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THE STEROLS AND TRITERPENES OF *SORGHUM VULGARE* GRAINS

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Abstract—A complex mixture containing more than 20 4-demethylsterols, 4-monomethylsterols and triterpenes was isolated from the grains of *Sorghum vulgare*. Seventeen of these compounds were identified using a combination of GLC, GC–MS, IR and NMR.

INTRODUCTION

The 4-demethylsterol content has been described for some members of the Gramineae [1–3] However those of *Sorghum vulgare* (*S. bicolor* (Linn) Moench) have not previously been reported, although we have detected small amounts of 24-dihydroobtusifoliol [4], a sterol originally identified in *Chlorella emersonii* [5] but apparently not previously found in higher plants.

Cycloartenol has been identified in maize [6, 7] and rice bran oil [8] but in the present study neither this nor any other 4,4-dimethylsterol [9] precursor could be detected in *S. vulgare* although relatively high levels of pentacyclic triterpenes were found. This apparent lack of 4,4-dimethylsterols and an abundance of pentacyclic triterpenes was also found in earlier studies on the Gramineae [10] and one species of *Euphorbia* [11].

RESULTS

The unsaponifiable lipid from *S. vulgare* grain was chromatographed on alumina and after elution of fractions containing hydrocarbons (22%) and tocopherols (24%), a third fraction containing sterol was eluted and treated with digitonin. The sterols and triterpenes obtained after regeneration from the digitonides were rechromatographed on alumina and the relevant fractions further separated by PLC into triterpenes, 4-monomethylsterols and 4-demethylsterols respectively which were analysed by GLC (Table 1). Acetates of the triterpene and sterol mixtures were prepared and further resolved by preparative argentation TLC before GLC analysis (Table 1).

Identification of the 4-demethylsterols

Argentation TLC of the acetates of the 4-demethylsterol fraction revealed three component bands co-chromatographing with marker cholesteryl (band 1, R_f 0·46), stigmasteryl (band 2, R_f 0·38) and 28-isofucosteryl (band 3, R_f 0·25) acetates respectively. GLC analysis of band 1 revealed two major peaks in approximately 2:1 ratio, the larger peak co-chromatographed with authentic sitosteryl acetate and the other with campesteryl acetate on all four GLC phases used. A third component (1%) had an identical R_t to cholesteryl acetate. An NMR spectrum of band 1 showed a multiplet at δ 5·34 ppm confirming a nuclear double bond and the IR spectrum revealed weak bands at 1670 and 840 cm^{-1} characteristic of Δ^5 unsaturation. The NMR spectrum was consistent with that reported for sitosterol [12, 13]. GC–MS confirmed the presence of cholesteryl acetate in this fraction by showing a molecular ion at *m/e* 428, a base peak at *m/e* 368 (M^+-acetate) and other important ions at *m/e* 255, 247 and 213. MS fragmentation patterns of the two main components of band 1 indicated

Table 1. Relative retention times of the sterol and triterpene acetates detected in *S. vulgare*. All R_t's are relative to cholesteryl acetate and figures in parentheses are for the free compounds relative to cholesterol

4-Demethylsteryl acetates	OV-17*	QF-1†	SE-33‡	Hieff 8B§	4-Monomethyl steryl acetates	OV-17*	QF-1†	SE-33‡
Cholesterol (1·00)	1·00	1·00	1·00	(1·00)	24-Dihydroobtusifoliol	1·38	1·45	1·38
Campesterol (1·28)	1·28	1·28	1·30	(1·33)	24-Methyl-lophenol	1·66	1·45	1·54
Stigmasterol (1·41)	1·41	1·29	1·40	(1·33)	24-Ethyl-lophenol	2·10	1·77	1·93
Sitosterol (1·63)	1·63	1·60	1·68	(1·59)	Obtusifoliol	1·42	1·45	1·35
Sterol A	1·10	1·03	1·08		24-Methylenelophenol (1·78)	1·76	1·45	1·52
24-Methylenecholesterol	1·31	1·28	1·25		24-Ethylidenelophenol (2·53)	2·50	1·84	2·03
24-Methylenecholest-7-en-3β-ol	1·64		1·41		Triterpene acetates δ-Amyrin (1·86)	1·63	1·94	1·51
Sterol B			1·54		β-Amyrin	1·55	1·79	1·42
28-Isofucosterol (1·80)	1·80	1·62	1·66	(1·83)	Pentacyclic Triterpene X (1·70)	1·60	1·51	1·41
24-Ethylidenecholest-7-en-3β-ol (1·91)	1·91	1·73	1·86	(2·10)	Lupeol (2·10)	1·88	1·86	1·60
					Pentacyclic Triterpene Y (2·27)	2·00	2·10	1·83

* 3% OV-17; 225°; N_2 40 ml/min; RR_t 5α cholestane to cholesterol and cholesteryl acetate 0·37 and 0·26 respectively.
† 2·5% QF-1; 205°; N_2 25 ml/min; RR_t 5α cholestane to cholesteryl acetate 0·21.
‡ 1% SE-33; 225°; N_2 25 ml/min RR_t 5α cholestane to cholesteryl acetate 0·34.
§ 1% Hieff 8B + 2% PVP; 220°; N_2 32 ml/min.

a C_{28} steryl acetate having a molecular ion at *m/e* 442 and a C_9H_{19} side chain and a C_{29} homologue with a molecular ion at *m/e* 456 and a $C_{10}H_{21}$ side chain. The relative intensities of these and other important ions confirmed the presence of campesteryl and sitosteryl acetates respectively. Saponification of this mixture yielded the free sterols which were again identified by GC–MS.

Band 2 appeared to be a single compound with an identical GLC R_t to stigmasteryl acetate. The MS showed a weak molecular ion at *m/e* 454, a base peak at *m/e* 394 (M^+-acetate), an ion for loss of an isopropyl group and acetate at *m/e* 351 (12%) characteristic of Δ^{22} unsaturated sterols and an ion at *m/e* 255 (46%) indicative of a steryl acetate having a $C_{10}H_{19}$ mono-unsaturated side chain. The IR spectrum showed weak absorption at 1735 cm^{-1} and strong absorption at 965 cm^{-1}, characteristic of a *trans* Δ^{22} bond. NMR spectroscopy confirmed the side chain unsaturation by having peaks at δ 5·1 ppm. A MS identical to that of stigmasterol was obtained after saponifying the steryl acetate from band 2.

GLC of band 3 revealed a complex mixture of at least six sterols and a complete separation of all the compounds could not be achieved on any of the GLC phases used. The major component (50%) had identical R_t's to authentic 28-isofucosteryl acetate [14]. In general, the GLC mobilities and the percentage composition of this fraction were very similar to those reported for the $\Delta^{24(28)}$ unsaturated 4-demethylsterols of oat seeds [15]. The IR spectrum of band 3 indicated the presence of one or more compounds with a 24-ethylidene group by the characteristic weak absorption at 820 cm^{-1} for a trisubstituted double bond. An additional weak band at 890 cm^{-1} suggested the presence of a small proportion of 24-methylenesterols. The NMR spectrum of band 3 had a multiplet at δ 5·34 ppm corresponding to the C-6 proton and a multiplet at 5·05 ppm for the proton at C-28 thus confirming Δ^5 and $\Delta^{24(28)}$ unsaturation respectively. GC–MS of band 3 showed the major component to be 28-isofucosteryl acetate and the base peak at *m/e* 296 together with the GLC R_t confirmed that it could not be fucosterol [16]. The peak eluting immediately after 28-isofucosteryl acetate was the isomer, 24-ethylidenecholest-7-en-3β-yl acetate (12%). Both these steryl acetates gave identical MS fragments but variations in the relative intensities of the ions at *m/e* 356 (M^+-part of side chain), 313 (M^+-SC) and 296 (M^+-part of side chain + acetate) supported the assignments.

The MS of two compounds eluting just before 28-isofucosteryl acetate (Table 1) indicated that they were the acetates of 24-methylenecholesterol (10%) and its isomer 24-methylenecholest-7-en-3β-ol (8%) respectively. Both steryl acetates had a molecular ion at *m/e* 440 and identical fragmentation patterns but with differing intensities

of ions at *m/e* 380 (M^+-acetate), 356 and 313 in agreement with data published for the TMS derivatives of these sterols [15]. Two minor sterols (A and B Table 1) were not identified.

Identification of the 4-monomethylsterols

The 4α-methylsteryl acetates from *S. vulgare* were resolved into three bands by argentation TLC. Band 1 had an R_f of 0·38; band 2, R_f 0·2 co-chromatographed with 24-ethylidenelophenyl acetate; and band 3, the most polar, had an R_f of 0·08. Band 1 was resolved by GLC into two almost equal peaks having R_t's indicating that they may be 24-methyl- and 24-ethyllophenyl acetate. GC–MS confirmed that the first peak eluted was 24-methyllophenyl acetate with a molecular ion at *m/e* 456 (100%) and an ion at *m/e* 269 (90%) indicative of a steryl acetate with a C_9 saturated side chain. The MS of the second component showed a molecular ion at *m/e* 470 and a fragmentation pattern indicating it to be 24-ethyllophenyl acetate. The MS and GLC data for the major sterols of band 1 were in agreement with those reported previously for 24-methyl- and 24-ethyl-lophenol [17, 18]. A third minor component of this mixture (15%) was found by GC–MS to have a molecular ion at *m/e* 470 (18%) a base peak at *m/e* 395 ($M^+ - Me + acetate$) and a fragmentation pattern and GLC mobility which showed it to be 24-dihydroobtusifoliol [4].

Band 2 was found to consist almost entirely of a compound co-chromatographing with authentic 24-ethylidenelophenyl acetate on GLC. The IR spectrum showed weak absorption at 820 cm^{-1} indicating a trisubstituted double bond and the MS showed a molecular ion at *m/e* 468 (8%), a base peak at *m/e* 327 ($M^+ - SC + 2H$) and an ion at *m/e* 370 (56%) confirming side chain unsaturation at $\Delta^{24(28)}$. The overall fragmentation pattern was consistent with that reported for 24-ethylidenelophenol [18].

GLC of band 2 also showed a minor peak which co-chromatographed with authentic obtusifoliol. Ions attributable to this compound were masked on the MS of band 2 but we have identified obtusifoliol by GC–MS in 3-day-old seedlings of *S. vulgare*[25] and on the basis of this and the GLC evidence in Table 1, the minor component of band 2 is obtusifoliol.

GLC of band 3 revealed a single peak with R_t's similar to those published for 24-methylenelophenyl acetate [19]. MS showed a molecular ion at *m/e* 454 (7%) with a base peak at *m/e* 327 ($M+ - SC$) and a fragmentation pattern very similar to that described for 24-methylenelophenyl acetate.

Identification of the triterpenes

GLC of the triterpene fraction on OV-17 prior to acetylation showed four component peaks. The major compound (65%) had a R_t the same as cycloartenol on both OV-17 and SE-33. A second peak Y (12%) eluted just after the major peak had a similar R_t to 24-methylenecycloartanol. GC–MS revealed a molecular ion at *m/e* 426 for the major component but the absence of an ion at *m/e* 286, characteristic of a 9β19-cyclopropane ring [20], indicated that this compound could not in fact be cycloartenol. The overall fragmentation pattern and lack of an ion at *m/e* 300 in the MS of compound Y indicated that this was probably a pentacyclic triterpene and not 24-methylenecycloartanol. Following acetylation, the four components of the triterpene fraction were separated by argentation TLC. The acetate of the major triterpene was the most polar compound having an R_f of 0·28 (band 4). The R_f values of the remaining three bands were 0·66 (band 1), 0·59 (triterpene Y) and 0·50 (triterpene X). Triterpenes X and Y have not yet been identified.

Band 4 gave a single peak on all GLC phases used with the same R_t as lupeyl acetate. The NMR spectrum was identical to that of lupeyl acetate and showed peaks at δ 4·69 and 4·56 ppm integrating for the two protons of the methylene group. Other peaks were observed at δ 2·01, 1·68, 1·54, 1·03, 1·25, 0·93, 0·82 and 0·79 ppm. The IR spectrum of band 4 showed strong absorptions at the following frequencies: 2960; 2940; 2860; 1730; 1360; 1240; 1170; 1105; 1023; 1015; 975; 940; and 895 cm^{-1}. The MS of band 4 was similar to that obtained for authentic lupeyl acetate showing a molecular ion at *m/e* 468 (42%), ions at *m/e* 425 (5%) and *m/e* 365 (8%) for loss of the isopropylidene and isopropylidene plus acetate groups respectively. A base peak was observed at *m/e* 189 with intense ions at *m/e* 204 (50%) and 218 (37%).

Band 1 was found to have the same R_f as β-amyrin acetate on TLC. It showed a broadly

based asymmetric peak on GLC comprising at least two incompletely resolved components, a minor peak (10%) appearing as a shoulder on the leading edge of the principle peak had an R_t suggesting that it was β-amyrin acetate. MS were taken repeatedly across the principle GLC peak and even though the components were not fully resolved, spectra adequate for interpretation were obtained. MS were obtained for two compounds, both having a molecular ion at *m/e* 468 and the same fragmentation pattern but differing in relative intensities. The MS of the minor shoulder peak revealed a base peak at *m/e* 218 which is the typical retro-Diels–Alder fragment characteristic of $\Delta^{12(13)}$ triterpenes of the β- and α-amyrin type [21]. On the basis of both the GLC and MS evidence it is concluded that this triterpene was β-amyrin acetate. The MS taken at the apex of the principle peak showed the most important fragment at *m/e* 205 with ions of equal intensity at *m/e* 189 (50%) and 218 suggesting [22] that this compound is an isomer of β-amyrin having a $\Delta^{13(18)}$ bond and is probably δ-amyrin.

DISCUSSION

It is generally considered (see Ref. [9]) that sterols are synthesised in higher plants by a complex of inter-connected pathways leading from cycloartenol through 4,4-dimethylsterols to 4-monomethylsterols and ending in the 4-demethylsterols. The sterols found in *S. vulgare* seeds can be fitted into this concept very readily with 24-methylenecholest-7-en-3β-ol and 24-methylenecholesterol situated on the pathway to campsterol and 24-ethylidene-lophenol, 24-ethylidenecholest-7-en-3β-ol and 28-isofucosterol on the route to sitosterol. It now seems likely that the 4-demethylsterol intermediates of the campesterol pathway can be alkylated at C-28 and diverted to the sitosterol pathway. Thus 24-methylenecholesterol is possibly alkylated to give 28-isofucosterol and converted to sitosterol in *S. vulgare* since Baisted [23] showed that in *Euphorbia peplus* 28-isofucosterol was converted to sitosterol and stigmasterol and Van Aller *et al.* [24] showed that in *Pinus pinea* 24-methylenecholesterol was converted not only to campesterol but also to 28-isofucosterol.

The grain of *S. vulgare* contains comparatively high concentrations of the 4-demethylsterols usually found in higher plants together with some 4-monomethylsterols but 4,4-dimethylsterols were not detected. This suggests that at some point during seed maturation the supply of substrate for 4,4-dimethylsterol synthesis is reduced resulting in the depletion of the 4,4-dimethylsterols. Though cycloartenol was not detected this cannot be taken as evidence that it plays no part in sterol biosynthesis in *S. vulgare*.

Pentacyclic triterpenes reach comparatively high levels in *S. vulgare* grain (65·6 μg/g). Since hydrocarbons, including squalene, do not increase during maturation [25], this implies that squalene may be diverted from sterol to pentacyclic triterpene synthesis. Baisted [26] reached similar conclusions when working on pea seeds. The suggested route from squalene 2,3-oxide to obtusifoliol lies through cycloartenol. Neither cycloartenol nor any similar sterol with a 9β 19-cyclopropane ring was found in *S. vulgare* grain or in a wide variety of grasses [10]. If regulation of sterol biosynthesis generally takes place at or close to squalene 2,3-oxide the absence of cycloartenol and other 4,4-dimethylsterols from seeds could be explained.

EXPERIMENTAL

GLC was with 2 m × 1·5 mm glass columns, the operating conditions are given in Table 1. IR spectra were recorded with the samples dissolved in CS_2 in 0·1 mm microcells. Sterols isolated in sufficient quantity were also examined as nujol and Kel-f mulls. NMR spectra were determined at 90 MHz using either TMS or pure $CHCl_3$ to provide the lock signal and TMS as internal standard with $CDCl_3$ solvent. GC–MS was carried out on an AEI MS.30 at 70 eV.

Materials and lipid extractions. S. vulgare grain was obtained from Gunson Seeds Ltd. Batches of grain (20 × 50 g) were homogenized in Me_2CO with an Ultra Turrex then refluxed in Me_2CO (50 ml/g) for 3 hr in soxlet extractors. After refluxing, the homogenate was stirred with $CHCl_3$–MeOH [27] overnight. All lipid extracts were evaporated to dryness *in vacuo* then resuspended in Et_2O, filtered, evaporated and dried in a desiccator flushed with N_2 then bulked. 0·005% BHT as anti-oxidant [28] was routinely added to extracting solvents. As a precaution, standard sterols and triterpenes were incubated with concentrated BHT solutions (0·5%) and examined by NMR and MS, no detectable degradation was observed.

Column chromatography. Bulk lipid was preliminarily purified on a 3·5 × 3·75 cm (10 g) column of Silic AR CC_7 200–325 mesh (Mallinckrodt). A clear yellow oil (30 g/kg) was eluted with 500 ml $CHCl_3$ and saponified for 90 min in 8% KOH in 80% MeOH under N_2. Unsaponifiable lipid (1·7 g/kg) was separated on Woelm anionotropic alumina (Brockmann grade

III). The petrol fraction containing squalene and hydrocarbons and the 2% Et_2O in petrol fraction were both discarded. A third fraction (850 mg) eluted with 40% Et_2O in petrol contained sterols and triterpenes. After digitonin precipitation [29] and regeneration by DMSO [30] the crude sterols and triterpenes were re-chromatographed on alumina with stepwise elution using petrol and 2, 4, 6, 9 and 20% Et_2O in petrol [31].

TLC. Column fractions and digitonin precipitations were monitored by TLC using precoated Camlab Si gel G plates. Sterols and triterpenes were visualized by spraying with 50% H_2SO_4 and heating at 110°. For preparative work compounds were visualized by spraying with 0·1% ethanolic berberine hydrochloride and viewed under UV. Column fractions containing sterols and triterpenes were separated on Si gel G plates (1 mm, 20 × 40 cm) developed with $CHCl_3$–EtOH (49:1). Argentation TLC of steryl acetates [32] was on 0·75 mm Chromolay grooved plates impregnated with 10% $AgNO_3$ and developed in pure $CHCl_3$.

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