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INVESTIGATIONS ON THE Δ^{23}-, $\Delta^{24(28)}$- and Δ^{25}-STEROLS OF *ZEA MAYS*

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Key Word Index—*Zea mays*, Gramineae, sterols, sterol biosynthesis, cyclolaudenol, cyclosadol, 24-methylenecycloartanol, 24-methylcholesta-5,*E*-23-dien-3β-ol, 24-methylcholesta-5,24(28)-dien-3β-ol, 24-methylcholesta-5,25-dien-3β-ol, 24-methylcholest-5-en-3β-ol, 24-ethylcholest-5-en-3β-ol

Abstract—The sterols of *Zea mays* shoots were isolated and characterized by TLC, HPLC, GC/MS and ^{1}H NMR techniques In all, 22 4-demethyl sterols were identified and they included trace amounts of the Δ^{23}-, Δ^{24}- and Δ^{25}-sterols, 24-methylcholesta-5,*E*-23-dien-3β-ol, 24-methylcholesta-5,*Z*-23-dien-3β-ol, 24-methylcholesta-5,25-dien-3β-ol, 24-ethylcholesta-5,25-dien-3β-ol and 24-ethylcholesta-5,24-dien-3β-ol In the 4,4-dimethyl sterol fraction, cycloartenol and 24-methylenecycloartanol were the major sterol components but small amounts of the Δ^{23}-compound, cyclosadol, and the Δ^{25}-compound, cyclolaudenol, were recognized These various Δ^{23}- and Δ^{25}-sterols may have some importance in alternative biosynthetic routes to the major sterols, particularly the 24β-methylcholest-5-en-3β-ol component of the C_{28}-sterols Radioactivity from both [2-^{14}C]MVA and [methyl-^{14}C]methionine was incorporated by *Z mays* shoots into the sterol mixture Although 24-methylene and 24-ethylidene sterols were relatively highly labelled, the various Δ^{23}- and Δ^{25}-sterols contained much lower levels of radioactivity, which is possibly indicative of their participation in alternative sterol biosynthetic routes (24*R*)-24-Ethylcholest-5-en-3β-ol (sitosterol) had a significantly higher specific activity than the 24-methylcholest-5-en-3β-ol indicating that the former is synthesized at a faster rate

INTRODUCTION

The sterol compositions of *Zea mays* seeds, coleoptiles and shoots have been extensively studied [1–11] 24α-Ethylcholest-5-en-3β-ol (**1a**, sitosterol)* is the major component while the 24-methylcholesterol (campesterol) is now recognized to be a mixture of 24α-methylcholest-5-en-3β-ol (**2a**) and 24β-methylcholest-5-en-3β-ol (**3a**) [12–14], a situation which occurs in other plants [12, 15] The 24α-methylcholest-5-en-3β-ol (**2a**) component is believed [14, 16, 17] to be synthesized by a sequence (Scheme 1) involving isomerization of a 24-methylene sterol intermediate (e g **4a**) to a Δ^{24}-compound (**5a**) followed by stereospecific reduction to the 24α-methyl sterol (**2a**) In a similar way, the 24α-ethyl sterol (**1a**) is envisaged to arise from 24-ethylcholesta-5,*Z*-24(28)-dien-3β-ol (**6a**, isofucosterol) via 24-ethylcholesta-5,24-dien-3β-ol (**7a**) [16–20]

Evidence for an involvement of Δ^{24}-sterols (**5a** and **7a**) in 24α-alkyl sterol biosynthesis has been provided by the labelling patterns of phytosterols obtained after incorporation of [2-^{14}C, (4*R*)4-$^3H_1$]mevalonic acid [14, 18–21], [24-^{3}H]lanosterol [17] and [24-^{3}H]cycloartenol [22] Also, several Δ^{24}-sterols have been identified in various plants [23–25] However, convincing evidence from labelling studies showing the formation of Δ^{24}-sterols or their reduction to 24α-alkyl sterols remains elusive [20]

The 24β-methyl sterols of the algae from the order Chlorococcales are synthesized via a 25-methylene intermediate (Scheme 1) [16] and it was suggested [17, 25] that the 24β-methyl sterols found in some higher plants might also arise from a 24β-methyl-Δ^{25}-sterol such as cyclolaudenol (**8g**) [26]

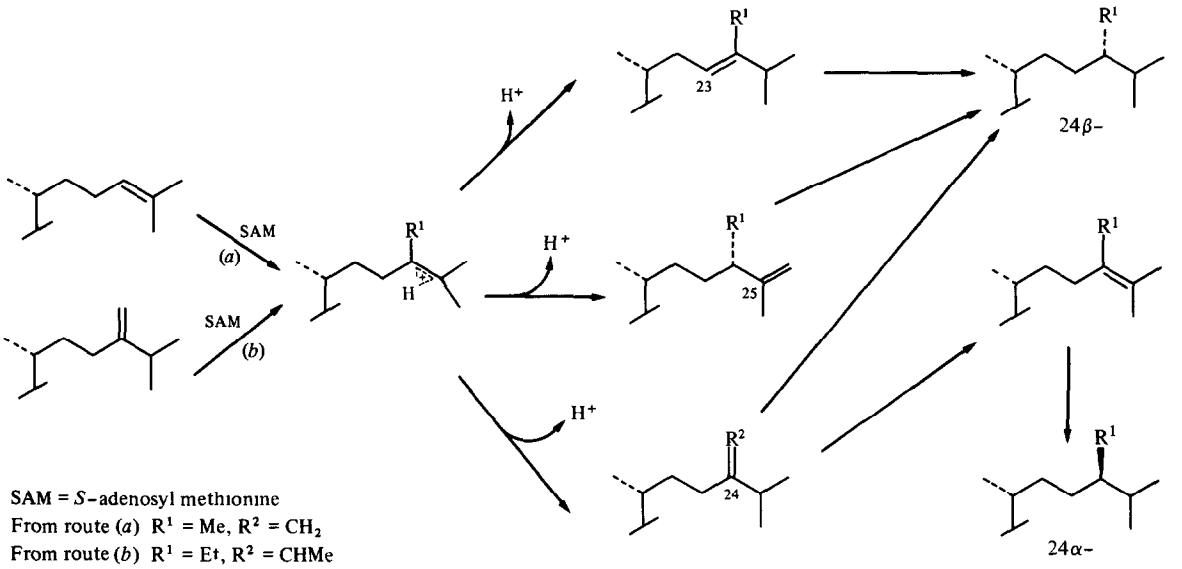
Recent investigations [10, 11] of the sterols of *Z mays* seeds and coleoptiles have resulted in the identification of a series of Δ^{23}-sterols including 24-methylcholesta-5,23-dien-3β-ol (**9a**) and cyclosadol (**9g**). This led to the suggestion [10, 13] that a Δ^{23}-sterol might be the immediate precursor to the 24β-methyl sterol (Scheme 1) In support of this suggestion, incubation of *Z mays* coleoptile microsomes in the presence of cycloartenol (**10g**) and [methyl-^{14}C]-*S*-adenosyl methionine gave labelled 24-methylenecycloartanol (**4g**) and cyclosadol (**9g**) [13] However, no trace of labelled cyclolaudenol (**8g**) was detected, nor were any other Δ^{25}-sterols identified in the coleoptile sterol mixture, and it was concluded [13] that 24β-methyl sterol biosynthesis in maize coleoptiles proceeds by the Δ^{23}-intermediate pathway Concurrently with the studies of Benveniste [13] we were also investigating the origins of the 24α- and 24β-methyl sterols of *Z mays* shoots [14, 27] We now report the identification of trace amounts of Δ^{23}- and Δ^{25}-sterols, and describe studies on their labelling from [2-^{14}C]mevalonic acid (MVA) and [methyl-^{14}C]methionine

RESULTS AND DISCUSSION

Sterol composition of Z mays *shoots*

The 4-demethyl sterols were isolated from 9-day-old maize shoots, acetylated and separated into eight bands by silver nitrate-silica gel TLC (see Experimental) GC/MS

*The C-24 configuration of sterols will be assigned, where known, as 24α- and 24β- in this paper, 24α- corresponds to 24*R*- and 24β- to 24*S*-



Scheme 1 Postulated routes for the formation of the side chains of 24α- and 24β-alkyl sterols

examination of these fractions permitted the identification of the sterols listed in Table 1 Many of the sterols have been reported previously as constituents of *Z mays* tissues [1–11] Several 5α-stanols were detected as minor constituents of the sterol mixture (Table 1) Stanols do not appear to be of very common occurrence in higher plants but they have been reported previously in maize seedlings [2]

Evidence was obtained for two Δ^{25}-sterols which were identified as 24-methylcholesta-5,25-dien-3β-ol (**8a**) and 24-ethylcholesta-5,25-dien-3β-ol (**12a**) The acetate of the former sterol (**8b**) was in band 2 from the silver nitrate-silica gel TLC, which also contained 24-methyl-5α-cholesta-7,24(28)-dien-3β-yl acetate (**4d**) This band ran slightly ahead of 24-methylcholesta-5,24(28)-dien-3β-yl acetate (**4b**) The mass spectra of **4b** and **8b** were very similar but the fragmentation peak at *m/z* 296, due to the loss of part of the side chain, was prominent (relative intensity 39%) in the mass spectrum of **4b** However, this ion was considerably weaker (relative intensity 11%) in the spectrum of **8b** as was the case with an authentic sample of this compound (codisteryl acetate [28]) These facts permit the identification of **8b** but the C-24 configuration remains unassigned due to lack of pure material for further investigation Sterol **8b**, with the 24β-configuration, has previously been reported as a constituent of the alga *Codium fragile* [28]

The acetate of the other Δ^{25}-sterol (**12b**) was in band 3, which also contained **9b** and **6b** Compound **12b** was obtained from this mixture by reverse-phase HPLC The mass spectra of **12b** and of the derived free sterol **12a** and the TMSi-ether of **12a** were all in agreement with its identification as 24-ethylcholesta-5,25-dien-3β-ol (**12a**) while the melting point of **12b** suggested that it was the 24β-isomer, clerosteryl acetate [29,30], although this could not be verified by ^{1}H NMR due to lack of sample Sterol **12a** was first identified in some species of the Verbenaceae and Cucurbitaceae [29,30] but it has also been reported in *Calendula officinalis* [31], *Brassica napus* [32] and *Kalanchoe diagremontiana* [15] indicating that it may be more widespread in higher plants than hitherto suspected We have previously speculated [14] that the *Z mays* 24α-ethyl sterol (**1a**) might be accompanied by a small amount of its 24β-ethyl epimer (clionasterol) on the basis of the ^{3}H ^{14}C atomic ratio obtained after incubation of *Z mays* shoots with [2-^{14}C, (4*R*)4-$^3H_1$]MVA Sterol **12a** could be a putative precursor of the 24β-ethylcholest-5-en-3β-ol

The HPLC separation of the steryl acetates in the above fraction also provided a compound identified as 24-methylcholesta-5,*E*-23-dien-3β-yl acetate (**9b**) by the appearance of a characteristic fragmentation ion at *m/z* 283 in its mass spectrum This ion arises by cleavage of the C-20, C-22 bond in Δ^{23}-sterols [10] Sterol **9a** was first reported as a constituent of *Z mays* coleoptiles by Scheid and Benveniste [10] and it was assumed [11,13] to have the *E*-23-configuration from a comparison of its ^{1}H NMR spectrum with that of synthetic 4,4,14α-trimethylcyclo-5α-ergost-*E*-23-dien-3β-yl acetate (**9h**), and on the basis of GLC retention time data [11] We have now confirmed that **9b** (mp 126–128 5°) from *Z mays* does indeed have the *E*-23-configuration by direct comparison with synthetic samples of 24-methylcholesta-5,*E*-23-dien-3β-yl acetate (**9b**, mp 127–128°) and 24-methylcholesta-5,*Z*-23-dien-3β-yl acetate (**13b**, mp 119–125°) These compounds were prepared by iodine isomerization of 24-methylcholesta-5,24(28)-dien-3β-yl acetate (**4b**) and purified by silver nitrate-silica gel TLC and HPLC The *E*- and *Z*-isomers were easily differentiated by their 400 MHz ^{1}H NMR spectra (Table 2) as predicted by Itoh *et al* [11] from their study of ^{1}H NMR spectra of cyclosadyl acetate (**9h**) and the model compounds 3,4-dimethyl-*E*-2-pentene and its *Z*-isomer The most significant differences between the spectra of **9b** and **13b** are in the chemical shifts for H-23 and H-25 but diagnostically significant differences can also be noted for the H-18, H-21, H-26 and H-27, and H-28 signals Similar ^{1}H NMR spectra have recently been reported for the synthetic free sterols **9a** and **13a** [33] Compound **9b** obtained from *Z mays* had an identical ^{1}H NMR spectrum to synthetic **9b** (Table 2) thus confirming

Table 1 The demethyl sterol composition of *Z mays* shoots

Sterol	Amount (μg/g fr wt)	Composition (%)
Cholest-5-en-3β-ol (**11a**)	0 88	0 53
5α-Cholestan-3β-ol (**11e**)	0 04	0 02
24-Methylcholest-5-en-3β-ol(**2a** and **3a**)*	25 69	15 61
24-Methyl-5α-cholestan-3β-ol (**2e**)†	0 83	0 50
24-Methylcholesta-5,22-dien-3β-ol (**15a**)†	0 61	0 37
24-Methylcholesta-5,24(28)-dien-3β-ol (**4a**)	4 35	2 64
24-Methyl-5α-cholesta-7,24(28)-dien-3β-ol (**4c**)	0 86	0 52
24-Methylcholesta-5,*E*-23-dien-3β-ol (**9a**)	3 46	2 10
24-Methyl-5α-cholesta-7,*E*-23-dien-3β-ol (**9c**)	0 50	0 30
24-Methylcholesta-5,25-dien-3β-ol (**8a**)†	0 74	0 45
24-Methylcholest-7-en-3β-ol (**2c**)†	trace	trace
24-Ethylcholest-5-en-3β-ol (**1a**)‡	64 29	39 06
24-Ethyl-5α-cholestan-3β-ol (**1e**)†	2 00	1 22
24-Ethyl-5α-cholest-22-en-3β-ol (**16e**)†	0 45	0 27
24-Ethyl-5α-cholest-7-en-3β-ol (**1c**)†	0 45	0 27
24-Ethylcholesta-5,22-dien-3β-ol (**16a**)‡	45 35	27 55
24-Ethylcholesta-5,*Z*-24(28)-dien-3β-ol (**6a**)	10 79	6 55
24-Ethyl-5α-cholesta-7,*Z*-24(28)-dien-3β-ol (**6c**)	1 51	0 92
24-Ethylcholesta-5,25-dien-3β-ol (**12a**)†	0 98	0 60
24-Ethylcholesta-5,24-dien-3β-ol (**7a**)	0 31	0 19
Other sterols, possibly including 24-methylcholesta-5,*Z*-23-dien-3β-ol (**13a**) and 24-ethylcholesta-5,*E*-24(28)-dien-3β-ol (**14a**)	0 51	0 31

*Shown by its ^{1}H NMR spectrum to be a mixture of the 24α- and 24β-methyl epimers in the approximate ratio 3 7 [14]

†The C-24 configuration of these sterols could not be determined due to insufficient pure material for ^{1}H NMR analysis

‡Shown by ^{1}H NMR spectroscopy to be predominantly the 24α-ethyl epimer [13, 14]

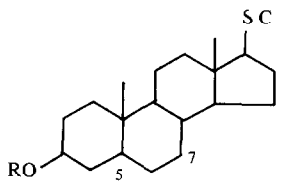
Table 2 ^{1}H NMR spectral data for synthetic compounds **9b** and **13b**

	H-18	H-19	H-21	H-26 and H-27	H-28	H-25	H-23	H-6
24-Methylcholsta-5,*E*-23-dien-3β-yl acetate (**9b**)	0 683*s*	1 017*s*	0 887*d**	0 896*d*†	1 543*s*	2 234*septet*†	5 136*t*	5 376*d*
24-Methylcholesta-5,*Z*-23-dien-3β-yl acetate (**13b**)	0 679*s*	1 017*s*	0 902*d*	0 948*d*	1 603*s*	2 788*septet*	5 028*t*	5 376*d*

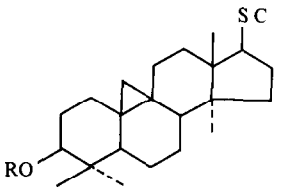
*J = 6 5 Hz
†J = 7 0 Hz
‡J = 7 0 Hz

the identification of 24-methylcholesta-5,*E*-23-dien-3β-ol (**9a**) We noted that although synthetic **9b** and **13b** could be separated by reverse-phase HPLC, they did not show any significant separation by GLC on Hi-EFF8B, OV-1 or OV-17 stationary phases Thus, the use of GLC data alone does not appear to provide adequate evidence for assigning the configuration of Δ^{23}-sterols (cf ref [11])

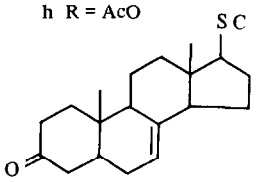
A trace component chromatographing on silver nitrate-silica gel in band 4 (i e less polar than **9b**) had a similar R_f and mass spectrum to synthetic 24-methylcholesta-5,*Z*-22-dien-3β-yl acetate (**13b**) The three other steryl acetates in band 4 had RR_fs and mass spectra allowing their tentative identifications as 24-methyl-5α-cholesta-7,23-dien-3β-yl acetate (**9d**), 24-ethylcholesta-5,*E*-24(28)-dien-3β-yl acetate (**14b**, fucosteryl acetate) and 24-ethyl-5α-cholesta-7,*Z*-24(28)-dien-3β-yl acetate (**6d**) Compounds **6c** and **9c** have been reported previously in *Z mays* [10, 13] and fucosterol (**14a**) was demonstrated as a constituent of some Solanaceae seed oils and rice bran oil [34] Confirmation of the side-chain double bond configurations of **9d**, **13b** and **14b** required ^{1}H NMR spectral analysis but this was not possible because the small amounts available prevented their further purification However, it now seems reasonable to assume that **9d** had the 23-*E*-configuration as observed for **9b** and the other Δ^{23}-sterols of *Z mays* [11] Sterols **13a** and **14a** may be natural products of *Z mays* produced in trace amounts during the sterol side-chain alkylation reactions However, it is also possible that **13b** and **14b** are artefacts since it has been observed that fucosteryl acetate (**14b**) can



a R = H, Δ^5
b R = AcO, Δ^5
c R = H, Δ^7
d R = AcO, Δ^7
e R = H
f R = AcO



g R = H
h R = AcO



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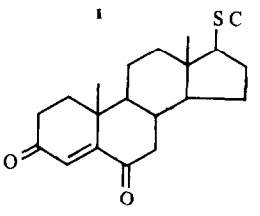

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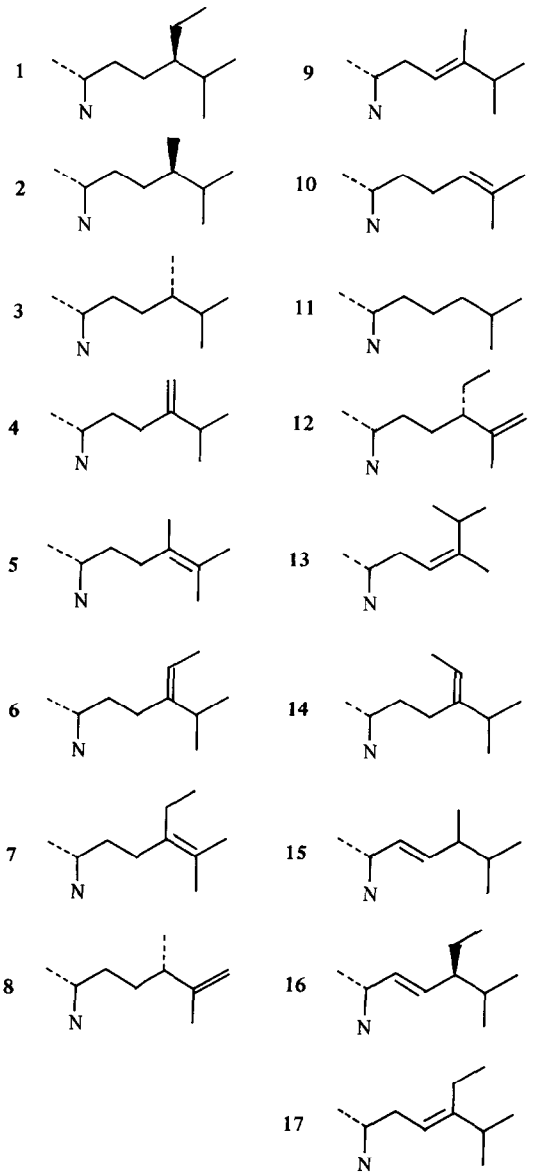

be formed by isomerization of isofucosteryl acetate (**6b**) during silver nitrate-silica gel TLC [32]

Two other very minor constituents in *Z mays* shoots provisionally identified from the mass spectra of the acetates were 24-methylcholesta-5,22-dien-3β-ol (**15a**) and 24-ethylcholesta-5,24-dien-3β-ol (**7a**) The latter sterol is significant due to its implication in 24α-ethyl sterol (**1a** and **16a**) biosynthesis It is suggested [19] that **7a** arises by isomerization of isofucosterol (**6a**) The very low concentration of **7a** compared to **6a** implies that the equilibrium of this isomerization must favour **6a** and/or that **7a** may be rapidly reduced to yield **1a** Such a situation may account for the failure to obtain significant labelling in **7a** when *Z mays* shoots were incubated with labelled MVA in both the present study and a previous investigation [20]

The 4,4-dimethyl sterols were acetylated and separated by silver nitrate-silica gel TLC (see Experimental) to yield five bands which were eluted and their constituents identified by GC/MS (Table 3) With the exception of cyclolaudenol (**8g**), the other compounds have been reported previously in *Z mays* [1–3, 11, 13] Cyclolaudenyl acetate (**8h**) and 24-methylenecycloartanyl acetate (**4h**) have very similar GLC retention times and mass spectra and are consequently difficult to differentiate when present as minor constituents in a mixture They also tend to co-chromatograph on silver nitrate-silica gel TLC with several solvent systems However, we observed that authentic **8h** ran slightly ahead of **4h** if freshly distilled, ethanol-free chloroform with 2% diethyl ether was employed Using this TLC system the *Z mays* 4,4-dimethyl steryl acetates yielded a minor component with the same R_f as authentic cyclolaudenyl acetate (**8h**) The GLC, RR_t and mass spectrum of this material agreed with the corresponding data for authentic **8h** and thus permitted the identification of cyclolaudenol (**8g**) as a trace constituent of *Z mays* The C-24 configuration could not be determined in this instance but a 24β-methyl has been

Table 3 The 4,4-dimethyl sterol and triterpene composition of *Z mays* shoots

Compound	Amount (μg/g fr wt)	Composition (%)
α-Amyrin	3 31	46 3
β-Amyrin	1 85	25 9
Cyclosadol (**9g**)	0 08	1 1
Cycloartenol (**10g**)	0 82	11 5
Cyclolaudenol (**8g**)	0 17	2 4
24-Methylenecycloartanol (**4g**)	0 92	12 9

assigned to this isolated from other sources [35, 36]

The incorporation of [2-^{14}C]*mevalonic acid and* [*methyl*-^{14}C]*methionine into the sterols of* Z mays *shoots*

To obtain further evidence for the production of Δ^{23}- and Δ^{25}-sterols in maize, the incorporation of [2-^{14}C]MVA and [methyl-^{14}C]methionine into these compounds was investigated after allowing maize shoots to imbibe solutions of these labelled substrates (see Experimental) The labelled non-saponifiable lipids recovered from each incubation were fractionated by preparative silica gel TLC to give the 4,4-dimethyl sterols and 4-demethyl sterols which were then acetylated, mixed with the appropriate steryl acetate carriers, and separated by TLC on silver nitrate-silica gel to give the constituent bands indicated in Tables 4 and 5

As found in previous studies, the cycloartenyl acetate (**10h**) and 24-methylenecycloartanyl acetate (**4h**) were extensively labelled from [2-^{14}C]MVA (Table 4) However, a small but significant amount of radioactivity also co-chromatographed with both cyclosadyl acetate (**9h**) and cyclolaudenyl acetate (**8h**) With [methyl-^{14}C]methionine as the substrate most of the radioactivity was associated with 24-methylenecycloartanyl acetate (**10h**) but small amounts of radioactivity were again present in cyclolaudenyl acetate (**8h**) and in cyclosadyl acetate (**9h**) obtained after further purification by HPLC (Table 4) It therefore appears from this evidence that, although Δ^{23}- and Δ^{25}-sterols (**9g** and **8g**) are produced at the first transmethylation step, the favoured product is the $\Delta^{24(28)}$-sterol (**4g**) and this has been confirmed with *Z mays* cell-free preparations [13, 27]

The 4-demethyl sterols were acetylated, carrier steryl acetates added and the mixture was submitted to preparative TLC on silver nitrate-silica gel to yield eight radioactive fractions (Table 5) The most polar band contained only 24-methylcholesta-5,24(28)-dien-3β-yl acetate (**4b**) which was apparently labelled from both precursors Band 2 was a mixture of 24-methylcholesta-5,25-dien-3β-yl acetate (**8b**) and 24-methyl-5α-cholesta-7,24(28)-dien-3β-yl acetate (**4d**) The latter compound was anticipated to be labelled as it is the likely precursor of **4b** In order to determine if **8b** was labelled, additional carrier **4d** and **8b** were added to the radioactive material and the acetates hydrolysed The recovered free sterols **4c** and **8a** were then oxidized to yield 24-methyl-5α-cholesta-7,24(28)-dien-3-one (**4i**) and 24-methylcholesta-4,25-dien-3,6-dione (**8j**), respectively With both the [2-^{14}C]MVA and the [methyl-^{14}C]methionine labelled samples, product **4i** retained the larger proportion of the recovered radioactivity thus revealing that sterol **4c** was the major labelled component of the band 2 material However, radioactivity also accompanied **8j** thus indicating low incorporation of the precursors into the Δ^{25}-sterol **8a**

The mixture of steryl acetates (**6b**, **9b** and **12b**) in band 3 (Table 5) could not be resolved by TLC Since it was

Table 4 Incorporation of [2-^{14}C]MVA and [methyl-^{14}C]methionine into the 4,4-dimethyl sterols of *Z mays*

Band	Component	Incorporation (dpm) [2-^{14}C]MVA	Incorporation (dpm) [Methyl-^{14}C]methionine
1	α-, β-Amyrin acetates	18 900	
2	Cyclosadyl acetate (**9h**)	8710(3550)*	170†
3	Cycloartenyl acetate (**10h**)	65 560	—
4	Cyclolaudenyl acetate (**8h**)	6210(1610)*	670
5	24-Methylenecycloartanyl acetate (**4h**)	36 550	9460

The labelled 4,4-dimethyl sterols were acetylated, carrier **4h**, **8h**, **9h** and **10h** added (1 0 mg of each), and the mixture was separated by TLC on silver nitrate-silica gel

*Dpm accompanying **9h** and **8h**, respectively, after a second purification on silver nitrate-silica gel TLC

†Dpm recovered after a further purification on reverse-phase HPLC (Lichrosorb RP-18, MeOH at 1 5 ml/min)

Table 5 Incorporation of [2-^{14}C]MVA and [methyl-^{14}C]methionine into the 4-demethyl sterols of *Z mays*

Band	Components*	Incorporation (dpm) [2-^{14}C]MVA	Incorporation (dpm) [Methyl-^{14}C]methionine
1	24-Methylcholesta-5,24(28)-dien-3β-yl acetate (**4b**)	26010	37770
2	24-Methylcholesta-5,25-dien-3β-yl acetate (**8b**) 24-Methyl-5α-cholesta-7,24(28)-dien-3β-yl acetate (**4d**)	6380	9810
3	24-Methylcholesta-5,*E*-23-dien-3β-yl acetate (**9b**) 24-Ethylcholesta-5,25-dien-3β-yl acetate (**12b**) 24-Ethylcholesta-5,*Z*-24(28)-dien-3β-yl acetate (**6b**)	33000	135050
4	24-Ethyl-5α-cholesta-7,*Z*-24(28)-dien-3β-yl acetate (**6d**)	9800	27600
5	24-Methylcholesta-5,24-dien-3β-yl acetate (**5b**) 24-Ethylcholesta-5,24-dien-3β-yl acetate (**7b**)	4970	4640
6	24-Ethylcholesta-5,22-dien-3β-yl acetate (**16b**)	7990	—
7	24-Methylcholest-5-en-3β-yl acetate (**2b** and **3b**) 24-Ethylcholest-5-en-3β-yl acetate (**1b**)	85450	218070†
8	24-Methyl-5α-cholestan-3β-yl acetate (**2f**) 24-Ethyl-5α-cholestan-3β-yl acetate (**1f**)	9500	—

The labelled 4-demethyl sterols were acetylated, carrier **4b**–**9b** and **12b** added and the mixture was separated by silver nitrate-silica gel TLC as described in the Experimental

*Composition checked by GLC analysis (OV-17)

†This included **16b** which was eluted together with **2b** and **1b**

important to establish that the Δ^{23}- (**9b**) and Δ^{25}- (**12b**) compounds were labelled from the radioactive precursors, the application of HPLC was examined for the separation of these compounds It was found that the steryl acetates were well resolved on a reverse-phase HPLC column and they were eluted in the order **9b**, **12b** and **6b** A sample of the labelled band 3 material from the [methyl-^{14}C]methionine incubation was separated by HPLC with collection of samples (2 ml) which were assayed for radioactivity A histogram plot of the recovered radioactivity in each fraction showed that small, but discrete, radioactive peaks coincided with the mass peaks of **9b** and **12b** but the bulk of the radioactivity coincided with the mass peak for **6b**

Further preparative HPLC analysis of the band 3 samples from both the [2-^{14}C]MVA and the [methyl-^{14}C]methionine incubations with collection of the three steryl acetate constituents showed that in each case small but significant amounts of radioactivity were associated with the purified 24-methylcholesta-5,*E*-23-dien-3β-yl acetate (**9b**) and 24-ethylcholesta-5,25-dien-3β-yl acetate (**12b**) thus substantiating the production of these Δ^{23}- and Δ^{25}-sterols in *Z mays* shoots The majority of the recovered radioactivity was in the 24-ethylcholesta-5,*Z*-24(28)-dien-3β-yl acetate (**6b**), which agrees with previous reports [13, 17, 19, 20, 22] of the labelling of sterol **6a** after incubation of maize and other plants with radioactive precursors

The radioactivity in band 4 co-chromatographed with 24-ethyl-5α-cholesta-7,*Z*-24(28)-dien-3β-yl acetate (**6d**) on both silver nitrate-silica gel TLC and on reverse-phase HPLC and indeed **6c** is a constituent of the *Z mays* sterols (Table 1) However, we have observed that synthetic 24-ethylcholesta-5,*E*-23-dien-3β-yl acetate (**17b**) co-chromatographs with **6b** in both the TLC and HPLC systems used in this work Although **17a** was not detected as a natural constituent of the *Z mays* sterols, it can be envisaged that this compound may be formed during the second transmethylation reaction leading to the 24-ethyl sterols in a manner analogous to the formation of **9a** in the first transmethylation reaction (see Scheme 1) To investigate the possible formation of **17a**, carrier **6d** and **17b** were added to the radioactive sample containing **6d** which was obtained after HPLC of the band 4 sample from the [methyl-^{14}C]methionine incubation The steryl acetates (12700 dpm) were saponified and the recovered free sterols oxidized to yield 24-ethyl-5α-cholesta-7,*Z*-24(28)-dien-3-one (**6i**, 4170 dpm) produced from **6c** and 24-ethylcholesta-4,23-dien-3,6-dione (**17j**, 730 dpm) obtained from **17a** The presence of the bulk of the recovered radioactivity in **6i** was in accord with the anticipated labelling of **6c**, which is a presumed precursor of **6a**, and hence of 24α-ethylcholest-5-en-3β-ol (**1a**) The recovery of a small amount of radioactivity co-chromatographing with **17j** suggests that 24-ethylcholesta-5,*E*-23-dien-3β-ol (**17a**) may indeed be a natural product but at levels which were too low to permit its recognition in the present GC/MS analysis of the *Z mays* sterols However, 24-ethyl-Δ^{23}-sterols have previously been reported as constituents of a sponge [37] and the euglenid alga *Eutreptia viridis* [38]

The recovery of radioactivity in band 5 indicated the possible labelling of the C_{28}- and C_{29}-Δ^{24}-steryl acetates (**5b** and **7b**) which chromatographed in this band Since compounds **5a** and **7a** are considered to play a key role in 24α-alkyl sterol synthesis, their labelling was investigated by further purification of compounds **5b** and **7b** by HPLC The radioactivity recovered in **5b** and **7b**, from both the [2-^{14}C]MVA and the [methyl-^{14}C]methionine labelled samples, was very low as found in a previous study [20] However, in the examination of the maize shoot sterols (Table 1) no evidence was found for **5a** while only a trace amount of **7a** was identified Thus, although the total radioactivity accumulated in these sterols was very low, they may have had specific activities comparable to some of the more abundant labelled sterols The Δ^{24}-

sterols **5a** and **7a** may therefore be transient intermediates formed by isomerization of $\Delta^{24(28)}$-sterols **4a** and **6a**, respectively, prior to rapid reduction to **2a** and **1a**, respectively

The 24-ethylcholesta-5,22-dien-3β-yl acetate (**16b**) recovered from the incubations with both [2-^{14}C]MVA and [methyl-^{14}C]methionine had a considerably lower specific activity than the 24-ethylcholest-5-en-3β-yl acetate (**1b**) in accord with the reported precursor–product relationship of **1a** and **16a** [39, 40]

The steryl acetates in band 7 from the [2-^{14}C]MVA incubation were a mixture of 24-methylcholest-5-en-3β-yl acetate (**2b** and **3b**) and stigmast-5-en-3β-yl acetate (**1b**) in the approximate ratio 1 2 5 To determine if the C_{28}-and C_{29}-sterols incorporated radioactivity from [2-^{14}C]MVA in proportion to their respective masses in the mixture, the steryl acetates of band 7 were separated by reverse-phase HPLC The ratio of radioactivity recovered in the C_{28}-steryl acetate (**2b** and **3b**, 12 070 dpm) compared to the radioactivity in the C_{29}-steryl acetate (**1b**, 38 950 dpm) was 1 3 2 Thus it appears that the C_{29}-sterol (**1b**) had a somewhat higher specific activity than the C_{28}-sterol (**2b** and **3b**), and the specific activity ratio for **2b** + **3b** **1b** was approximately 1 1 3 This indicates that the C_{29}-sterol (**1a**) was synthesized at a somewhat faster rate than the C_{28}-sterol (**2a** + **3a**) under the particular conditions employed A similar conclusion followed from the analysis of the radioactivity incorporated into the sterols when [methyl-^{14}C]methionine was the precursor (see *Experimental* for details) The C_{28}-sterol (**2b** + **3b**) had a specific activity of 9460 dpm/mg while the C_{29}-sterol (**1b**) had a specific activity of 25 600 dpm/mg When a correction is made to allow for the incorporation of two ^{14}C atoms due to the transfer of two methyl groups from [methyl-^{14}C]methionine into **1b** compared with only one ^{14}C atom incorporated into **2b** + **3b**, then it is apparent that the C_{28}- and C_{29}-sterols were synthesized in the ratio 1 1 4

The apparently slower rate of synthesis of C_{28}-sterol (**2a** + **3a**) compared to C_{29}-sterol (**1a**) may be correlated with the extent of labelling of the various precursors of these compounds The 24-methylene sterols (**4b** and **4h**) were comparatively highly labelled as were the 24-ethylidene sterols (**6b** and **6d**) These facts suggest that the major portion of the 24-methylene sterol produced in the first transmethylation is utilized as the substrate for the second transmethylation to yield 24-ethylidene sterols which are the precursors to the 24α-ethyl sterols (**1a** and **16a**)

The 24-methyl sterol of *Z mays* shoots is a 3 7 mixture of the 24α- and 24β-epimers (**2a** and **3a**, respectively) [14] The former compound (**2a**) is thought to be produced from a 24-methylene sterol precursor [14, 17] However, the isomer **3a** has been suggested to arise by reduction of either a Δ^{23}-sterol [13] or a Δ^{25}-sterol [14, 17, 26] The identification of trace amounts of Δ^{23}- and Δ^{25}-sterols in maize is compatible with both routes operating in 24β-methyl sterol production in this plant Moreover, the low incorporation of radioactivity from precursors into these compounds indicates a slow rate of synthesis of these sterols and hence of the final 24β-methyl sterol product (**3a**), as was observed However, the stereospecific reduction of a 24-methylene sterol precursor to yield 24β-methyl sterol, as occurs in yeast and some algae [16], cannot be excluded with the evidence currently available It seems that an unequivocal decision on the relative importance of the various pathways involving $\Delta^{24(28)}$-, Δ^{23}- and Δ^{25}-sterols in 24β-methyl sterol production, and indeed the role of Δ^{24}-sterols in 24α-alkyl sterol synthesis, must now await the use of plant cell-free preparations capable of catalysing the postulated sterol interconversions shown in Scheme 1

EXPERIMENTAL

All mps were taken on a Reichert hot stage apparatus and are uncorr TLC was on silica gel G or 10% $AgNO_3$-silica gel G, compounds were located by viewing under UV after spraying with 0 005% beberine–HCl in EtOH, for preparative work compounds were eluted from the silica gel with Et_2O HPLC was performed using an Altex model 110A pump, peaks were detected by UV at 206 or 210 nm, the columns (250 × 4 6 mm) used were Lichrosorb RP18 and Ultrasphere-ODS GC/MS was on a VG 70–70 F coupled to a Finnegan 2400 Incos Data System, 3% OV-17 or 3% OV-1 columns at 270–280° were routinely used for GC ^{1}H NMR spectra were determined in $CDCl_3$ with TMS as the reference compound by either Dr B E Mann, Department of Chemistry, University of Sheffield or by the Department of Chemistry, University of Liverpool Scintillation counting employed 0 7% butyl-PBD in toluene as the counting cocktail

Isolation of sterols Zea mays (Caldera 535) seeds were germinated in trays on moist cotton wool and paper for 8–9 days and the shoots excised at their base The shoots (645 g) were exhaustively extracted with Me_2CO to yield the total lipid, which was hydrolysed by reflux with 8% KOH in aq 85% EtOH to yield the non-saponifiable lipid (274 mg) The non-saponifiable lipid was separated by prep *TLC* (silica gel, *CHCl$_3$–EtOH*, 49 1) to give the 4,4-dimethyl sterols (12 8 mg), 4-monomethyl sterols (10 5 mg) and 4-demethyl sterols (125 8 mg)

Analysis of the 4,4-dimethyl sterols The 4,4-dimethyl sterols were acetylated (Ac_2O–pyridine) and the acetates (13 4 mg) separated by 10% $AgNO_3$–silica gel TLC developed twice with freshly distilled EtOH-free $CHCl_3$–Et_2O (49 1) Five bands were observed after spraying with berberine in EtOH and viewing under UV light These were eluted with Et_2O and submitted to GC/MS analysis (3% OV-17 at 268°, *RR$_t$* cholesterol acetate = 1 00)

Band 1 24-methylenecycloartanyl acetate (**4h**), R_f 0 36, *RR$_t$* 1 98, MS *m/z* (rel int) 482 $[M]^+$ (7), 467 (12), 422 (100), 407 (96), 379 (31), 357 (4), 353 (15), 300 (45), 297 (42)

Band 2 cyclolaudenyl acetate (**8h**), R_f 0 41, *RR$_t$* 1 96, MS *m/z* (rel int) 482 $[M]^+$ (13), 467 (13), 422 (100), 407 (80), 379 (33), 357 (6), 353 (17), 300 (45), 297 (39)

Band 3 cycloartenyl acetate (**10h**), R_f 0 57, *RR$_t$* 1 80, MS *m/z* (rel int) 478 $[M]^+$ (20), 453 (18), 408 (100), 393 (86), 365 (30), 357 (6), 339 (40), 286 (62), 297 (25)

Band 4 cyclosadyl acetate (**9h**), R_f 0 62, *RR$_t$* 2 00, MS *m/z* (rel int) 482 $[M]^+$ (1), 467 (7), 422 (100), 407 (77), 379 (10), 353 (4), 325 (61), 300 (27), 297 (11)

Band 5 β-amyrin acetate, R_f 0 79, *RR$_t$* 1 62, MS *m/z* (rel int) 468 $[M]^+$ (2), 218 (100), 203 (45), 189 (19), α-amyrin acetate, *RR$_t$* 1 82, MS *m/z* (rel int) 468 $[M]^+$ (4), 218 (100), 203 (22), 189 (24)

Analysis of the 4-demethyl sterols The demethyl sterol fraction was acetylated (Ac_2O–pyridine) and the steryl acetates (129 mg) were separated into 8 bands by prep TLC on 10% $AgNO_3$-silica gel developed with EtOH-free $CHCl_3$–Et_2O (49 1) The bands were eluted and analysed by GC/MS (3% OV-17 at 260°, *RR$_t$* cholesteryl acetate = 1 00)

Band 1 R_f 0 10; *RR$_t$* 1 35, 24-methylcholesta-5,24(28)-dien-3β-yl acetate (**4b**), MS *m/z* (rel int) 380 $[M - Ac]^+$ (100), 365 (14), 296 (39), 281 (14), 259 (8), 255 (9), 253 (17), 228 (9), 213 (20)

Band 2 R_f 0 14, *RR$_t$* 1 34, 24-methylcholesta-5,25-dien-3β-yl-acetate (**8b**), MS *m/z* (rel. int) 380 $[M - Ac]^+$ (100), 365 (12), 296

(11), 283 (7), 259 (9), 255 (12), 228 (7), 213 (16), *RR*$_t$ 1 61, 24-methylcholesta-7,24(28)-dien-3β-yl acetate (**4d**), MS *m/z* (rel int) 440 $[M]^+$ (9), 425 (12), 380 (5), 365 (7), 356 (27), 342 (4), 313 (100), 296 (6), 273 (7), 255 (26), 227 (16), 213 (32)

Band 3 R_f 0 23, *RR*$_t$ 1 35, 24-methylcholesta-5, *E*-23-dien-3β yl acetate (**9b**), MS *m/z* (rel int) 380 $[M-Ac]^+$ (61), 365 (5), 296 (5), 283 (67), 255 (7), 253 (27), 213 (8), 81 (87), 55 (100), *RR*$_t$ 1 63, 24-ethylcholesta-5,25-dien-3β-yl acetate (**12b**), MS *m/z* (rel int) 394 $[M-Ac]^+$ (98), 379 (7), 315 (3), 296 (5), 281 (6), 255 (13), 253 (16), 228 (9), 213 (16), 55 (100), *RR*$_t$ 1 80, 24-ethylcholesta-5,*Z*-24(28)-dien-3β-yl acetate (**6b**, isofucosteryl acetate), MS *m/z* (rel int) 394 $[M-Ac]^+$ (14), 379 (3), 296 (100), 281 (18), 253 (8), 229 (10), 213 (13)

Band 4 R_f 0 28, *RR*$_t$ 1 36, probably 24-methylcholesta-5,*Z*-23-dien-3β-yl acetate (**13b**), MS *m/z* (rel int) 380 $[M-Ac]^+$ (73), 365 (7), 296 (5), 283 (75), 253 (27), 227 (5), 213 (8), *RR*$_t$ 1 62, 24-methyl-5α-cholesta-7,*E*-23-dien-3β-yl acetate (**9d**), MS *m/z* (rel int) 440 $[M]^+$ (4), 425 (3), 394 (98), 380 (4), 379 (12), 343 (10), 313 (53), 283 (41), 273 (6), 255 (7), 253 (21), 227 (7), 215 (6), 213 (6), *RR*$_t$ 1 69, probably 24-ethylcholesta-5,*E*-24(28-dien-3β-yl acetate (**14b**, fucosteryl acetate), MS *m/z* (rel int) 394 $[M-Ac]^+$ (44), 379 (7), 296 (100), 281 (19), 253 (14), 228 (10), 213 (11), *RR*$_t$ 2 08, 24-ethyl-5α-cholesta-7,*Z*-24(28)-dien-3β-yl acetate (**6d**), MS *m/z* (rel int) 454 $[M]^+$ (2), 439 (3), 379 (2), 356 (43), 341 (5), 313 (100), 296 (6), 288 (8), 281 (5), 273 (5), 255 (11), 253 (8), 227 (9), 213 (17)

Band 5 R_f 0 35, *RR*$_t$ 1 14, 24-methylcholesta-5,22-dien-3β-yl acetate (**15b**), MS *m/z* (rel int) 380 $[M-Ac]^+$ (86), 365 (6), 337 (6), 282 (7), 267 (2), 255 (54), 253 (8), 228 (9), 213 (13), *RR*$_t$ 1 87, 24-ethylcholesta-5,24-dien-3β-yl acetate (**7b**), MS *m/z* (rel int) 394 $[M-Ac]^+$ (27), 379 (5), 296 (75), 281 (15), 255 (8), 253 (19), 228 (10), 213 (16)

Band 6 R_f 0 48, *RR*$_t$ 1 38, 24-ethylcholesta-5,22-dien-3β-yl acetate (**16b**), MS *m/z* (rel int) 394 $[M-Ac]^+$ (96), 379 (5), 351 (15), 282 (7), 255 (47), 253 (9), 228 (11), 213 (12)

Band 7 R_f 0 55, *RR*$_t$ 1 00, cholest-5-en-3β-yl acetate (**11b**), MS *m/z* (rel int) 368 $[M-Ac]^+$, 353 (12), 260 (11), 255 (27), 253 (8), 247 (10), 213 (7), *RR*$_t$ 1 26, 24-methylcholest-5-en-3β-yl acetate (**2b** and **3b**), MS *m/z* (rel int) 382 $[M-Ac]^+$ (100), 367 (19), 340 (2), 274 (14), 261 (14), 255 (16), 213 (15), *RR*$_t$ 1 55, 24-ethylcholest-5-en-3β-yl acetate (**1b**), MS *m/z* (rel int) 296 $[M-Ac]^+$ (100), 381 (17), 354 (2), 288 (12), 275 (12), 255 (15), 213 (14)

Band 8 R_f 0 61, *RR*$_t$ 1 02, 5α-cholestan-3β-yl acetate (**11f**), MS *m/z* (rel int) 430 $[M]^+$ (10), 370 (12), 355 (2), 335 (4), 318 (1), 275 (25), 215 (100), *RR*$_t$ 1 27, 24-methyl-5α-cholestan-3β-yl acetate (**2f**), MS *m/z* (rel int) 444 $[M]^+$ (28), 429 (5), 384 (27), 369 (21), 330 (2), 276 (33), 275 (28), 261 (7), 215 (100), *RR*$_t$ 1 40, 24-ethyl-5α-cholest-22-en-3β-yl acetate (**16f**), MS *m/z* (rel int) 456 $[M]^+$ (19), 442 (2), 396 (3), 353 (21), 344 (16), 315 (16), 275 (4), 257 (46), 255 (9), 229 (5), 215 (20), *RR*$_t$ 1 56, 24-ethyl-5α-cholestan-3β-yl acetate (**1f**), MS *m/z* (rel int) 458 $[M]^+$ (34), 398 (22), 383 (24), 344 (2), 276 (25), 275 (16), 257 (5), 215 (100), *RR*$_t$ 1 84, 24-ethyl-5α-cholest-7-en-3β-yl acetate (**1d**), MS *m/z* (rel int) 456 $[M]^+$ (100), 441 (19), 396 (10), 381 (10), 315 (7), 273 (14), 255 (98), 229 (29), 213 (44)

Further analysis of the band 3 steryl acetates The steryl acetates (9 8 mg) were separated by reverse-phase HPLC on an Ultrasphere 5 ODS column (250 × 5 mm) eluted with MeOH–H_2O (19 1) at 1 1 ml/min with 0 5–1 0 mg steryl acetate per injection Three compounds (3A, 3B, 3C) were collected separately and shown to be 93–100% pure by GLC on 3% OV-17 Compound 3A (1 2 mg), 24-methylcholesta-5,*E*-23-dien-3β-yl acetate (**9b**), mp 126–128 5° (lit [10] mp 122–124°), MS see above, ^{1}H NMR (400 MHz, $CDCl_3$) δ 0 683 (3H, *s*, H-18), 0 888 (3H, *d*, *J* = 6 5 Hz, H-21), 0 986 (6H, *d*, *J* = 6 8 Hz, H-26 and H-27), 1 017 (3H, *s*, H-19), 1 543 (3H, *s*, H-28), 2 005 (3H, *s*, acetate), 2 235 (1H, *septet*, *J* = 7 0 Hz, H-25), 4 615 (1H, *m*, H-3α), 5 136 (1H, *t*, *J* = 7 0 Hz, H-23), 5 374 (1H, *d*, *J* = 5 Hz, H-6) Compound 3B (0 4 mg), 24-ethylcholesta-5,25-dien-3β-yl acetate (**12b**), mp 125–127° (lit [29] mp 124–127°), MS as above Compound 3B was hydrolysed (8% KOH in 85% EtOH) and the free sterol had MS *m/z* (rel int) 412 $[M]^+$ (100), 397 (21), 394 (69), 379 (26), 328 (16), 314 (41), 299 (42), 283 (23), 281 (22), 273 (27), 271 (80), 255 (37), 253 (39), 229 (30), 213 (58) The TMSi-ether prepared by addition of 100 μl *N,O*-bis(trimethylsilyl)acetamide had MS *m/z* (rel int) 484 $[M]^+$ (25), 469 (7), 394 (30), 379 (14), 355 (26), 213 (10), 129 (100) These MS are close to those published [32] for the 24-ethylcholesta-5,25-dien-3β-ol isolated from *Brassica napus* Compound 3C (4 1 mg) 24-ethylcholesta-5,*Z*-24(28)-dien-3β-yl acetate (**6b**, isofucosteryl acetate), mp 132–135° (lit [41] mp 131–133°), MS as above

Incorporation of [2-^{14}C]*MVA into* Z mays *sterols* Excised 12-day-old shoots (110) were allowed to imbibe a soln of [2-^{14}C]MVA (10 μCi) over a period of 24 hr The non-saponifiable lipid (9 54 × 10^5 dpm) was then extracted and separated by prep TLC (silica gel, $CHCl_3$–EtOH, 49 1) into the 4,4-dimethyl sterols (2 43 × 10^5 dpm) and 4-demethyl sterols (2 93 × 10^5 dpm) Each of these fractions was acetylated (pyridine–Ac_2O) and, after addition of appropriate carrier Δ^{23}-, $\Delta^{24(28)}$-, Δ^{24}- and Δ^{25}-steryl acetates, the mixtures were separated into their component bands by prep TLC (10% $AgNO_3$–silica gel, EtOH-free $CHCl_3$–Et_2O, 49 1, 1 or 2 developments) and the component identifications checked by GLC (Tables 4 and 5)

(a) *Oxidation of radioactive 4-demethyl sterols in band* 2 Compounds **8b** (1 9 mg) and **4d** (2 1 mg) were added to a portion of the band 2 material (4100 dpm) and the mixture hydrolysed (8% KOH in 85% aq EtOH) The recovered free sterols were oxidized [42] and the two products separated by TLC on silica gel ($CHCl_3$–EtOH, 49 1) 24-Methylcholesta-4,25-diene-3,6-dione (1 4 mg, **8j**, 220 dpm, R_f 0 44, GLC (SE-30) *RR*$_t$ 2 36, UV λ_{max}^{EtOH} nm 255, MS *m/z* (rel int) 410 $[M]^+$ (13), 341 (24), 327 (10), 311 (14), 283 (14), 270 (9), 259 (7), 243 (10) 24-Methyl-5α-cholesta-7,24(28)-dien-3-one (0 8 mg, **4i**, 862 dpm), R_f 0 61, GLC (SE-30) *RR*$_t$ 1 46, UV λ_{max}^{EtOH} nm 221, MS *m/z* (rel int) 396 $[M]^+$ (5), 381 (9), 312 (27), 297 (8), 283 (4), 269 (100), 255 (5), 244 (13), 229 (21)

(b) *Separation of the radioactive 4-demethyl steryl acetates of band 3 by HPLC* The steryl acetates in band 3 were separated by reverse-phase HPLC on Lichrosorb RP-18 (10 μm) eluted with MeOH–H_2O (19 1, 1 0 ml/min) and the peaks collected corresponding, in order of elution, to **9b** (630 dpm), **12b** (570 dpm) and **6b** (12 170 dpm)

(c) *Analysis of the labelled 4-demethyl steryl acetates of band* 5 The band 5 material was rechromatographed on $AgNO_3$-silica gel TLC and then submitted to reverse-phase HPLC on Lichrosorb RP 18 (MeOH, 0 55 ml/min) to yield 24-methylcholesta-5,24-dien-3β-yl acetate (**5b**, 50 dpm) and 24-ethylcholesta-5,24-dien-3β-yl acetate (**7b**, 60 dpm) (purity checked by GLC)

(d) *Analysis of the 4-demethyl steryl acetates in band* 7 A portion of this material was separated by reverse-phase HPLC on Lichrosorb RP 18 (MeOH–H_2O, 49 1, 1 0 ml/min) to yield 24-methylcholest-5-en-3β-yl acetate (**2b** and **3b**, 12 070 dpm) and 24-ethylcholest-5-en-3β-yl acetate (**1b**, 38 950 dpm) Each of these components was shown to be 95% pure by GLC

Incorporation of [*methyl*-^{14}C]*methionine into* Z mays *sterols* Excised 8-day-old shoots (*ca* 350) were allowed to imbibe a soln of [methyl-^{14}C]methionine (155 μCi) and the non-saponifiable lipids (8 07 × 10^5 dpm) extracted after 24 hr TLC (silica gel, $CHCl_3$–EtOH, 49 1) gave the 4,4-dimethyl sterols (1 944 × 10^4 dpm) and the 4-demethyl sterols (5 93 × 10^5 dpm) These were acetylated, mixed with appropriate carrier Δ^{23}-, $\Delta^{24(28)}$- and Δ^{25}-steryl acetates and separated by TLC on $AgNO_3$-silica gel as

described above (Tables 4 and 5)

(a) *Further analysis of the labelled 4-demethyl steryl acetates in band 2* Carrier **8b** (2 5 mg) and **4d** (4 8 mg) were added and the mixture was hydrolysed and the free sterols (**8a** and **4c**) were oxidized [42] as described above for the corresponding fraction recovered from the [2-^{14}C]MVA incubation The products were separated by silica gel TLC to yield 24-methyl-5α-cholesta-7,24(28)-dien-3-one (**4i**), 2900 dpm, mp 133–137°, R_f 0 53, GC (SE-30) *RR*$_t$ 1 46, UV λ_{max}^{EtOH} nm 221, MS *m/z* (rel int) 396 $[M]^+$ (6), 381 (9), 312 (28), 297 (9), 283 (4), 269 (100), 255 (5), 244 (12), 229 (20), and 24-methylcholesta-4,25-dien-3,6-dione (**8j**), 340 dpm, mp 125–132°, R_f 0 38, GLC (SE-30) *RR*$_t$ 2 37, UV λ_{max}^{EtOH} nm 253, MS *m/z* (rel int) 410 $[M]^+$ (17), 395 (3), 341 (32), 326 (10), 311 (18), 297 (4), 283 (18), 270 (9), 257 (15), 243 (12)

(b) *HPLC analysis of the 4-demethyl steryl acetates of band 3* Appropriate carriers (**6b**, **9b** and **12b**) were added to a portion of the radioactive band 3 material and the mixture was separated by reverse-phase HPLC on Ultrasphere 5 ODS eluted with MeOH–H_2O (19 1) at 1 0 ml/min The three steryl acetates collected in order of elution were 24-methylcholesta-5,*E*-23-dien-3β-yl acetate (**9b**, 320 dpm), 24-ethylcholesta-5,25-dien-3β-yl acetate (**12b**, 1050 dpm) and 24-ethylcholesta-5,*Z*-24(28)-dien-3β-yl acetate (**6b**, 17 280 dpm) The first two components were shown to be pure by GLC, **6b** was 92% pure and contained contaminating **12b** In a second HPLC run similar to that above, 2 0 ml samples were collected and assayed for radioactivity A histogram plot showed that radioactive peaks corresponded to the mass peaks of compounds **9b**, **12b** and **6b**

(c) *HPLC of the 4-demethyl steryl acetates in band 4* To the radioactive sample (24 300 dpm) was added 24-ethylcholesta-7,*Z*-24(28)-dien-3β-yl acetate (**6d**, 1 8 mg) and the mixture separated by HPLC on Ultrasphere 5 ODS (MeOH–H_2O, 46 1, 1 ml/min) One major radioactive component (12 740 dpm) was obtained which co-chromatographed with **6d** To this was added a further 1 2 mg **6d** and 1 mg 24-ethylcholesta-5,23-dien-3β-yl acetate (**17d**) and the mixture saponified The recovered sterols were then oxidized [42] and the products purified by TLC as described above 24-Ethyl-5α-cholesta-7,*Z*-24(28)-dien-3-one (**6i**, 0 6 mg, 4170 dpm), R_f 0 59, GLC (SE-30) *RR*$_t$ 1 86, UV λ_{max}^{EtOH} nm 218, MS *m/z* (rel int) 410 $[M]^+$, 395 (2), 312 (33), 297 (9), 283 (4), 269 (100), 244 (17), 229 (18) 24-Ethylcholesta-4,23-diene-3,6-dione (**17j**, 0 8 mg, 730 dpm), R_f 0 37, *RR*$_t$ 2 69, UV λ_{max}^{EtOH} nm 250, MS *m/z* (rel int) 424 $[M]^+$ (6), 327 (3), 313 (12), 311 (5), 284 (7), 283 (7), 243 (5)

(d) *HPLC separation of the Δ^{24}-steryl acetates in band 5* The steryl acetates (4640 dpm) in band 5 were separated by reverse-phase HPLC on Ultrasphere 5 ODS (MeOH–H_2O, 19 1, 1 0 ml/min) to provide 24-methylcholesta-5,24-dien-3β-yl acetate (**5b**, 150 dpm) and 24-ethylcholesta-5,24-dien-3β-yl acetate (**7b**, 490 dpm)

(e) *Analysis of the 4-demethyl steryl acetates in band 7* This band (218 070 dpm) contained a mixture of **1b**, **2b** + **3b**, and **16b** which had shown poor separation on the $AgNO_3$-silica gel TLC No carrier steryl acetates were added to this fraction A portion of the sample was separated by reverse-phase HPLC on Ultrasphere 5 ODS (MeOH–H_2O, 19 1, 1 0 ml/min) to give a mixture (20 140 dpm) of **2b** + **3b** (40%) and **16b** (60%) and a sample (70 920 dpm) containing 85% **1b**, 9% **16b** and 6% **2b** + **3b** The former mixture was subjected to $AgNO_3$-silica gel TLC to yield **2b** + **3b** (sp act 10 440 dpm/mg) and **16b** (sp act 5520 dpm/mg) From the sp act values it was calculated that **1b** had sp act 24 840 dpm/mg To substantiate these values, a second sample of the band 7 material was separated by $AgNO_3$-silica gel TLC to give **16b** (4700 dpm/mg) and a mixture of **1b** and **2b** + **3b** which was separated by reverse-phase HPLC on Ultrasphere 5 ODS 26 350 dpm/mg) and **2b** + **3b** (sp act 8490 dpm/mg) These two procedures showed reasonable agreement and average sp act values (dpm/mg) were **1b** 25 600, **2b** + **3b** 9460, **16b** 5110

Synthesis of Δ^{23}-steryl acetates Cyclosadyl acetate (**9h**) was prepared by isomerization of 24-methylenecycloartanyl acetate (**4h**) by reflux with I_2 in C_6H_6 [42–44] It was purified by TLC on $AgNO_3$-silica gel (redistilled $CHCl_3$–Et_2O, 49 1) to remove other products (e g **5h** and **8h**) I_2 isomerization of 24-methylcholesta-5,24(28)-dien-3β-yl acetate (**4b**) gave 24-methylcholesta-5,*E*-23-dien-3β-yl acetate (**9b**) and 24-methylcholesta-5,*Z*-dien-3β-yl acetate (**13b**) together with other products (**5b**, **8b**) Compounds **9b** and **13b** were purified by TLC on $AgNO_3$-silica gel (redistilled $CHCl_3$–Et_2O, 97 3) and HPLC on Lichrosorb RP 18 (MeOH, 1 0 ml/min), ^{1}H NMR Table 2, GLC *RR*$_t$s 3% OV-1 (275°) **9b** 1 24, **13b** 1 22, 3% OV-17 (280°) **9b** 1 30, **13b** 1 29, 1% HiEFF 8B (245°) **9b** 1 29, **13b** 1 33 Details of these syntheses will be published elsewhere

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