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24-METHYLENEPOLLINASTANOL AND 24-DEHYDROPOLLINASTANOL—TWO STEROLS OF POLLEN

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Key Word Index—Labiatae; Cruciferae; mixed pollens; phytosterols; 24-methylenepollinastanol; 24-dehydropollinastanol; 31-norcycloartenol.

Abstract—The esterified and unesterified sterol fractions of bee-gathered mixed pollens were examined, and total sterol composition was determined. Two new sterols of pollens, 14α-methyl-9β,19-cyclo-5α-cholest-24-en-3β-ol (24-dehydropollinastanol) and 14α-methyl-5α-ergost-24(28)-en-3β-ol (24-methylenepollinastanol) were isolated and identified. Both sterols were found primarily in the esterified sterol fraction, and 24-methylenepollinastanol accounted for 43% of the sterols of this fraction. 24-Dehydropollinastanol and four other sterols which also contain a 9β,19-cyclopropane ring were found only in the esterified sterol fraction. 24-Methylenecholesterol was the major sterol of the unesterified sterol fraction.

INTRODUCTION

24-Methylenecholesterol is often the principal sterol in pollen from a number of species of plants [1–5]. It is the major sterol of pollens examined from various species of plants belonging to the Gramineae, Leguminosae, Cactaceae, Cruciferae, Rosaceae and Salicaceae [6]. The first new and unusual sterol to be isolated from a pollen was identified as 14α-methyl-9β,19-cyclo-5α-cholestan-3β-ol (**5b**) and appropriately given the trivial name of pollinastanol [7]. Although a large number of sterols have been isolated from pollen, it still remains a source for new and unusual sterols.

During our efforts to isolate and identify chemicals that attract and elicit pollen collection by foraging honey bees, *Apis mellifera*, we isolated and identified the unesterified and esterified sterols from bee-collected pollen from Spain. Although the sample consisted of a mixture of pollens the principal free sterol was 24-methylenecholesterol.

This paper reports the isolation and identification of two new sterols from pollen, 24-methylenepollinastanol (**1b**) and 24-dehydropollinastanol (**2b**), and also 31-norcycloartenol (**3b**). Their possible role in sterol biosynthesis is discussed.

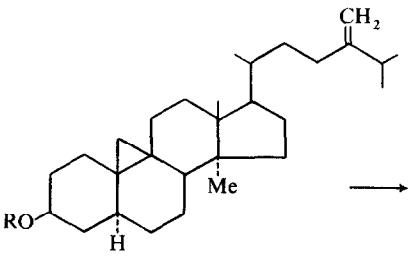
RESULTS AND DISCUSSION

From 300 gm of the mixed pollens we obtained approximately 1.5 and 0.5 g of sterol acetates from the esterified and unesterified sterol fractions, respectively. The relative quantities of sterol acetates from the esterified sterol fraction, as eluted from the $AgNO_3$-Unisil column in order of their increasing polarity, were: 1.9% pollinastanol (**5a**), trace amounts (<0.5%) of 31-norcycloartanol (**6a**) and 24-methylpollinastanol (**4a**), 4.6% monoene Δ^5-sterols, 1.4% 31-norcycloartenol (**3a**), 5.9% 24-dehydropollinastanol (**2a**), 43% 24-methylenepollinastanol (**1a**), and 43% 24-methylenecholesterol. Further analyses of the monoene Δ^5-sterol acetates by GLC and GC–MS showed that the mixture consisted of the following sterol acetates: 9% cholesterol, 35% campesterol, 6% stigmasterol and 38% sitosterol. This fraction still contained about 6% pollinastanol acetate and 6% of a compound that appeared, from MS data, to be a C_{29} Δ^7-sterol acetate. Interestingly, all of the sterols containing the 9β,19-cyclopropane ring except for 24-methylenepollinastanol were found only in the esterified sterol fraction.

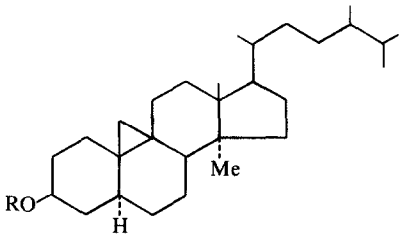
The relative percentages of the acetates of the sterols from the unesterified sterol fraction were: 18% monoene Δ^5-sterols, 9% isofucosterol, 9% 24-methylenepollinastanol, and 64% 24-methylenecholesterol. The 18% monoene Δ^5-sterol acetates consisted of 3% cholesterol acetate, 22% campesterol acetate, 2% stigmasterol acetate, 68% sitosterol acetate and 3% of a C_{29} Δ^7-sterol acetate.

This is the first isolation and identification of 24-dehydropollinastanol (**2b**) and 24-methylenepollinastanol (**1b**) from pollen, and to the best of our knowledge this is the first isolation of **2b** from any source. 24-Methylenepollinastanol was previously identified in triparanol-treated cells of *Chlorella emersonii* [8], in *Astasia longa* [9] and also in the esterified and tightly-bound sterol fraction from peel of banana [*Musa paradisiaca* (= *sapientum*)] [10] and, as in the banana peel, it was present predominantly in the esterified sterol fraction.

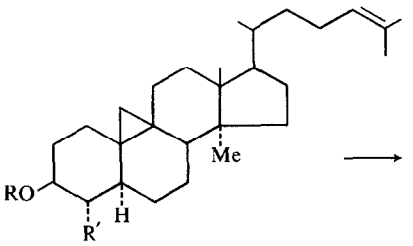
The structures of **2b** and **3b** and their acetates **2a** and **3a**, respectively, were accordingly established. The acetates of both compounds gave only slightly different R_fs on a $AgNO_3$-Si gel plate though separable by column chromatography. Their IR and NMR spectra confirmed



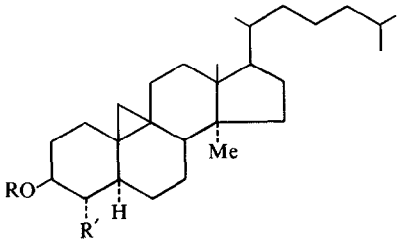
1a R = Ac
1b R = H



4a R = Ac
4b R = H



2a R = Ac; R′ = H
2b R = H; R′ = H
3a R = Ac; R′ = Me
3b R = H; R′ = Me



5a R = Ac; R′ = H
5b R = H; R′ = H
6a R = Ac; R′ = Me
6b R = H; R′ = Me

the presence of a cyclopropane ring and their NMR spectra also indicated that both contained a Δ^{24}-bond [11]. The MS of the faster and slower moving sterol acetates exhibited molecular ions at *m/e* 454 and 440, respectively and base peaks at *m/e* 69 and other similar fragmentation patterns. These results suggested that the compounds differed from each other by one carbon atom and placed the additional carbon on the sterol nucleus for the more apolar acetate with a MW of 454, preferably on ring A and at C-4 and has the structure of 31-norcycloartenol acetate (**3a**). Its slightly faster TLC mobility and the separation of the two doublets of the two hydrogens of the cyclopropane ring in the NMR spectra by only 22 Hz compared with 28 Hz for the other 9,19 cyclo sterols without a substituent at C-4 further supported this assignment. Its physical properties also agreed with the reported values for the acetate of 31-norcycloartenol isolated from the pollen from cactus *Carnegiea gigantea.* [12]. Further, the conversion by hydrogenation of sterol **3b** to a compound that agrees by TLC, GLC [13], mp and MS data [14], to that of 31-norcycloartanol (**6b**) confirms the structures of **3a** and **3b**.

Similarly the results indicated that the slightly more polar sterol acetate with a molecular ion at *m/e* 440 had the structure of a 24-dehydropollinastanol acetate (**2a**) and the free sterol that of **2b**. The conversion by hydrogenation of the free sterol **2b** to pollinastanol (**5b**) confirms the structures of **2a** and **2b**.

The major bee-collected mixed pollen was from the plant family Labiatae and represents about 48% of the pollen mixture. Since such a relatively large quantity of 24-methylenepollinastanol was isolated from the mixed pollens, it is likely that 24-methylenepollinastanol is derived from the pollen from this plant family.

All of the sterols detected can be fitted into the same biosynthetic scheme. The conversion of pollinastanol to cholesterol by *Nicotiana tabacum* has been demonstrated [15] and the finding of cycloartenol and 31-norcycloartanol in pollen from *Taraxacum officinale* has led to the conclusion that these compounds and pollinastanol are intermediates in the conversion of cycloartenol to cholesterol [14]. The 24-methylenepollinastanol may act as a precursor of C_{28} and C_{29} sterols in pollen as suggested for sterol formation in *Chlorella emersonii* [8]. The 24-dehydropollinastanol could be simply the C-31 demethylation product of 31-norcycloartenol and, as such, is an intermediate in the conversion of cycloartenol to pollinastanol, namely: cycloartenol → 31-norcycloartenol → 24-dehydropollinastanol → pollinastanol. Since the biosynthesis of typical plant sterols requires the introduction of an alkyl group into the side-chain and Δ^{24}-sterols serve as precursors, it could be a precursor for 24-methylpollinastanol and other C-24 methyl sterols. It is possible that 24-dehydropollinastanol serves as an intermediate in both pathways.

At present there is little available knowledge of the physiological functions or role of sterols in pollen. Our investigation of the sterols in pollen are a result of our interest in the role of pollen sterols in honey bee nutrition. Even though insects generally require dietary sterols for growth and development, such a requirement has not yet definitely been demonstrated for the honey bee. Pollens are certainly a rich source of sterols, and all bees feed almost exclusively on pollen and nectar. Comparative chemical and biochemical studies on the composition of the sterols of a single pollen source on which honey bees can be reared and on both the esterified and unesterified sterols of various developmental stages of bees reared on the single pollen source are necessary for determining the role of pollen sterols in relation to sterol requirement for bees. The present study has resulted in the identification of new sterols from pollen and adds to

the compendium of pollen sterols. This will certainly enhance the identification and the determination of the role of sterols in bees in which they may be present in minute quantities. Such information may also serve in determining what role, if any, pollen sterols play in the process of pollination and plant reproduction.

EXPERIMENTAL

Mps were taken on a Kofler block and are corr. Specific optical rotations were determined in *ca* 1% soln in $CHCl_3$ at 23°. NMR spectra were recorded at 60 MHz with TMS as an int. stand. in $CDCl_3$. For MS the compounds were introduced directly into the ion chamber or into the ion chamber through the GLC column; the GLC system used was 0.75% SE-30. The ionization energy was 70 eV. GLC analyses at 235° were made on a gas-chromatograph equipped with an Ar ionization detector and Ar as carrier with cholesterol or cholesterol acetate as an int. stand. [13]. With free sterols and sterol acetates the RR_t is relative to cholesterol and cholesterol acetates, respectively. Column packings were prepared from 100 to 120 mesh Gas Chrom P according to ref. [16] and the stationary phase used was 0.75% SE-30.

Pollens. The rich spring bee-collection of pollens from natural habitat in Spain included pollens from at least 13 plant families. The major pollens (estimated % by vol.) were from the following plant families: 48% Labiatae, 16% Cruciferae, 10% Leguminosae (mainly, *Trifolium*), 7% Geraniaceae (*Erodium*), 5% Compositae Tubuliflorae and 2% Ranunculaceae.

Pollen extraction and separation of esterified and unesterified sterols. The mixed pollens (300 g) were mechanically stirred and extracted at room temp. with $CHCl_3$–MeOH, then with 50% aq. MeOH. The residue from the combined extracts was partitioned between $CHCl_3$ and H_2O and from the $CHCl_3$ phase 24 g of hexane soluble residue was obtained. Chromatography of the residue over Woelm neutral Al_2O_3 activity grade II using the following solvents, hexane, hexane–C_6H_6(1:1), C_6H_6, C_6H_6–Et_2O(3:1) and Et_2O gave hydrocarbons, sterol esters and waxes, triglycerides, alcohols and sterols respectively. The sterol ester and wax fraction was saponified and the esterified sterols were similarly isolated. The esterified and unesterified sterols were separately acetylated with Ac_2O in C_5H_5N.

Separation and identification of sterol acetates. The steryl acetates derived from the ester fraction were chromatographed over a 20% $AgNO_3$-Unisil* column (1.9 × 36 cm), and the column was eluted with 50 ml hexane followed by 100 ml vol. (50 ml vol. collected) containing 1, 2, 3, 4, 5, 6, 7, 8 and 9% Et_2O in hexane. The fractions were monitored by GLC and TLC ($AgNO_3$-Si gel) and showed the following distribution of sterol acetates: hexane–Et_2O (97:3) (1st portion) pollinastanol, 24-methylpollinastanol, 31-norcycloartanol, monoene Δ^5-sterols; hexane–Et_2O (97:3) (2nd portion) monoene Δ^5-sterols, 31-norcycloartenol, 24-dehydropollinastanol; hexane–Et_2O (24:1), hexane–Et_2O (19:1) (1st portion) 24-methylenepollinastanol; hexane–Et_2O (19:1) (2nd portion) hexane–Et_2O (47:3–91:9) 24-methylenecholesterol. The pure steryl acetates were obtained by rechromatography. The unesterified sterol acetates were similarly separated and purified. Recrystallization of the combined 24-methylenecholesterol acetate (605 mg) from Me_2CO–MeOH gave 590 mg of pure acetate mp 133–134°, $[\alpha]_D^{23}$ −47°; lit. [17] mp 136°, $[\alpha]_D^{22}$ −42°. Recrystallization of 24-methylenepollinastanol acetate from Me_2CO–MeOH gave 575 mg of **1a**, mp 52–52.5°, $[\alpha]_D^{23}$ +28°, IR $\nu_{max}^{CS_2}$ cm^{-1} 3040 (cyclopropane), 1735 (acetate), 1635 and 885 (=CH_2); MS, $C_{31}H_{50}O_2$, *m/e* (rel. int.): 454 (M^+ 11), 439 (M^+-Me, 10), 394 (M^+-acetate, 100), 379 (M^+-Me-acetate, (80), 269 (M^+-acetate-side chain, 39) and other fragments similar to those previously reported [10]. NMR (δ) two doublets centered at 0.46 and 0.075 (2H, cyclopropane), 2.0 (3H, —OCMe), and 4.7 (2H, =CH_2).

The identities and physical properties of the other sterol acetates thus obtained were: 24-dehydropollinastanol acetate (**2a**), mp 81–82°, $[\alpha]_D^{23}$ +38°, IR $\nu_{max}^{CS_2}$ cm^{-1} 3040 (cyclopropane), 1733 (acetate); MS, $C_{30}H_{48}O_2$, *m/e* (rel. int.): 440 (M^+, 5), 380 (M^+-acetate, 37), 365 (M^+-acetate-Me, 23), 311 [M^+-acetate-CH_2CH=CMe, 7], 269 (M^+-acetate-side chain, 26), 227(11), 205 (10), 189 (12), 175 (40), 173 (30), 159 (41), 147 (51), 145 (38), 135 (38), 133 (42), 95 (84), 93 (65), 81 (73), 70 (50), 69[CH_2-CH=CMe_2, 100], 55 (86), 43 (86), and 41 (70); NMR (δ) two doublets centered at 0.46 and 0.06 (2H, cyclopropane), 1.68 and 1.60 (6H, C-26 and C-27), 2.0 (3H,—OCMe); 31-norcycloartenol acetate (**3a**), mp 92–93° $[\alpha]_D^{23}$ +58°, lit. [18], mp 92–94° $[\alpha]_D$ +66°; lit. [12], mp 93–95° $[\alpha]_D^{20}$ +63°; IR $\nu_{max}^{CS_2}$ cm^{-1}, 3040 (cyclopropane), 1733 (acetate); MS, $C_{31}H_{50}O_2$, *m/e* (rel. int.): 454 (M^+, 9), 439 (M^+-Me, 8), 394 (M^+-acetate, 79), 379 (M^+-Me-acetate), 286 (14), 283 (M^+-side chain-acetate, 13), and other fragments similar to those previously reported [12]. NMR (δ) two doublets centered at 0.43 and 0.18 (2H, cyclopropane), 1.7 and 1.63 (6H, C-26 and C-27), 2.05 (−OCMe); isofucosterol acetate (41 mg), mp 134–135°; lit. [19] mp 130.5–131°. The apolar stanol acetates namely, pollinastanol acetate (**5a**), 31-norcycloartanol acetate (**6a**), and 24-methypollinastanol acetate were inseparable. However, as the free stanols, **6b** could be separated from **5b** and **4b** (see sterols).

Sterols and stanols. Saponification of the respective steryl acetate and its recrystallization from dil. MeOH gave: 24-methylenecholesterol as plates, mp 148–149°, $[\alpha]_D^{23}$ −51°; lit. [17], mp 142°, $[\alpha]_D^{22}$ −35; 24-methylenepollinastanol(**1b**) as needles, mp 115–116° $[\alpha]_D^{23}$ +47°, lit. [10], mp 115–117°; IR $\nu_{max}^{CS_2}$ cm^{-1} 3610 (OH), 3040 (cyclopropane), 3080, 1658 and 885 (=CH_2); MS $C_{29}H_{48}O_2$, *m/e* (rel. int.): 412 (M^+, 22), 397 (M^+-Me, 42), 394 (M^+-H_2O, 40), 379 (M^+-Me—H_2O, 43), and other fragments similar to those previously reported [8, 10]; NMR (δ) two doublets centered at 0.46 and 0.08 (2H, cyclopropane), and 4.7 (2H, =CH_2); 24-dehydropollinastanol (**2b**) as needles, mp 112–113°, $[\alpha]_D^{23}$ +58°; IR $\nu_{max}^{CS_2}$ cm^{-1} 3610 (OH), 3040 (cyclopropane); MS $C_{28}H_{46}O$, *m/e* (rel. int.): 398 (M^+, 36), 383 (M^+-Me, 52), 380 (M^+-H_2O, 43), 365 (M^+-Me—H_2O, 43), 286 (M^+-side chain, 30), 270 (13), 268 (M^+-H_2O-sidechain, 12), 231 (17), 217 (12), 205 (28), 175 (35), 173 (28), 161 (30), 159 (27), 147 (46), 135 (32), 133 (43), 123 (27), 121 (47), 109 (51), 107 (52), 105 (41), 95 (76), 81 (63), 69[M^+-CH_2CH=CMe_2, 100], 55 (77), 43 (19) and 41 (67); NMR (δ) two doublets centered at 0.47 and 0.07 (2H, cyclopropane) 1.68 and 1.61 (6H, C-26 and C-27); 31-norcycloartenol (**3b**) as needles, mp 103–104°, $[\alpha]_D^{23}$ +38°, lit. [18] mp 112–114°, $[\alpha]_D$ +48; GC–MS, $C_{29}H_{48}O$, *m/e* (rel. int.): 412 (M^+, 23), 397 (M^+-Me, 42), 394 (M^+-H_2O, 40), 379 (M^+-Me—H_2O, 42), 283 (M^+-H_2O-sidechain), 69 [CH_2CH=CMe_2, 100]. The saponification of the stanyl acetate mixture from the esterified sterols and chromatography over neutral Al_2O_3 yielded 3 mg pure 31-norcycloartanol (**6b**) and 27 mg of a 9:1 mixture of pollinastanol–24-methylpollinastanol, mp 107–109°. The differences in our observed mps and/or $[\alpha]_D$ values for pollinastanol and 24-methylenecholesterol from the lit. citations probably result from the differences in the degree of purity, solvent used for recrystallization, or physical method used in determining optical rotations. Insufficient material prevented us from recrystallizing 31-norcycloartenol to a constant mp.

Hydrogenation of **1b**, **2b** *and* **3b**. The hydrogenation of **1b**, **2b** and **3b** in EtOAc with PtO_2 at room temp. and atmos. pres. yielded, respectively: 24-methylpollinastanol (**4b**), mp 106–107°, $[\alpha]_D^{26}$ +46°; pollinastanol (**5b**), mp 108–109°, $[\alpha]_D^{26}$ +51 (lit. [7] mp 95°, $[\alpha]_D^{20}$ +35); and 31-norcycloartanol (**6b**), mp 132–133°, $[\alpha]_D^{28}$ +48°; (lit. [14] mp 129–132°; $[\alpha]_D^{27}$ +57°).

RR_t and R_f values of steryl and stanyl acetates. The steryl acetates RR_ts from an SE-30 solumn and R_f values on $AgNO_3$-Si gel developed in C_6H_6–hexane (3:1) respectively were: cholesterol (1.0, 0.54); stigmastanol (1.43, 0.58); 31-norcycloartanol (1.39, 0.59); 24-methylpollinastanol (1.51, 0.59); pollinastanol (1.16, 0.59); 31-norcycloartenol (1.47, 0.50); 24-dehydropollinastanol

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(1.28, 0.49); fucosterol (1.63, 0.48); isofucosterol (1.68, 0.42); 24-methylenepollinastanol (1.47, 0.39): 24-methylenecholesterol (1.28, 0.30).

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