The literature reveals however the presence of trioxygenated coumarins in 31 of the 50 genera which have been studied [3]. Moreover, although 5,7,8-trioxygenated coumarins have been isolated from seven of the eight *Ruta* examined, 6,7,8-trimethoxycoumarin has also been reported from three of these [3, 4]. The same coumarin has indeed been found in three species of *Zanthoxylum* [3,5,6] but isopimpinellin (5,7,8) has been isolated from *Z. belizense* and *Z. suberosum* [3], phellopterin (5,7,8) and pimpinellin (5,6,7) from *Z. usambarense* [7] while fraxinol (5,6,7) and 5,6,7-trimethoxycoumarin occur in *Z. integrifolium* [5].

EXPERIMENTAL

IR spectra were recorded in CCl_4, UV spectra in EtOH and NMR spectra in $CDCl_3$.

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4α-METHYLERGOSTA-8,24(28)-DIEN-3β-OL, A MINOR STEROL IN THE SEED OF HORSE CHESTNUT

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Key Word Index—*Aesculus hippocastanum*; Hippocastanaceae; seed oil; unsaponifiable matter; 4α-methyl sterol; 4α-methylergosta-8,24(28)-dien-3β-ol.

Abstract—From ripe horse chestnut seed the 4α-methyl sterol fraction was isolated representing 4.5% of the unsaponifiable matter, i.e. 3 mg% of the seed. This fraction was investigated by capillary GC and combined GC–MS. It contains at least 12 components, of which 5 were identified as: obtusifoliol, 4α-methylergosta-8,24(28)-dien-3β-ol, gramisterol, cycloeucalenol and citrostadienol. The distribution of these five 4α-methyl sterols in the seed was also determined and they represent about 90% of the investigated fraction. 4α-Methylergosta-8,24(28)-dien-3β-ol up to now been found in higher plants only in traces, while in this fraction it was found in the amount of about 5%.

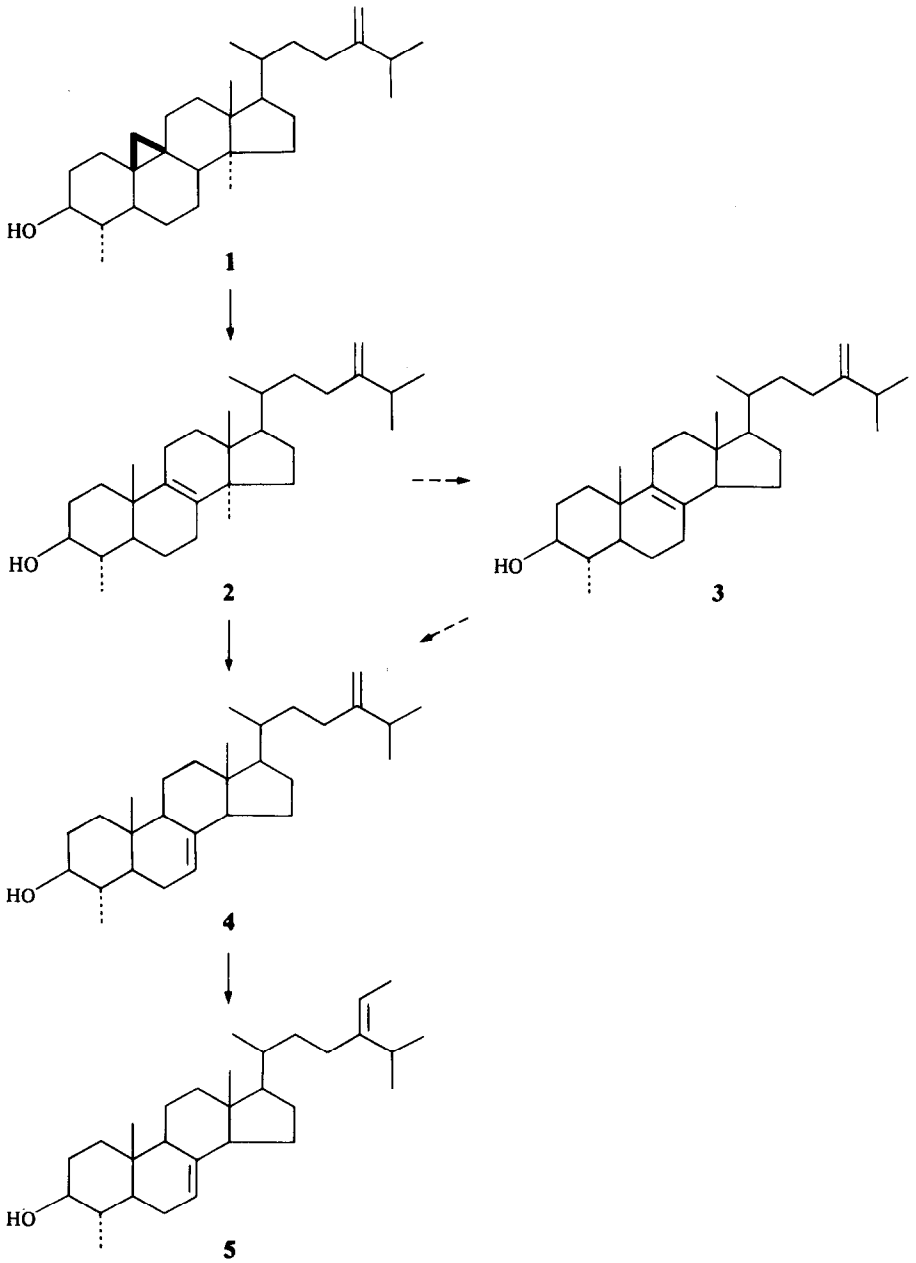
INTRODUCTION

According to present knowledge, the biosynthesis of stigmasterol and sitosterol, the two most common sterols in higher plants, takes place via 4-methyl sterols in such a generally accepted way that obtusifoliol (**2**) is a precursor of gramisterol (**4**) [1]. Only recently Schmit and Benveniste [2] while investigating sterol biosynthesis detected in bramble cell culture traces of 4α-methylergosta-8,24(28)-dien-3β-ol (**3**) and suggested that in higher plants, obtusifoliol (**2**) is first demethylated in position C-14 to give **3** and that the Δ^8 double bond of **3** is then isomerized to yield **4** (Scheme 1). 4α-Methylergosta-8,24(28)-dien-3β-ol (**3**) had been previously found in yeast [3, 4] and in some types of fungi [5], but not in higher plants. However, up to now few experimental data have been published which could confirm this theory and accordingly we investigated the 4-methyl sterol fractions of certain plant oils [6]. We succeeded in detecting traces of **3** only in rapeseed oil, which does not completely confirm the accepted hypothesis of the participation of **3** in sterol biosynthesis. Our investigations were continued with horse chestnut seed which, regarding the quantitative ratio of the Δ^5 and Δ^7 sterols [7] shows considerable deviations from the ratio determined for most higher plants [8]. In this paper we present the results of qualitative and quantitative analysis of the 4-methylsterol fraction of horse chestnut seed.

RESULTS AND DISCUSSION

The 4α-methyl sterol fraction was isolated from unsaponifiable matter of horse chestnut seed oil by conventional methods and then analysed by GC and combined GC–MS. By analysis on a glass capillary column (OV-101) we detected at least 12 components of which 5 components, on the basis of retention time and mass spectral data [5, 9–11], were identified as: cycloeucalenol (**1**), obtusifoliol (**2**), 4α-methylergosta-8,24(28)-dien-3β-ol (**3**), gramisterol (**4**) and citrostadienol (**5**). Because all these compounds, except **3**, are common in the plant kingdom the mass spectra and details of their identification are not given. GC–MS data for **3** were identical to the published data for a sterol identified in one species of fungus [5].

The qualitative and quantitative compositions of the 4α-methyl sterol fraction are given in Table 1. The results of quantitative analysis have shown that this fraction presents 4.5% of the unsaponifiable matter, i.e. 3 mg per 100 g of dry seed and that the 5 identified sterols represent 90% of the fraction. Compound **3** amounted to 5% of the 4α-methyl sterol fraction which is 0.15 mg per 100 g of the dry seed. This is the first time that sterol **3** has been found in higher plants in amounts greater than traces. However, current results do not completely explain the role of **3** in the biosynthesis of sterols in higher plants because **3** was found in a relatively small number of higher plants. This can be explained in two ways: (a) by the existence of various sterol biosynthetic routes in higher plants of which all do not include **3**, and (b) compound **3** is present



Scheme 1.

Table 1. Composition of the 4α-methyl sterol fraction of horse chestnut seed

Compound	M_r	Methylene indices	Quantity μg/g	Quantity dry seed
Obtusifoliol (2)	426	32.13	5.5	18.1*
4α-Methylergosta-8,24(28)-dien-3β-ol (3)	412	32.26	1.5	5.1
Unidentified	—	32.35	1.3	4.3
Unidentified	—	32.50	tr	tr
Gramisterol (4)	412	32.55	6.1	20.1
Cycloeucalenol (1)	426	32.68	1.2	4.2
Unidentified	—	33.06	tr	tr
Unidentified	—	33.19	tr	tr
Unidentified	—	33.23	tr	tr
Unidentified	—	33.39	0.9	2.9
Unidentified	—	33.55	0.6	2.1
Citrostadienol (5)	426	33.74	12.9	42.6

tr, Trace.
*As a percentage of 4α-methyl sterols (area % by GLC).

in most higher plants in amounts which cannot be detected by present analytical methods. In order to solve this dilemma and to obtain the final answer as to the role of **3** in sterol biosynthesis in higher plants, it would be interesting to foresee in which higher plants the accumulation of **3** could be expected. It is known that **3** has, up to now, been found in measurable amounts only in horse chestnut seed in which Δ^7-sterols predominate over Δ^5-sterols [7] and this indicates that the accumulationof **3** should be expected in those higher plants which show a high ratio of Δ^7-sterols to Δ^5-sterols and are therefore closer to lower plants. This hypothesis is supported by the findings of Schmit and Benveniste [2] who determined, *in vitro*, the dependence between the accumulation of **3** and the inhibition of Δ^5-sterol biosynthesis.

EXPERIMENTAL

Plant material. The horse chestnut seed originated from the Leskovac region (Yugoslavia). The ripe seed was picked in the second half of September and was dried in air until the moisture content had decreased to less than 10%.

Isolation of unsaponifiable matter. Chestnut flour (5 kg) was extracted 3 × with a double amount of petrol (10 l.) at room temp. Upon removing the solvent, the oily residue was dried in a desiccator for 24 hr over H_2SO_4. A clear oil (150 g) of yellow–greenish colour, was separated by filtration from a ppt formed during oil drying and saponified as described previously [7]. From 100 g of this oil 2.2 g of unsaponifiable matter were obtained.

Isolation and analysis of the 4α-methyl sterol fraction. 4α-Methyl sterols were isolated from unsaponifiable matter by TLC on silica gel (0.5 mm) impregnated with 0.03% 2,7-dichlorofluorescin. Development was performed in hexane–Et_2O (1:1) for 1 hr. The 4α-methylsterol band (R_f = 0.35) was located under UV light (254 nm), scraped off and then quantitatively extracted with Et_2O. From 0.6 g of unsaponifiable matter 27 mg of 4α-methylsterols were obtained. In this way the isolated 4α-methylsterols were analysed by GLC and combined GC–MS as described previously [7].

Mass spectral data. 4α-Methylergosta-8,24-(28)-dien-3β-ol (**3**); MS *m/z* (assignment, rel. int.): 412 $[M]^+$ (100), 397 $[M-Me]^+$ (40), 397 $[M-Me-H_2O]^+$ (3), 313 $[M-Me-C_6H_{12}]^+$ (7), 287 $[M-SC]^+$ (3), 286 $[M-SC-H]^+$ (6), 285 $[M-SC-2H]^+$ (18), 269 $[M-SC-H_2O]^+$ (9), 271 $[M-Me-C_7H_{14}-H_2O]^+$ (9), 260 $[M-SC-C_2H_3]^+$ (8), 259 $[M-SC-C_2H_4]^+$ (12), 245 $[M-SC-Me-C_2H_3]^+$ (14), 243 $[M-SC-C_2H_2-H_2O]^+$ (14), 241 $[M-SC-C_2H_4-H_2O]^+$ (16), 227 $[M-SC-C_2H_3-Me-H_2O]^+$ (17).

4α-Methylergosta-8,24(28)-dien-3β-yl trimethylsilyl ether (**3a**); MS *m/z* (assignment, rel. int.): 484 $[M]^+$ (100), 469 $[M-Me]^+$ (20), 394 $[M-TMSiOH]^+$ (16), 379 $[M-Me-TMSiOH]^+$ (18), 357 $[M-SC-2H]^+$ (9), 267 $[M-SC-2H-TMSiOH]^+$ (6), 269 $[M-SC-TMSiOH]^+$ (8), 243 $[M-SC-C_2H_2-TMSiOH]^+$ (8), 241 $[M-SC-C_2H_4-TMSiOH]^+$ (14), 227 $[M-SC-C_2H_3-Me-TMSiOH]^+$ (15).

Nomenclature. Obtusifoliol = 4α,14α-dimethyl-24-methylene-5α-cholest-8-en-3β-ol, cycloeucalenol = 4α,14α-dimethyl-9,19-cyclo-24-methylene-5α-cholestan-3β-ol, gramisterol = 4α-methyl-24-methylene-5α-cholest-7-en-3β-ol, citrostadienol = 4α-methyl-24-ethylidene-5α-cholest-7-en-3β-ol.

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FREE STEROLS OF CITRUS ROOTS

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Key Word Index—*Citrus kharna*; *Citrus medica*; *Citrus reticulata*; *Poncirus trifoliata*; Rutaceae; citrus; root sterols; 4-desmethylsterols; campesterol; sitosterol; stigmasterol; cholesterol.

Abstract—Free 4-desmethylsterols from fibrous roots of 6 citrus rootstocks were identified by combined gas chromatography and mass spectrometry as campesterol, stigmasterol, sitosterol and cholesterol (minor component). No isofucosterol was present.

INTRODUCTION

Investigations involving the quantitation of sterols from citrus (and other woody plants) are few and have been limited to tentative identification of individual sterols by co-chromatography with authentic standards on GC [1, 2]. Individual sterols affect membrane permeability in plants to different extents [3, 4] and changes in sterol composition appear to play a significant role in the response of roots to salinity [1], mycorrhyzal infection [2] and other environmental factors such as light intensity [5], hormone treatment [6] and temperature [7]. Therefore, it seemed appropriate to formally identify the free 4-desmethylsterol components of citrus roots to verify observed differences in composition in response to such environmental factors. In citrus this necessity is accentuated by the fact that isofucosterol has been tentatively identified in one investigation [2], but appears to be absent in another investigation [1] on roots of the same citrus rootstocks.

RESULTS AND DISCUSSION

GC of free 4-desmethylsterols from fibrous roots of all 6 citrus rootstocks examined showed the presence of only 4 main compounds. These corresponded in relative retention time to the authentic sterols cholest-5-en-3β-ol (cholesterol), 24ξ-methylcholest-5-en-3β-ol (campesterol), 24ξ-ethylcholest-5-en-3β-ol (sitosterol), and 24-ethylcholesta-5,22-dien-3β-ol (stigmasterol) with the latter three compounds accounting for greater than 96% of the total sterols (Table 1). An additional peak with a relative (to 5α-cholestane) retention time greater than that of sitosterol and of similar order to that calculated for isofucosterol (24-ethylidenecholest-5-en-3β-ol) was also present in very small amounts (usually less than 1%). GC-MS of trifluoroacetate derivatives of the citrus sterols, scanned over the range m/z 50–500, gave a total ion chromatogram with peaks at the same relative retention times as the sterol standards. Cholesterol (RR_t = 1.27), campesterol (RR_t = 1.51) and sitosterol (RR_t = 1.75) trifluoroacetates showed characteristic ions of $[M-114]^+$ (as base peak) due to the loss of CF_3COOH and $[M-129]^+$ due to the further loss of the methyl at C-21. The abundance of the $[M-114]^+$ ion of stigmasterol (RR_t = 1.60) was reduced due to the production of minor peaks for $[M-142]^+$ due to the elimination of the 2C unit at C-24 (C_2H_4) and $[M-157]^+$ (the additional loss of the C-21 methyl). Ions for $[M-112]^+$ and $[M-141]^+$ indicated fragmentation of the side chain promoted by the double bond at C-22. In addition, all sterols showed ions m/z 255 and 213 due to the loss of side-chain and cleavage of ring D, indicative of the perhydrocyclopentanophenanthrene ring structure.

The peak observed running after sitosterol did not show an $[M-114]^+$ ion at m/z 394 that might be expected for isofucosterol nor other ions characteristic of the 4-desmethylsterols and is thus not isofucosterol. Serial