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BIOSYNTHESIS OF $\Delta^{5,23}$ STEROLS IN ETIOLATED COLEOPTILES FROM *ZEA MAYS*

F. SCHEID, M. ROHMER* and P. BENVENISTE

Institut de Botanique, Laboratoire de Biochimie Végétale, E.R.A. du C.N.R.S. No. 487, 28 rue Goethe, 67083, Strasbourg Cédex, France; *Ecole Nationale Supérieure de Chimie de Mulhouse, 3 rue Alfred Werner, 68093, Mulhouse, France

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Key Word Index—*Zea mays*; Gramineae; Δ^{23} sterols; cycloartenol-C-24-methyltransferase.

Abstract—In addition to the previously found ergosta-5, *E*-23-dien-3β-ol and 5α-ergosta-7, *E*-23-dien-3β-ol, the following Δ^{23} sterols have been identified in etiolated maize coleoptiles: cyclosadol, 4α, 14α-dimethyl-5α-ergosta-8, *E*-23-dien-3β-ol, 4α, 14α-dimethyl-9β, 19-cyclo-5α-ergosta-8, *E*-23-dien-3β-ol and 4α-methyl-5α-ergosta-7, *E*-23-dien-3β-ol. The incubation of maize coleoptile microsomes in the presence of cycloartenol and of [^{14}C-methyl]*S*-adenosyl methionine gave a mixture of labelled 24-methylene cycloartanol and cyclosadol. No trace of cyclolaudenol could be detected in these conditions. It is suggested that Δ^{23} sterols are products of the C-24 methyltransferase reaction and they probably do not arise from a $\Delta^{24} \rightarrow \Delta^{23}$ isomerization occurring at a later stage of the biosynthesis. The Δ^{23}-sterols may play an intermediary role in the biosynthesis of 24-methyl sterols in this plant material.

INTRODUCTION

Etiolated maize coleoptiles represent an actively growing organ. In order to study relationships existing between cell elongation and sterol biosynthesis, we proceeded first to a careful analysis of the sterols contained in this material. In addition to the typical sterols encountered in most higher plants: sitosterol, campesterol and stigmasterol, we recently reported the discovery of two unusual sterols: ergosta - 5, *E* - 23 - dien - 3β - ol (**1**) and 5α - ergosta - 7, *E*-23-dien-3β - ol (**2**) which together constituted 11% of the total sterols [1]. The presence of **1** and **2** in our material raised two questions: (a) how are $\Delta^{5,23}$-sterols biosynthesized; and (b) are they intermediates of 24-methyl sterol biosynthesis in maize etiolated coleoptiles? Two possible answers could be given to the first question. Firstly the Δ^{23}-bond could be derived from the isomerization of the $\Delta^{24(28)}$-bond present in sterol intermediates all along the biosynthetic pathway and secondly, the Δ^{23}-bond could arise from the C-24 methyltransferase reaction [2]. The answer to the second question would constitute an important contribution to the elucidation of the mechanism of the formation of 24-methyl sterols in higher plants since the passage through Δ^{23}-sterols could constitute an alternative pathway to the generally postulated $\Delta^{24(28)} \rightarrow \Delta^{24(25)}$ isomerization and $\Delta^{24(25)}$ reduction scheme [3]. Results obtained in the present study give a partial answer to these questions in showing that: (a) Δ^{23}-sterols are present in addition to $\Delta^{24(28)}$-sterols all along the biosynthetic scheme; (b) Δ^{23}-sterols are probably the products of the cycloartenol - C - 24 - methyltransferase reaction; and (c) the time labelling of $\Delta^{5,23}$-sterols are compatible with an intermediary role in 24-methylcholesterol biosynthesis.

Nomenclature: ergosta-5, *E*-23-dien-3β-ol (**1**); 5α-ergosta-7, *E*-23-dien-3β-ol (**2**); 4α-methyl-5α-ergosta-7, *E*-23-dien-3β-ol (**3**); 4α, 14α-dimethyl-9β, 19-cyclo-5α-ergost-*E*-23-en-3β-ol (**4**); 4α, 14α-dimethyl-5α-ergosta-8, *E*-23-dien-3β-ol (**5**); cyclosadol: 4, 4, 14α-trimethyl-9β, 19-cyclo-5α-ergost-*E*-23-en-3β-ol (**6**); cyclolaudenol: 4, 4, 14α-trimethyl-9β, 19-cyclo-5α-ergost-25-en-3β-ol; cycloartenol: 4, 4, 14α-trimethyl-9β, 19-cyclo-5α-cholest-24-en-3β-ol; lanosterol: 4, 4, 14α-trimethyl-5α-cholesta-8, 24-dien-3β-ol; 24-methylenecycloartanol: 4, 4, 14α-trimethyl-9β, 19-cyclo-5α-ergost-24(28)-en-3β-ol; cycloeucalenol: 4α, 14α-dimethyl-9β, 19-cyclo-5α-ergost-24(28)-en-3β-ol; obtusifoliol: 4α, 14α-dimethyl-5α-ergosta-8, 24(28)-dien-3β-ol; 24-methylenelophenol: 4α-methyl-5α-ergosta-7, 24(28)-dien-3β-ol; 24-ethylidenelophenol: 4α-methyl-5α-stigmasta-7, *Z*-24(28)-dien-3β-ol; episterol: 5α-ergosta-7, 24(28)-dien-3β-ol; isofucosterol: stigmasta-5, *Z*-24(28)-dien-3β-ol; Δ^7-avenasterol: 5α-stigmasta-7, *Z*-24(28)-dien-3β-ol; 24-dehydropollinastanol: 14α-methyl-9β, 19-cyclo-5α-cholest-24-en-3β-ol; 24-methylenepollinastanol: 14α-methyl-9β, 19-cyclo-5α-ergost-24(28)-en-3β-ol.

RESULTS

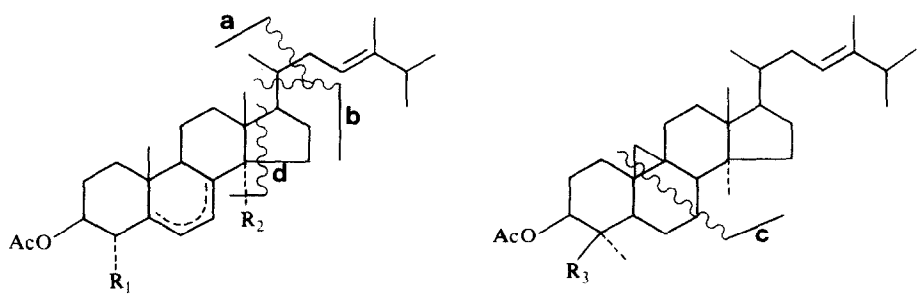
1. *Identification of* Δ^{23} - 4α - *methyl- and* Δ^{23} - 4,4 - *dimethyl sterols*

As described previously [1], etiolated maize coleoptiles contained two Δ^{23}-sterols: ergosta - 5, *E* - 23 - dien - 3β - ol (**1**) and 5α - ergosta - 7, *E* - 23 - dien-

3β - ol (**2**). In order to check for the presence of precursors of **1** and **2**, we performed a careful analysis of the components of the 4α-methyl and the 4, 4-dimethyl sterol fractions. The 4α-methyl steryl acetate fraction was submitted to argentation TLC as described in the Experimental. In addition to the known compounds contained in this fraction [4–6], 24-methylenelophenyl acetate (R_f 0.16), cycloeucalenyl plus obtusifoliyl acetates (R_f 0.30), 24 - ethylidenelophenyl acetate (R_f 0.36), two additional bands were detected at R_f 0.46 and 0.62. The corresponding compounds, **3** and **4** plus **5** respectively, were submitted to GC/MS analysis. The 4, 4-dimethyl steryl acetates were also separated by argentation TLC. In addition to the known compounds occurring in this fraction: 24-methylenecycloartanyl acetate (R_f 0.14), cycloartenyl acetate (R_f 0.29) and α- plus β-amyrin acetates (R_f 0.61), a very small band appeared at R_f 0.38. The corresponding compound (**6**) was submitted to GC/MS analysis. Table 1 assembles the mass spectral data of the new compounds. These were identified as 4α - methyl - 5α - ergosta - 7, *E* - 23 - dien - 3β - yl **3**-acetate; 4α, 14α - dimethyl - 9β, 19 - cyclo - 5α - ergost - *E* - 23 - en - 3β - yl **4**-acetate; 4α, 14 - dimethyl - 5α - ergosta - 8, *E* - 23 - dien - 3β - yl **5**-acetate and 4, 4, 14α - trimethyl - 9β, 19 - cyclo - 5α - ergost - *E* - 23 - en - 3β - yl **6**-acetate (cyclosadol). The identifications were based on the presence in the mass spectra of a fragment (**a**) $[M-C_7H_{13}-Ac]^+$ corresponding to the cleavage of the C-20, C-22 bond allylic to the C-23 double bond. This fragment was absent or had a very low intensity in sterols not possessing the Δ^{23}-bond. Identification of **3** was unambiguous since its mass spectrum displayed very typical features: in particular the fragmentation (**b**) $[M-\text{lateral chain}-2H]^+$ was characteristic of a Δ^7-sterol containing a double bond in the lateral chain [7]. Moreover, the mass spectrum was identical to that of the acetate of 4α - methyl - 5α - ergosta - 7, *E*-23 - dien - 3β - ol identified recently in maize germ oil [8]. Identification of **4** was also without ambiguity since this compound showed the very typical fragment (**c**) corresponding to the cleavage of the cyclopropane ring [9]. Identification of **5** was only tentative since as for most 14α - methyl - Δ^8 - sterols, the mass spectrum of **5** did not display any characteristic fragmentation. Nevertheless the postulated structure for **5** was highly probable with respect to its TLC and GC behavior. Finally, **6** was identified by its mass spectrum which displayed the characteristic features of 9β, 19 - cyclopropyl sterols (fragment **c**) [9], and was identical to that of the acetate of authentic cyclosadol [8, 10]. The configuration of the Δ^{23}-double bond present in products **1–6** was not determined in this study. However as discussed previously by Itoh *et al.* [8], the ^{1}H NMR signals of the C-28 methyl and C-25 methine protons for the acetates of **1** and **2** [1] were consistent with the corresponding signals of the

Table 1. Mass spectra of the 4-desmethyl-, 4α-methyl- and 4, 4 - dimethyl - Δ^{23} - steryl acetates obtained from maize coleoptiles

	$[M]^+$	$[M-60]^+$	a	a − 60	b − 60	b − 2H	b − H − 60	c	d − 60
Ergosta - 5, 23 - dien - 3β - yl **1**-acetate	440(0)	380(100)	343(0)	283(100)	255(10)	313(0)	253(35)	—	213(10)
5α - Ergosta - 7, 23 - dien - 3β - yl **2**-acetate	440(15)	380(11)	343(19)	283(60)	255(12)	313(100)	253(22)	—	213(21)
4α - Methyl - 5α - ergosta - 7, 23 - dien - 3β - yl **3**-acetate	454(12)	394(18)	357(20)	297(64)	269(12)	327(100)	267(72)	—	227(25)
4α, 14α - Dimethyl - 9β, 19 - cyclo - 5α - ergost - 23 - en - 3β - yl **4**-acetate	468(0)	408(75)	—	311(50)	283(5)	—	—	300(10)	241(18)
4α, 14α - Dimethyl - 5α - ergosta - 8, 23 - dien - 3β - yl **5**-acetate	468(15)	408(20)	—	311(10)	283(15)	—	—	—	241(23)
4, 4, 14α - Trimethyl - 9β, 19-cyclo - 5α - ergost - 23-en-3β - yl **6**-acetate	482(0)	422(64)	—	325(71)	297(29)	—	—	300(10)	255(?)



R_1, R_2, R_3 = H or Me

acetate of synthetic 4, 4, 14α - trimethyl - 9β, 19 - cyclo - 5α - ergost - *E* - 23 - en - 3β - ol [8]. Hence **1** and **2** should also have the *E*-configuration of their Δ^{23}-bond. The acetates of **3** and **6** were shown to be identical (GC, MS) to the acetates of 4α - methyl - 5α-ergosta - 7, *E* - 23 - dien - 3β - ol and 4, 4, 14α-trimethyl - 9β, 19 - cyclo - 5α - ergost - *E* - 23 - en -3β-ol, respectively, which were identified recently in maize germ oil [8]. Thus **3** and **6** also have the *E*-configuration of their Δ^{23}-bond. We shall assume that the configuration of the Δ^{23}-bond of **4** and **5** is also *E*.

2. *Sterol composition of etiolated maize coleoptiles*

The relative amounts of individual sterols in each of the three classes: 4-desmethyl, 4α-methyl and 4, 4-dimethyl sterols are reported in Table 2. The mixture of campesteryl, sitosteryl and stigmasteryl acetates was submitted to argentation TLC with washed chloroform as solvent in order to separate stigmasteryl acetate from the acetates of campesterol and sitosterol. These latter were further analysed by HPLC which allowed the separation of campesteryl acetate from sitosteryl acetate. Finally the purified compounds, as well as authentic samples of (24*R*)- and (24*S*) - 24 - methylcholesteryl acetates, were submitted to ^{1}H NMR (360 MHz) (Table 3). The results obtained, when compared with those in the literature [11–13], showed unambiguously that the 24-methylcholesterol fraction was in fact a mixture (1 : 1) of (24*R*)- and (24*S*) - 24 - methylcholesterol. By contrast the sitosteryl acetate fraction was shown to

Table 2. Sterol composition of etiolated maize coleoptiles

Sterol	Quantity (μg/g dry wt)	
4-Desmethyl sterols		
Cholesterol	25	1.5*
(24*R*) - 24 - Methylcholesterol	140	8.5
(24*S*) - 24 - Methylcholesterol	140	8.5
Sitosterol	400	24
Stigmasterol	650	39
Ergosta - 5, 23 - dien - 3β - ol (**1**)	150	9
Ergosta - 7, 23 - dien - 3β - ol (**2**)	30	2
Isofucosterol	40	2.5
Δ^7-Avenasterol	8	0.5
24-Methylenecholesterol	10	0.5
Episterol	tr.	—
Δ^7-Stigmastenol	tr.	—
Δ^7-Campestenol	tr.	—
Miscellanous	15	1
Total 4-desmethyl sterols	1650	
4α-Methyl sterols		
24-Methylenelophenol	1.5	8†
Cycloeucalenol	0.5	3
Obtusifoliol	3	18
24-Ethylidenelophenol	1.5	9
4α - Methyl - 5α - ergosta - 7, 23 - dien - 3β - ol (**3**)	6	35
4α, 14α - Dimethyl - 9β, 19 - cyclo - 5α - ergost - 23 - en - 3β - ol (**4**) plus 4α, 14α - Dimethyl - 5α - ergosta - 8, 23 - dien - 3β - ol (**5**)	3.5	20
4,4-Dimethyl sterols		
24-Methylenecycloartanol	4.5	18‡
Cycloartanol	6	24
Cyclosadol (**6**)	0.5	2.5
β-Amyrin	8.5	35
α-Amyrin	5.5	20
Total 4, 4-dimethyl sterols plus 4α-methyl sterols	42.0	

*As a percentage of 4-desmethyl sterols.
†As a percentage of 4α-methyl sterols.
‡As a percentage of 4, 4-dimethyl sterols.

Table 3. ^{1}H NMR (360 MHz) spectral shifts (δ) of the proton signals of (24*R*)- and (24*S*) - 24 - methylcholesteryl acetates

	C-18	C-19	C-21	C-26	C-27	C-28
(24*R*) - 24 - Methylcholesteryl acetate*	0.676 *s*	1.017 *s*	0.909 *d*, $J = 6.5$‡	0.849 *d*, $J = 6.5$	0.800 *d*, $J = 7$	0.770 *d*, $J = 7$
(24*S*) - 24 - Methylcholesteryl acetate*	0.676 *s*	1.017 *s*	0.913 *d*, $J = 6.5$	0.849 *d*, $J = 6.5$	0.775 *d*, $J = 7$	0.771 *d*, $J = 7$
(24*RS*) - 24 - Methylcholesteryl acetate†	0.675 *s*	1.017 *s*	0.909 and 0.917 *d*'s, $J = 6.5$	0.852 *d*, $J = 6.5$	0.800 and 0.781 *d*'s, $J = 7$	0.773 *d*, $J = 7$

*Authentic samples.
†Compound extracted from maize coleopiles.
‡Coupling constants in Hz.

contain (24*R*) - 24 - ethylcholesteryl acetate and the stigmasteryl acetate fraction only (24*S*) - 24 - ethylcholesta - 5, 22 - dien - 3β - yl acetate.

3. *Biosynthesis of* Δ^{23}*-sterols*

The [1-^{14}C]acetate of [5-^{14}C]mevalonate was fed to maize coleoptile segments for various times (from 0.5 to 8 hr). All Δ^{23}-sterols identified above were shown to be labelled. After acetylation with [^{3}H]acetic anhydride, specific radioactivity was measured as described previously [14] after extensive purification of the sterols. The specific radioactivity obtained for 4-desmethyl steryl acetates extracted from etiolated maize coleoptiles segments incubated for 4 hr in the presence of [1-^{14}C]acetate are reported in Table 4. Specific radioactivity of Δ^{23}-steryl acetates was shown to be much higher than that of the mixture of campesteryl and sitosteryl acetates but lower than that of 24-methylenecholesteryl acetate. This result was obtained regardless of the time of the incubation [15].

4. In vitro *study of the transfer of the methyl group of S-adenosyl methionine to various* Δ^{24}*-sterols*

It has been suggested before [1] that Δ^{23}-sterols could originate either from $\Delta^{24(28)}$-sterols, themselves products of the *S*-adenosyl methionine - (SAM) - sterol - *C* - 24 - methyltransferase activity, or by the SAM - *C* - 24 - methyltransferase reaction. To gain information concerning this point, maize coleoptile microsomes were incubated in the presence of [^{14}C-methyl]SAM (100 μM) and cycloartenol (100 μM) for 1 hr. The products of the C-24 methylation were expected to be 24-methylenecycloartanol, cyclolaudenol and cyclosadol for the following reasons: 24-methylenecycloartanol has been shown to be produced under such conditions [16, 17], cyclolaudenol has been reported previously in the same material [3] and cyclosadol has been identified in maize seed oil [10]. After argentation TLC, cycloartenyl acetate, cyclosadyl acetate (1500 cpm) and the mixture of presumed cyclolaudenyl plus 24-methylenecycloartanyl acetates (5500 cpm) were separated. Cyclolaudenyl- and 24-methylenecycloartanyl acetates were differentiated using a procedure described previously [16] based on osmium tetroxide treatment of the mixture. This yielded 25, 26 - dihydroxy - 4, 4, 14α - trimethyl - 9β, 19 - cyclo - 5α - ergostan - 3β - yl **7**-acetate and 24, 28 - dihydroxy - 4, 4, 14α - trimethyl - 9β, 19 - cyclo - 5α - ergostan - 3β-yl **8** - acetate which were readily separated by TLC. Whereas most of the initial radioactivity was associated with **8**-acetate, no trace of radioactivity could be detected in **7**-acetate showing clearly that in our system only 24-methylenecycloartanol and cyclosadol were the products of the SAM - cycloartenol - C - 24 - methyltransferase reaction. Figure 1 shows the evolution of the radioactivity associated with 24-methylenecycloartanol and cyclosadol with the incubation time: the ratio of the radioactivities associated with cyclosadol and 24-methylenecycloartanol was constant for short incubation periods and decreased after longer incubation periods. This feature would not be in agreement with a precursor–product relationship between 24-methylenecyclo-

artanol and cyclosadol (the latter arising from the former by isomerization of the $\Delta^{24(28)}$-bond). Thus our results suggested that cyclosadol is really one of the products of the C-24 methyltransferase reaction. In order to obtain additional information concerning the *in vitro* synthesis of Δ^{23}-sterols, we performed a study of the influence of the sterol substrate structure on the yield of 24-alkylated products and on the relative amount of Δ^{23} - 24 - alkylated sterols. For this purpose an acetone powder was prepared from microsomes. A buffer suspension of the acetone powder was incubated in the presence of [^{14}C-methyl]SAM (100 μM) and various Δ^{24}-substrates at different concentrations. Incubations were performed for 2 hr. Analytical details concerning the identification of 24-alkylated compounds are given in the Experimental. The evolution of the rate of the reaction with increasing amounts of the different substrates is shown in Fig. 2. Table 5 summarizes the results obtained and shows that at saturating concentrations of the sterol substrates, cycloartenol is a much better substrate than lanosterol confirming previous studies [16, 17]. Surprisingly, 31 - nor - cycloartenol is not as good a substrate as 31 - nor - lanosterol. Table 5 also gives the relative percentages of the Δ^{23}- and $\Delta^{24(28)}$-compounds present in the mixture of 24-alkylated products of the C-24 methyltransferase reaction. The data showed that the relative percentages of the Δ^{23}-sterols were dependent upon the substrate used and that desmosterol gave reproducibly the lowest yield of Δ^{23}-sterols.

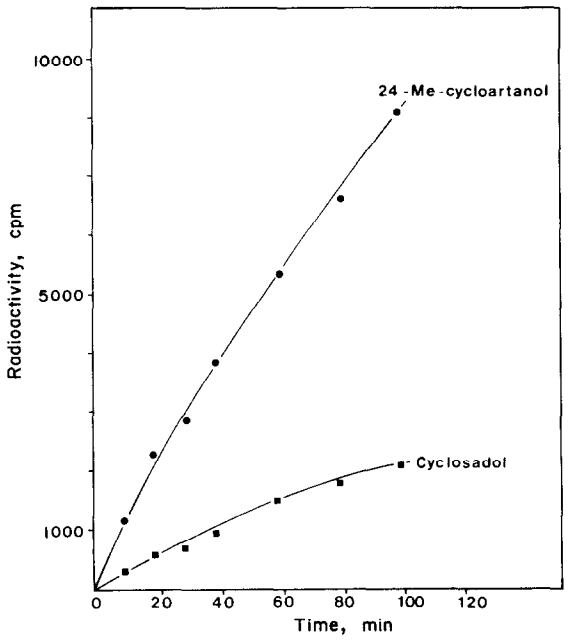


Fig. 1. Evolution of the radioactivity associated with 24-methylenecycloartanol and cyclosadol with incubation time. [^{14}C-methyl]SAM (100 μM), cycloartenol (50 μM).

DISCUSSION

In addition to the ubiquitously occurring higher plant sterols [18], etiolated maize coleoptiles contained the following Δ^{23}-sterols: cyclosadol (**6**); 4α, 14α - dimethyl - 5α - ergosta - 8, *E* - 23 - dien - 3β - ol (**5**); 4α, 14α - dimethyl - 9β, 19 - cyclo - 5α - ergost - *E* - 23 - en - 3β - ol (**4**); 4α - methyl - 5α - ergosta - 7, *E* - 23 - dien - 3β - ol (**3**); ergosta - 5, *E* - 23 - dien - 3β - ol (**1**); and 5α - ergosta - 7, *E* - 23 - dien - 3β - ol (**2**). As cyclosadol has been suggested to result from the SAM C-24 methylation of cycloartenol, it is possible to propose the existence, in addition to the classical pathway involving $\Delta^{24(28)}$-sterols, of a secondary route involving Δ^{23}-sterols (Scheme 1). At this time it is still not possible to decide whether there are connections between the $\Delta^{24(28)}$- and the Δ^{23}-sterol pathways, possibly through the existence of an enzyme capable of isomerizing $\Delta^{24(28)}$-sterols into Δ^{23}-sterols.

A possible role as intermediates of 24-methyl sterol biosynthesis has been suggested for Δ^{23}-sterols in our material [1]. Some experimental evidence suggested that (24*R*) - 24 - methylcholesterol (campesterol) could arise from 24-methylenecholesterol, through isomerization of the $\Delta^{24(28)}$-band to the $\Delta^{24(25)}$-bond and enzymatic hydrogenation of this latter compound [3, 18, 19]. However, the 24-methylcholesterol found in higher plants often consists of a mixture of epimers at C-24: (24*R*) - 24 - methylcholesterol (campesterol) and (24*S*) - 24 - methylcholesterol (22-dihydrobrassicasterol) [12, 13]. This was also the case for 24-methylcholesterol isolated in the present study which was shown to contain a mixture (1 : 1) of the 24*R*- and

Table 4. Specific radioactivities of 4-demethylsteryl acetates isolated from maize coleoptile segments incubated in the presence of [1-^{14}C]acetic acid for 4 hr

4-Desmethyl steryl acetates	Specific radioactivities (μCi/mol)
24-Methylenecholesteryl acetate	1.20
Isofucosteryl acetate	2.00
Δ^{23}-Steryl **1**- and **2**-acetate	0.20
Campesteryl- and sitosteryl acetates	0.01*
Stigmasteryl acetate	0.01*
5α - Stigmast - 7 - en - 3β - yl- and 5α - ergost - 7 - en - 3β - yl-acetates	1.00

*These numbers correspond to low but significative values of the ^{14}C label incorporated.

Table 5. Characteristics of the SAM - Δ^{24} - sterol C-24 methyltransferase measured with various Δ^{24}-substrates

Substrate	νm (nmol/hr/mg protein)	% incorporation into $\Delta^{24(28)}$-sterols	% incorporation into Δ^{23}-sterols
Cycloartenol	0.66*	75	25
Lanosterol	0.13	55	45
31 - Nor - cycloartenol	0.23	65	35
31 - Nor - lanosterol	0.36	70	30
Desmosterol	0.21	90	10
30, 31 - Bisnor - cycloartenol	0.44	75	25

*The incubations were performed as described in the Experimental. Values plotted in Fig. 2 for saturating concentrations (50 μm) of the Δ^{24}-sterol substrates have been used.

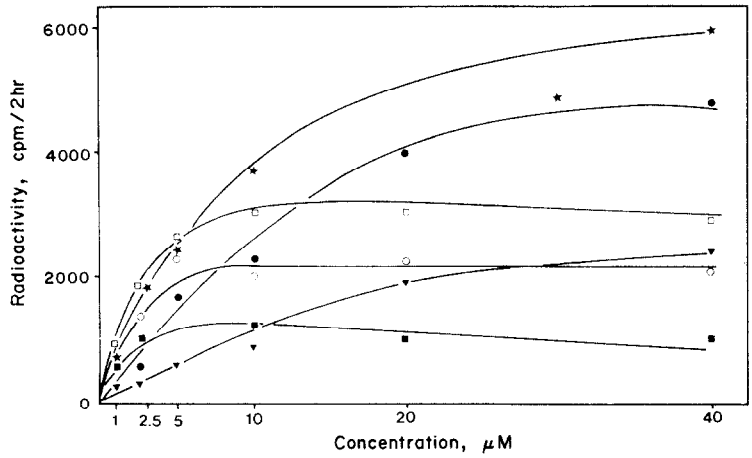


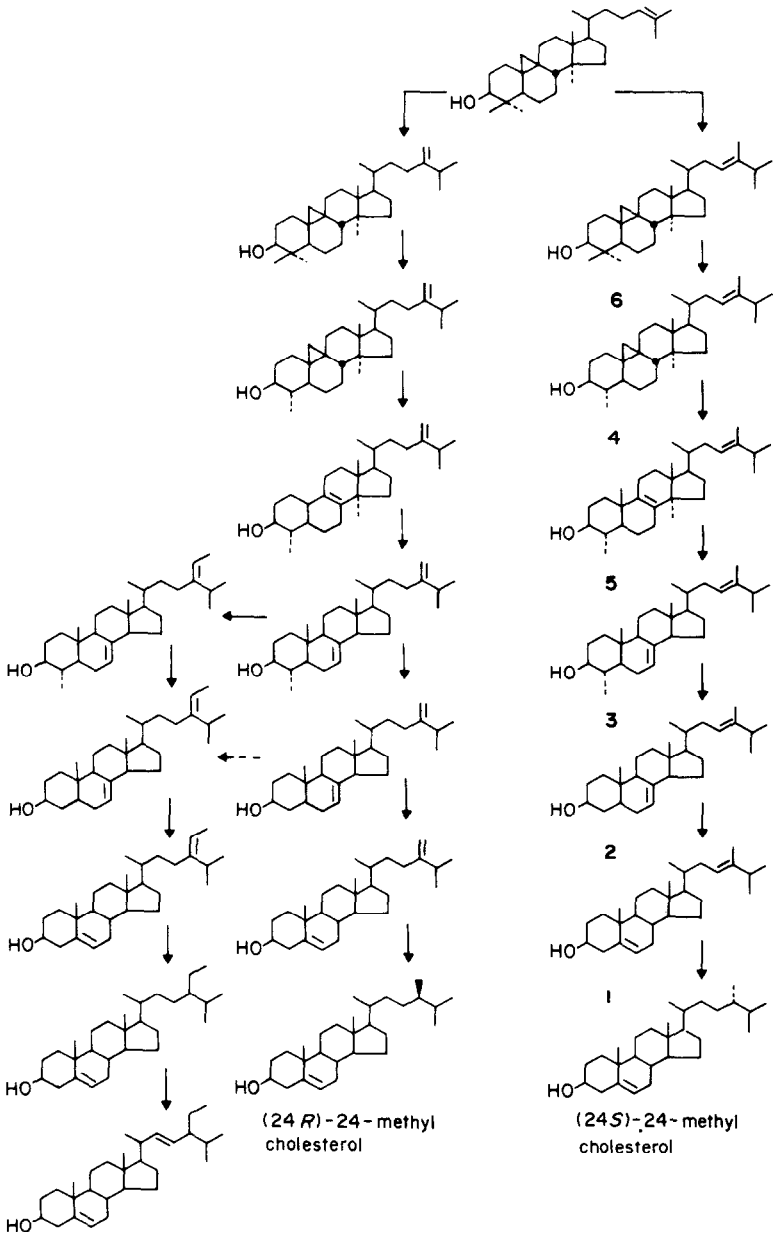
Fig. 2. Evaluation of the rate of the C-24 methylation with increasing amounts of the different substrates. The acetone powder is incubated in the presence of [^{14}C-methyl]SAM (80 μM) and of the Δ^{24}-sterol for 2 hr.

24*S*-epimers. It has been suggested that the (24*S*) - 24-methylcholesterol could arise from a different pathway from that involving (24*R*) - 24 - methylcholesterol production. This pathway could involve the intermediacy of 24 - methyl - Δ^{25} - sterols such as cyclolaudenol [3, 20, 21]. As our material did not contain either cyclolaudenol or any other 24 - methyl - Δ^{25} - sterols, but did contain several 24 - methyl - Δ^{23} - sterols, we suggest that these latter could be intermediates of the biosynthesis of (24*S*) - 24 - methylcholesterol in our material. This suggestion would be in agreement with the labelling of 24 - methyl - Δ^{23} - sterols observed in the present study. Scheme 2 describes two hypothetical routes for (24*R*)- and (24*S*) - 24 - methyl sterol biosynthesis. We assume that a unique enzyme lacking specificity would hydrogenate the Δ^{23}- and the $\Delta^{24(25)}$-bonds. If this last reaction proceeds by a *trans* addition of a proton and a hydride ion [22], the configuration at C-24 of the two compounds resulting from the hydrogenation of the Δ^{23}- and the $\Delta^{24(25)}$-bonds will be opposite. If this hypothesis is true, the existence of (24*S*) - 24 - methyl sterols would result from the lack of specificity of the SAM - sterol - Δ^{24} - C - 24 - methyltransferase which yielded both cyclosadol and 24-methylene cycloartanol or from the occurrence of two specific methyl transferases. Regardless of the real situation, the methyl transferase(s) presents some interesting features in our material. This work showed that it discriminated 9β, 19 - cyclopropyl, Δ^{24}-sterols from $\Delta^{8,24}$-sterols (cycloartenol being a much better substrate than lanosterol) when the substrates contained two methyls at C-4. However, this ability was greatly reduced when the substrates possessed only a 4α-methyl (31 - nor - cycloartenol being as efficiently or even less efficiently methylated than 31 - nor - lanosterol). This intriguing feature has been observed in other materials [Benveniste, P., unpublished] and remains unexplained.

EXPERIMENTAL

Plant material. Maize seeds (*Zea mays* cv INRA 258) were allowed to germinate in the dark at 25°. The coleoptiles were excised after 6 days of germination.

Isolation and identification of sterols. Most of the techniques used in this work have been described previously [23]. The *RR*$_t$'s (OV-17, cholesterol, *RR*$_t$ 1.0) on GC for the acetates of the 4-desmethyl-, 4α-methyl- and 4, 4-dimethyl sterols isolated in this study were: campesteryl acetate,

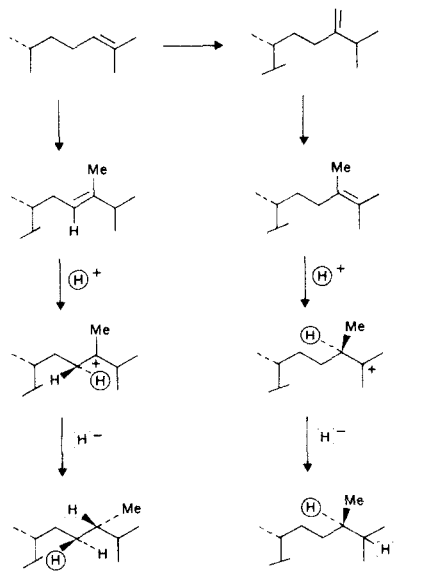


Scheme 1.

1.68; stigmasteryl acetate, 1.81; sitosteryl acetate, 2.05; Δ^7-campestenyl acetate, 1.98; Δ^7-stigmastenyl acetate, 2.42; campestanyl acetate, 1.70; 22-dehydrostigmastanyl acetate, 1.84; stigmastanyl acetate, 2.08; ergosta - 5, 23 - dien - 3β - yl **1**-acetate, 1.73; 5α - ergosta - 7, 23 - dien - 3β - yl **2**-acetate, 2.05; isofucosterylacetate, 2.23; Δ^7-avenasteryl acetate, 2.64; 24-methylenelophenyl acetate, 2.25; cycloeucalenyl acetate, 2.24; obtusifoliyl acetate, 1.89; 24-ethylidenelophenyl acetate, 2.94; 4α - methyl - 5α - ergosta - 7, 23 - dien - 3β - yl **3**-acetate, 2.25; 4α, 14α - dimethyl - 5α - ergosta - 8, 23 - dien - 3β - yl **5**-acetate, 1.90; 4α, 14α - dimethyl - 9β, 19 - cyclo - 5α - ergost - 23 - en - 3β - yl **4**-acetate, 2.21; 24-methylenecycloartanyl acetate, 2.64; cycloartenyl acetate, 2.39; α-amyrin acetate, 2.37; β-amyrin acetate, 2.10; cyclosadyl acetate, 2.61.

Radioactivity pulses. The coleoptiles (10 g) were cut into segments (1 cm) and immersed in a medium (50 ml) containing saccharose (0.1 M) and the mineral salts of Heller's soln diluted 10-fold [24]. The labelled precursor: sodium [1-^{14}C]acetate (50 mCi/mmol, 50 μCi for each assay) or [5-^{14}C]mevalonic acid (12 mCi/mmol, 10 μCi for each assay) was added. The incubation was conducted in darkness, at 25°, and with smooth stirring. The mixture was filtered and the segments were rinsed with iced H_2O and, finally, lyophilized.

Analytical procedure. The isolation of 4, 4-dimethyl-, 4α-methyl- and 4-desmethylsteryl acetates has been described previously [23]. Each of the three classes of acetates was analysed by GC, and the total amount of sterols present in each class was quantified. Analytical argentation TLC, in



Scheme 2.

which cyclohexane–toluene (3:2) was the developing solvent and migration was for 15 hr, was performed on each class of steryl acetate and the bands obtained were analysed by GC. There were four bands of 4, 4-dimethylsteryl acetates corresponding in order of decreasing polarity to 24-methylenecycloartanyl acetate (R_f 0.14), cycloartenyl acetate (R_f 0.29), cyclosadyl **6**-acetate (R_f 0.38), and a mixture of α- and β-amyrin acetates (R_f 0.61). There were six bands of 4α-methylsteryl acetates corresponding in order of decreasing polarity to 24-methylenelophenyl acetate (R_f 0.16), cycloeucalenyl- and obtusifoliyl acetate (R_f 0.30), 24-ethylidenelophenyl acetate (R_f 0.36), 4α - methyl - 5α - ergosta, 7, 23 - dien - 3β - yl **3**-acetate (R_f 0.46), 4α, 14 - dimethyl - 5α - ergosta - 8, 23 - dien - 3β - yl **5**-acetate and 4α, 14α - dimethyl - 9β, 19 - cyclo - 5α - ergost - 23 - en - 3β - yl **4**-acetate and 24-dihydrocycloeucalenyl acetate (R_f 0.77). There were four bands of 4-desmethylsteryl acetates corresponding in order of decreasing polarity to 24-methylenecholesteryl acetate and episteryl acetate (R_f 0.14), isofucosteryl acetate and Δ^7-avenasteryl acetate (R_f 0.29), ergosta - 5, 23 - dien - 3β - yl **1**-acetate and 5α - ergosta - 7, 23 - dien - 3β - yl **2**-acetate (R_f 0.35), campesteryl-, sitosteryl- and stigmasteryl acetates (R_f 0.54) and stanyl acetates (R_f 0.65). Argentation TLC, using EtOH free $CHCl_3$ as the developing solvent, was used to separate stigmasteryl acetate (R_f 0.55) from campesteryl- and sitosteryl acetates (R_f 0.65) and from Δ^7-stigmasteryl- and Δ^7-campesteryl acetates (R_f 0.73). When the sterols were extracted following an incorporation of [^{14}C]acetate or [^{14}C]mevalonate, the analytical techniques used were identical to those described above except that [^{3}H]Ac_2O (20 mCi/mmol) was used to acetylate the ^{14}C-labelled sterols. Specific radioactivity was calculated from the $^{14}C/^3H$ ratio of the doubly labelled steryl acetates as described earlier [14].

Separation of 24-methyl cholesteryl acetate from sitosteryl acetate. The separation was performed by HPLC using a μ-Bondapax C_{18} column (4 × 150 mm, Waters) and a mixture of MeOH–H_2O (95:5) as elution solvent (1.5 ml/min, pressure: 750 psi).

SAM - C - 24 - methyltransferase assays. Maize coleoptiles (100 g) were ground in a mortar at 0° with 1 vol. of medium containing: 0.1 M Tris–HCl, 1 mM EDTA, 10 mM mercaptoethanol, 0.5 M sucrose and 0.5% BSA, final pH 7.5. The homogenate was squeezed through four layers of gauze and filtered on nylon cloth (50 μm). The filtrate was centrifuged at 6000 *g* for 15 min to remove mitochondria. The supernatant was centrifuged for another 60 min at 100000 *g* to sediment out microsomes. Soluble supernatant was removed and the microsomal pellets were suspended in a medium containing 0.1 M Tris–HCl, 5 mM mercaptoethanol, pH 7.5 and were washed by a second centrifugation at 100000 *g* and the pellet obtained was resuspended in the same medium as before. Protein was determined by the method of Lowry *et al.*[25] with BSA used as a standard. Membrane protein concentrations of *ca* 1 mg/ml were generally used. The washed microsomes could be used directly for methyltransferase assays. In some cases, it was necessary to use Me_2CO powders. For this purpose the washed microsomes were mixed with 20× their vol. of iced Me_2CO (−10°) for 15 min. The suspension was centrifuged at 10000 *g* for 20 min, the pellets were dried under N_2 and finally suspended in a medium containing 0.1 M Tris–HCl and 5 mM mercaptoethanol, pH 7.5. The SAM-Δ^{24}-sterol C-24 methyltransferase assay was conducted as described previously [17]. The reaction mixture contained the enzyme (0.3 ml), [^{14}C-methyl]SAM (80 μM), the sterol substrate (50 μM) and Tween 80 (0.05%). Incubations were conducted at 30° for 2 hr and were stopped by addition of 1 vol. of ethanolic KOH (20%). The resulting mixture was extracted with hexane (3 × 3 vol.). The extracts were pooled, the solvent evaporated and the residue chromatographed on TLC using CH_2Cl_2 as developing solvent (two runs). Radioactivity associated with 4, 4-dimethyl-, 4α-methyl- and 4-desmethyl sterols was measured after elution of these compounds from the Si gel in a liquid scintillation spectrometer. When a more precise identification of the products of the enzymatic assay was needed, the 4, 4-dimethyl fraction resulting from the incubation of cycloartenol was acetylated and the acetates were purified using argentation TLC as described above. 24-Methylenecycloartanyl- and cyclosadyl acetates, products resulting of the C-24 methylation of cycloartenol, were identified as described above. The same procedure was used when lanosterol, 31 - nor - cycloartenol, 31 - nor - lanosterol, 24-dehydropollinastanol, and desmosterol were incubated in place of cycloartenol. The expected products were $\Delta^{24(28)}$-sterols (respectively, 24 - methylene - 24 - dihydrolanosterol, cycloeucalenol, obtusifoliol, 24-methylenepollinastanol and 24-methylenecholesterol) and their Δ^{23}-isomers (respectively, 4, 4, 14α - trimethyl - 5α - ergosta - 8, 23 - dien - 3β - ol, **4**, **5**, 9β, 19 - cyclo - 5α - ergost - 23 - en - 3β - ol and **1**). All these compounds were identified by their chromatographic behavior and by comparison with authentic samples.

Separation of 24-methylenecycloartanol and cyclolaudenol. Since the acetates of these two compounds were not separated in our chromatographic conditions, the radioactive fraction containing these two products was diluted with unlabelled samples of 24-methylenecycloartanyl- and cyclolaudenyl acetates, and then the mixture was treated with OsO_4 as described previously [16]. The two diols obtained: 25, 26 - dihydroxy - 4, 4, 14α - trimethyl-9β, 19 - cyclo - 5α - ergostan - 3β - yl **7**-acetate and 24, 28 - dihydroxy - 4, 4, 14α - trimethyl - 9β, 19 - cyclo - 5α - ergostan - 3β - yl **8**-acetate were readily separated by TLC using $CHCl_3$–MeOH (92:8) as developing solvent. **7**- and **8**-acetates were identified by their MS.

Authentic materials. 24-Methylenecholesterol and 24-dehydropollinastanol were extracted from a commercial source of pollen (Amylex–Heuprophax) of unknown composition. 31 - Nor - lanosterol and desmosterol were extracted from *Fontumia latifolia* and 31 - nor - cycloartenol from pollen of *Carnegia gigantea* supplied by Dr. L. N. Standifer (Tucson, U.S.A.). 24-Methylenelophenol and 24-methylenecycloartanol were the kind gift of Dr. Itoh (Tokyo, Japan). Cyclosadol was a gift from Dr. H. Pinhas (Paris, France) and cyclolaudenol was supplied by Dr. A. S. Narula (Canberra, Australia).

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