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THE SYNTHESIS OF 24-METHYLENECYCLOARTANOL, CYCLOSADOL AND CYCLOLAUDENOL BY A CELL FREE PREPARATION FROM *ZEA MAYS* SHOOTS*

NEIL L. A. MISSO and L. JOHN GOAD

Department of Biochemistry, The University of Liverpool, P.O. Box 147, Liverpool, L69 3BX, U.K.

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Key Word Index—*Zea Mays*; Gramineae; sterols; sterol synthesis; *S*-adenosyl methionine–cycloartenol methyl transferase; cycloartenol; 24-methylenecycloartanol; cyclosadol; cyclolaudenol.

Abstract—A cell free microsomal preparation of *Zea mays* shoots has been employed to investigate the products of the *S*-adenosyl methionine–cycloartenol methyl transferase reaction. Using [methyl-^{14}C]SAM as the substrate the radioactive products were characterized by a combination of chromatographic and chemical degradation procedures. The major product was the $\Delta^{24(28)}$-compound, 24-methylenecycloartanol, but small amounts of the Δ^{23}-sterol, cyclosadol, and the Δ^{25}-sterol, cyclolaudenol, were also positively identified. The transmethylation mechanisms responsible for the elaboration of these sterols were investigated using [methyl-$^{2}H_3$]SAM as the substrate and identifying the products by GC/MS. The 24-methylenecycloartanol contained only two deuterium atoms as expected. However, both the cyclolaudenol and the cyclosadol retained all three of the deuterium atoms originally present in the transferred [methyl-$^{2}H_3$] group. This shows that neither compound was formed by isomerization of preformed 24-methylenecycloartanol but must have arisen by proton elimination from either C-27 or C-23, respectively, of the intermediate cation produced during transfer of the methyl group to the cycloartenol substrate.

INTRODUCTION

Stigmast-5-en-3β-ol (**1a**, sitosterol), stigmasta-5,22-dien-3β-ol (**2a**, stigmasterol) and 24-methylcholest-5-en-3β-ol (**3a** and **4a**, campesterol) are the most commonly encountered sterols in higher plants [1–3]. The 24α-ethyl compounds **1a** and **2a** may be the predominant, if not the only constituents of the 24-ethyl fraction. However, in several higher plants the 24-methylcholest-5-en-3β-ol is now recognized to be a mixture of the 24α-methyl (**3a**) and 24β-methyl (**4a**) epimers [4, 5]. In *Zea mays* coleoptiles [6] and shoots [7] the 24β-methyl epimer (**4a**) was shown by ^{1}H NMR spectroscopy to constitute 50–70% of the 24-methyl sterol fraction. The 24α-methyl sterol (**3a**) is thought to arise from a 24-methylene precursor (e.g. **5a**) which is isomerized to a Δ^{24}-sterol (**6a**) prior to stereospecific reduction to yield **3a** [3, 8–10]. By contrast, two alternative routes have been considered for the production of the 24β-methyl sterol (**4a**). The occurrence in a few higher plants of the Δ^{25}-sterol cyclolaudenol (**7b**), which has the 24β-configuration, first led to the proposal that this compound might act as the precursor to other Δ^{25}-sterols which are reduced to yield the 24β-methyl sterols, such as **4a** [1, 10]. However, the isolation [6] of ergosta-5,*E*-23-dien-3β-ol (**8a**) and other Δ^{23}-sterols, such as cyclosadol (**8b**) [11] from *Z. mays* tissues, prompted the alternative suggestion [6, 12] that 24β-methyl sterols may arise by stereospecific reduction of the Δ^{23}-bond of these sterol precursors. To substantiate this latter route Scheid *et al.* [12] have shown that incubation of *Z. mays* coleoptile microsomes with cycloartenol (**9b**) and *S*-adenosyl [methyl-^{14}C] methionine gave labelled 24-methylenecycloartanol (**5b**) as the major product accompanied by a small amount of labelled cyclosadol (**8b**). No labelled cyclolaudenol (**7b**) was detected and it was concluded that Δ^{23}-sterols may act as intermediates in 24-methyl sterol biosynthesis [12].

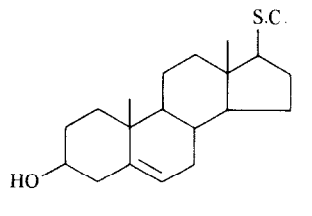
We have examined the sterols of *Z. mays* shoots and identified the Δ^{23}- and Δ^{25}-sterols cyclosadol (**8b**), cyclolaudenol (**7b**), ergosta-5,*E*-23-dien-3β-ol (**8a**) and 24-methylcholesta-5,25-dien-3β-ol (**7a**) as well as the $\Delta^{24(28)}$-compounds 24-methylenecycloartanol (**5b**) and ergosta-5,24(28)-dien-3β-ol (**5a**) [13]. Moreover, all these compounds were labelled after incubation of *Z. mays* shoots with either [2-^{14}C]MVA or [methyl-^{14}C]methionine although the 24-methylene compounds were much more heavily labelled than the Δ^{23}- and Δ^{25}-sterols suggesting that the latter compounds may be on minor biosynthetic routes [13].

To substantiate the production of both Δ^{23}- and Δ^{25}-sterols in *Z. mays* shoots and to elucidate the mechanism of their formation we have used a cell free preparation which can methylate cycloartenol (**9b**). We now describe the results of these studies in this paper.

RESULTS AND DISCUSSION

Microsomal preparations obtained from bramble cells [14] and *Z. mays* coleoptiles [15–17] were previously shown to possess *S*-adenosyl methionine (SAM)–cycloartenol methyl transferase activity. Preliminary experiments were, therefore, undertaken to find the best methods for the preparation of cell free homogenates of

*This paper is dedicated to Professor E. Lederer on the occasion of his 75th birthday.



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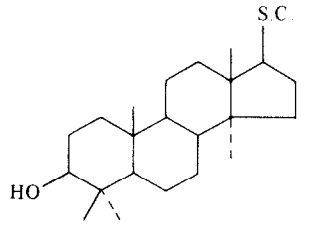

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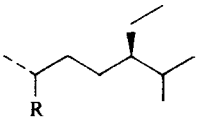

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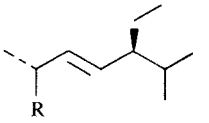

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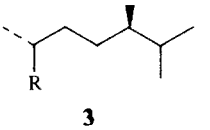

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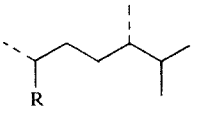

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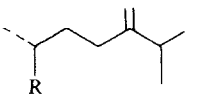

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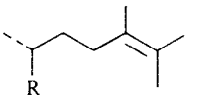

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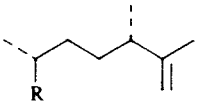

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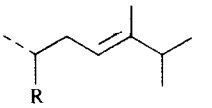

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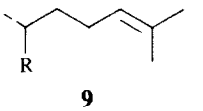

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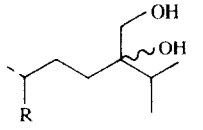

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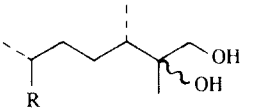

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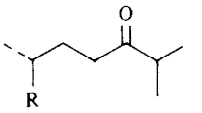

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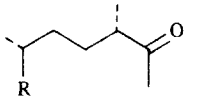

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Z. mays shoots and to ascertain the optimum incubation conditions for measuring SAM–cycloartenol methyl transferase activity. Details of the homogenization procedure, preparation of the 10 500 *g* microsomal fraction and the incubation conditions for enzyme assay are given in the Experimental.

When the microsomal preparation was incubated with cycloartenol and [methyl-^{14}C]SAM for varying times it was found that incorporation of radioactivity into the recovered 4,4-dimethyl sterol fraction increased during the first 3–4 hr but then showed only a further small increase up to 18 hr. However, between 6 and 18 hr there was a marked increase in the incorporation into an unidentified polar material which remained at the origin during TLC of the non-saponifiable lipid. For this reason subsequent incubations were restricted to 6 hr or less.

The microsomal SAM–cycloartenol methyl transferase showed maximum activity at pH 7.4 but it was virtually unaffected by addition of mercaptoethanol. When cycloartenol (**9b**) was omitted from the incubation the incorporation of radioactivity into the 4,4-dimethyl sterol fraction was only 13–25% of that obtained in the presence of cycloartenol. Presumably there was some endogenous cycloartenol (or other sterol substrate) in the microsomal

preparation but addition of cycloartenol (**9b**) was required for full expression of the methyl transferase activity. No methyl transferase activity was observed when a boiled microsomal preparation was used thus showing the absence of any non-enzymatic methylation of cycloartenol. A comparison of the methyl transferase activities in microsomal preparations obtained from 5 and 8 day old shoots suggested that the methyl transferase activity was greater in the 5 day old shoots as the incorporation of radioactivity from [methyl-^{14}C]SAM into the 4,4-dimethyl sterol fraction was more than twice that obtained with the older shoots.

In order to characterize the products of the SAM–cycloartenol methyl transferase reaction five large scale incubations were conducted using microsomal preparations obtained from 4–6 day old *Z. mays* shoots. A total of 10 μCi [methyl-^{14}C]SAM and 8.6 mg cycloartenol was used in these incubations. Upon termination of each incubation the non-saponifiable lipid was extracted and the labelled 4,4-dimethyl sterols obtained by prep. TLC on silica gel. The combined 4,4-dimethyl sterols (9.54×10^5 dpm) were then acetylated, mixed with carrier 24-methylenecycloartanyl acetate (**5c**), cyclolaudenyl acetate (**7c**) and cyclosadyl acetate (**8c**) and a small portion of the mixture separated by silver nitrate–silica gel TLC. An autoradiogram showed that most of the radioactivity co-chromatographed with 24-methylenecycloartanyl acetate (**5c**) but radioactive bands also clearly co-chromatographed with the marker samples of cyclolaudenyl acetate (**7c**) and cyclosadyl acetate (**8c**). In addition, small amounts of radioactivity were associated with three bands which were more polar than **5c** but the identity of these compounds is not known. The remaining labelled 4,4-dimethylsteryl acetates were separated by prep. silver nitrate–silica gel TLC to yield the compounds chromatographing with 24-methylenecycloartanyl acetate (**5c**, 7.90×10^5 dpm) cyclolaudenyl acetate (**7c**, 8.58×10^3 dpm) and cyclosadyl acetate (**8c**, 2.85×10^4 dpm).

The radioactivity associated with cyclosadyl acetate represented 3.2% of the [methyl-^{14}C]SAM incorporated into the 4,4-dimethyl sterol fraction. This is somewhat less than the proportion incorporated (25%) into this compound in the study conducted by Scheid *et al.* [12] using a microsomal preparation from *Z. mays* coleoptiles. In order to establish that radioactivity was actually associated with this Δ^{23}-steryl acetate, synthetically prepared cyclosadyl acetate (**8c**, 22 mg) was added to the radioactive material (28 000 dpm) and the mixture crystallized to constant sp. act. (1280, 1500, 1640, 1590 dpm/mg for successive crystallizations). These results confirmed that the Δ^{23}-sterol, cyclosadol (**8b**) was indeed a product of the SAM–cycloartenol methyl transferase reaction in *Z. mays* shoots.

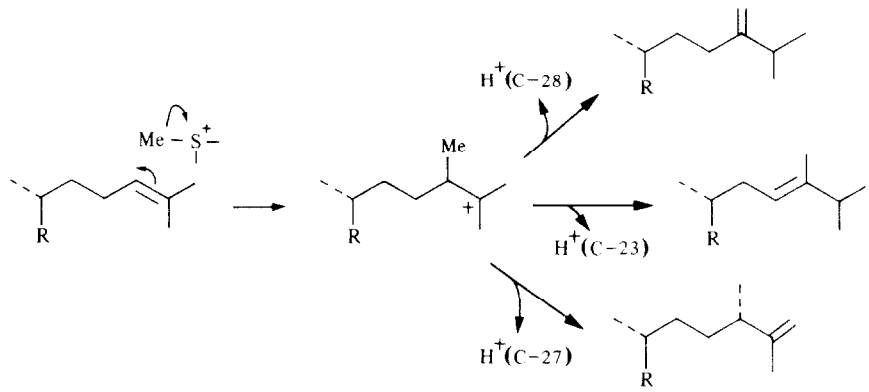
The 24-methylenecycloartanyl acetate (**5c**) accounted for 88.4% of the radioactivity in the recovered 4,4-dimethyl steryl acetates while 1.0% was associated with cyclolaudenyl acetate (**7c**) and the remainder (7.4%) was in the three unidentified polar compounds. To verify that compounds **5c** (2850 dpm/mg) and **7c** (120 dpm/mg) were labelled they were subjected to the osmium tetroxide oxidation procedure which we have used previously to differentiate between these two compounds [18, 19]. Both the 24,28-dihydroxy-24-methylcycloartanyl acetate (**10c**, 30 000 dpm/mg) derived from **5c** and the 25,27-dihydroxy-24-methylcycloartanyl acetate (**11c**, 77 dpm/mg) obtained from **7c** retained the bulk of the radioactivity associated with the parent compounds.

The crystallized 24,28-diol (**10c**) and 25,27-diol (**11c**) were cleaved by treatment with sodium periodate to give the corresponding norketones **12c** and **13c**, respectively, which were purified by prep. TLC and crystallized to constant sp. act. The conversion of **10c** into 24-oxo-cycloartanyl acetate (**12c**, 2 dpm/mg) resulted in loss of radioactivity as expected since **10c** is labelled at C-28. This carbon is introduced from [methyl-^{14}C]SAM in the methyl transferase reaction to produce 24-methylene-cycloartanol (**5b**). By contrast the 25-oxo-27-nor-24-methylcycloartanyl acetate (**13c**, 84 dpm/mg) derived from **11c** retained all its radioactivity. This is the predicted result if the starting labelled compound was indeed cyclolaudenol (**7c**) since the labelled C-28 methyl group introduced into **7c** during the transmethylation sequence is retained in **13c**.

The above results demonstrated quite convincingly that radioactive cyclolaudenol (**7b**) was produced together with **5b** and **8b** by the *Z. mays* shoot microsomal preparation. These findings are in accord with our previous identification of various Δ^{23}- and Δ^{25}-sterols in maize shoots and their *in vivo* labelling from [2-^{14}C]MVA and [methyl-^{14}C]methionine [7, 13]. However, our results apparently contrast with the observations of Scheid *et al.* [12] who could find no evidence for cyclolaudenol (**7c**) production using a microsomal preparation from etiolated *Z. mays* coleoptiles. Also, as already mentioned, their preparation gave a higher yield of cyclosadol (**8b**) than did our system. These differences may perhaps be explained by the use of different strains of *Z. mays*, the use of green shoots as opposed to etiolated coleoptiles or the differences in the experimental techniques used for the preparation of the cell free homogenates and the incubations.

In both our work and that of Scheid *et al.* [12] the major product was 24-methylenecycloartanol (**5b**). This is not surprising since not only is **5b** the presumed precursor to 24α-methylcholest-5-en-3β-ol (**3a**) but it is also the precursor of 24-methylenelophenol which is the substrate [3, 14] for the second transmethylase reaction which leads ultimately to the 24α-ethyl sterols **1a** and **2a**. Stigmast-5-en-3β-ol (**1a**), stigmasta-5,22-dien-3β-ol (**2a**) and **3a** together comprise *ca* 75% of the total demethyl sterols of *Z. mays* shoots [13]. Therefore, it seems reasonable that the precursor (**5b**) of these compounds should be the major product of the initial SAM–cycloartenol methyl transferase reaction.

With the identification of $\Delta^{24(28)}$-, Δ^{23}- and Δ^{25}-sterols as the products of incubation of cycloartenol (**9b**) with the *Z. mays* shoot microsomal preparation it was of interest to elucidate the mechanisms of formation of these compounds. It is now clear [2, 3, 8, 20] that the 24-methylene group of **5b** arises by loss of a proton from C-28 of the cation produced upon methylation of cycloartenol (**9b**) (Scheme 1). It has also been shown [18] that cyclolaudenol (**7b**) is produced in rhizomes of the fern *Polypodium vulgare* by loss of a proton from C-27 of the intermediate cation (Scheme 1). Two mechanisms can be envisaged for the production of the Δ^{23}-compound cyclosadol (**8b**) [6]. It can arise either by loss of a C-23 proton from the precursor cation or it can be formed by isomerization of preformed 24-methylenecycloartanol (**5b**) (Scheme 1). The various mechanisms can be differentiated by following the fate of the hydrogen atoms of the methyl group derived from SAM which is introduced



Scheme 1. Alkylation mechanisms for the conversion of cycloartenol into 24-methylenecycloartanol, cyclolaudenol and cyclosadol.

at C-24 in the transmethylation reaction. Clearly, only two of the original hydrogens will be retained in the 24-methylenecycloartanol (**5b**) and, hence, in the Δ^{23}-sterol, cyclosadol (**8b**), if it is derived by isomerization of the former. Conversely, all three of the original methyl hydrogens will be retained in cyclosadol (**8b**), and in cyclaudenol (**7b**), if they are formed by appropriate proton elimination from C-23 or C-27, respectively, of the precursor cation (Scheme 1).

In order to determine which of these mechanisms operated in *Z. mays* *S*-adenosyl[methyl-$^2H_3$]methionine was synthesized by incubating yeast in the presence of [methyl-$^2H_3$]methionine [21–23]. Positive ion FAB-MS of the purified [methyl-$^2H_3$]SAM showed a molecular ion at *m/z* 402 and fragmentation ions at 301 and 136 which established that the compound did contain three deuterium atoms in the methyl group. Ions at *m/z* 399 and 298 showed that the sample also contained *ca* 25% of unlabelled SAM.

The [methyl-$^2H_3$]SAM was mixed with [methyl-^{14}C]SAM and two incubations were set up with added cycloartenol (**9b**) and microsomal preparations from *Z. mays* shoots. The [methyl-^{14}C]SAM was added to (a) verify that the microsomal preparation was enzymically active, (b) to enable the products to be located during prep. TLC by radioscanning, and (c) to allow the quantities of products to be calculated. From the sp. act. of the [methyl-$^2H_3$]SAM and [methyl-^{14}C]SAM mixtures used for the two incubations (111 and 222 dpm/nmol, respectively) and the incorporation of ^{14}C into the 4,4-dimethyl sterol fractions it was estimated that there was produced *ca* 300 μg of methylated products. Acetylation of these sterols and prep. TLC on silver nitrate–silica gel yielded 24-methylenecycloartanyl acetate (**5c**, 270 μg), cyclolaudenyl acetate (**7c**, 5 μg) and cyclosadyl acetate (**8c**, 1 μg) which were analysed by GC/MS to determine their deuterium content (Fig. 1).

The 24-methylenecycloartanyl acetate (**5c**) sample

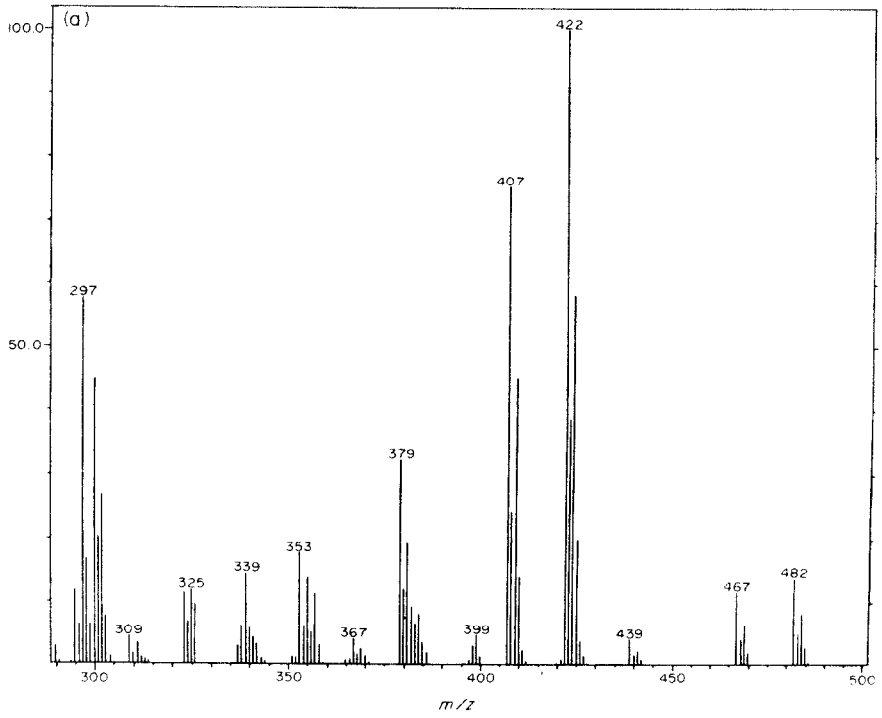


Fig. 1a.

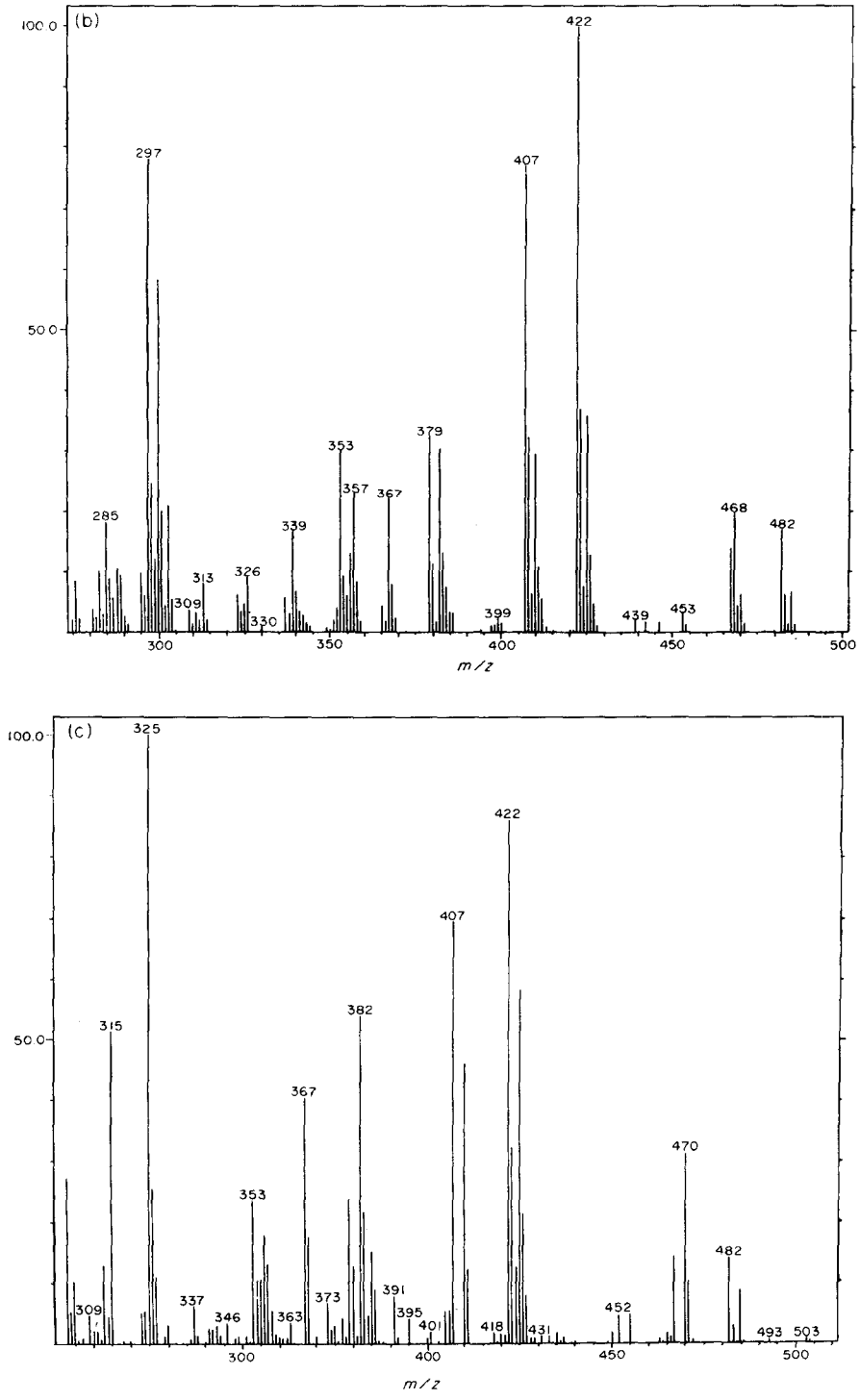


Fig. 1. Mass spectra of (a) 24-methylenecycloartanyl acetate (**5c**), (b) cyclolaudenyl acetate (**7c**) and (c) cyclosadyl acetate (**8c**) derived from an incubation of [methyl-$^2H_3$]SAM and cycloartenol (**9c**) with a microsomal preparation of *Z. mays* shoots.

showed one major peak with a mass spectrum which confirmed its identity (Fig. 1a). There were clearly two components in the sample; one was unlabelled **5c** (M^+ at m/z 482) while the other showed M^+ at m/z 484. Other ions in the mass spectrum of **5c** at m/z 467, 439, 422, 407, 379, 353 and 300 were accompanied by ions 2 a.m.u. higher thus showing that the second component was 24-[28-$^2H_2$]methylenecycloartanyl acetate which comprised *ca* 40% of the mixture. This result agrees with the expected mechanism of 24-methylenecycloartanol (**5b**)

biosynthesis in which one of the C-28 hydrogen atoms derived from SAM is lost in order to stabilize the intermediate cation (Scheme 1).

The labelled cyclolaudenyl acetate (**7c**) sample showed, on GC/MS analysis, a component (Fig. 1b) which was identified as a mixture of unlabelled **7c** (M^+ at *m/z* 482) accompanied by a trideuterated species of **7c** (M^+ at *m/z* 485). The unlabelled material had fragmentation ions at 467, 422, 407, 379, 367, 353, 300 and 297 permitting its identification as **7c**. With the exception of the ions at *m/z* 367 and 297, which arise by loss of the side chain, all the other ions were accompanied by ions which were 3 a.m.u. higher and which, therefore, provided conclusive evidence for the identification of [28-$^2H_3$]cyclolaudenyl acetate (**7c**), which constituted *ca* 30% of the sample. The retention of all three deuterium atoms present in the initial [methyl-$^2H_3$]SAM showed that **7b** could not be formed by isomerization of 24-methylenecycloartanol (**5b**) but must be produced by proton loss from the intermediate cation as shown in Scheme 1. The present result therefore confirms this mechanism for cyclolaudenol production which was previously indicated by incorporation studies with *P. vulgare* employing [methyl-^{3}H,^{14}C]methionine [18].

The cyclosadyl acetate sample (**8c**) showed upon GC/MS analysis a very small peak with a RR_t corresponding to authentic **8c**. The mass spectrum of this material again showed it to be a mixture of unlabelled compound accompanied by a deuterated species. The unlabelled sample had a molecular ion at *m/z* 482 and fragmentation ions at 467, 422, 407, 379, 367, 353, 325 and 300 (Fig. 1c). This mass spectrum, with the strong ion at *m/z* 325, which is characteristic of **8c** [11, 12] confirmed the identification of cyclosadyl acetate (**8c**). The presence of ions at *m/z* 485 $[M]^+$, 470, 425, 410, 382, 356 and 303 which were 3 a.m.u. higher than the corresponding ions of unlabelled **8c**, demonstrated that a trideuterated sample of **8c** was also present. The absence of ions 3 a.m.u. higher than *m/z* 367 and 325, which arise by loss of the side chain, showed that the three deuterium atoms were located, as expected, at C-28. The labelled component was, therefore, identified as [28-$^2H_3$]cyclosadyl acetate (**8c**) and it represented *ca* 40% of the cyclosadyl acetate sample. The retention of three deuterium atoms in the cyclosadol (**8b**) demonstrated that it was formed directly by C-23 proton elimination from the carbonium ion (Scheme 1) and that it was not produced by isomerization of 24-methylenecycloartanol (**5b**).

It will be interesting to establish if **5b**, **7b** and **8b** are the products of one transmethylase enzyme or if three specific enzymes are responsible for their production. The unlabelled **5b**, **7b** and **8b** identified in the samples recovered from the incubations probably represent preformed endogenous compounds present in the microsomal preparation. These results, therefore, provide further evidence for the occurrence of both Δ^{23}- and Δ^{25}-sterols as natural products in *Z. mays* shoots [13]. However, it remains to be established if either or both of these sterol types can fulfil their postulated role as precursors of the 24β-methylsterols. The alternative possibility is that they are merely the minor products of an aberrent C-24 transmethylation reaction which do not undergo any further side chain modification.

EXPERIMENTAL

Exptal methods were generally as described previously [13]. GC/MS was performed on a VG 70-70F instrument coupled to a Finnegan 2400 Incos Data System, spectra were taken at 70 eV. GC employed a 3% OV-17 column operated at 280°. *S*-Adenosyl-L-[methyl-^{14}C]methionine (0.5 or 61 mCi/mmol) was obtained from Amersham International, U.K. [methyl-$^2H_3$]Methionine was synthesized as described previously [24, 25].

Preparation of Z. mays shoot cell free homogenate. Seeds of *Z. mays* (Calder 535) were soaked overnight then placed in trays on moist cotton wool and paper and allowed to germinate and grow under normal day light conditions for 4–6 days. The shoots were homogenized by grinding in a chilled mortar and pestle at 5° with medium (1 ml/g shoots) which consisted of 0.1 M Tris–HCl buffer, pH 8.0, 0.5 M mannitol, 1 mM EDTA, 0.5% bovine serum albumin, 10 mM mercaptoethanol and PVP (1 mg/ml). The homogenate was filtered through four layers of gauze and the filtrate centrifuged for 5 min at 1000 *g*. The pellet was discarded and the supernatant centrifuged at 10 000 *g* for 10 min. The supernatant was recentrifuged at 105 000 *g* for 60 min to give the microsomal pellet which was resuspended in an appropriate vol. of 0.1 M Tris–HCl buffer, pH 7.4, 4 mM $MgCl_2$ and 2 mM mercaptoethanol.

Microsomal incubations. Three large scale incubations contained 18 ml microsomal preparations, 5 μmol cycloartenol (**9b**) (emulsified in 1.5 ml buffer containing 15 μl Tween 80) 5.0 μmol [methyl-^{14}C]SAM (0.5 μCi/μmol) in a total vol. of 20 ml. Two other incubations contained half these quantities. The incubations were maintained at 30° with shaking for 6 hr and the non-saponifiable lipid was then extracted from each in the usual manner.

Isolation of the 4,4-dimethyl steryl acetates. Prep. TLC on silica gel ($CHCl_3$–EtOH, 49:1) of each non-saponifiable lipid gave the labelled 4,4-dimethylsterols which were combined (9.54×10^5 dpm) and acetylated (Ac_2O–pyridine). After addition of 24-methylenecycloartanyl acetate (**5c**, 10 mg), cyclolaudenyl acetate (**7c**, 8 mg) and cyclosadyl acetate (**8c**, 8 mg) the mixture was separated by prep. TLC on 10% $AgNO_3$ silica gel (developed twice with EtOH free $CHCl_3$–Et_2O, 49:1). Elution of the radioactive bands gave unknown A (R_f 0.16, 1814×10^4 dpm), unknown B plus C (R_f 0.25–0.34, 4.30×10^4 dpm), 24-methylenecycloartanyl acetate (**5c**, R_f 0.45, 7.90×10^5 dpm), cyclolaudenyl acetate (**7c**, R_f 0.52, 8.58×10^4 dpm), unknown (**9c**, R_f 0.66, 384×10^3 dpm) and cyclosadyl acetate (**8c**, R_f 0.71, 2.85×10^4 dpm).

Oxidation of **5c** *and* **7c**. Carrier **5c** was added to a portion of the radioactive 24-methylenecycloartanyl acetate (**5c**) and carrier **7c** was added to the whole of the radioactive cyclolaudenyl acetate (**7c**). Compounds **5c** (51 mg, 1.46×10^5 dpm) and **7c** (59 mg, 7.19×10^3 dpm) were each dissolved in 3.5 ml pyridine, 75 mg OsO_4 in 1.5 ml pyridine was added and the mixtures were left at room temp. for 16 hr [18, 19]. Sodium metabisulphite (500 mg) in 3 ml H_2O was added to each mixture and stirred for 2 hr. After addition of 100 ml H_2O the products were extracted with Et_2O (4 × 100 ml). The Et_2O extracts were washed, dried over Na_2SO_4 and the Et_2O removed to yield diols **10c** (58 mg) and **11c** (65 mg), respectively. These were purified by TLC on silica gel ($CHCl_3$–MeOH, 92:8) and crystallized once from MeOH. 24,28-Dihydroxy-24-methylcycloartanyl acetate (**10c**, mixture of 24*R*- and 24*S*-epimers), 36.5 mg; sp. act. 3000 dpm/mg; R_f 0.61; mp 182.5–186.5° (lit. [19] 183–184°); MS *m/z* (rel. int.): 516 $[M]^+$ (0.5), 498 (2), 483 (3), 456 (8), 438 (19), 423 (22), 413 (5), 410 (3), 395 (7), 369 (7), 357 (6), 334 (4), 316 (10), 297 (25), 215 (9), 203 (31), 175 (48), 95 (100). 25,27-Dihydroxy-24-methylcycloartanyl acetate (**11c**, mixture of 25*R*- and 25*S*-epimers), 43.8 mg; sp. act. 77 dpm/mg; R_f 0.50 and 0.53; mp 192–195° (lit. [19] 197–199° and 200–203°); MS: identical to MS of **10c** except that ion at *m/z* 413 was absent. Compounds **10c** (25 mg) and **11c** (40 mg) were each dissolved in 7 ml dioxan, 70 mg $NaIO_3$ in 3.5 ml H_2O added

and the reaction mixtures stirred at room temp. for 17.5 hr. After addition of 80 ml H_2O the products were extracted into Et_2O (4 × 100 ml), dried over Na_2SO_4 and the solvent removed. The resulting oxo-compounds were purified by prep. TLC on silica gel ($CHCl_3$–MeOH, 49:1) and crystallized from MeOH to constant sp. act. 24-Oxo-cycloartanyl acetate (**12c**); 23 mg; sp. act. 2 dpm/mg; mp 123–125° (lit. [19] 119–121°); R_f 0.62; GC RR_t 2.33 (3% OV-1), 3.07 (3% OV-17); MS *m/z* (rel. int.): 484 $[M]^+$ (1), 469 (0.5), 424 (13), 409 (13), 381 (5), 355 (5), 302 (3), 297 (9), 255 (2), 203 (14), 84 (66), 56 (100). 25-Oxo-27-nor-24-methylcycloartanyl acetate (**13c**), 37 mg, sp. act. 84 dpm/mg; mp 145–147° (lit. [19] 138–140°); R_f 0.55; GC RR_t 2.34 (3% OV-1), 3.13 (3% OV-17); MS *m/z* (rel. int.): 484 $[M]^+$ (1), 469 (1), 424 (34), 409 (39), 381 (12), 355 (16), 302 (5), 297 (26), 255 (5), 203 (29), 95 (87), 55 (100).

Preparation of S-adenosyl-[methyl-$^2H_3$]methionine. Yeast (*Saccharomyces cerviseae*) was grown in a medium containing 10 g KH_2PO_4, 5 g K_2HPO_4, 2 g $(NH_4)_2SO_4$, 1 g sodium citrate, 0.3 g $MgCl_2 \cdot 6H_2O$, 0.1 g $MnSO_4 \cdot 4H_2O$, 0.1 g $ZnSO_4 \cdot 7H_2O$, 0.1 g $CaCl_2 \cdot 2H_2O$ and 15 g glucose per l. To 100 ml of medium in a 500 ml flask was added 76 mg [methyl-$^2H_3$]methionine (M^+ at *m/z* 152, 100% trideuterated) and 1 g activated dry yeast added and the culture placed in an orbital incubator at 30°. After 24 hr a further 1 g glucose in 5 ml medium was added and the incubation was terminated after 48 hr. The cells were harvested by centrifugation (36 000 rpm, 10 min) and washed twice with H_2O. The cells (4.9 g wet wt) were extracted with 10 ml 1.5 N perchloric acid with stirring for 1 hr [23]. After centrifugation (9000 *g* 20 min) the supernatant was retained and the residue was re-extracted with 5 ml 1.5 N perchloric acid.

The supernatants were combined and small quantities of solid $KHCO_3$ added, with cooling and stirring, until the soln was *ca* pH 4.0; the $KClO_4$ ppt was removed by filtration. The soln was applied to an Amberlite CG-50 (100–200 mesh) column (150 × 10 mm) eluted first with H_2O (300 ml) and the SAM then eluted with 0.05 M H_2SO_4. Fractions (5 ml) were collected and the concn of SAM estimated by UV (260 nm, ε 15 400 [26]). The fractions containing SAM (72 μmol) were combined, adjusted to pH 2.5–3.0 with freshly pptd $BaCO_3$ and centrifuged. The supernatant was applied to a Dowex 1-HCO_3 column (50 × 10 mm) in order to remove any contaminating *S*-adenosylhomocysteine [26]. SAM passes straight through this column and fractions (5 ml) were collected, monitored by UV, and those containing SAM (57.4 μmol) combined and adjusted to pH 2.8 with 0.05 M H_2SO_4. A portion of the [methyl-$^2H_3$]SAM (7 μmol) was lyophilized to dryness and analysed by positive ion FAB-MS with glycerol as the solvent; *m/z* (rel. int.): 402 $[M]^+$ (12), 301 $[M - CH_2CH_2CH(NH_2)COOH + H]^+$ (25), 136 [M − (ribose + methionine) + 2H]$^+$ (100). Unlabelled SAM had FAB-MS ions at *m/z* 399 $[M]^+$, 298 and 136.

Incubation of [methyl-$^2H_3$]SAM with Z. mays microsomes. Two large scale incubations were carried out. The first contained 19.0 ml microsomal suspension, 10 μmol cycloartenol emulsified with 20 μl Tween 80 in 2.0 ml buffer, 25 μmol [methyl-$^2H_3$]SAM and 0.02 μ mol [methyl-^{14}C] SAM (1.25 μCi) in a final vol. of 25 ml. The second incubation contained 18 ml microsomal suspension, 20 μmol cycloartenol in 27 μl Tween 80 and 5 ml buffer, 25 μmol [methyl-$^2H_3$]SAM and 0.04 μmol [methyl-^{14}C]SAM (2.5 μCi) in a final vol. of 28 ml. The incubations were maintained at 30° for 6 hr with shaking. The non-saponifiable lipid was extracted and the 4,4-dimethyl sterols (1.16×10^5 dpm) obtained by prep. TLC on silica gel ($CHCl_3$–EtOH, 49:1). These were acetylated (Ac_2O–pyridine) and separated by prep. TLC on 10% $AgNO_3$–silica gel (two developments with EtOH free $CHCl_3$–Et_2O, 49:1) and bands eluted which corresponded to 24-methylenecycloartanyl acetate (**5c**, R_f 0.57, 9.39×10^4 dpm), cyclolaudenyl acetate (**7c**, R_f 0.62, 1.86×10^3 dpm) and cyclosadyl acetate (**8c**, R_f 0.82, 360 dpm). These compounds were then analysed by GC/MS (3% OV-17 at 280°) to determine their deuterium content (Fig. 1).

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