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EFFECTIVE SEPARATION OF STEROL C-24 EPIMERS BY REVERSED-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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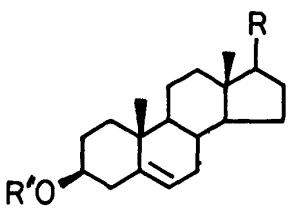
Separation of typical C-24 epimers of sterol benzoates was effectively achieved by reversed-phase HPLC under the controlled temperature. It turned out that the retention time of each 24α-epimer is appreciably shorter than that of the corresponding 24β-epimer.

KEYWORDS —— reversed-phase HPLC; C-24 epimeric sterol; campesterol; dihydrobrassicasterol; sitosterol; clionasterol; crinosterol; brassicasterol; stigmasterol; poriferasterol

Many marine and terrestrial sterols contain an alkyl group at the C-24 position, and several epimeric pairs are known. Separation and stereochemical assignment of these epimers have important biosynthetic and synthetic implications.[1)]

An attempt at gas chromatographic separation of C-24 epimers of steranes and sterols has previously been reported,[2)] but it seems rather impractical because of the very long retention time. Lately, use of high performance liquid chromatography (HPLC) using various normal phase and reversed-phase columns was developed to separate various sterol derivatives, and many examples of successful application of this technique have been reported so far.[3)] In 1982, Djerassi et al.[4)] reported the effective separation of C-24 epimers of several Δ^{22}-sterols by reversed-phase HPLC using a Whatman Partisil M9 10/50 ODS-2 column. However, the corresponding saturated compounds could not be separated even by repeated HPLC. Recently we have successfully performed the separation of a C-24 epimeric pair of methylsterols possessing a cyclopropane ring at the $C_{(5)}$-$C_{(10)}$ position and a saturated side chain by reversed-phase HPLC using a TSK-GEL LS-410A ODS column and obtained each epimer in a few milligrammes scale.[5)] In connection with this finding, we examined the separation and HPLC mobilities of typical C-24 epimeric sterols[6)] such as campesterol (1a), dihydrobrassicasterol (2a), sitosterol (3a), clionasterol (4a), crinosterol (5a), brassicasterol (6a), stigmasterol (7a), and poriferasterol (8a).

The above sterols were treated with benzoyl chloride in pyridine as usual and the resulting benzoates (1b, 2b, 3b, 4b, 5b, 6b, 7b, and 8b) were subjected to reversed-phase HPLC[7)] employing a TSK-GEL ODS-120A column with various solvent systems, among which chloroform-acetonitrile, hexane-isopropanol-acetonitrile, and methylene chloride-acetonitrile systems gave satisfactory results as shown in Table 1.



(a: R' = H, b: R' = CO-C_6H_5)

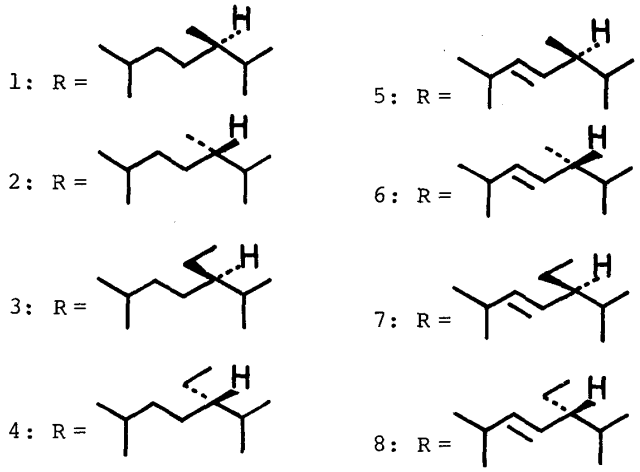


Table 1. Relative Retention Times of 24-Epimeric Sterol Benzoates on Reversed-Phase HPLC at 20°C

Condition: column, TSK-GEL ODS-120A (25cm x 4.6 mm i.d.); flow rate, 0.6 ml/min; detector setting, UV 240 nm.

Eluant / Compounds[a] (C-24 config.)	$CHCl_3$-CH_3CN (2:8)	Hexane-*i*PrOH-CH_3CN (5:15:80)	CH_2Cl_2-CH_3CN (2:8)
	Mobility[b]	Mobility[b]	Mobility[b]
1b (R/α)	1.05	1.14	1.06
2b (S/β)	1.13	1.21	1.14
3b (R/α)	1.05	1.22	1.08
4b (S/β)	1.08	1.24	1.11
5b (S/α)	0.85	0.90	0.84
6b (R/β)	1.01	1.04	1.02
7b (S/α)	1.04	1.15	1.05
8b (R/β)	1.08	1.18	1.08
	82.0[c] (min)	85.2[c] (min)	111.8[c] (min)

a) A pair of C-24 epimers was co-injected every time.

b) Retention times of epimers are relative to ergosterol benzoate, which was taken as an internal standard (1.00).

c) Averaged retention time of ergosterol benzoate.

The most satisfactory separation of C-24 epimers was observed with chloroform - acetonitrile (2:8) at 20°C, where the epimeric pairs of 24-methyl sterols (1b and 2b, 5b and 6b) were completely separated as illustrated in Fig. 1. The pairs of 24-ethyl sterols (3b and 4b, 7b and 8b) were only partially separated (Fig. 1), but the preparative separation of these epimeric pairs was possible with repeated HPLC. However, separation of the epimers became less effective gradually as the operation temperature was elevated. For instance, at 26°C the pairs of 24-ethyl sterols were inseparable, although the 24-methyl sterols could still be separated at this temperature.

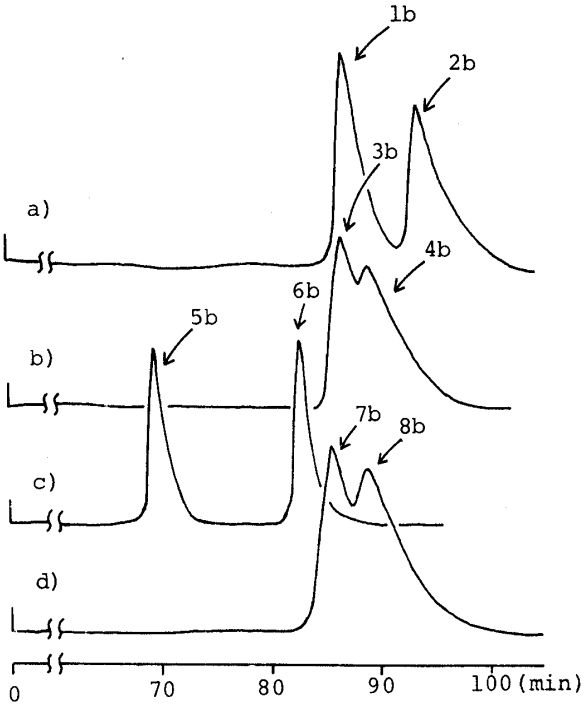


Fig. 1. HPLC Chromatogram of C-24 Epimeric Mixtures of Sterol Benzoates

a) Campesterol benzoate (1b) and dihydrobrassicasterol benzoate (2b).

b) Sitosterol benzoate (3b) and clionasterol benzoate (4b).

c) Crinosterol benzoate (5b) and brassicasterol benzoate (6b).

d) Stigmasterol benzoate (7b) and poriferasterol benzoate (8b).

Condition: the same as given in Table 1; solvent, chloroform-acetonitrile (2:8); temperature, 20°C.

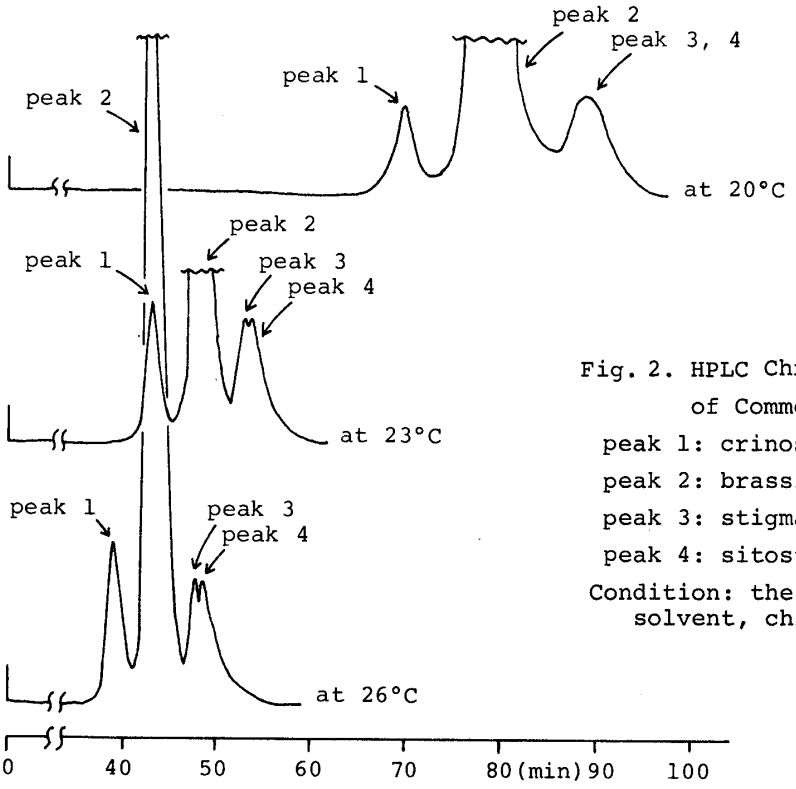


Fig. 2. HPLC Chromatogram of the Benzoate of Commercial Brassicasterol

peak 1: crinosterol benzoate (5b) (4%).

peak 2: brassicasterol benzoate (6b) (84%).

peak 3: stigmasterol benzoate (7b) (6%).

peak 4: sitosterol benzoate (3b) (6%).

Condition: the same as given in Table 1; solvent, chloroform-acetonitrile (2:8).

On the other hand, the hexane-*i*PrOH-acetonitrile system gave a better result for the separation of the pairs of homologous 24-methyl and 24-ethyl compounds (1b and 3b, 2b and 4b, 5b and 7b, 6b and 8b).[8]

It must be emphasized that the 24α-epimers (1b, 3b, 5b, and 7b) are eluted faster than the corresponding 24β-epimers (2b, 4b, 6b, and 8b) throughout the solvent systems examined (see Table 1). Thus the comparison of HPLC mobilities may be helpful for the determination of the side chain stereochemistry in 24-epimeric sterols having other types of nuclei. Here, one must be careful of the effect of the temperature during HPLC experiments in order to achieve a better separation.

Next, as an example of the application we examined the purity of commercial brassicasterol[9] (purchased from Tama Biochemical Co., Ltd.). A sample of the benzoate, derived in the usual way, showed four peaks (approximate ratio, 4 : 84 : 6 : 6) on HPLC at 26°C as shown in Fig. 2. Preparative HPLC of this benzoate on a reversed-phase column (TSK-GEL ODS-120A) using chloroform-acetonitrile (2:8) at 26°C led to the isolation of each component, and peaks 1, 2, 3, and 4 were identified as crinosterol benzoate (5b), brassicasterol benzoate (6b), stigmasterol benzoate (7b), and sitosterol benzoate (3b), respectively, by MS and HPLC comparisons with the corresponding authentic samples. It is of interest to note that in this case peak 3 (7b) and peak 4 (3b) overlapped with each other at lower temperature (Fig. 2).

Summarizing the above findings, the reversed-phase HPLC is generally accessible for the separation of C-24 epimers of sterol benzoates with a C-24 alkyl substituent, and also it may be utilized for the assignment of the C-24 stereochemistry.

REFERENCES AND NOTES

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2) J. R. Maxwell, A. S. Mackenzie, and J. K. Volkman, *Nature*, 286, 694 (1980). It takes more than 500 min to separate C-24 epimers of 24-Me and 24-Et-5α-cholestanol.
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5) S. Kadota, S. Matsuda, H. Suehara, and T. Kikuchi, *Chem. Pharm. Bull.*, 32, 1256 (1984).
6) All the sterol samples employed in this study are synthetic ones. Y. Fujimoto, M. Kimura, F. A. M. Khalifa, and N. Ikekawa, *Chem. Pharm. Bull.*, in press.
7) HPLC and preparative HPLC were performed on a Waters Associates ALC/GPC 201D compact type liquid chromatograph.
8) For instance, mixtures of (1b, 3b, 5b, and 7b) and (2b, 4b, 6b, and 8b) showed three peaks and four peaks, respectively, with this solvent system at 20°C, while they gave two peaks and three peaks, respectively, with the chloroform-acetonitrile system.
9) Co-occurrence of brassicasterol (6a) and its C-24 epimer (5a) (ca. 10-40%) in the seed oil of some *Brassica* and *Raphanus* species has been reported. See T. Matsumoto, N. Shimizu, S. Asano, and T. Itoh, *Phytochemistry*, 22, 1830 (1983).

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