Phytochemistry, 1977, Vol. 16, pp. 459–463. Pergamon Press. Printed in England.

VARIATIONS IN STEROL AND TRITERPENE CONTENTS OF DEVELOPING *SORGHUM BICOLOR* GRAINS

MICHAEL A. PALMER* and BERNARD N. BOWDEN

Department of Applied Biology, Chelsea College, University of London, Hortensia Road, Chelsea, London SW10 0QX, England

(*Revised received* 18 *October* 1976)

Key Word Index—*Sorghum bicolor*; Gramineae; biosynthesis; sterols; triterpene esters; lupanol; multiflorenol; amyrins.

Abstract—The free, esterified and glycosylated sterols and the pentacyclic triterpene esters of developing *Sorghum bicolor* grains were analysed by GLC and GC–MS. All the pentacyclic triterpenes were completely esterified but were not detected until 24 days after anthesis. Lupanol, multiflorenol, α-amyrin and isoarborinol were identified in the mature grains as components of the triterpene fraction but no 4,4-dimethylsterols could be found at any stage of development. A sixfold increase in total sterol per grain occurred during development. At 8 days after anthesis, 28-isofucosterol was found to be the second most abundant steryl ester. Campesterol was the major steryl glycoside and obtusifoliol was the major 4-monomethylsterol.

INTRODUCTION

More than twenty sterols and triterpenes have been isolated from the mature grains of *Sorghum bicolor* [1] but neither cycloartenol nor any other 4,4-dimethylsterol was found. By contrast, relatively high concentrations of pentacyclic triterpenes were present and all were completely esterified [2].

Baisted [3] reported that pea seeds incorporated mevalonic acid-[2-^{14}C] into cycloartenol at early stages of their development but in the mature seeds radioactivity was recovered in β-amyrin. This implies that a common precursor such as squalene-2,3-oxide may be diverted from sterol to pentacyclic triterpene synthesis. We suggested [1] that a similar switching mechanism may operate in *S. bicolor* and that this may help to explain the apparent absence of 4,4-dimethylsterols and the abundance of pentacyclic triterpenes reported for the grains of a variety of grasses [4]. The results presented here support the view that pentacyclic triterpene biosynthesis may occur in developing seeds at the expense of sterol biosynthesis [5]. It seems that no detectable cyclization of squalene-2,3-oxide to pentacyclic triterpenes occurs during the development of *S. bicolor* grains until at least 12 days after anthesis. However, despite the absence of pentacyclic triterpenes from the immature grains of *S. bicolor*, 4,4-dimethylsterols could not be detected.

* Present address—Department of Biochemistry, The University of Liverpool, P.O. Box 147, Liverpool L69 3BX, England.

RESULTS AND DISCUSSION

Triplicate batches of grain were harvested from plants of *S. bicolor* at intervals of 8, 12, 24, and 48 days after anthesis (DFA). Between 24 and 48 DFA a reciprocal increase in dry weight and a decrease in fresh weight of the grains was observed (Table 1). The grains were sorted into their respective age groups after harvesting and were dried *in vacuo* before homogenization and Soxhlet extraction first with acetone and then with

Table 1. The fresh weight, dry weight and lipid content of developing *S. bicolor* grains

	Age of grains (Days after anthesis) 8	12	24	48
Fr. wt g/100 grains	1.34	4.40	6.40	6.21
Dry wt g/100 grains	0.27	1.19	3.26	4.30
Neutral lipid				
mg/g dry wt	29.5 (±1.3)	19.3 (±1.5)	28.6 (±1.1)	34.7 (±0.9)
mg/100 grains	8.1	23.0	98.3	149.0
Glycolipid				
mg/g dry wt	9.3 (±1.3)	5.4 (±0.9)	3.5 (±0.1)	1.9 (±0.4)
mg/100 grains	2.6	6.5	11.4	8.1
Phospholipid				
mg/g dry wt	6.8 (±1.7)	5.1 (±0.7)	3.1(±0.3)	1.6 (±0.2)
mg/100 grains	1.8	6.1	10.0	6.9

Figures in parenthesis are standard errors of mean, 3 replicates.

chloroform–methanol (2:1). The lipid obtained after each of the two extractions was combined to give a total lipid fraction. The various total lipid fractions were divided into two portions. The first was separated on silicic acid columns into neutral lipid, glycolipid and phospholipid (Table 1). The steryl glycosides were isolated from the glycolipid by PLC and subjected to methanolysis. The neutral lipid was separated on alumina columns into fractions containing steryl and triterpene esters and free sterols. The free sterols were purified via their digitonides and the esters were cleaved by saponification. The second portion of total lipid was saponified directly and the total sterols and triterpenes were isolated by alumina column chromatography and PLC.

Analysis of the unsaponifiable lipid from the 8 and the 12 DFA grains (Table 2) gave no evidence for the presence of either pentacyclic triterpenes or 4,4-dimethylsterols. By 24 DFA however a significant concentration (47 μg/100 grains) of pentacyclic triterpene was present which continued to accumulate to a final concentration of 339 μg in the 48 DFA grains. The pentacyclic triterpenes of the 24 and 48 DFA grains were completely esterified.

GLC analysis of the triterpenes from the 48 DFA grains (Table 3) indicated the presence of similar compounds to those previously found in the grains of the parent generation [1]. The unsaponifiable lipid from 500 g (fr. wt) of 48 DFA grain was chromatographed on alumina giving a crude sterol and triterpene fraction which was subsequently treated with digitonin. The sterols and triterpenes recovered from the digitonides were further purified on an alumina column and by PLC. The triterpenes were acetylated and resolved into four component bands by argentation PLC. GLC of the least polar band (R_f 0.66) (Table 3) showed that this was a mixture of at least four compounds. Further analysis by GC–MS showed that each of these compounds had a MW of 468. The MS corresponding to the first peak and the last peak eluted from the GLC column (Table 3) were almost the same. Each showed a base peak at *m/e* 218. They were identified on the basis of GLC and MS evidence as β- and α-amyrin respectively [6]. The MS for the major component (74%) of this mixture indicated that it was δ-amyrin [7]. The fourth triterpene eluted immediately after δ-amyrin on GLC (Table 3) and it gave an MS showing an intense ion at *m/e* 301. This is a characteristic

Table 2. The percentage composition of the total sterols and triterpenes and the glycosylated sterols of developing *S. bicolor* grains

	Age of grains (days after anthesis)			
Total* 4-Demethylsterols	8	12	24	48
Cholesterol	0.8	0.7	0.5	0.2
Campesterol	24.9	27.0	28.5	31.2
Stigmasterol	27.4	26.5	16.9	17.7
Sitosterol	35.2	35.5	44.2	42.7
28-Isofucosterol	9.8	8.3	7.6	6.2
24-Ethylidene-5α-cholest-7-en-3β-ol	1.9	1.9	2.3	2.0
μg/g dry wt	940 (±7.0)	614 (±4.0)	320 (+7.0)	308 (±7.0)
μg/100 grains	258	731	1042	1324
Glycosylated 4-demethylsterols				
Cholesterol	11.3	9.5	6.6	6.0
Campesterol	36.6	29.5	28.1	24.8
Stigmasterol	21.0	20.0	16.3	10.0
Sitosterol	31.1	40.7	48.6	54.6
μg/g dry wt	31 (±1.0)	16 (± 0.5)	9 (±1.0)	2 (±0.5)
μg/100 grains	8	19	30	9
† Total-4-monomethylsterols				
Obtusifoliol	42.1	30.0	34.6	11.7
24-Methylenelophenol	25.4	41.5	17.2	31.6
24-Ethylidenelophenol	32.5	28.5	48.2	56.7
μg/g dry wt	16 (±0.5)	10 (±0.5)	8 (±0.5)	4 (±0.5)
μg/100 grains	4	12	25	17
‡ Total pentacyclic triterpenes				
Lupanol	N/D	N/D	9.0	16.3
δ-Amyrin	N/D	N/D	6.4	15.4
Lupeol	N/D	N/D	44.6	55.9
Multiflorenol	N/D	N/D	40.0	12.4
μg/g dry wt	—	—	14 (±1.0)	79 (±2.0)
μg/100 grains	—	—	47	339

Figures in parenthesis are standard errors of the mean, 3 replicates * comprises free and esterified 4-demethylsterols. † Found exclusively in the free form. ‡ Found exclusively esterified.

Table 3. R_f values and retention times of the pentacyclic triterpene acetates isolated from mature *S. bicolor* grains

Triterpene acetate	R_f value§	GLC stationary phase OV-17*	QF-1†	SE-33‡
β-amyrin	0.66	1.55	1.79	1.42
δ-amyrin	0.66	1.63 (1.86)	1.94	1.51
isoarborinol	0.66	1.74	2.00	1.60
α-amyrin	0.66	2.00	2.20	1·73
Lupanol (X)	0.50	1.60 (1.70)	1.51	1.41
Lupeol	0.28	1.88 (2.10)	1.86	1.60
Multiflorenol (Y)	0.59	2.00 (2.27)	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. § 10% $AgNO_3$ impregnated Si-gel developed in pure $CHCl_3$.
All R_t's are relative to cholesteryl acetate and figures in parenthesis are for the free compound relative to cholesterol.

fragmentation of 13,14-dimethylated triterpenes. Equally intense ions at *m/e* 453 (100%) and 393 for loss of a methyl group and a methyl group plus acetate respectively, supported our view that this was isoarborinyl acetate [7]. The presence of the pentacyclic triterpene fernenol in *S. bicolor* is also a possibility. Both isoarborinol and its stereoisomer, fernenol, have similar MS fragmentation patterns and are reported to be constituents of *Sorghum* and other species of the Andropogoneae [4].

Each of the remaining triterpene bands obtained after argentation PLC gave a single peak on all three GLC phases used (Table 3). The most polar band (R_f 0.28) was identified as lupeyl acetate and was quantitatively the most abundant triterpene found in the 48 DFA grain (Table 2). The R_f RR_t and MS of the other two triterpene bands were very similar to those of triterpenes X and Y previously found in the parent grains of *S. bicolor* [1]. The triterpene acetate from the 48 DFA grains (R_f 0.59) corresponding to triterpene Y gave an MS having peaks at *m/e* 315 (3%), 301 (4%) and 262 (49%) respectively. These ions represented fragments containing the acetoxy group as they were shifted to *m/e* 273 (3%), 259 (29%) and 220 (71%) in the free compound. An intense ion at *m/e* 205 observed in the MS of both the free and acetylated compound (63% and 52% respectively) probably comprised rings D and E. The overall MS data suggested that this compound was multiflorenol, a Δ^7 unsaturated pentacyclic triterpene.

The MS obtained for triterpene X (R_f 0.5) showed few intense peaks above *m/e* 150. Molecular ions at *m/e* 428 and 470 were obtained for the free and acetylated compound respectively. Weak ions observed in the MS of the acetylated derivative at *m/e* 427 (M^+-43) and 367 (M^+-Ac + 43) indicated the loss of an isopropyl group from the molecule. This fragmentation is indicative of a saturated triterpene of the lupane series. Intense ions at *m/e* 205 (100%), 191 (70%) and 123 (100%) supported this assignment [7]. From the overall GLC and MS evidence, triterpene X was identified as lupanol. This 3β compound does not appear to have been previously reported in a living organism. Both multiflorenol and 24-methylenecycloartanol have similar GLC R_t's [1] but GC–MS analysis of the triterpene fraction from 24 DFA grains indicated that the enhanced concentration of multiflorenol found at this stage (Table 2) was not due to the presence of 24-methylenecycloartanol.

One possible explanation for the absence of any free pentacyclic triterpenes from the grains of *S. bicolor* and the failure to detect cycloartenol is that cyclization is mediated by protein bound complexes [8]. The transformations involved in the biosynthesis of 4-monomethylsterols from squalene-2,3-oxide may occur in *S. bicolor* utilising triterpene–cyclase or triterpene–carrier protein complexes [9]. If such intermediates were tightly bound, the sterol moiety may be resistant to organic solvent extraction. Further, the pentacyclic triterpenes of *S. bicolor* were extracted solely as their esters and it may be that these compounds are released from analogous squalene-2,3-oxide-protein complexes after cyclization as a result of esterification. They may alternatively be esterified by active esterases immediately upon release from the cyclase. It is notable that 24-methylenepollinastanol was found to be the major component of the tightly bound sterols and the sterol esters of *Musa sapientum* but only a trace was found to occur in the free form [10].

The total 4-demethylsterols of *S. bicolor* grains increased continuously throughout development (Table 2) rising from 258 to 1324 μg/100 grains between 8 and 48 DFA. The highest rate of sterol accumulation (118 μg/day) occurred between 8 and 12 DFA and was accompanied by a high stigmasterol:sitosterol ratio. Between 12 and 24 DFA, total 4-demethylsterols increase by 311 μg/100 grains. Most of this increase (200 μg) appeared to be due to the accumulation of sitosterol. If sitosterol is the major precursor of stigmasterol [11], these results suggest that a marked decrease in the activity of sitosterol C-22, C-23 dehydrogenase takes place after 12 DFA. The steryl glycosides showed a rise and fall throughout the development period (Table 2) similar to that reported previously for *Calendula officinalis* seeds [12]. At 8 DFA campesterol appeared to be the major steryl glycoside (36.6%) but as time progressed, the proportion of campesterol decreased so that by 48 DFA the percentage composition of the steryl glycosides was similar to that observed with the grains of the parent plants. Neither 28-isofucosterol nor its Δ^7 isomer were found as glycosides in developing *S. bicolor* grains. It is possible that these

Table 4. The percentage composition of the free and the esterified 4-demethylsterols of developing *S. bicolor* grains

Free 4-demethylsterols	Age of grains (Days after anthesis) 8	12	24	48
Cholesterol	0.5	0.8	0.7	0.8
Campesterol	26.5	28.3	29.7	26.5
Stigmasterol	27.9	24.7	20.7	18.4
Sitosterol	38.5	40.5	42.7	48.6
28-Isofucosterol	5.7	4.4	5.1	4.4
24-Ethylidene-5α-cholest-7-en-3β-ol	0.9	1.3	1.1	1.3
Proportion of total sterol occurring in the free form	94·2%	96·7%	93·8%	91·3%
Esterified 4-demethylsterols				
Cholesterol	11.9	12.5	4.9	3.7
Campesterol	18.8	23.2	27.0	26.2
Stigmasterol	11.7	13.3	9.2	12.2
Sitosterol	38.7*	39.8*	47.3	48.8
28-Isofucosterol	19.0	11.3	11.1	9.1
24-Ethylidene-5α-cholest-7-en-3β-ol	t	t	t	t
Proportion of total sterol occurring esterified	5.8%	3.3%	6.2%	8.7%

* Includes approximately 8% stigmastanol. t = traces.

sterols may be destroyed or isomerized by the acid conditions of methanolysis [13]. The steryl esters comprised 5.8% of the total sterol at 8 DFA and 8.7% at 48 DFA. At 8 DFA an exceptionally high proportion of the esterified sterol was 28-isofucosterol (19%) compared with the free form (5.7%). Similarly steryl esters contained a higher relative proportion of cholesterol (11.9%) than the free sterol fraction (0.5%). In general, the proportions of free 28-isofucosterol and cholesterol showed little change throughout development but the proportions of these sterols in the ester fractions decreased significantly (Table 4). The marked variation in the percentage composition of the free and esterified sterols in the young grains was also accompanied by a qualitative difference. GLC analysis on QF-1 of the steryl esters from the 8 and 12 DFA grains indicated the presence of approximately 8% stigmastanol [14], but this sterol did not appear to exist in the free form, nor did it appear as a glycoside. Stigmastanol was not found at all in the 24 and 48 DFA grains but in germinating *S. bicolor* grains [15] this sterol re-appeared together with 5α-stigmast-22-en-3β-ol.

EXPERIMENTAL

Grains of *S. bicolor* (Linn.) Moench Pers DC36 purchased from Gunsons Seeds Ltd were sown in pots at 20° and grown under fluorescent lighting until 40 days old. The young plants (about 45 cm tall) were transferred to a glasshouse under natural light conditions. The following dates were recorded:
(a) The date that the inflorescence of each plant emerged (about 180 days after sowing).
(b) The date that all the florets had exserted anthers. This date was designated anthesis and occurred on average 6 days after emergence.

Lipid extraction. Batches of grain (15–25 g fr. wt) were harvested, sorted and dried. Each batch was homogenized and refluxed first in Me_2CO and then in $CHCl_3$–MeOH (2:1). BHT antioxidant (0.005%) was added to all extracting solvents [16].

Column chromatography. Aliquots of lipid were separated on Mallinkrodt SilicAR CC$_7$ silicic acid 200–325 mesh into fractions containing neutral lipid, glycolipid and phospholipid. The neutral lipid was further separated into hydrocarbon, triterpene ester with steryl ester and free sterol fractions on Al_2O_3 columns (Woelm anionotropic, Brockmann grade III). Unsaponifiable lipid was separated into triterpene, 4-monomethylsterol and 4-demethylsterol fractions respectively on Al_2O_3 columns eluted with petrol containing increasing amounts of Et_2O [17].

Digitonin precipitation. The free sterols obtained from Al_2O_3 column chromatography were pptd with 1% digitonin in 60% EtOH and regenerated with DMSO [18]. Triterpenes are incompletely pptd from digitonin [17].

Saponification. Aliquots of total lipid and the steryl ester with triterpene ester fractions were refluxed for 90 min in 8% KOH in 80% MeOH under N_2.

TLC and PLC. This was carried out as described by Palmer and Bowden [2]. The glycolipids were separated on Si gel developed with $CHCl_3$–MeOH–HOAc (65:25:8). Triterpenes were acetylated and separated on Si gel plates impregnated with 10% $AgNO_3$ and developed in pure $CHCl_3$.

Methanolysis. The steryl glycosides were cleaved with 0.65 M methanolic HCl prepared from dry MeOH and acetyl chloride (20:1).

GLC. Glass columns (2 m × 1.5 mm) were used packed with either 3% OV-17, 2.5% QF-1 or 1% SE-33. Operating conditions are given in Table 3. Quantitative determinations were made on OV-17 calibrated with standard cholesterol. The trivial names of the 24α epimers campesterol and sitosterol are used throughout the text but it is now recognised that variable amounts of the 24β epimers may be present in higher plants [19].

GC–MS. Multiflorenylacetate: *m/e* 468 M^+(2), 453(2), 408(3), 262(49), 202(23), 315(3), 301(4), 241(13), 229(18), 218(8), 205(52), 191(10), 189(16), Isoarborinyl acetate: *m/e* 468(M^+–35), 453(100), 408(22), 393(100), 383(4), 315(10), 301(100), 289(10), 241(90), 229(28), 218(10), 205(28), 191(28), 189(25), Lupanyl acetate: *m/e* 470(M^+–4), 455(3), 410(3), 395(31), 427(2), 367(2), 287(28), 274(35), 259(31), 245(17), 231(16); 218(32), 205(100), 191(70), 189(40), 123(100).

Acknowledgements—We thank the University of London for financial support, Drs L. J. Goad for triterpene standards and

A. Macleod and A. Wilkinson for GC–MS determined at Queen Elizabeth College. We also thank the curator Mr. A. Patterson and the staff of the Chelsea Physic Garden for help with the cultivation of *S. bicolor*.

REFERENCES

1. Palmer, M. A. and Bowden, B. N. (1975) *Phytochemistry* **14**, 2049.
2. Palmer, M. A. and Bowden, B. N. (1975) *Phytochemistry* **14**, 1813.
3. Baisted, D. J. (1971) *Biochem. J.* **124**, 375.
4. Ohmoto, T., Ikuse, M. and Natori, S. (1970) *Phytochemistry* **9**, 2137.
5. Grunwald, C. (1975) *Ann. Rev. Plant. Physiol.* **26**, 209.
6. Karliner, J. and Djerassi, C. (1966) *J. Am. Chem. Soc.* **88**, 1945.
7. Budzikiewicz, H., Wilson, J. M. and Djerassi, C. (1963) *J. Am. Chem. Soc.* **85**, 3688.
8. Rees, H. H., Goad, L. J. and Goodwin, T. W. (1968) *Biochem. J.* **107**, 417.
9. Lenton, J. R., Hall, J., Smith, A. R. H., Ghisalberti, E. L., Rees, H. H., Goad, L. J. and Goodwin, T. W. (1971) *Arch. Biochem. Biophys.* **143**, 664.
10. Knapp, F. F., Phillips, D. O., Goad, L. J. and Goodwin, T. W. (1972) *Phytochemistry* **11**, 3497.
11. Bennett, R. D., Heftmann, E., Preston, W. H. Jr. and Haun, J. R. (1963) *Arch. Biochem. Biophys.* **103**, 74.
12. Kasprzyk, Z., Pyrek, J. and Turowska, G. (1968) *Acta. Biochim. Polon.* **15**, 149.
13. Palmer, M. A. (1975) Ph. D. thesis, University of London.
14. Kemp, R. and Mercer, E. I. (1968) *Biochem. J.* **110**, 111.
15. Palmer, M. A. and Bowden, B. N. In press.
16. Wren, J. and Szczepanowska, A. (1964) *J. Chromatog.* **14**, 405.
17. Goad, L. J. and Goodwin, T. W. (1966) *Biochem. J.* **99**, 735.
18. Issidorides, C. H., Kitagawa, I. and Mosettig, E. (1962) *J. Org. Chem.* **27**, 4693.
19. Nes, W. R., Krevitz and Behzadan, S. (1976) *Lipids* **11**, 118.