Phytochemistry, Vol. 21, No. 8, pp. 1969–1974, 1982.
Printed in Great Britain.

0031-9422/82/081969-06$03.00/0
Pergamon Press Ltd.

7-OXO-24ξ(28)-DIHYDROCYCLOEUCALENOL, A POTENT INHIBITOR OF PLANT STEROL BIOSYNTHESIS

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(*Received* 8 *December* 1981)

Key Word Index—*Zea mays*; Gramineae; *Rubus fruticosus*; Rosaceae; plant sterol biosynthesis inhibitors; cycloeucalenol-obtusifoliol isomerase; 7-oxo-24ξ(28)-dihydrocycloeucalenol; cellular and enzymatic targets at 7-oxo-24ξ(28)-dihydrocycloeucalenol.

Abstract—Cycloeucalenol-obtusifoliol isomerase from higher plant cells catalyses the opening of the cyclopropane ring of cycloeucalenol yielding obtusifoliol. 7-Oxo-24ξ(28)-dihydrocycloeucalenol was not a substrate but behaved like a potent inhibitor of the isomerase. The inhibition was reversible and highly specific; the inhibitor needed the presence of the 7-oxo group, the cyclopropane ring and the absence of a 4β-methyl group to be active. Other enzymes involved in plant sterol biosynthesis such as 2, 3-oxidosqualene-cycloartenol cyclase and *S*-adenosyl methionine cycloartenol C-24 methyltransferase were not inhibited by 7-oxo-24ξ(28)-dihydrocycloeucalenol. *In vivo* treatment of a suspension of bramble cells growing in a liquid medium with 7-oxo-24ξ(28)-dihydrocycloeucalenol resulted in a strong accumulation of 9β, 19-cyclopropyl sterols confirming that the main cellular target of the inhibitor is the cycloeucalenol-obtusifoliol isomerase.

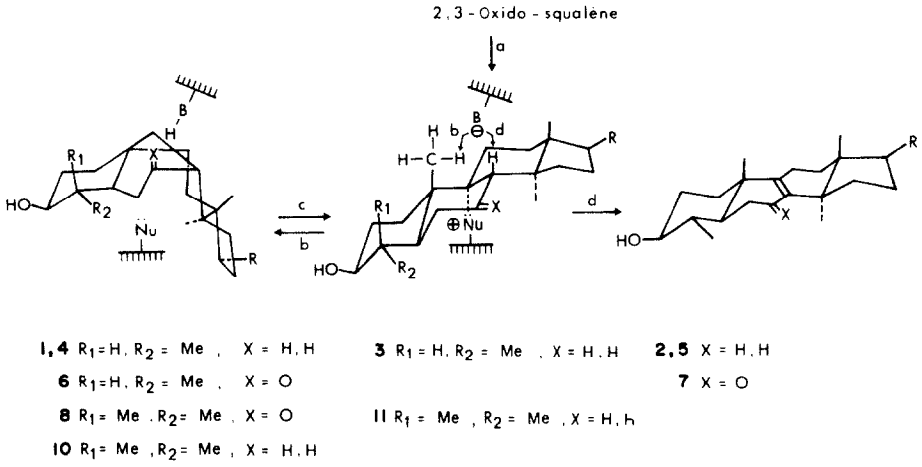
INTRODUCTION

9β, 19-Cyclopropyl sterols, are considered to be intermediates of sterol biosynthesis in photosynthetic eucaryotes [1]. As the sterols contained in these organisms do not contain a cyclopropane ring, there must be an enzyme that opens the cyclopropane ring of precursor cyclopropyl sterols. Such an enzyme, the cycloeucalenol **1**-obtusifoliol-**2**-isomerase has been discovered in our laboratory [2], it catalyses the opening of the 9β, 19-cyclopropane of **1** yielding **2** [2]. Compounds **1** and **2** are two 4α-methyl sterols ubiquitously found in the Plant Kingdom [1]. Some properties of this isomerase enzyme have been recently studied [2–5]. It has been shown in particular that the opening of the cyclopropane ring can be considered as the biochemical equivalent of an acid catalysed isomerization [4, 5] and that the acidic residue of the enzyme involved in the protonation of the cyclopropane ring would be located as indicated in Scheme 1 [4]. In order to explain the mechanism of action of the isomerase, the involvement of the intermediate **3** during catalysis has been suggested (Scheme 1) [1, 6]. In an attempt to trap **3** or to change the stereoelectronic factors involved in its formation and determining its reactivity we have planned to modify the B-ring of the 9β, 19-cyclopropyl sterol substrates. The introduction of a keto group at C-7 was considered since the presence of the carbonyl group in intermediate **3** could strongly labilize the 8β-H resulting possibly in an easier elimination of this proton and in the formation of a thermodynamically favored α, β-unsaturated ketone. As shown elsewhere [1, 2] the reduction of the $\Delta^{24(28)}$-double bond of **1** did not change the capacity of the resulting 24(28)dihydrocycloeucalenol (**4**) to be a substrate of the COI, thus 7-oxo-24ξ(28)-dihydrocycloeucalenol (**6**) was expected to be a substrate for the isomerase and was supposed to give 7-oxo-24ξ(28)-dihydroobtusifoliol (**7**). Compound **6** was synthesized and was incubated in the presence of the isomerase obtained from maize seedlings. The results described below show that, surprisingly, the cyclopropane ring of **6** is not opened by the isomerase. In addition, **6** is shown to be a potent and highly specific inhibitor of the isomerase both *in vitro* and *in vivo*.

Nomenclature: Cycloeucalenol = 4α, 14α-dimethyl-9β, 19-cyclo-5α-ergost-24(28)-en-3β-ol (**1**); Obtusifoliol = 4α, 14α-dimethyl-5α-ergosta-8,24(28)-dien-3β-ol (**2**); 4α,14α, 24ξ(28)-trimethyl-9β,19-cyclo-5α-cholestane-3β-ol (**4**); 4α, 14α,24ξ(28)-trimethyl-5α-cholest-8-en-3β-ol (**5**); 7-oxo-4α, 14α,24ξ(28)-trimethyl-9β,19-cyclo-5α-cholestan-3β-ol (**6**); 7-oxo-4α,14α,24ξ(28)-trimethyl-5α-cholest-8-en-3β-ol (**7**); 7-oxo-4,4,14α-trimethyl-9β,19-cyclo-5α-cholestan-3β-ol (**8**); Cycloartenol = 4,4,14α-trimethyl-9β,19-cyclo-5α-cholestan-3β-ol (**10**); 24-methylenepollinastanol = 14α-methyl-9β,19-cyclo-5α-ergost-24(28)-en-3β-ol (**12**); Cyclofontumienol = 4α,14α-dimethyl-9β,19-cyclo-5α-stigmast-*Z*-24(28)-en-3β-ol (**13**); 14α(24ξ)-24-dimethyl-9β,19-cyclo-5α-cholestan-3β-ol (**14**); 14α-methyl,(24ξ)-24-ethyl-9β,19-cyclo-5α-cholestan-3β-ol (**15**).

RESULTS

In parallel experiments 7-oxo-24ξ(28)-dihydrocycloeucalenol (**6**) and 24ξ(28)-dihydrocycloeucalenol (**4**) were incubated with a microsomal preparation of the isomerase. Whereas **4** was trans-



1,4 R_1=H, R_2 = Me, X = H,H — **3** R_1 = H, R_2 = Me, X = H,H — **2,5** X = H,H

6 R_1=H, R_2 = Me, X = O — **7** X = O

8 R_1 = Me, R_2 = Me, X = O — **11** R_1 = Me, R_2 = Me, X = H,H

10 R_1 = Me, R_2 = Me, X = H,H

Scheme 1. Reactions catalysed by the isomerase: **1** → **3** → **2** and by the 2,3-oxidosqualene cycloartenol cyclase: 2,3-oxidosqualene → **11** → **10**. Nu = nucleophile.

formed into 24ξ(28)-dihydroobtusifoliol (**5**) with a yield higher than 70%, no trace of 7-oxo-24ξ(28)-dihydroobtusifoliol (**7**) was detectable in the case where **6** was incubated. The absence of any trace of **7** formed during the incubation of **6** was confirmed unambiguously by the use of techniques (TLC and GC) which allowed a clear separation of **6** from an authentic sample of **7** and indicated that no product co-migrating with **7** appeared in the chromatograms. The absence of **7** did not however exclude the formation of other products (e.g. $\Delta^{9(11)}$-derivative resulting from the opening of the cyclopropane ring and not separable from **6** by TLC. In order to assess this point, **4** and **6** were incubated with the isomerase in pure ^2H_2O, and the products of the incubation analysed by GC/MS. Whereas 0.7 ^2H atom was incorporated per mol of **5** formed [2], no trace of ^2H was shown to be incorporated in the fraction containing **6** after GC/MS analysis. Thus the cyclopropane ring of **6** was not opened.

Inhibition of the isomerase by 7-oxo-24ξ(28)-dihydrocycloeucalenol (**6**) *and by 7-oxo-sterols*

Incubation of **4** with the isomerase was performed in the presence of various concentrations of **6**. Results obtained (Fig. 1, curve a) indicated that the activity of the isomerase was strongly inhibited by **6**. From curve (a) it was possible to calculate the concentration of **6** (I_{50}) which produced a 50% inhibition of the isomerase in the presence of **4** (100 μM). This value was reproducibly 0.5×10^{-6} M. Under the assay conditions used in this study where the concentrations of the substrate was close to its K_m value (100 μM), I_{50} values are of the order of the inhibition constants and the value found for **6** indicates that the isomerase has a much higher affinity for **6** than for its

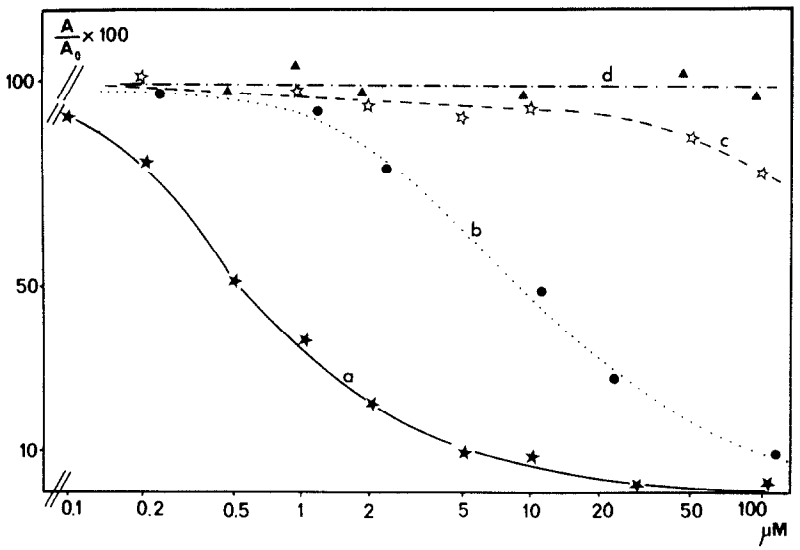


Fig. 1. Inhibition of isomerase by **6** [(★) curve a], **7** [(●) curve b], **8** [(☆) curve c], **9** [(▲) curve d]. A_0: Activity measured without 7-oxo sterols. A: Activity measured in the presence of 7-oxo sterol. Concentration of **4**: 100 μM.

substrate **4**. In order to determine the specificity of the inhibition of the isomerase by **6**, we have performed incubations in the presence of 7-oxo-24ξ(28)-dihydroobtusifoliol (**7**), 7-oxo-24-dihydrocycloartenol (**8**) and 7-oxo-cholesterol (**9**). As shown in Fig. 1, **7** (curve b) was a relatively good inhibitor of the isomerase ($I_{50} = 10\ \mu M$), **8** (curve c) was slightly inhibitory ($I_{50} \sim 300\ \mu M$) and **9** (curve d) was not inhibitory at the highest concentration tested in this work. Thus **6** was by far the best inhibitor of this group of molecules.

Action of **6** *on* 2, 3-*oxidosqualene cycloartenol* (**10**) *cyclase and on SAM-cycloartenol C-24 methyltransferase*

In order to check if the inhibition of the isomerase by **6** resulted from a specific or a non-specific interaction of **6** with the isomerase, **6** was tested as an inhibitor of the 2,3-oxido squalene cycloartenol cyclase [7, 8] and of the SAM-cycloartenol C-24 methyltransferase [9, 10], two enzymes involved in plant sterol biosynthesis. These two enzymes were considered because the substrate of the methyltransferase is also a 9β, 19-cyclopropyl sterol and since it has been suggested [8] that the conversion of 2, 3-oxidosqualene into cycloartenol catalysed by the cyclase could involve the formation of an intermediate (**11**) very similar to **3** [8]. Results shown in Fig. 2 indicate that under conditions where **6** strongly inhibited the isomerase (curve a), it did not affect the cyclase (curve b) and the methyltransferase at any of the concentrations tested in this work.

Reversibility of the inhibition of the isomerase by **6**

In the case of a strongly inhibitory molecule such as **6**, it may be asked whether the interaction of the molecule with the active site of the enzyme is reversible or irreversible. In order to assess this point, two identical samples of microsomes (0.6 ml ~ 5 mg protein) were preincubated for 35 min at 30°, in the presence of a strongly inhibitory concentration of **6** (1 μM). Then one of the two samples was incubated in the presence of the substrate (**4**) (100 μM = K_m) resulting in an 85% inhibition of the isomerase (first assay). After a 10 fold dilution of the second sample of the preincubated microsomes, resulting in a concentration of **6** (0.1 μM) only slightly inhibitory (Fig. 2), the isomerase was measured by addition of **4** (100 μM = K_m) (second assay). Finally a third sample of microsomes identical to the preceding samples was preincubated in the absence of **6** and diluted as above, then the isomerase was measured after addition of **4** (100 μM) plus **6** (0.1 μM) (third assay). If **6** was an irreversible inhibitor the residual enzymatic activity measured in the second assay must be much lower than in the third assay. The results obtained showed that in fact the enzymatic activity measured in the second assay was 99% that of the third assay, demonstrating that the inhibition was fully reversible. Such a conclusion was reached whatever preincubation period (35 min–4 hr) was used.

Inhibition of sterol biosynthesis in suspension cultures of bramble cells

At the end of the exponential phase of growth, [1-^{14}C]acetic acid (100 μCi ~ 0.2 mM) and **6** (4 μM) were given to suspension cultures of bramble cells for a period of 4 hr. Compound **6** was solubilized in tergitol (final concentration: 10 mg/l.). A control incubation containing tergitol but lacking **6** was performed. After 4 hr of incorporation, the cells were harvested, lyophilized and finally extracted as described in the Experimental. The different classes of sterols were separated, purified and their radioactivity was measured. The results obtained are summarized in Table 1 and lead to the following conclusions. (i) The biosynthesis of 24-methylenecholesterol, isofucosterol and sitosterol plus campesterol (major sterols contained in bramble cells [11]) was dramatically reduced in the cells treated with **6**. (ii) A new 4-desmethyl sterol not present in the control was

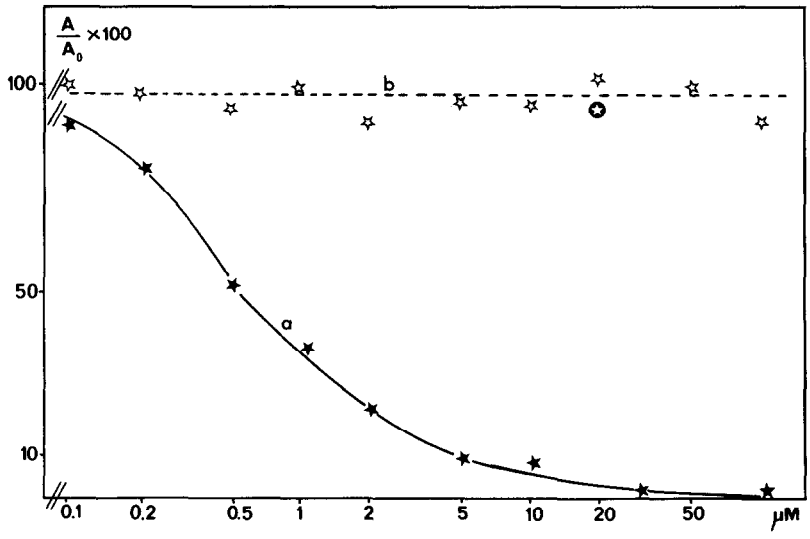


Fig. 2. Inhibition of isomerase [(★) curve a], of 2, 3-oxidosqualene cyclase [(☆) curve b] and of the SAM cycloartenol methyltransferase (✪) by **6** in the presence of **4** (100 μM).

Table 1. Effect of 7-oxo-24ξ, 28-dihydrocycloeucalenol (**6**) on sterol biosynthesis in suspension cultures of bramble cells*

	Control	Treated
4-Desmethyl sterols		
24-Methylenecholesterol	105†	8.5
Isofucosterol	400	25
Sitosterol plus campesterol	8.5	1
24-Methylenepollinastanol (**12**)	0	55
Total radioactivity in 4-desmethyl sterols	515	90
4α-Methyl sterols		
24-Methylenelophenol	35	25
Cycloeucalenol (**1**) plus obtusifoliol‡	50	550
Cyclofontumienol (**13**)	0	60
24-Ethylidenelophenol	15	0
Unidentified sterols	25	0.5
Total radioactivity in 4α-methyl sterols	125	625
4, 4-Dimethyl sterols		
24-Methylenecycloartanol	300	250
Cycloartenol	45	100
α- plus β-amyrin	90	40
Total radioactivity in 4, 4-dimethyl sterols	435	390

*The sterol biosynthetic capability of bramble cells was measured by incubation of the cells in the presence of [1-^{14}C]acetic acid (100 μCi) in the presence (treated) or absence (control) of **6**. Compound **6** was solubilized in tergitol (final concentration in the medium: 10 mg/l.). Details are given in the Experimental.

†10^{-3} cpm/g dry wt.

‡After separation of **1** and obtusifoliol as their epoxy acetates, the bulk of the radioactivity was found to be associated with the monoepoxy acetate of **1**, whereas the diepoxy acetate of obtusifoliol was only slightly radioactive.

detected in the cells treated with **6**. This new sterol was shown to be identical to 24-methylenepollinastanol (**12**). (iii) A large accumulation of 4α-methyl sterols was observed in treated cells. The radioactivity was essentially associated with the mixture of cycloeucalenol (**1**), obtusifoliol (**2**) and a new compound not present in the control which was identical to cyclofontumienol (**13**). After separation of **1** and **2** as their epoxy-acetates [12], the bulk of the radioactivity was associated with the monoepoxyacetate of **1** whereas negligible radioactivity remained with the diepoxyacetate of **2**. Only low radioactivity was detected in 24-methylene and 24-ethylidenelophenols. Identification of **1**, **12** and **13** in treated cells by mass spectrometry is described in the Experimental. (iv) No significant differences could be observed between control cells and treated cells concerning the biosynthesis of 4,4-dimethylsterols. These observations lead to the conclusion that the *in vivo* target of **6** could be the isomerase since considerable amounts of **1**, which is the substrate of this enzyme, as well as other cyclopropyl sterols such as **12** and **13** accumulated in **6** treated cells. By contrast all the compound occurring after **1** in the biosynthetic scheme leading to sitosterol were only slightly labelled. These results are similar to those obtained previously when Tridemorph is used as inhibitor in place of **6** [11].

When **6** was given to bramble cells at the onset of culture and for a longer period of time (2 weeks), the same compounds (**1**, **12**, and **13**) as above were identified. In addition (24ξ)-24-methyl-**14** and (24ξ)-24-ethylpollinastanol (**15**) were detected by GC/MS analysis and by comparison of the data obtained with those obtained with authentic samples [11].

DISCUSSION

The results reported in this paper clearly show that the introduction of a carbonyl group at C-7 changes the reactivity of 24ξ(28)-dihydrocycloeucalenol (**4**) since the 9β,19-cyclopropane ring of 7-oxo-24ξ-(28)dihydrocycloeucalenol (**6**) is not opened by the isomerase. Moreover **6** becomes a powerful inhibitor of the enzyme. To explain these observations it is suggested that the presence of the carbonyl group at C-7 could stabilize the 9β,19-cyclopropane ring in releasing strains in the B-ring [13] resulting in a decreased reactivity of the cyclopropane ring towards acids, as shown in recent work [5]. In addition the carbonyl group at C-7 could compete with the cyclo-

propane ring for the protons of the electrophilic site -B-H of the enzyme (Scheme 1), the group -B-H interacting preferentially with the oxo group rather than with the cyclopropane ring. Such an interaction (H bonding) would be in good agreement with the fact that inhibition of the isomerase by **6** was shown to be reversible. This last feature and the lack of incorporation of ^2H into **6** when the incubations were performed in ^2H_2O would exclude a mechanism of inhibition involving an interaction of the active site of the isomerase with an intermediate such as **3** resulting from the opening of the cyclopropane ring (Scheme 1).

The inhibition of the isomerase by **6** was highly specific since among the four 7-oxo sterols **6–9**, **6** was by far the most efficient ($I_{50} = 0.5 \times 10^{-6}$ M). The fact that **8**, which differs from **5** by the presence of an additional 4β-methyl group, was strikingly less inhibitory ($I_{50} = 300 \times 10^{-6}$ M) than **6** shows clearly that the presence of the 4β-methyl group strongly hinders the interaction of 7-oxo-9β,19-cyclopropyl sterols with the isomerase and was in agreement with the fact that 9β, 19-cyclopropyl sterols possessing a 4β-methyl group were not substrates of the isomerase [1, 3]. As shown above, **7** which differs from **6** by its flat conformation, was 20 times less inhibitory ($I_{50} = 10 \times 10^{-6}$ M). This shows that the bent conformation of 9β, 19-cyclopropyl sterols is a prerequisite for a strong interaction with the isomerase. The high specificity of the inhibition of the isomerase by **6** also resulted from the fact that **6** did not inhibit the 2, 3-oxidosqualene-cycloartenol cyclase and the SAM-cycloartenol-C-24-methyltransferase.

Finally, the inhibitory properties of **6** could be of physiological interest. Results reported in this work have shown that **6** modified profoundly the biosynthesis of sterols in suspension cultures of bramble cells when this compound was added in the culture medium. From the structure of the compounds which accumulated, it can be concluded that the cellular target of **6** *in vivo* is the isomerase thus confirming the results of the enzymatic study. Similar results have been obtained in our laboratory [11] with Tridemorph, a systemic fungicide. This last molecule when added to the medium of suspension cultures of bramble cells leads to a spectacular accumulation of 9β, 19-cyclopropyl sterols [1]. However Tridemorph has some phytotoxicity [14] which does not seem to be correlated with the inhibition of sterol biosynthesis but rather with secondary effects in other metabolic pathways. [Bladocha, M. and Benveniste, P., unpublished]. In contrast, the present study shows that the inhibition of the isomerase by **6** is highly specific, thus **6** seems to be a valuable molecular tool to evaluate the physiological influence of the accumulation of 9β, 19-cyclopropyl sterols in plant cells in conditions where side effects could be minimized. Whatever the mechanism of action of **6** may be, this compound can be considered a potent and new inhibitor of plant sterol biosynthesis.

EXPERIMENTAL

Most of the techniques used in the present work have been described previously [2, 11].

Preparation of subcellular fractions. Maize seeds (*Zea mays*, var. Inra 258) were soaked for 5 hr in H_2O before being planted in moist Vermiculite. They were grown for 72 hr at 25°. The seedlings were harvested and the embryos excised. The embryos (70 g) were ground in an 'Ultra-turrax' homogenizer at 0° with 3 vol. of medium containing: 0.1 M Tris–HCl, 0.3% BSA, 10 mM mercaptoethanol, 0.5 M sucrose, 4 mM $MgCl_2$, final pH 7.5. The homogenate was squeezed through 2 layers of cheese cloth and centrifuged at 2000 *g* for 6 min. The supernatant was centrifuged at 105 000 *g* for 1 hr. Soluble supernatant was removed and the microsomal pellets were suspended in a medium containing: 0.1 M Pi buffer pH 7.4, 5 mM mercaptoethanol, 2 mM $MgCl_2$, and dispersed in a Potter–Elvehjem homogenizer. The protein concn was *ca* 10 mg/ml and the final pH was 7.4.

Enzymatic assays. The isomerase was assayed as described previously [2]. The incubation mixture contained 0.5 ml of maize seedlings microsomes, the substrate (**1** or **4**) (100 μM), and various concns of the inhibitor in a total vol. of 0.6 ml (final pH 7.4). Before the incubation, the substrate and the inhibitor were emulsified in aq. Tween 80 in order to obtain a final concn of Tween of 0.1%. This amount of detergent has been shown not to inhibit the isomerase. The incubations were carried out at 30° for 1 hr and were stopped by the addition of 1 vol. KOH in EtOH. 2, 3-Oxidosqualene cycloartenol cyclase [7] and SAM cycloartenol C-24-methyltransferase [9, 10] were assayed as described previously.

Analytical procedure. The incubation mixture was extracted ×3 with petrol (50 ml). Combined extracts were dried over Na_2SO_4, evaporated under red. pres. and separated by TLC with CH_2Cl_2 as the solvent (2 runs). The band of 4α-methyl sterols was scraped off and eluted. The 4α-methyl sterols were analysed by GC (SE 30). The substrates (**1**, **4**) were unambiguously separated from the products (**2**, **5**) of the reaction and their concns calculated using a computer linked to the gas chromatograph. When the incubation mixture contained 7-oxo-24ξ(28)-dihydrocycloeucalenol (**6**) this was separated from the anticipated 7-oxo-24ξ(28)-dihydroobtusifoliol (**7**) by the following procedure. After TLC (CH_2Cl_2–MeOH, 96:4) of the petrol extract, 7-oxo compounds (R_f 0.20) were readily separated from the 4-desmethyl- and the 4α-methyl sterol fraction. The 7-oxo compounds were acetylated and the acetates were separated using TLC (CH_2Cl_2). In these conditions the 7-acetate (R_f 0.11) was unambiguously separated from 6-acetate (R_f 0.18). When the incubations of **1** and **6** were performed in H_2O, the presence of ^2H in **2**, **7** and the recovered **6** was checked by GC/MS of the acetates after separation by argentation chromatography as described previously [2].

Acetate of 24ξ(28)-dihydrocycloeucalenol (**4**). Cycloeucalenol (**1**) (600 mg) was hydrogenated in cyclohexane in the presence of PtO_2 and the recovered products were acetylated. After crystallization, the acetate of **4** (560 mg) was recovered, mp 101.5–103.5° (lit. 112–113°) [15].

Acetate of 7-oxo-24ξ(28)-dihydrocycloeucalenol (**6**). Compound **6** was synthesized according to a method described by Lawrie *et al.* [13]. The acetate of **4** (500 mg) was dissolved in $CHCl_2$ (500 ml) and treated at −78° by ozonized oxygen for 1 hr. After treatment by 1 M $FeSO_4$ (200 ml) at room temp. and conventional work-up, a mixture of acetates was isolated and chromatographed on prep. TLC (CH_2Cl_2). The fraction (R_f 0.23–0.44) was recovered and was chromatographed again on analytical TLC (cyclohexane–EtOAc, 85:15) resulting in pure **6**-acetate (105 mg), mp 205.5–206.5° (lit. 196°) [13]. IR ν_{max} cm^{-1}: 1720, 1680, 1250. GC/MS, *m/z* (rel. int.): 484(21), 469(31), 466(2), 451(4), 424(61), 409(29), 406(40), 357(24), 317(33), 297(28), 275(100). ^1H NMR

(250 MHz, $CDCl_3$): δ 0.051 (1H, *d*, $J = 6$ Hz, H-19), 0.462 (1H, *d*, $J = 6$ Hz, H-19), 0.776 (3H, *d*, $J = 7$ Hz, H-28), 0.803 (3H, *d*, $J = 6.5$ Hz, H-21), 0.833 (3H, *s*, H-32), 0.847 (9H, *d*, $J = 7$ Hz, H-26, H-27, H-30), 0.919 (3H, *s*, H-18), 2.32–2.47 (2H, *m*, H-6), 2.805 (1H, *s*, $W_{1/2} = 3$ Hz, H-8β), 4.550 (1H, *m*, H-3α). The acetate of **6** was saponified by boiling methanolic KOH (6%) for 1 hr under reflux. After conventional work-up, **6** was obtained, mp 166–168° (MeOH); GC (1% SE-30): 98% purity. GC/MS, *m/z* (rel. int.): 442(18), 427(30), 424(31), 409(23), 406(11), 315(16), 275(100).

Acetate of 7-oxo-24ξ,28-dihydroobtusifoliol (**7**). The acetate of **6** (7 mg) was treated by a mixture of pure HOAc and conc. HCl (20:1) (6 ml) for 3 hr under reflux. After conventional work-up, the products were purified by TLC (CH_2Cl_2). The major band (R_f 0.115) corresponded to the acetate of **7** (4 mg), mp 178–180°. UV λ_{max} nm: 247, ϵ 6500 (cyclohexane). IR ν_{max} cm^{-1}: 1720, 1655, 1585, 1250 [16]. GC/MS, *m/z* (rel. int.): 484(43), 469(100), 424(6), 409(6), 357(17), 343(13), 241(7), 229(21). ^1H NMR (250 MHz, $CDCl_3$): δ 0.672 (3H, *s*, H-18), 0.781 (3H, *d*, $J = 6$ Hz, H-28), 0.806 (3H, *d*, $J = 6$ Hz, H-21), 0.862 (9H, *d*, $J = 5.5$ Hz, H-26, H-27, H-30), 0.927 (3H, *s*, H-32), 1.195 (3H, *s*, H-19), 2.20–2.33 (2H, *m*, H-11), 2.45–2.52 (2H, *m*, H-6), 4.389 (1H, *m*, H-3α) [16]. The acetate of **7** was saponified using boiling methanolic KOH (6%) for 1 hr under reflux. After conventional work-up, **7** was obtained, mp 155–158°; GC/MS, *m/z* (rel. int.): 442(27), 427(100), 409(66), 229(23).

Acetate of 7-oxo-24-dihydrocycloartenol (**8**). The acetate of **8** was synthesized using the same procedure as for the acetate of **6**. The acetate of 24-dihydrocycloartanol (**10**) was used as the starting product (200 mg). Using the same purification techniques as for the acetate of **6**, purified acetate of **8** was obtained (66 mg), mp 208.5–210° (MeOH) (lit 210) [4, 13]. IR ν_{max} cm^{-1}: 1715, 1680, 1250; GC/MS, *m/z* (rel. int.): 484(22), 469(33), 451(3), 424(43), 409(27), 371(15), 331(24), 311(16), 261(100). ^1H NMR (250 MHz, $CDCl_3$): δ 0.205 (1H, *d*, $J = 6$ Hz, H-19), 0.528 (1H, *d*, $J = 6$ Hz, H-19), 0.813 (3H, *s*, H-31), 0.833 (3H, *s*, H-32), 0.868 (6H, *d*, $J = 8$ Hz, H-26, H-27), 0.895 (3H, *d*, $J = 7$ Hz, H-21), 0.910 (6H, *s*, H-30, H-18), 2.235–2.329 (2H, *m*, H-6), 2.763 (1H, *s*, $W_{1/2} = 3$ Hz, H-8β), 4.624 (1H, *dd*, $J = 12$ Hz, $J_2 = 5$ Hz, H-3α). The acetate of **8** was saponified as described above yielding **8**. GC (1% SE-30): 98% purity.

Biosynthesis of sterols in the presence of **6**. The suspension cultures of bramble cells used in the present work have been described previously [11]. At the end of the exponential phase of growth the cells were incubated for 4 hr in the presence of [1-^{14}C]HOAc (100 μCi, 0.2 mM) and of **6** (4 μM). Tergitol (final concn in the medium: 10 mg/l.) was added to improve the solubilization of **6** in the incubation medium and its penetration into the cells [17]. A control incubation containing tergitol but lacking **6** was performed. The cells were harvested by centrifugation and lyophilized. The dry matter was extracted ×3 under reflux with CH_2Cl_2–MeOH (2:1), the pooled extracts were evaporated under red. pres. and the residue saponified by boiling methanolic KOH (6%) for 1 hr under reflux. The non-saponifiable matter was isolated using conventional work-up and was separated on TLC using CH_2Cl_2 (2 runs). 4, 4-Dimethyl-, 4α-methyl- and 4-desmethyl sterols were analysed as described previously [11]. Briefly the acetates of each group of sterols were submitted to argentation TLC (hexane–toluene, 6:4). The 4,4-dimethyl steryl acetate fraction of **6** treated cells was identical to that of control cells and gave in order of increasing polarity: α- plus β-amyrin acetates, cycloartenyl acetate and 24-methylenecycloartanyl acetate. The 4α-methyl steryl acetate fraction of **6** treated cells contained: cyclofontumienyl (**13**) acetate, 24-ethylidenelophenyl acetate, **1**- plus **2**-acetates, and 24-methylenelophenyl acetate; **13**-acetate was not present in control 4α-methyl steryl acetates. The 4-desmethyl steryl fraction from treated cells contained campesteryl- plus sitosteryl acetates, isofucosterylacetate, 24-methylenepollinastanyl **12**-acetate, and 24-methylenecholseteryl acetate; **12**-acetate was not present in control 4-desmethyl steryl acetates. Separation of isofucosteryl- and **12**-acetates was improved by argentation TLC using commercial $CHCl_3$ as solvent. The radioactivity incorporated into each individual steryl acetate was measured using a liquid scintillation spectrometer. **12**-acetate, GC/MS, *m/z* (rel. int.): 454(8), 439(6), 394(100), 379(98), 310(10), 300(10), 269(60), 227(20). **13**-acetate, GC/MS, *m/z* (rel. int.): 482(6), 467(5), 422(100), 407(87), 384(5), 324(6), 300(9), 283(30), 241(16) [18]. **14**-acetate, GC/MS, *m/z* (rel. int.): 456(7), 441(6), 396(86), 381(100), 269(89), 302(9), 227(11). **15**-acetate, GC/MS, *m/z* (rel. int.): 470(6), 455(6), 410(100), 395(94), 269(93), 316(10), 227(16).

Acknowledgements—We are indebted to Professor Massy-Westropp, Adelaide, Australia, for a generous gift of tallow wood (*Eucalyptus microcorys*). We thank Dr. Pierrette Nave for GC/MS, Mr. Roland Graff for NMR spectra and Professor D. Arigoni, Zurich, Switzerland, for stimulating discussions.

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