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Studies on the Constituents of Orchidaceous Plants. III.[1] Isolation of Non-conventional Side Chain Sterols from *Nervilia purpurea* SCHLECHTER and Structure Determination of Nervisterol[2]

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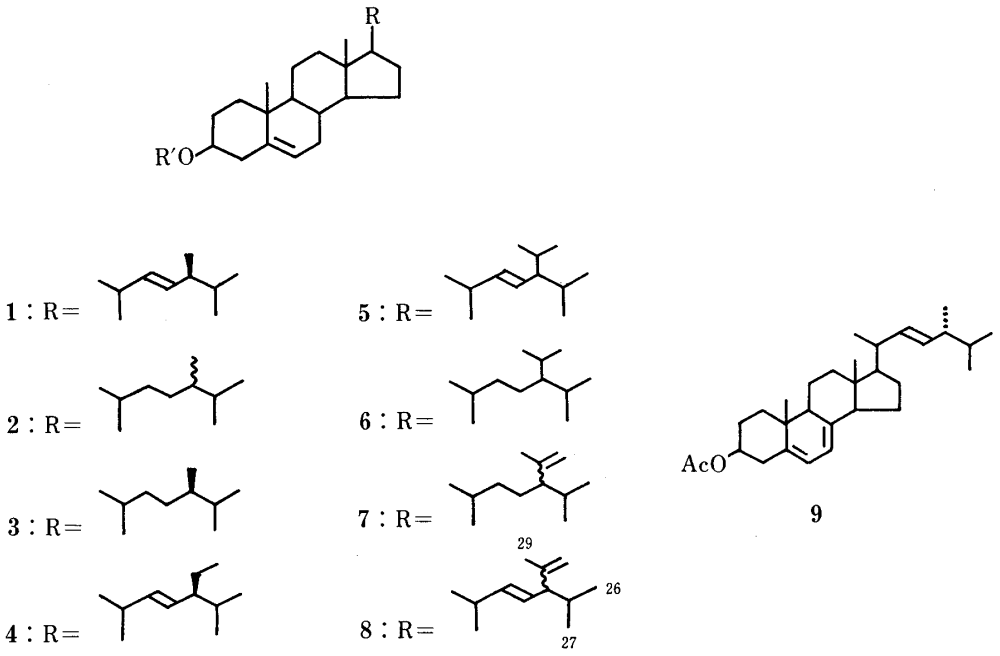
From the neutral fraction of the ether extract of *Nervilia purpurea* SCHLECHTER (Orchidaceae), unusual side chain sterols (**5a**, **6a**, and **7a**), previously reported as the constituents of marine invertebrates, were isolated along with 24-epibrassicasterol, 24ξ-methylcholesterol, ergosterol, and stigmasterol. A new non-conventional side chain sterol, named nervisterol (**8a**), was also isolated and its structure was elucidated.

Keywords——*Nervilia purpurea*; Orchidaceae; non-conventional side chain sterol; nervisterol; 22-dehydro-24-isopropylcholesterol; 24-isopropylcholesterol; 24ξ-isopropenylcholesterol; 24-epibrassicasterol; GC-MS; ^{1}H-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 also the isolation and structure elucidation of new triterpenes from substance MA (a triterpene mixture) obtained from *N. purpurea*.[1] This paper deals with the isolation and structure determination of non-conventional side chain sterols, including a new sterol (**8a**) named nervisterol, from *N. purpurea*.[2]

The sterol mixture[3] obtained from the ether 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), although thin layer chromatography (TLC) revealed only a single spot. The mass chromatogram obtained by the GC-MS method is reproduced in Fig. 1, which proved the substance to consist of at least eight components, corresponding to the molecular formulae $C_{28}H_{46}O$ (**1a**: M^+ *m/z* 398), $C_{28}H_{48}O$ (**2a**: M^+ *m/z* 400), $C_{28}H_{44}O$ (**9a**: M^+ *m/z* 396), $C_{29}H_{48}O$ (**4a**: M^+ *m/z* 412), $C_{30}H_{48}O$ (**8a**: M^+ *m/z* 424), $C_{30}H_{50}O$ (**5a**: M^+ *m/z* 426), $C_{30}H_{50}O$ (**7a**: M^+ *m/z* 426), and $C_{30}H_{52}O$ (**6a**: M^+ *m/z* 428).

The sterol mixture was acetylated as usual and the resulting acetate mixture was carefully separated by column chromatography on 20% silver nitrate-impregnated silica gel[4] with benzene–hexane (1 : 5) as the eluent, and some of the fractions were further separated by preparative TLC on 20% silver nitrate-impregnated silica gel plates[5] to afford 24-epibrassicasteryl acetate (**1b**), 24ξ-methylcholesteryl acetate (**2b**, impure), stigmasteryl acetate (**4b**), ergosteryl acetate (**9b**), S_4-*O*-acetate (**5b**), S_5-*O*-acetate (**6b**), S_{5B}-*O*-acetate (**7b**), and nervisteryl acetate (**8b**). Among these, **4b** and **9b** were identified by direct comparisons with corresponding authentic samples by means of the proton nuclear magnetic resonance (^{1}H-NMR) and MS methods,[6] while **2b** was found to be indistinguishable from authentic campesteryl acetate (**3b**) by GC and GC-MS analyses. However, the stereochemistry at the C-



a : R'=H, **b** : R'=Ac

Chart 1

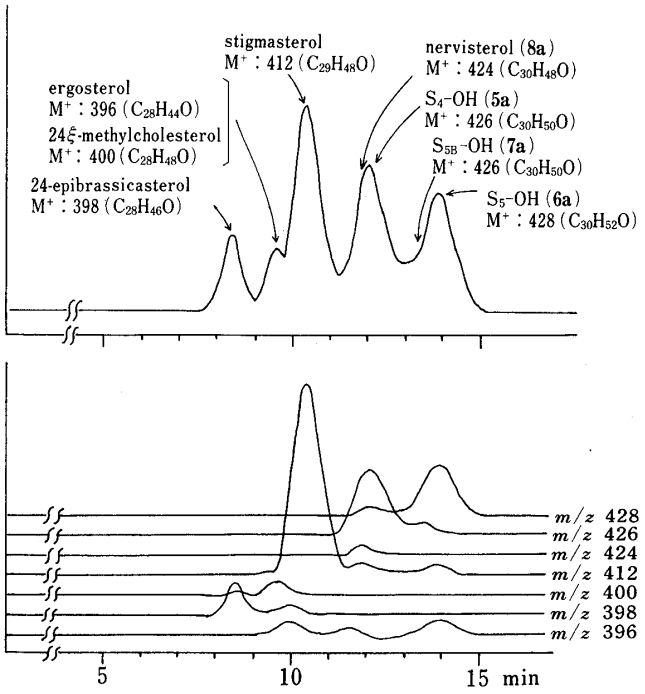


Fig. 1. Gas Chromatogram and Mass Chromatogram of the Sterol Mixture Obtained from *N. purpurea* (2% OV-17 Column)

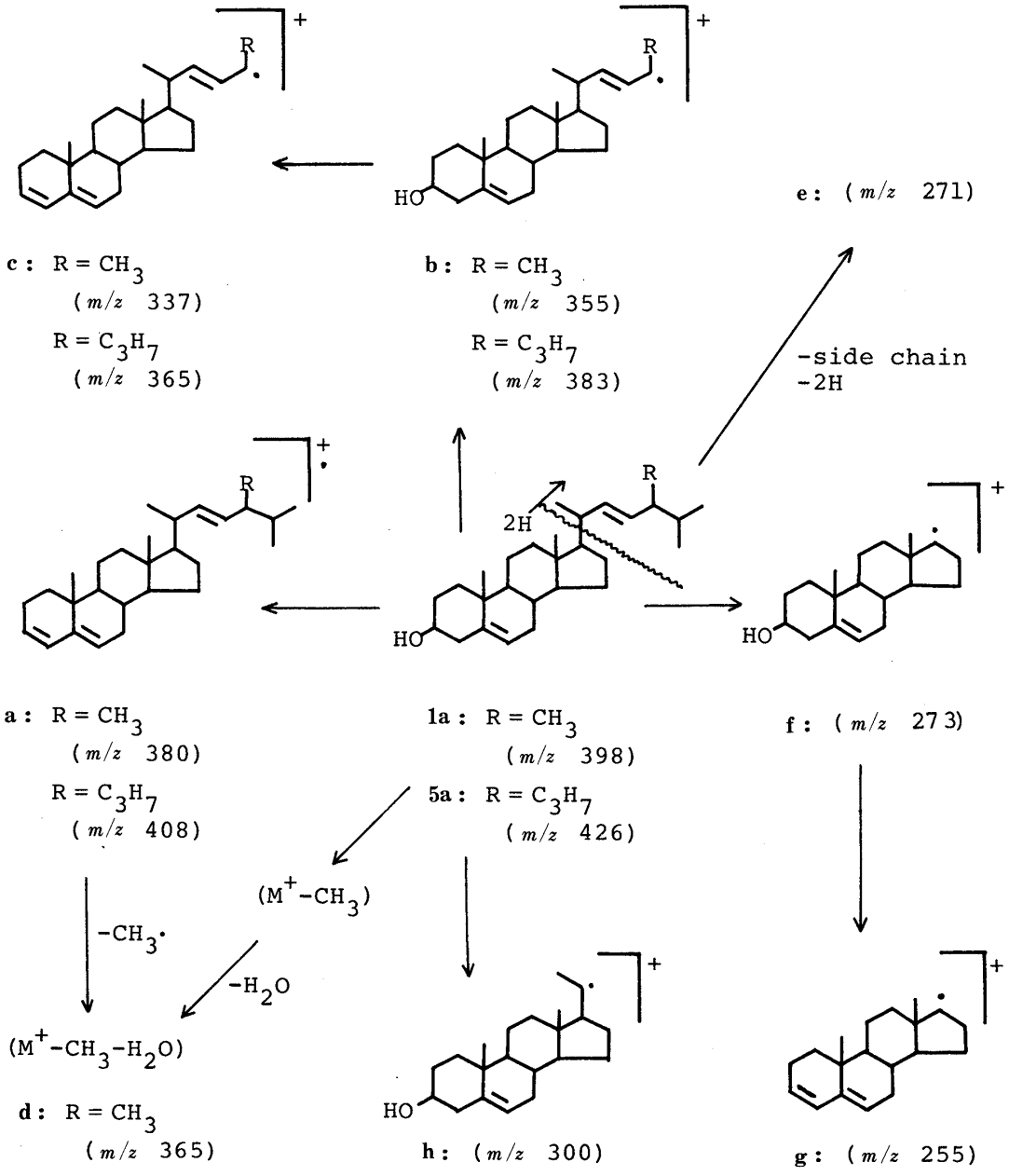
24 position in **2b** remains uncertain.

Alkaline hydrolysis of the above acetates (**1b**, **5b**, **6b**, and **7b**) gave the corresponding free sterols, **1a**, S_4-OH (**5a**), S_5-OH (**6a**), and S_{5B}-OH (**7a**), respectively. Sterol **1a**, mp 147—149 °C, showed significant fragment peaks in the MS as shown in Chart 2, and finally it was identified as 24-epibrassicasrerol (**1a**) by ^{1}H-NMR comparison with an authentic sample.[7]

S_4-OH (**5a**), mp 168—171 °C, $[\alpha]_D$ −45.7 °, and S_5-OH (**6a**), mp 134—136 °C, $[\alpha]_D$ −42 °, were determined to have the molecular formulae $C_{30}H_{50}O$ and $C_{30}H_{52}O$, respectively, by

high-resolution MS measurements. The ^{1}H-NMR spectrum of **5a** exhibited a pair of double doublets at δ 5.02 and 5.14 (J=15, 9 Hz) due to *trans*-oriented olefin protons and a broad doublet at δ 5.36 due to another olefinic proton together with signals arising from a hydroxyl-bearing methine, five secondary methyl groups, and two tertiary methyl groups, and the MS (Fig. 2a) showed significant fragment peaks at *m/z* 408 (**a**), 383 (**b**), 365 (**c**), 300 (**h**), 273 (**f**), 271 (**e**), and 255 (**g**), which could be reasonably explained by the fragmentations shown in Chart 2.[8] On the other hand, the ^{1}H-NMR spectrum of S_5-OH (**6a**) was similar to that of S_4-OH (**5a**), but it was characterized by the absence of two of the olefinic protons at δ 5.02—5.14. The MS of **6a** (Fig. 2b) exhibited noticeable signals at *m/z* 343 and 317 which could be ascribed to the fragment ions **i** and **j** (Chart 3),[9] respectively, along with peaks due to **a'**, **d'**, **f**, and **g**.

From the above findings, S_4-OH and S_5-OH were deduced to be 22-dehydro-24-isopropylcholesterol (**5a**) and 24-isopropylcholesterol (**6a**), respectively. These sterols (**5a** and



c: R = CH_3 (*m/z* 337); R = C_3H_7 (*m/z* 365)

b: R = CH_3 (*m/z* 355); R = C_3H_7 (*m/z* 383)

e: (*m/z* 271)

a: R = CH_3 (*m/z* 380); R = C_3H_7 (*m/z* 408)

1a: R = CH_3 (*m/z* 398)

5a: R = C_3H_7 (*m/z* 426)

f: (*m/z* 273)

d: R = CH_3 (*m/z* 365)

h: (*m/z* 300)

g: (*m/z* 255)

Chart 2

6a) have recently been found as constituents of an Australian sponge of the genus *Pseudaxinyssa* by Hofheinz and Oesterhelt,[10] who obtained 0.2—0.3 mg each of pure **5a** and **6a** by repeated preparative GC. Eventually, S_4-OH and S_5-OH were identified as **5a** and **6a**, respectively, by direct comparisons with an authentic sample (approximately 7 : 3 mixture) of **5a** and **6a** by means of GC and GC-MS.

S_{5B}-OH (**7a**), mp 126—128 °C, is one of the minor components of the sterol mixture and its molecular formula, $C_{30}H_{50}O$, was confirmed by high-resolution MS. The ^{1}H-NMR spectrum of **7a** exhibited new signals ascribable to a vinyl methyl (δ 1.56) and a terminal methylene group (δ 4.73 and 4.60), suggesting the presence of an isopropenyl group in **7a**, and the MS (Fig. 2c) showed fragment peaks at *m/z* 328 (**k**), 314 (**l**), and 299 (**m**), which are diagnostic of C-25 unsaturated sterols,[11] together with peaks due to **a'**, **b'**, **d'**, **e**, and **g** (Chart 3).

From these spectral data and the molecular formula, S_{5B}-OH should be 24-isopropenylcholesterol (**7a**), though the stereochemistry at the C-24 position is uncertain. This sterol has recently been obtained as the 24-epimeric mixture from a Caribbean sponge, *Verongia cauriformis*, by the combination of silver nitrate–silica gel TLC, reversed-phase high performance liquid chromatography (HPLC), and preparative GC by Djerassi and coworkers,[12] who also synthesized **7a** from fucosterol and isolated one of the C-24 epimers in an almost pure state. As shown in Table I, the ^{1}H-NMR properties of our sample (**7a**) did not

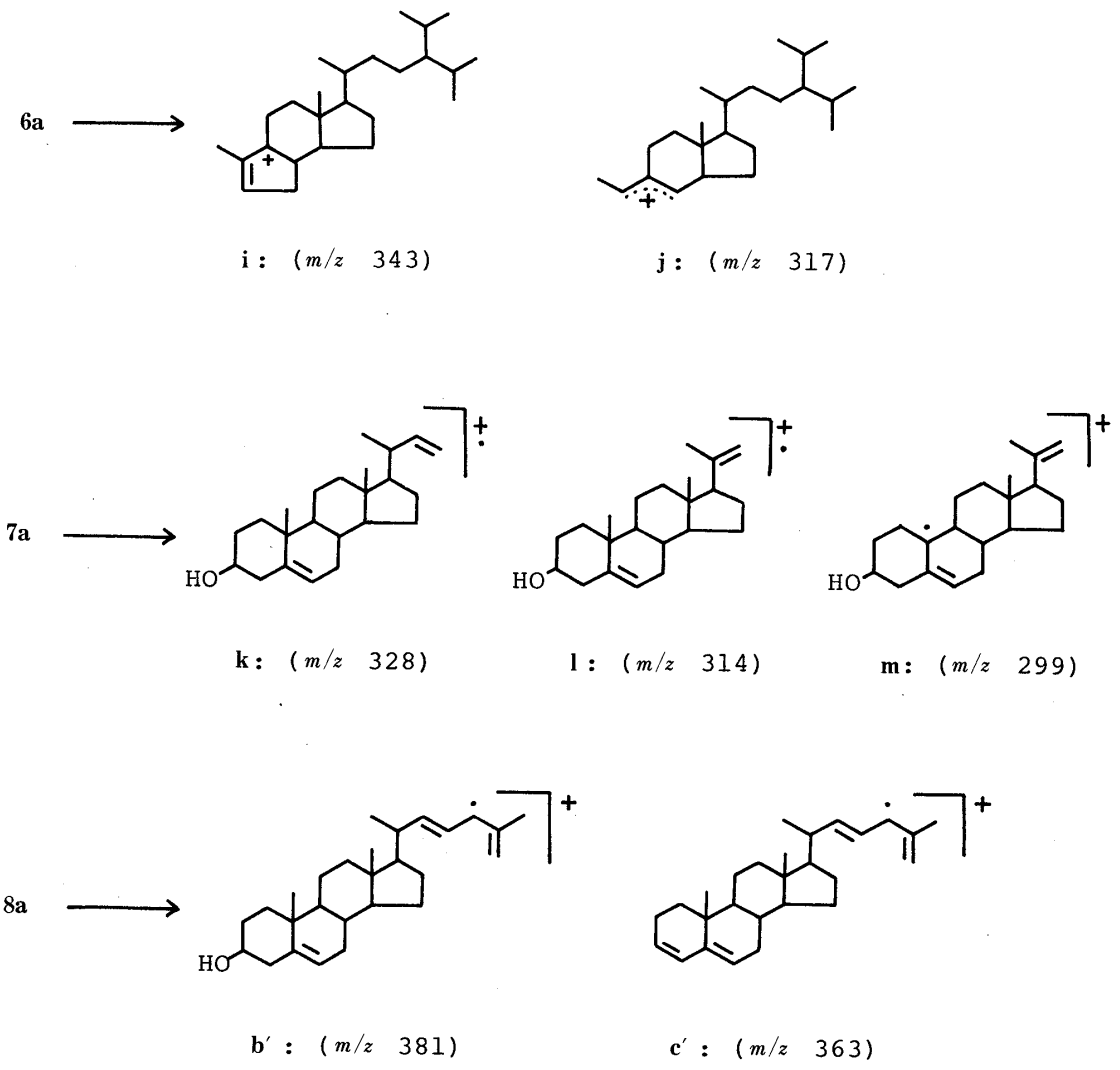


Chart 3

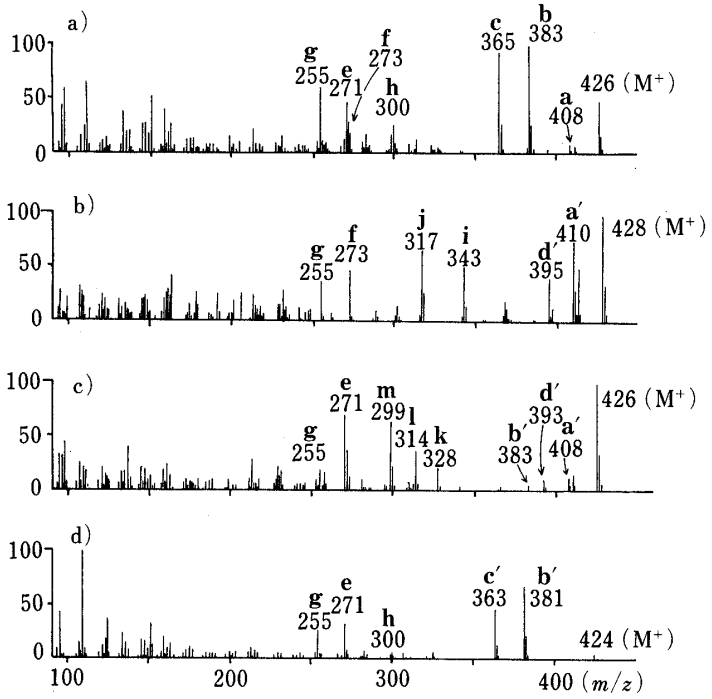


Fig. 2. Mass Spectra of Non-conventional Side Chain Sterols from *N. purpurea*

a) S_4-OH (**5a**), b) S_5-OH (**6a**), c) S_{5B}-OH (**7a**), and d) nervisterol (**8a**).
(**a′**, **b′**, **c′**, and **d′**: ions analogous to **a**, **b**, **c**, and **d**, respectively).

TABLE I. ^{1}H-NMR Spectral Data for 24ξ-Isopropenylcholesterol (**7a**) and Nervisterol (**8a**) from *Nervilia purpurea* and for Reference Compounds

Compounds	18-Me	19-Me	21-Me	26- and 27-Me	29-Me	Others
7a (24ξ)	0.666	1.006	0.911[a]	0.802,[a] 0.922[a]	1.566	3.52 (1H, m, C$\underline{H}$–OH), 4.60, 4.73 (each 1H, s, C=C$\underline{H}_2$), 5.35 (1H, br d, J=5.5 Hz, 6-H)
7a[b] (24·R+S)	0.666 (77%) 0.672 (23%)	1.006	0.908[a]	0.803,[a] 0.925[a]	1.564	
7a[c] (24ξ)	0.672	1.005	0.907[a]	0.798,[a] 0.903[a]	1.559	
8a (24ξ)	0.696	1.010	1.004	0.821, 0.835	1.652	3.65 (1H, m, C$\underline{H}$–OH), 4.70 (2H, m, C=C$\underline{H}_2$), 5.24 (2H, m, 22-H and 23-H), 5.39 (1H, br d, J=5.5 Hz, 6-H)

δ values in $CDCl_3$. *a*) Assignments may be interchanged in each compound. *b*) Natural 24-isopropenylcholesterol obtained from *Verongia cauriformis* (ref. 12). *c*) One of the C-24 epimers of synthetic isopropenylcholesterol (ref. 12).

coincide with those of Djerassi's synthetic sample, but were identical with those of the other epimer. Thus, S_{5B}-OH (**7a**) might be epimeric with Djerassi's synthetic 24ξ-isopropenylcholesterol, although direct comparison could not be performed.

Nervisteryl acetate (**8b**), mp 187—189 °C, $[\alpha]_D$ −55.2 °, showed the molecular ion peak at m/z 466 ($C_{32}H_{50}O_2$) in the MS, and its ^{1}H-NMR spectrum showed signals at δ 2.03 due to an acetyl group and δ 4.67 due to an acetoxyl-bearing methine group.

Alkaline hydrolysis of **8b** yielded nervisterol (**8a**), mp 175—177 °C, $[\alpha]_D$ −47.9 °, whose composition was proved to be $C_{30}H_{48}O$ by high-resolution MS measurement. It showed a characteristic infrared (IR) absorption due to a terminal methylene group at 895 cm^{-1} and characteristic ^{1}H-NMR signals of a vinyl methyl (δ 1.652) and an olefinic methylene group

(δ 4.70, multiplet), indicating that nervisterol also has an isopropenyl group. Furthermore, the ^{1}H-NMR spectrum showed a broad doublet at δ 5.39, typical of the olefinic proton of Δ^5-sterols,[13] and a multiplet at δ 5.24 due to two olefinic protons, along with signals arising from a hydroxyl-carrying methine, three secondary methyl groups, and two tertiary methyl groups (Table I). The MS of **8a** exhibited significant peaks assignable to the fragment ions **b'**, **c'**, **e**, **g**, and **h**, accompanied by the molecular ion peak at *m/z* 424, as shown in Fig. 2d.

On the basis of the above spectral data and the molecular formula, the structure of nervisterol was concluded to be **8a**. Confirmation was provided by the selective hydrogenation of nervisteryl acetate (**8b**) in the presence of tristriphenylphosphinerhodium chloride[14] in benzene, leading to the formation of 22-dehydro-24-isopropylcholesteryl acetate (**5b**). The identity of the product with **5b** was confirmed by direct GC, GC-MS, and ^{1}H-NMR comparisons. Thus, the structure of nervisterol was established to be 22-dehydro-24-isopropenylcholesterol (**8a**) except for the configuration at the C-24 position. The stereochemistry of **8a** and **7a** is under investigation.

Various non-conventional side chain sterols have so far been isolated from many marine sources,[15] but our present results provide the first example of the isolation of unusual side chain sterols from a terrestrial source. The distribution of these unusual side chain sterols in terrestrial plant species and its biological implications are of particular interest.

Experimental

Melting points were determined with a Kofler-type apparatus and are uncorrected. Optical rotations were measured on a JASCO DIP-4 automatic polarimeter in chloroform solution at 22 °C. IR spectra were recorded on a JASCO IRA-2 spectrometer in KBr disc and ^{1}H-NMR spectra were taken on a Varian Associates XL-200 spectrometer in $CDCl_3$ solution with tetramethylsilane as an internal standard; chemical shifts are recorded in δ values. GC analyses were done on a Shimadzu GC-6A gas chromatograph using a 2 m glass column (3 mm i.d.) packed with 2% OV-17 on Gas-Chrom Q at a column temperature of 280 °C. Nitrogen was employed as a carrier gas at a flow rate of 40 ml/min. MS measurements were done on a JEOL D-300 mass spectrometer using a direct inlet system or a GC injection system. GC-MS operating conditions were as follows: column, 2% OV-17 on Gas-Chrom Q (1 m × 2 mm i.d. glass tube); column temperature, 280 °C; injection temperature, 300 °C; ionization energy, 70 eV; accelerating voltage, 3 kV. The 20% $AgNO_3$–silica gel for column chromatography was prepared according to Ghosh's description.[4] Preparative TLC was carried out on Merck Kieselgel 60 $PF_{254+366}$ or on 20% $AgNO_3$–Kieselgel $PF_{254+366}$ plates[5] developed with ether–hexane (5 : 95); extraction of substances from the silica gel was done with ether–hexane (1 : 1) and the solutions were concentrated *in vacuo*. For drying organic solutions, anhydrous $MgSO_4$ was used.

Isolation and Properties of Sterol Acetates——Sterol mixture (96 mg),[3] obtained previously from the ether extract of dried herbs of *N. purpurea*, was treated with acetic anhydride (1 ml) and pyridine (1 ml) overnight at room temperature. Usual work-up and recrystallization from ether–MeOH yielded an acetate mixture (100 mg), which was chromatographed on 20% $AgNO_3$–silica gel (160 g) with benzene–hexane (1 : 9). The first eluate, upon recrystallization from MeOH, gave S_5-*O*-acetate (**6b**: 24-isopropylcholesteryl acetate) (3.5 mg), colorless leaves, mp 108—110 °C. MS *m/z*: 470 (M^+), 410 ($M^+ - 60$, base peak), 395, 302, 289, and 255. ^{1}H-NMR δ: 0.676 (3H, s, 18-CH_3), 0.826, 0.840 (each 3H, d, J=6.7 Hz, isopropyl), 0.862 (6H, d, J=6.7 Hz, isopropyl), 0.941 (3H, d, J=6.4 Hz, 21-CH_3), 1.018 (3H, s, 19-CH_3), 2.03 (3H, s, OAc), 4.63 (1H, m, C<u>H</u>–OAc), and 5.39 (1H, br d, J=5.5 Hz, 6-H). The second eluate (4 mg) was a mixture of 24ξ-methylcholesteryl acetate (**2b**), S_4-*O*-acetate (**5b**), and S_5-*O*-acetate (**6b**) (approximate ratio of 1 : 1 : 8 by GC analysis). **2b** was identical with campesteryl acetate (**3b**) by GC and GC-MS comparisons with an authentic sample (**3b**), but the stereochemistry at the C-24 position in **2b** was uncertain. **5b** and **6b** were identified by GC and GC-MS analyses.

The third eluate, upon recrystallization from MeOH, gave S_4-*O*-acetate (**5b**: 22-dehydro-24-isopropylcholesteryl acetate) (7.5 mg), colorless plates, mp 153—156 °C, $[\alpha]_D$ −51.3° (c=0.38). MS *m/z*: 468 (M^+), 408 ($M^+ - 60$, base peak), 365, 282, and 255. ^{1}H-NMR δ: 0.70 (3H, s, 18-CH_3), 0.77, 0.78 (each 3H, d, J=6.7 Hz, isopropyl), 0.83 (6H, d, J=6.7 Hz, isopropyl), 1.02 (3H, s, 19-CH_3), 1.03 (3H, d, J=6.7 Hz, 21-CH_3), 2.04 (3H, s, OAc), 4.62 (1H, m, C<u>H</u>–OAc), 5.02, 5.15 (each 1H, dd, J=15, 9 Hz; 22-H and 23-H), and 5.39 (1H, br d, J=5.5 Hz, 6-H). The fourth eluate, upon recrystallization from MeOH, gave colorless plates (28 mg), mp 138—142 °C, $[\alpha]_D$ −43.7° (c=0.67), identified as stigmasteryl acetate (**4b**) by GC, MS, and ^{1}H-NMR comparisons with an authentic sample (**4b**). The fifth eluate, upon recrystallization from MeOH, gave 24-epibrassicasteryl acetate (**1b**) (7 mg), colorless leaves, mp 157—158 °C, $[\alpha]_D$ −54.5° (c=0.27). MS *m/z*: 440 (M^+), 380 ($M^+ - 60$, base peak), 365, 337, 282, and 255. ^{1}H-NMR δ: 0.691 (3H,

s, 18-CH_3), 0.816, 0.835 (each 3H, d, J=6.7 Hz, isopropyl), 0.910, 1.003 (each 3H, d, J=6.8 Hz, 28- and 21-CH_3), 1.020 (3H, s, 19-CH_3), 2.03 (3H, s, OAc), 4.64 (1H, m, C$\underline{H}$–OAc), 5.20 (2H, m, 22-H and 23-H), and 5.41 (1H, br d, J=5.5 Hz, 6-H).

The sixth eluate, upon recrystallization from MeOH, gave S_{5B}-*O*-acetate (**7b**: 24ξ-isopropenylcholesteryl acetate) (1 mg). MS *m/z*: 468 (M^+), 408 (M^+-60, base peak), 393, 255, and 253. ^{1}H-NMR δ: 0.661 (3H, s, 18-CH_3), 0.801, 0.897, 0.918 (each 3H, d, J=5.8 Hz, isopropyl and 21-CH_3), 1.015 (3H, s, 19-CH_3), 1.562 (3H, s, vinyl CH_3), 2.03 (3H, s, OAc), 4.60 (1H, m, C$\underline{H}$–OAc), 4.60, 4.77 (each 1H, s, C=C$\underline{H}_2$), and 5.41 (1H, br d, J=5.5 Hz, 6-H).

The last eluate (20 mg) gave a mixture of two components and it was further separated by preparative TLC on 20% $AgNO_3$–silica gel plates with ether–hexane (5:95) as the eluent. The less polar fraction was recrystallized from ether–MeOH to give nervisteryl acetate (**8b**) (5.3 mg), colorless needles, mp 187—189 °C, $[\alpha]_D$ −55.2° (c=0.21). MS *m/z*: 466 (M^+), 406 (M^+-60), 363 (base peak), 282, 255, and 253. ^{1}H-NMR δ: 0.693 (3H, s, 18-CH_3), 0.797, 0.821 (each 3H, d, J=6.7 Hz, isopropyl), 1.004 (3H, d, J=6.7 Hz, 21-CH_3), 1.020 (3H, s, 19-CH_3), 1.652 (3H, s, vinyl CH_3), 2.03 (3H, s, OAc), 4.67 (1H, m, C$\underline{H}$–OAc), 4.70 (2H, m, C=C$\underline{H}_2$), 5.23 (2H, m, 22-H and 23-H), and 5.37 (1H, br d, J=5.5 Hz, 6-H). The more polar fraction was recrystallized from MeOH to give ergosteryl acetate (**9b**) (0.5 mg), colorless leaves, mp 169—172 °C. Its identity was confirmed by GC, MS and ^{1}H-NMR comparisons with an authentic sample.

24-Epibrassicasterol (1a)——The acetate **1b** (4 mg) was refluxed with 3% KOH–MeOH (1 ml) for 20 min and the reaction mixture was worked up as usual. Recrystallization of the product from MeOH afforded 24-epibrassicasterol (**1a**) (3.5 mg), colorless plates, mp 147—149 °C. High-resolution MS: Found 398.3585, Calcd for $C_{28}H_{46}O$ (M^+) 398.3548. The identity of this product was proved by direct GC, MS, and ^{1}H-NMR comparisons with authentic **1a**.

S_4-OH (5a: 22-Dehydro-24-isopropylcholesterol)——Alkaline hydrolysis of **5b** (4.5 mg) in the same way as above and recrystallization of the product from MeOH gave S_4-OH (**5a**) (3 mg), colorless plates, mp 168—171 °C, $[\alpha]_D$ −45.7° (c=0.28). High-resolution MS: Found 426.3878, Calcd for $C_{30}H_{50}O$ (M^+) 426.3861. ^{1}H-NMR δ: 0.700 (3H, s, 18-CH_3), 0.770, 0.778 (each 3H, d, J=6.8 Hz, isopropyl), 0.841 (6H, d, J=6.7 Hz, isopropyl), 1.013 (3H, s, 19-CH_3), 1.029 (3H, d, J=6.7 Hz, 21-CH_3), 3.52 (1H, m, C$\underline{H}$–OH), 5.02, 5.14 (each 1H, dd, J=15, 9 Hz, 22-H and 23-H), and 5.36 (1H, br d, J=5.5 Hz, 6-H). This product was identified as 22-dehydro-24-isopropylcholesterol by GC and GC-MS comparisons with authentic **5a**.

S_5-OH (6a: 24-Isopropylcholesterol)——Alkaline hydrolysis of **6b** (2.9 mg), followed by recrystallization from MeOH, gave S_5-OH (**6a**) (2.2 mg), colorless plates, mp 134—136 °C, $[\alpha]_D$ −42.0° (c=0.20). This product was identified as 24-isopropylcholesterol (**6a**).[16] High-resolution MS: Found 428.4032, Calcd for $C_{30}H_{52}O$ (M^+) 428.4018. ^{1}H-NMR δ: 0.679 (3H, s, 18-CH_3), 0.826, 0.841 (each 3H, d, J=6.7 Hz, isopropyl), 0.863 (6H, d, J=6.7 Hz, isopropyl), 0.943 (3H, d, J=6.4 Hz, 21-CH_3), 1.010 (3H, s, 19-CH_3), 3.53 (1H, m, C$\underline{H}$–OH), and 5.37 (1H, br d, J=5.5 Hz, 6-H).

S_{5B}-OH (7a: 24ξ-Isopropenylcholesterol)——The acetate **7b** (1 mg) was treated with 5% KOH–MeOH (0.5 ml), and after purification by preparative TLC, the product was recrystallized from MeOH to give S_{5B}-OH (**7a**) (0.5 mg), colorless leaves, mp 126—128 °C. High-resolution MS: Found 426.3908, Calcd for $C_{30}H_{50}O$ (M^+) 426.3861. ^{1}H-NMR: see Table I.

Nervisterol (8a)——Nervisteryl acetate (**8b**) (2.5 mg) was refluxed with 5% KOH–MeOH (1 ml) for 30 min and the reaction mixture was worked up as usual. Recrystallization of the product from MeOH gave nervisterol (**8a**) (2 mg), colorless needles, mp 175—177 °C, $[\alpha]_D$ −47.9° (c=0.19). High-resolution MS: Found 424.3695, Calcd for $C_{30}H_{48}O$ (M^+) 424.3705. IR ν cm^{-1} (KBr): 3450, 965, and 895. ^{1}H-NMR: see Table I.

Catalytic Hydrogenation of Nervisteryl Acetate (8b)——Nervisteryl acetate (**8b**) (0.8 mg) was hydrogenated in the presence of tristriphenylphosphinerhodium chloride (0.5 mg) in dry benzene (0.3 ml) for 12 h. The reaction mixture was subjected to preparative TLC with ether–hexane (5:95) as the eluent and the product was then recrystallized from MeOH to give colorless plates (**5b**) (0.5 mg), mp 147—150 °C. MS *m/z*: 468 (M^+), 408 (M^+-60, base peak), 365, 282, and 255. This product was identified as 22-dehydro-24-isopropylcholesteryl acetate (**5b**) by GC, GC-MS, and ^{1}H-NMR comparisons.

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