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24-METHYLENE-25-METHYLCHOLESTEROL IN *PHASEOLUS VULGARIS* SEED: STRUCTURAL RELATION TO BRASSINOSTEROIDS*

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Key Word Index—*Phaseolus vulgaris*; Leguminosae; seed; sterol; brassinosteroid; 24-methylene-25-methylcholesterol; 24-ethyldesmosterol; clerosterol.

Abstract—24-Methylene-25-methylcholesterol, 24-ethyldesmosterol and clerosterol in addition to several common phytosterols were identified in immature seed of *Phaseolus vulgaris*. 24-Methylene-25-methylcholesterol is considered closely correlated biogenetically with 25-methyldolichosterone, a brassinosteroid recently isolated from the same plant material, because both of them have the same basic side-chain structure.

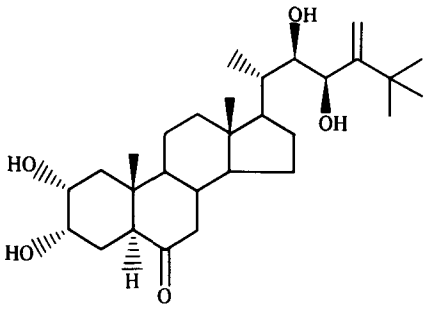
INTRODUCTION

Immature seed of *Phaseolus vulgaris* cv. Kentucky Wonder has recently been demonstrated to contain 25-methyldolichosterone (**1**) as one of the major brassinosteroids [1]. The structure of this brassinosteroid is unusual because it contains a tertiary butyl moiety in the side chain. We have also isolated 24-methylene-25-methylcholest-5-en-3β-ol (**3c**, 24-methylene-25-methylcholesterol) [2] and 24-methylene-25-methyl-5α-cholest-7-en-3β-ol (**4c**, 24-methylene-25-methyllathosterol) [3] from other higher plant sources, both of which carry a tertiary butyl moiety in the side chain. This prompted us to investigate the sterol constituents of the immature seed of *P. vulgaris* in order to examine the presence of sterols possessing a tertiary butyl group in the side chain. This paper describes the isolation and identification of **3c** and two other uncommon sterols, 24-ethylcholesta-5,24(25)-dien-3β-ol (**3g**, 24-ethyldesmosterol) and 24β(*S*)-ethylcholesta-5,25-dien-3β-ol (**3h**, clerosterol), besides several common phytosterols, in the immature seed of *P. vulgaris*. In relation to the sterol constituents, some aspects of biogenesis of brassinosteroids are discussed.

*Brassinosteroids in *Phaseolus vulgaris* Part IV. For Part III, see ref. [1].

RESULTS AND DISCUSSION

The sterol fraction obtained from the extract of the *P. vulgaris* seed was acetylated, and a portion (600 mg) of the steryl acetate was fractionated by argentation TLC into five fractions (referred to as fractions 1–5 in the order of mobility). Fraction 1 (R_f 0.64–0.77, 333 mg) was a mixture of two components which was then subjected to reverse-phase HPLC to give fractions 1A and 1B. Fraction 1A (mp 143–144°) was a 57:43 mixture of 24α-methylcholesterol (24α-**3a**, campesterol) acetate and 24β-methylcholesterol (24β-**3a**, 22-dihydrobrassicasterol) acetate. Fraction 1B was pure 24α-ethylcholesterol (**3d**, sitosterol) acetate (mp 122–123°). Fraction 2 (R_f 0.37–0.64, 119 mg) was 24α-ethyl-22*E*-dehydrocholesterol (**3e**, stigmasterol) acetate (mp 140–142°). Fraction 3 (R_f 0.25–0.37, 13 mg), on further purification by HPLC, afforded **3g**-acetate (mp 137–139°). Fraction 4 (R_f 0.10–0.25, 34 mg) was further fractionated by HPLC, giving fractions 4A and 4B Fraction 4A was **3h**-acetate (mp 128–129°) and fraction 4B (mp 137–139°) was 24*Z*-ethylidenecholesterol (24*Z*-**3f**, isofucosterol) acetate accompanied by small amounts of the acetates of 24*E*-ethylidenecholesterol (24*E*-**3f**, fucosterol) and 24*Z*-ethylidenelathosterol (24*Z*-**4f**, avenasterol). Fraction 5 (R_f 0.02–0.10, 11 mg) was subjected to HPLC which gave fractions 5A and 5B. Fraction 5A (mp 131–134°) was a mixture (*ca* 8:2) of the acetates of 24-



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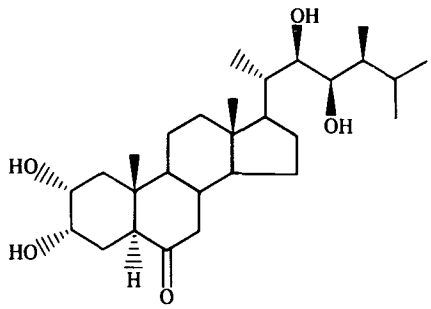

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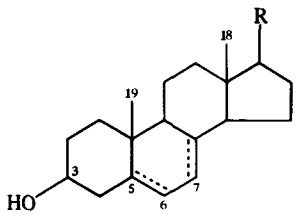
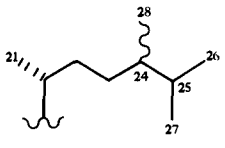
Side chains (R)



3 Δ^5

4 Δ^7, 5α-H



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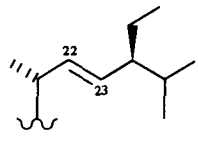

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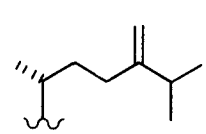

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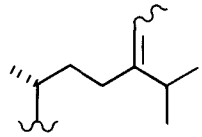

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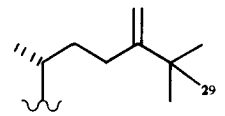

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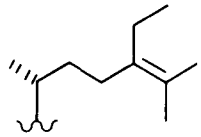

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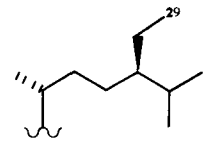

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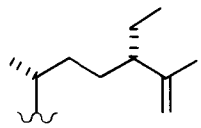

h

methylenecholesterol (**3b**) and 24-methylenelathosterol (**4b**), whereas fraction 5B was **3c**-acetate (mp 151–152°).

Identification of the above sterols was performed on the basis of argentation TLC, GC, MS and ^{1}H NMR data with the exception of 24*Z*-**4f** which was identified tentatively by argentation TLC and GC. The composition of *P. vulgaris* sterols was determined on the basis of argentation TLC, GC and ^{1}H NMR data as follows: 24α-**3a** (2.9%; relative RR_ts of the acetyl derivative to cholesteryl acetate on GC and HPLC are 1.31 and 1.14, respectively), 24β-**3a** (2.2%; 1.31, 1.14), **3b** (0.8%; 1.35, 0.82), **3c** (2.6%; 1.68, 0.96), **3d** (55.9%; 1.63, 1.26), **3e** (23.6%; 1.43, 1.06), 24*E*-**3f** (0.9%; 1.72, 1.01), 24*Z*-**3f** (8.9%; 1.81, 1.01), **3g** (1.1%; 1.95, 1.00), **3h** (0.7%; 1.63, 0.92), **4b** (0.2%; 1.61, 0.82) and 24*Z*-**4f** (0.3%; 2.15, 1.01). The 400 MHz ^{1}H NMR data of a mixture of **3b**- and **4b**-acetates, and the acetates of **3c**, **3g** and **3h** are shown in Table 1.

Thus, this study has demonstrated the presence of **3c**, **3g** and **3h**, as the minor sterol constituents as well as several common phytosterols, in the immature seed of *P. vulgaris*. The occurrence of sterol **3c** has hitherto been known only in some Cruciferae [2] and Cucurbitaceae [4], and its Δ^7-isomer (**4c**) occurs in *Sicyos angulatus* (Cucurbitaceae) [3]. Although the occurrence of **3g** in some Solanaceae [5, 6] and olive oil [7] has been reported, the present work seems to be the first instance for its unequivocal identification through high-resolution ^{1}H NMR. The occurrence of 24β-ethylsterol in higher-plants is known to be restricted, and only some plants from the Verbenaceae [8], Crassulaceae [9] and Cucurbitaceae [4, 10] have so far been reported to contain a 24β-ethylsterol **3h**.

The occurrence of 24-methylene-25-methylcholesterol (**3c**) in the immature seed of *Phaseolus* is interesting in relation to the biogenesis of brassinosteroids, because 25-methyldolichosterone (**1**), one of the major brassinosteroids in the seed, has the basic side-chain structure identical with that of **3c**. In addition to 25-methyldolichosterone, castasterone (**2**) has been found to be also one of the major brassinosteroids [11]. The occurrence of the basic sterol corresponding to castasterone, namely, cam-

Table 1. ^{1}H NMR chemical shifts ($CDCl_3$; 400 MHz)* of some sterols isolated from *Phaseolus vulgaris* seeds

Acetate	18-H_3 (*s*)	19-H_3 (*s*)	21-H_3 (*d*)	26-H_3	27-H_2 or 27-H_3	28-H_2	29-H_3 (*t*)	3β-OAc (*s*)	3α-H (*m*)	6-H or 7-H (*m*)
3b†	0.683	1.020	0.955(6.3)	1.023(*d*, 6.7) 1.029(*d*, 6.6)		4.657(*d*, 1.4) 4.713(*s*)	—	2.031	4.60(26)	5.38(10)
4b†	0.538	0.812	0.955(6.3)	1.023(*d*, 6.7) 1.029(*d*, 6.6)		4.657(*d*, 1.4) 4.713(*s*)	—	2.026	4.60(26)	5.15(11)
3c	0.688	1.021	0.964(6.6)		1.057(*s*)	4.660(*d*, 1.1) 4.833(*d*, 0.8)	1.057(*s*)	2.032	4.60(25)	5.38(10)
3g	0.682	1.020	0.969(6.6)		1.627(*s*) 1.633(*s*)	—	0.932(7.6)	2.032	4.60(25)	5.37(10)
3h	0.669	1.016	0.905(6.6)	1.567(*s*)	4.640(*d*, 2.8) 4.727(*s*)	—	0.801(7.4)	2.030	4.60(25)	5.37(10)

*Chemical shifts given in δ values from TMS; figures in parentheses denote coupling constants (*J* values) as for doublet and triplet signals, whereas half-width ($W_{1/2}$) values as for multiplet signals.

†Determined as a mixture (fraction 5A, see Text) of **3b**- and **4b**-acetates.

pesterol (24α-**3a**) was also demonstrated in this work. These findings suggest that there should be a close relationship in the biogenesis between sterols and brassinosteroids [12]. Major sterols in *Phaseolus* seed are those carrying a 24α-ethyl group (sitosterol and stigmasterol) and an ethylidene group (isofucosterol) which together account for 88% of the total sterol ammount. 24α-Ethyl- or 24-ethylidene-carrying brassinosteroids were also found in the same seed [13], but their contents were quite low (unpublished data). This fact indicates that, in *Phaseolus* seed, oxidation reactions leading to brassinosteroids are fairly selective for 24-methylsterols and 24-methylenesterols rather than 24-ethylsterols and 24-ethylidenesterols. This is in contrast to the case of a green alga, *Hydrodictyon reticulatum*, in which both the major sterol and brassinosteroid are of the 24α-ethyl type [14].

EXPERIMENTAL

Mps: uncorr. Argentation TLC (silica gel–$AgNO_3$, 4:1) was developed × 4 with CCl_4–CH_2Cl_2 (5:1). HPLC was carried out on an Altex Ultrasphere ODS column (Beckman; 5 μ; 25 cm × 10 mm i.d.) with MeOH as a mobile phase (flow rate, 4 ml/min) which was monitored by an RI detector. GC on OV-17 SCOT glass capillary column (30 m × 0.3 mm i.d.) was performed at the column temp. 260°. EIMS (70 eV) were recorded by means of probe injection. ^{1}H NMR spectra (400 MHz) were determined in $CDCl_3$ with TMS as int. standard. Acetylation was performed in Ac_2O–pyridine at room temp. overnight. Origin and extraction of the immature seed of *Phaseolus vulgaris* cv. Kentucky Wonder was described previously [1] and the sterol fraction was obtained according to ref. [14]. The following sterols; a mixture of 24α- and 24β-**3a**, **3b**, **3d**, **3e**, 24*E*-**3f**, 24*Z*-**3f**, 24*Z*-**4f** and **3h** [10], **3c** [2, 4] and **3g** [6], were used as the ref. specimens. The MS data of the acetates of **3c**, **3g** and **3h** isolated from *P. vulgaris* seed in this study are as follows

24-Methylene-25-methylcholesterol (**3c**) *acetate*. MS: *m/z* 394.3569 ($M^+ - HOAc$, $C_{29}H_{46}$, rel. int. 100%), 379.3334 ($C_{28}H_{43}$, 10%), 296.2422 ($C_{22}H_{32}$, 76%), 281.2255 ($C_{21}H_{29}$, 16%) 253.1925 ($C_{19}H_{25}$, 19%), 228.1915 ($C_{17}H_{24}$, 10%), 213.1679 ($C_{10}H_{21}$, 11%), 211.1502 ($C_{16}H_{19}$, 6%).

24-Ethyldesmosterol (**3g**) *acetate*. MS: *m/z* (rel. int.): 454 $[M]^+$ (0.3), 439 (0.4), 394 (52), 379 (5), 296 (100), 281 (19), 253 (15), 228 (11), 213 (12).

Clerosterol (**3h**) *acetate*. MS: *m/z* (rel. int.): 454 $[M]^+$ (5), 439 (3), 394 (100), 379 (9), 313 (3), 310 (2), 296 (4), 281 (5), 273 (4), 255 (8), 253 (12), 228 (10), 213 (15).

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