.2 mg), which was separated by preparative TLC (developed with MeOH–$CHCl_3$ (2:98)) into two fractions. The less polar fraction gave an oily keto aldehyde (**13**) (2.4 mg). MS *m/z*: 486 (M^+), 468, 457, 426, 359, 317, 299, 289, 222, 162. High-resolution MS: Found 486.3717, Calcd for $C_{31}H_{50}O_4$ (M^+) 486.3706; Found 426.3453, Calcd for $C_{29}H_{46}O_2$ 426.3486; Found 317.1776, Calcd for $C_{19}H_{25}O_4$ 317.1746; Found 289.1771, Calcd for $C_{18}H_{25}O_3$ 289.1797; Found 222.1247, Calcd for $C_{13}H_{18}O_3$ 222.1257; Found 162.1047, Calcd for $C_{11}H_{14}O$ 162.1041. The more polar fraction afforded an oily keto acid (**14**) (1 mg). MS *m/z*: 502 (M^+), 484, 442, 375, 333, 315, 289, 222, 162. High-resolution MS: Found 502.3644, Calcd for $C_{31}H_{50}O_5$ (M^+) 502.3656; Found 333.1717, Calcd for $C_{19}H_{25}O_5$ 333.1695; Found 222.1257, Calcd for $C_{13}H_{18}O_3$ 222.1257; Found 162.1055, Calcd for $C_{11}H_{14}O$ 162.1041.

The Keto Acid-8-d_1 (15: 24-Epimeric Mixture)——A mixture of the keto acid (**14**) (0.6 mg), MeOD (0.3 ml), and NaOD (7.5 N NaOD–D_2O) (0.1 ml) was refluxed for 3 h. After concentration *in vacuo*, the mixture was extracted with

CH_2Cl_2, dried, and concentrated. The residue was chromatographed on silica gel, eluting with MeOH–$CHCl_3$ (1 : 99), to afford an oily keto acid-8-d_1 (**15**) (0.2 mg). MS *m/z*: 461 (M^+), 443, 334, 316, 291, 181, 163. High-resolution MS: Found 461.3603, Calcd for $C_{29}H_{47}O_4D$ (M^+) 416.3614; Found 316.2035, Calcd for $C_{19}H_{38}O_3D$ 316.2031; Found 291.1605, Calcd for $C_{17}H_{21}O_4D$ 291.1610; Found 181.1190, Calcd for $C_{11}H_{15}O_2D$ 181.1220.

Acetylation of 24-Epicyclonervilasterol (3a)——A mixture of 24-epicyclonervilasterol (**3a**) (2 mg) was treated with acetic anhydride (0.1 ml) and pyridine (0.1 ml) and the reaction mixture was allowed to stand overnight at room temperature. The mixture was poured into ice-water and extracted with ether. The ether extract was washed successively with 5% HCl and dil. Na_2CO_3, dried, and concentrated. The residue was purified by preparative TLC (developed with $CHCl_3$) and the product was recrystallized from ether–MeOH to afford colorless needles (**3b**) (1.6 mg), mp 119—120 °C. IR ν_{max}: 1730 cm^{-1}. MS *m/z*: 452 (M^+), 392, 377, 364, 349, 285, 267, 225, 173. High-resolution MS: Found 452.3641, Calcd for $C_{31}H_{48}O_2$ (M^+) 452.3652. ^{1}H-NMR δ: 0.63 (1H, d, J=4.0 Hz, 19-H), 0.691 (6H, s, 18- and 32-CH_3), 0.819, 0.832 (3H, d, J=6.8 Hz, 26- and 27-CH_3), 0.912 (3H, d, J=6.92 Hz, 28-CH_3), 0.976 (3H, d, J=6.31 Hz, 21-CH_3), 2.002 (3H, s, OAc), 4.68 (1H, m, C<u>H</u>–OAc), 5.15, 5.25 (each 1H, dd, J=15, 7 Hz, 22- and 23-H), 5.43 (1H, m, C=C<u>H</u>–).

Collins Oxidation of 24-Epicyclonervilasterol (3a)——Collins reagent (9 mg) was added to a stirred solution of **3a** (2 mg) in dry CH_2Cl_2 (1 ml) and stirring was continued for 3 h at room temperature. The reaction mixture was chromatographed on silica gel with CH_2Cl_2 as the eluent and then recrystallized from MeOH to afford a ketone (**24**) (1.2 mg), colorless needles, mp 126—127 °C. IR ν_{max}: 1700 cm^{-1}. MS *m/z*: 408 (M^+), 393, 354, 310, 283, 281, 267, 265, 257, 241. High-resolution MS: Found 408.3395, Calcd for $C_{29}H_{44}O$ (M^+) 408.3390. ^{1}H-NMR δ: 0.713 (3H, s, 18-CH_3), 0.724 (3H, s, 32-CH_3), 0.824, 0.839 (each 3H, d, J=6.8 Hz, 26- and 27-CH_3), 0.917 (1H, d, J=6.8 Hz, 28-CH_3), 0.989 (3H, d, J=6.5 Hz, 21-CH_3), 2.52, 2.63 (each 1H, d, J=18 Hz, 4-H_2), 5.15, 5.26 (each 1H, dd, J=15, 7 Hz, 22- and 23-H), 5.54 (1H, m, C=C<u>H</u>–).

The Ketone-2,2,4,4-d_4 (21: 24-Epimeric Mixture)——A mixture of the ketone (**10**) (3 mg), CD_3OD (0.5 ml), and NaOD (7.5 N NaOD–D_2O) (0.1 ml) was refluxed for 1 h. After concentration *in vacuo*, the mixture was extracted with CH_2Cl_2, dried, and concentrated. Silica gel column chromatography of the residue with $CHCl_3$ gave a crystalline mass, which was recrystallized from MeOH to afford the ketone-2,2,4,4-d_4 (**21**) (2.1 mg), colorless needles, mp 135—137 °C. MS *m/z*: 412 (M^+) ($C_{29}H_{40}OD_4$), 397, 385, 369, 314, 287, 259, 245, 243. ^{13}C-NMR δ: 22.25 [C(5)], 24.24 [C(10)], 24.91 [C(1)], 31.92 [C(6)].

Sodium Borohydride Reduction of the Ketone-2,2,4,4-d_4 (21: 24-Epimeric Mixture)——$NaBH_4$ (3 mg) was added to a solution of the ketone-2,2,4,4-d_4 (**21**) (2 mg) in anhydrous MeOH (0.5 ml), then further $NaBH_4$ (3 mg) was added, and the mixture was stirred at room temperature for 1 h. After neutralization by addition of 5% HCl and evaporation of MeOH under reduced pressure, the mixture was diluted with water and extracted with CH_2Cl_2. The combined CH_2Cl_2 extracts were washed with brine, dried, and concentrated. Silica gel column chromatography of the residue with $CHCl_3$ gave a crystalline mass, which was recrystallized from MeOH to afford cyclonervilasterol-2,2,4,4-d_4 (**22**) (1.4 mg), mp 140—143 °C. MS *m/z*: 414 (M^+) ($C_{29}H_{42}OD_4$), 396, 381, 289, 271, 255, 229.

Acetylation of Dihydrocyclonervilasterol (5a)——Dihydrocyclonervilasterol (**5a**) (2 mg) was treated with acetic anhydride (0.1 ml) and pyridine (0.1 ml) overnight at room temperature. The reaction mixture was worked up in the usual manner to afford a crystalline mass, which was recrystallized from MeOH to give an acetate (**5b**) (1.7 mg), mp 82—84 °C. IR ν_{max}: 1730 cm^{-1}. MS *m/z*: 454 (M^+), 394, 379, 366, 313, 285, 267, 225, 173. High-resolution MS: Found 454.3818, Calcd for $C_{31}H_{50}O_2$ (M^+) 454.3808. ^{1}H-NMR δ: 0.63 (1H, d, J=4.0 Hz, 19-H), 0.671 (3H, s, 18-CH_3), 0.695 (3H, s, 32-CH_3), 0.775 (3H, d, J=5.49 Hz, 28-CH_3), 0.805, 0.852 (3H, d, J=6.8 Hz, 26- and 27-CH_3), 0.875 (3H, d, J=6.84 Hz, 21-CH_3), 2.007 (3H, s, OAc), 4.70 (1H, m, C<u>H</u>–OAc), 5.43 (1H, m, 11-H).

Collins Oxidation of Dihydrocyclonervilasterol (5a)——Collins reagent (10 mg) was added to a stirred solution of **5a** (2 mg) in dry CH_2Cl_2 (0.5 ml) and stirring was continued for 3 h at room temperature. The reaction mixture was worked up in the usual manner to afford a crystalline mass, which was recrystallized from MeOH to give a ketone (**26**) (1.2 mg), colorless needles, mp 117—118 °C. MS *m/z*: 410 (M^+), 395, 382, 313, 311, 283, 257, 255, 241, and 190. High-resolution MS: Found 410.3581, Calcd for $C_{29}H_{46}O$ (M^+) 410.3546. ^{1}H-NMR δ: 0.669, 0.724 (each 3H, s, 18- and 32-CH_3), 0.779 (3H, d, J=5.23 Hz, 28-CH_3), 0.809, 0.856 (each 3H, d, J=6.65 Hz, 26- and 27-CH_3), 0.888 (3H, d, J=5.86 Hz, 21-CH_3), 2.51, 2.61 (2H, d, J=18 Hz, 4-H_2), 5.53 (1H, m, 11-H).

Collins Oxidation of Dihydrocyclonervilasterol (4a: 24-Epimeric Mixture)——Collins reagent (30 mg) was added to a stirred solution of **4a** (9.5 mg) in dry CH_2Cl_2 (1 ml) and stirring was continued for 3 h at room temperature. The reaction mixture was chromatographed on silica gel with CH_2Cl_2 as the eluent and then recrystallized from MeOH to afford a ketone (**25**) (7 mg), mp 114—120 °C. IR ν_{max}: 1700 cm^{-1}. MS *m/z*: 410 (M^+), 395, 382, 367, 353, 313, 283, 265, 257, 241, 227, 190. High-resolution MS: Found 410.3472, Calcd for $C_{29}H_{46}O$ (M^+) 410.3546. ^{13}C-NMR δ: 22.43 [C(5)], 24.30 [C(10)], 25.03 [C(1)], 31.98 [C(6)].

The Ketone-2,2,4,4-d_4 (28: 24-Epimeric Mixture)——A mixture of the ketone (**25**) (3 mg), CD_3OD (0.5 ml), and NaOD (7.5 N NaOD–D_2O) (0.1 ml) was refluxed for 1 h. After concentration *in vacuo*, the mixture was extracted with CH_2Cl_2, and the extract was dried, and concentrated. Silica gel column chromatography of the residue with $CHCl_3$ gave a crystalline mass, which was recrystallized from MeOH to afford the ketone-2,2,4,4-d_4 (**28**) (2 mg), colorless needles, mp 114—116 °C. MS *m/z*: 414 (M^+) ($C_{29}H_{42}OD_4$), 399, 313, 287, 259, 245, 243, 194. ^{13}C-NMR δ: 22.27

[C(5)], 24.28 [C(10)], 24.88 [C(1)], 31.92 [C(6)].

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