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EFFECTIVE SEPARATION OF STEROL C-24 EPIMERS

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SUMMARY

Separation of C-24 epimeric pairs of 24-alkyl sterols has been achieved by reversed-phase high-performance liquid chromatography. Effects of the solvent system and temperature on the separation of these epimers are emphasized. This separation technique was effectively used for the profile analysis of sterol components from *Nervilia purpurea* and *N. aragoana*, demonstrating the characterization and stereochemical determination of the C-24 position of some new sterols. The application of gas–liquid and high-performance liquid chromatography to the separation of C-24 epimers of 23-substituted 24-alkyl sterols, including demethylgorgosterol, gorgosterol and brassinosteroid isomers, is briefly described.

INTRODUCTION

Most terrestrial and marine sterols have an alkyl group at the C-24 position, and in many cases they occur as C-24 epimeric mixtures. The separation and stereochemical assignment of these epimers have important biosynthetic, taxonomic and geochemical implications. Profile analysis of natural sterols is also important in the screening of natural resources as a starting material for the synthesis of biologically active steroids.

Since gas chromatography (GC) was developed as an essential method of steroid analysis[1], the separation of the epimers of C-24 alkyl sterols has always been a challenging aspect. NMR spectroscopy can be used for the assignment of the C-24 stereochemistry[2,3], but it cannot be applied to the analysis of natural sterol mixtures.

Attempts to separate C-24 epimers of steranes[4] and sterols[5] by GC on glass capillary columns have been reported, but the tremendous length of column required and the reproduction of columns that efficiently separated C-24 epimers have not been satisfactory. More recently, high-performance liquid chromatography (HPLC) has been applied to the separation of a number of naturally occurring steroids, and

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many examples of successful applications of HPLC to the separation of natural sterols have been published[6–9].

In 1981, Djerassi and co-workers[10] reported an effective separation of a couple of epimeric pairs of C-24 alkyl sterols with a 22(23) double bond by reversed-phase HPLC on a Whatman Partisil M9 10/50 ODS-2 column. However, under these conditions the corresponding pairs having a saturated side chain could not be separated.

Facile synthetic methods for C-24 stereoisomers of 24-methyl and ethyl sterols were developed in our laboratory[11,12]. This allowed us to attempt the separation of the epimers by chromatographic methods. While our initial attempts by glass capillary GLC were unsuccessful, we have recently found satisfactory HPLC conditions for the separation of the epimeric sterol benzoates on a reversed-phase column (TSK-Gel ODS-120A) (Toyosoda, Tokyo, Japan).

EXPERIMENTAL

Samples

All standard samples were prepared in our laboratory[11,12].

The benzoates were prepared by adding 4 μl benzoyl chloride to a solution of 0.5 mg sterol mixture in 40 μl pyridine. The mixture was stirred overnight at room temperature. After addition of water, the benzoate was extracted with dichloromethane. The benzoate fraction was purified by preparative thin-layer chromatography (TLC)[13].

For the preparation of the trimethylsilyl ethers, 100 μg of a sterol were dissolved in 30 μl trimethylsilylimidazole and the mixture was allowed to stand at room temperature for 30 min. A few μl of this sample were injected into the gas chromatograph.

For the bis-methaneboronate derivatization, a solution of 100 μl methaneboronic acid in dry 50 μl pyridine was added to 100 μg brassinosteroid. The mixture was heated at 60°C for 30 min. Several μl of this solution were injected into the gas chromatograph.

Instruments

A GC-7APrF chromatograph (Shimadzu, Kyoto, Japan) with a solventless inlet system (moving-needle type) and flame ionization detector was generally used. For Fig. 8, a VGA gas chromatograph–mass spectrometer (VG Analytical, Jasco International, Tokyo, Japan) with a split/splitless injector was used.

An ALC/GPC 201D compact-type high-performance liquid chromatograph (Waters, Division of Millipore, Tokyo, Japan) with an UV detector and a Toyosoda column (TSK-Gel ODS-120A) was used.

RESULTS AND DISCUSSION

The reversed-phase HPLC separation of four epimeric pairs of standard 24-alkyl sterols (Table I, **1**–**8**) was investigated with various solvent systems. Chloroform–acetonitrile, hexane–2-propanol–acetonitrile and dichloromethane–acetonitrile systems were found to be satisfactory solvent combinations (Table I). The most satisfactory separation of the C-24 epimers was achieved with chloroform–acetonitrile

TABLE I

RELATIVE RETENTION TIMES OF BENZOATES OF 24-EPIMERIC STEROLS IN REVERSED-PHASE HPLC

The values are expressed relative to ergosterol benzoate (1.00). Conditions: column, TSK-Gel ODS-120A (250 mm × 4.6 mm I.D.); flow-rate, 0.6 ml/min; temperature, 20°C; detection, 240 nm.

24-Alkyl sterol 3-benzoates	*Relative retention time*		
	Chloroform–acetonitrile (1:4)	*Hexane–isopropanol–acetonitrile (1:3:16)*	*Dichloromethane–acetonitrile (1:4)*
Campesterol (**1**) (24*R*/α)	1.05	1.14	1.06
Dihydrobrassicasterol (**2**) (24*S*/β)	1.13	1.21	1.14
Crinosterol (**3**) (24*S*/α)	0.85	0.90	0.84
Brassicasterol (**4**) (24*R*/β)	1.01	1.04	1.02
Sitosterol (**5**) (24*R*/α)	1.05	1.22	1.08
Clionasterol (**6**) (24*S*/β)	1.08	1.24	1.11
Stigmasterol (**7**) (24*S*/α)	1.04	1.15	1.05
Poriferasterol (**8**) (24*R*/β)	1.08	1.18	1.08
Averaged retention time of ergosterol benzoate (min)	82.0	85.2	111.8

(1:4) at 20°C. The benzoates of the epimeric pairs of 24-methyl sterols, campesteorl (**1**)/dihydrobrassicasterol (**2**) and crinosterol (**3**)/brassicasterol (**4**), were completely separated, as shown in Fig. 1[14]. The benzoates of the epimeric pairs of 24-ethyl sterols, sitosterol (**5**)/clionasterol (**6**) and stigmasterol (**7**)/poriferasterol (**8**), were only partially separated. Separation of these epimers was less effective at an higher temperature. For instance, at 26°C the pairs of 24-ethyl sterols were inseparable, although the 24-methyl sterols were still separated at this temperature.

For the separation of C-24 alkyl homologues, the hexane–2-propanol–acetonitrile (1:3:16) system gave better results than the chloroform–acetonitrile system. For example, a mixture of four 24β-alkyl sterol benzoates (**2, 4, 6** and **8**) gave four peaks with hexane–2-propanol–acetonitrile, whereas only three peaks were observed with chloroform–acetonitrile, as shown in Fig. 2.

Interestingly, the 24α-epimer was eluted before the corresponding 24β-epimer in all the solvent systems examined. Thus, a comparison of HPLC retention times may be helpful in the determination of the C-24 stereochemistry of 24-alkyl sterols having other types of steroid nuclei. The effect of temperature in HPLC must be taken into account in order to achieve the best separation.

The usefulness of this separation technique is illustrated in the analyses of the sterol componetns of *Nervilia purpurea* Schlechter and *N. aragoane* Gaud, which are used as a folk medicine "I-tiam-hong" in Taiwan, shown in Figs. 3 and 4[14]. The excellent separation of C-24 alkyl sterols as well as their C-24 epimers, seen in these figures, enabled us to identify these sterols and their C-24 stereochemistry. ^{1}H NMR spectroscopy and mass spectral (MS) studies of each sterol component isolated in this way gave useful information about their structures. Needless to say, a preliminary separation of a complex sterol mixture into various fractions by argentation chroma-

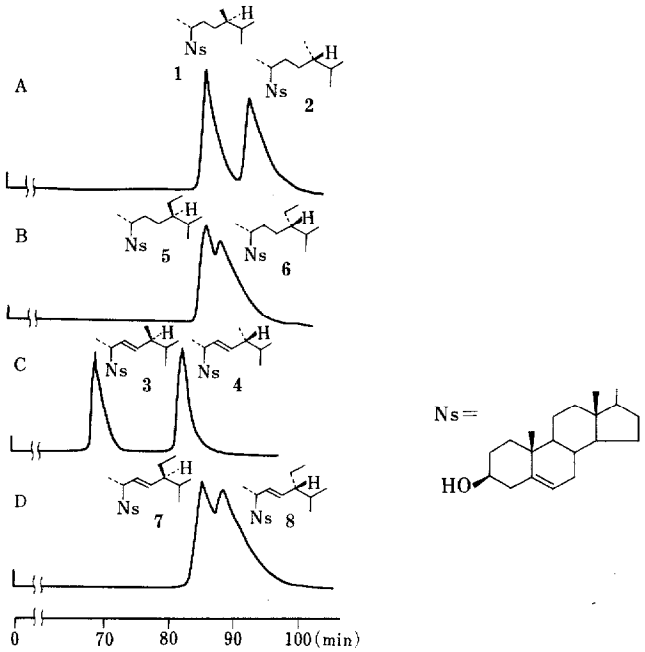

Fig. 1. HPLC separation of the benzoates of C-24 epimeric sterols. (A) Campesterol (**1**)/dihydrobrassicasterol (**2**); (B) sitosterol (**5**)/clionasterol (**6**); (C) crinosterol (**3**)/brassicasterol (**4**); (D) stigmasterol (**7**)/poriferasterol (**8**). Conditions: see Table I; eluent, chloroform–acetonitrile (1:4).

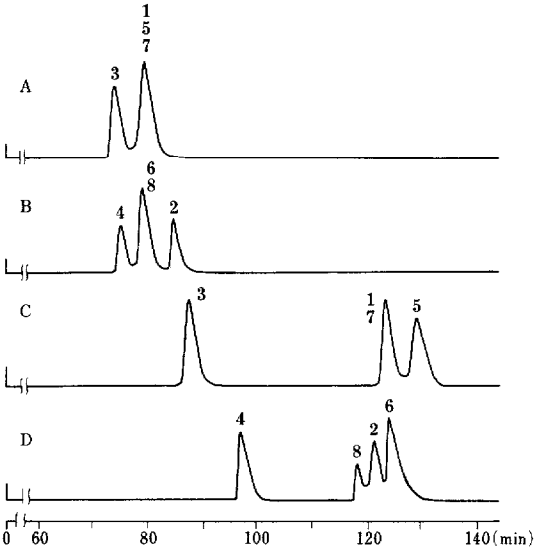

Fig. 2. HPLC separation of the benzoates of 24α- or 24β-24-alkyl sterols. (A) and (C) campesterol (**1**), crinosterol (**3**), sitosterol (**5**) and stigmasterol (**7**); (B) and (D) dihydrobrassicasterol (**2**), brassicasterol (**4**), clionasterol (**6**) and poriferasterol (**8**). Conditions: see Table I; (A) and (B), chloroform–acetonitrile (1:4); (C) and (D), hexane—2-propanol–acetonitrile (1:3:16).

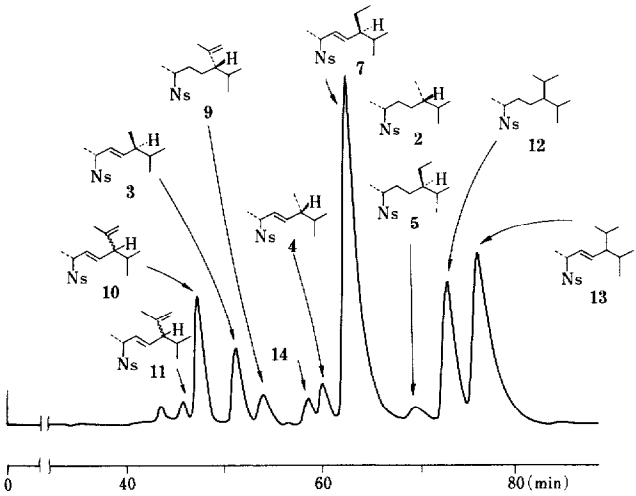

Fig. 3. HPLC separation of the benzoates of the sterol fraction of *Nervilia purpurea*. **9** = (24*S*)-24-isopropenylcholesterol; **10** = nervisterol; **11** = nervisterol isomer; **12** = 24-isopropylcholesterol; **13** = Δ^{22}-24-isopropylcholesterol; **14** = ergosterol. Conditions: see Table I; eluent, chloroform–acetonitrile (1:4).

tography and conventional capillary GLC (or GC–MS) analysis of the fractions may be recommended in certain cases, prior to the present HPLC separation.

A new sterol, 24-isopropenylcholesterol (**9**), was found in *N. purpurea*. For the determination of the C-24 configuration, both epimers were synthesized from fucosterol and separated by HPLC, using chloroform–acetonitrile (1:4) as the eluent. From a chromatographic comparison we concluded that the natural sterol is the 24α-epimer[15]. As expected, the 24α-epimer has a shorter retention time than that of the 24β-epimer. The C-24 configurations of the Δ^{22}-24-isopropenylcholesterols (**10** and **11**) are still not determined because of lack of standard samples.

A group of new 5,10-cyclopropyl-14-methylsterols (**15–20**) was also found in *N. purpurea*. In this group, epimeric pairs of 24-alkyl sterols, *e.g.*, **15/16**, occur in the

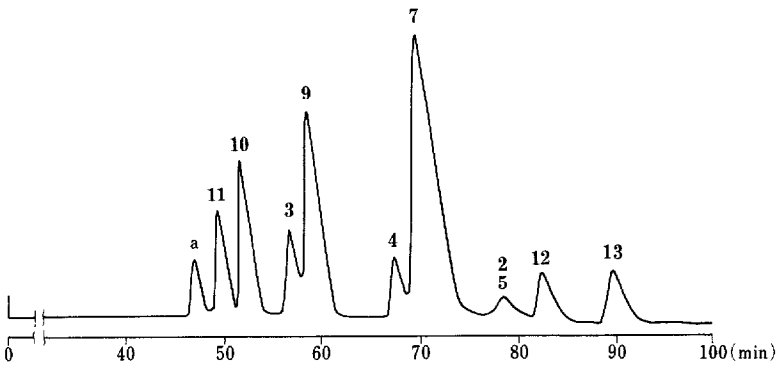

Fig. 4. HPLC separation of the benzoates of the sterol fraction of *Nervilia aragoana*. The sterol structures are shown in Fig. 3. Peak a was not identified. Conditions as in Fig. 3.

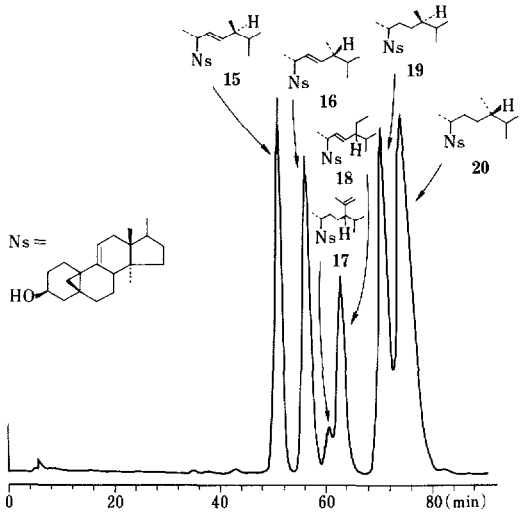

Fig. 5. HPLC separation of the 5,10-cyclopropyl-14-methylsterols from *Nervilia purpurea*. **15** = Cyclonervilasterol; **16** = 24-epicyclonervilasterol; **17** = neocyclonervilasterol; **18** = cyclohomonervilasterol; **19** = dihydrocyclonervilasterol; **20** = 24-epidihydrocyclonervilasterol. Conditions: column, Toyosoda TSK-Gel LS-410A ODS (300 mm × 7.5 mm I.D.); flow-rate, 2.0 ml/min; eluent, hexane–2–propanol–acetonitrile (1:3:16); detection, 225 nm; temperature, 20°C.

same plant. These epimers were also separated by HPLC, as shown in Fig. 5[16–18].

A series of new triterpenes, such as dihydrocycloeucalenol and dihydrocyclonervilol, which have a cyclopropane ring at the C-9/C-10 position and a 4α, 14α-

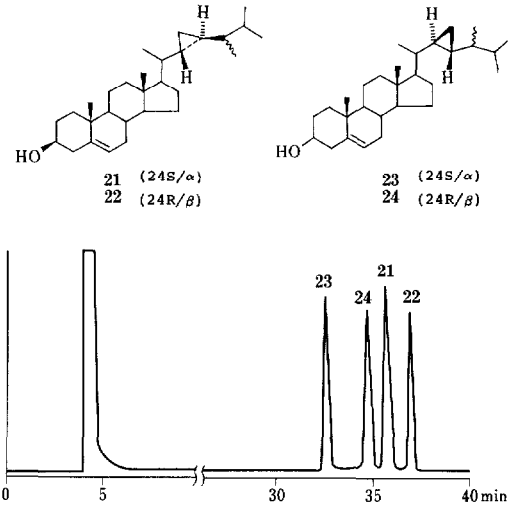

Fig. 6. GLC separation of the trimethylsilyl ethers of desmethylgorgosterol and its stereoisomers. **21** = (22*S*, 23*S*, 24*S*); **22** = (22*S*, 23*S*, 24*R*); **23** = (22*R*, 23*R*, 24*S*); **24** = (22SR, 23*R*, 24*R*) isomer. Conditions: glass capillary column, coated with OV-17 (45 m × 0.25 mm I.D.); oven temperature, 270°C

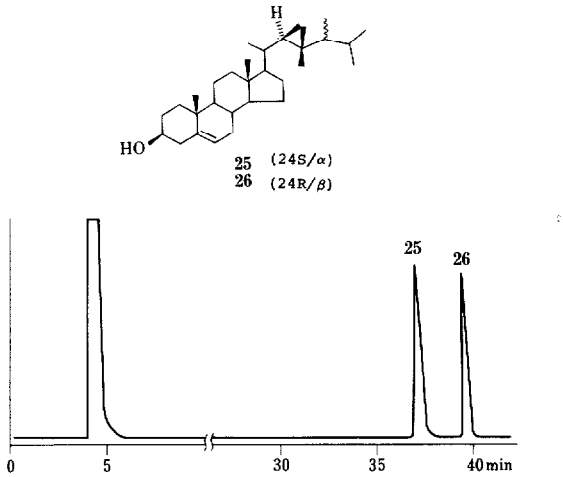

Fig. 7. GLC separation of the trimethylsilyl ethers of gorgosterol (**26**) and 24-epigorgosterol (**25**). Conditions: glass capillary column, coated with OV-101 (25 m × 0.25 mm I.D.); oven temperature, 170°C.

dimethyl group, were isolated from *N. purpurea*. These 24(*R*/α)- and 24(*S*/β)-epimers were also separated by HPLC[19].

In contrast with the aforementioned simple 24-alkyl sterols, C-24 epimers of 24-alkylsteroids which have a functional group at the C-23 position can be separated by GC. Four stereoisomers, (22*S*, 23*S*, 24*S*) (**21**), (22*S*, 23*S*, 24*R*) (**22**), (22*R*, 23*R*, 24*S*) (**23**) and (22*R*, 23*R*, 24*R*) (**24**, natural type) of 24-desmethylgorgosterol, which is a typical marine sterol, having a cyclopropane ring at the C-22/C-23 position, were synthesized in our laboratory[20,21]. These isomers were separated as their trimethylsilyl ethers by GC, using a glass capillary column, coated with OV-17, as shown in

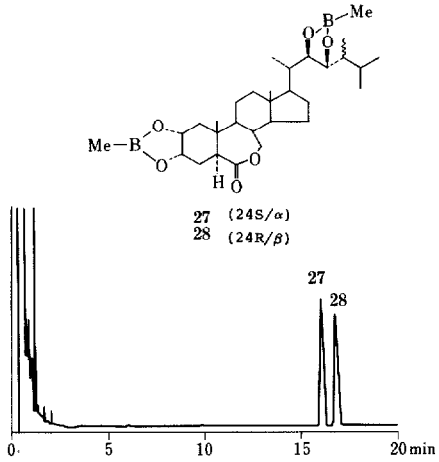

Fig. 8. HLC separation of bis-methaneboronate esters of brassinolide (**27**) and 24-epibrassinolide (**28**). Conditions: fused-silica capillary column, coated with OV-1 (12.5 m × 0.2 mm I.D.); programmed oven temperature 110–320°C (1-min hold at 110°C, then increased at 25°C/min). Me = methyl.

Fig. 6[20]. In this case, the 24(R/α)-isomer had a longer retention time than that of the 24(S/β) isomer. Gorgosterol (**26**) and its 24-epimer (**25**) were separated by the same method, the 24(S/β) epimer being eluted more rapidly than the 24(R/α)-epimer (Fig. 7) [22].

We have recently developed a new micro method for the analysis of brassinosteroids, a new series of steroidal plant growth hormones. In this case, a bis-methaneboronate derivative was used for GLC analysis[23]. Brassinolide (**27**) and 24-epibrassinolide (**28**) were separated as this derivative by a fused-silica capillary column, coated with OV-1 (Fig. 8). This method was applied to the identification of 24-epibrassinolide in the bee pollen of the broad bean, *Vicia faba* L[24].

HPLC with UV detection can be applied to the microanalysis of brassinosteroids. In this case, bis-naphthaleneboronate derivatives of brassinosteroids were employed in chromatography on a Shimadzu Shim-pack CLC-ODS (150 mm × 6.0 mm I.D.) column and 76% aqueous acetonitrile was the eluent. Unfortunately, under these conditions the derivatives of brassinolide and its 24-epimer were not separated[25].

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