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CYCLIZATION OF SQUALENE-2,3-EPOXIDE TO 10α-CUCURBITA-5,24-DIEN-3β-OL BY MICROSOMES FROM *CUCURBITA MAXIMA* SEEDLINGS*

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Key Word Index—*Cucurbita maxima*; Cucurbitaceae; cucurbitacin biosynthesis; oxidosqualene-cyclase; 10α-cucurbita-5,24-dien-3β-ol.

Abstract—Labelled 10α-cucurbita-5,24-dien-3β-ol was obtained from [3-^{3}H]squalene-2,3-epoxide incubated with microsomes of *Cucurbita maxima* seedlings. By contrast the lanostane triterpenoids [2-^{3}H]cycloartenol, [2-^{3}H]parkeol and their corresponding derivatives [2,12-^{3}H]11-ketocycloartenol and [2-^{3}H]24,25-dihydro-90α,11α-epoxyparkeol, incubated under the same conditions, gave no rearranged products with a cucurbitane skeleton. These results suggest that biosynthesis of cucurbitane triterpenoids from squalene-2,3-epoxide occurs through direct cyclization without the intermediacy of lanostane-type triterpenoids such as cycloartenol or parkeol. Labelled cycloartenol, α-amyrin, β-amyrin and 24-methylenecycloartanol were also obtained from [3-^{3}H]squalene-2,3-epoxide in the same enzymatic system yielding labelled 10α-cucurbitadienol.

INTRODUCTION

Different hypothetical routes of cucurbitacin biosynthesis from squalene-2,3-epoxide (**1**), through cyclization to the cation **2** and subsequent transformation of the intermediate **3**, have been suggested (Scheme 1) [1–3]: (i) direct rearrangement of **3** to 10α-cucurbitadienol (**4a**, route *a*); and (ii) formation of parkeol (**5a**, route *b*) or cycloartenol (**6a**, route *c*), which in turn could give, by backbone rearrangement, 10α-cucurbitadienol (**4a**).

Taking a chemical model into account it is apparent that, in parkeol (**5a**), the creation of a cationic centre next to the C-10 methyl group via the opening of the 9α,11α-epoxy-derivative **7a**, could favour the skeletal rearrangement to the 11α-hydroxy-10α-cucurbitadienol (**8b**, route *d*). However, when 9α,11α-epoxy-3β-acetoxy-5α-lanostane (**7b**) is treated with various Lewis acids in order to open the epoxide and induce 10β → 9β methyl migration, the reaction undergoes a different course and an 11-ketone derivative, together with the protosta-9(11),13(17)-diene, are formed [4, 40]. By contrast, the boron trifluoride–diethyl ether cleavage of 9α,11α-epoxyandrosta-4-en-3,17-dione [5] and 3β-acetoxy-9β,11β-epoxylanostan-7-one [6] have been reported as giving efficient 19(10β → 9β) methyl migration. For these latter compounds it has been claimed that the presence of the C-7 carbonyl group in ring B or the concomitant aromatization of ring A in the rearranged product, may act as an extra driving force to give the expected methyl migration [5, 6]. In higher plants an equivalent biochemical step could be represented by an enzymatic system, similar to cycloeucalenol-obtusifoliol isomerase [7], capable of protonating the epoxide function in **7a** and abstracting the C-6 proton to give **8b**.

Alternatively (route *e*), cycloartenol (**6a**), the precursor of phytosterols [2], could be enzymatically oxidized to 11-keto-cycloartenol (**9a**) and rearranged to yield 11-keto-cucurbitadienol (**10a**). Even though there is no chemical precedent for this particular skeletal rearrangement, it is evident that the C-11 carbonyl group totally destabilizes the incipient transition state having a carbocation character, with the positive charge placed at C-9. On the other hand, an appropriate enzymatic system could regiospecifically assist the concerted rearrangement (Me-10β → Me-9β, H-5 → H-10) and final elimination of the C-6 proton.

The suggested intermediacy of the oxygenated precursors **7a** and **9a** in cucurbitacin biosynthesis (routes *d* and *e*, respectively) could account for the presence of a C-11 keto group in almost all cucurbitacins so far isolated.

In a previous study of cucurbitacin biosyntheis [8], we showed that 10α-cucurbitadienol (**4a**) could be transformed into cucurbitacin C in *Cucumis sativus* seedlings whereas, under the same conditions, cycloartenol (**6a**) gave only the expected phytosterols and parkeol (**5a**) was recovered unaltered.

These results, while supporting the existence, in the Cucurbitaceae family, of an enzyme capable of converting

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*Part 2 in the series "Biosynthesis of Cucurbitacins". For Part I see ref. [8].

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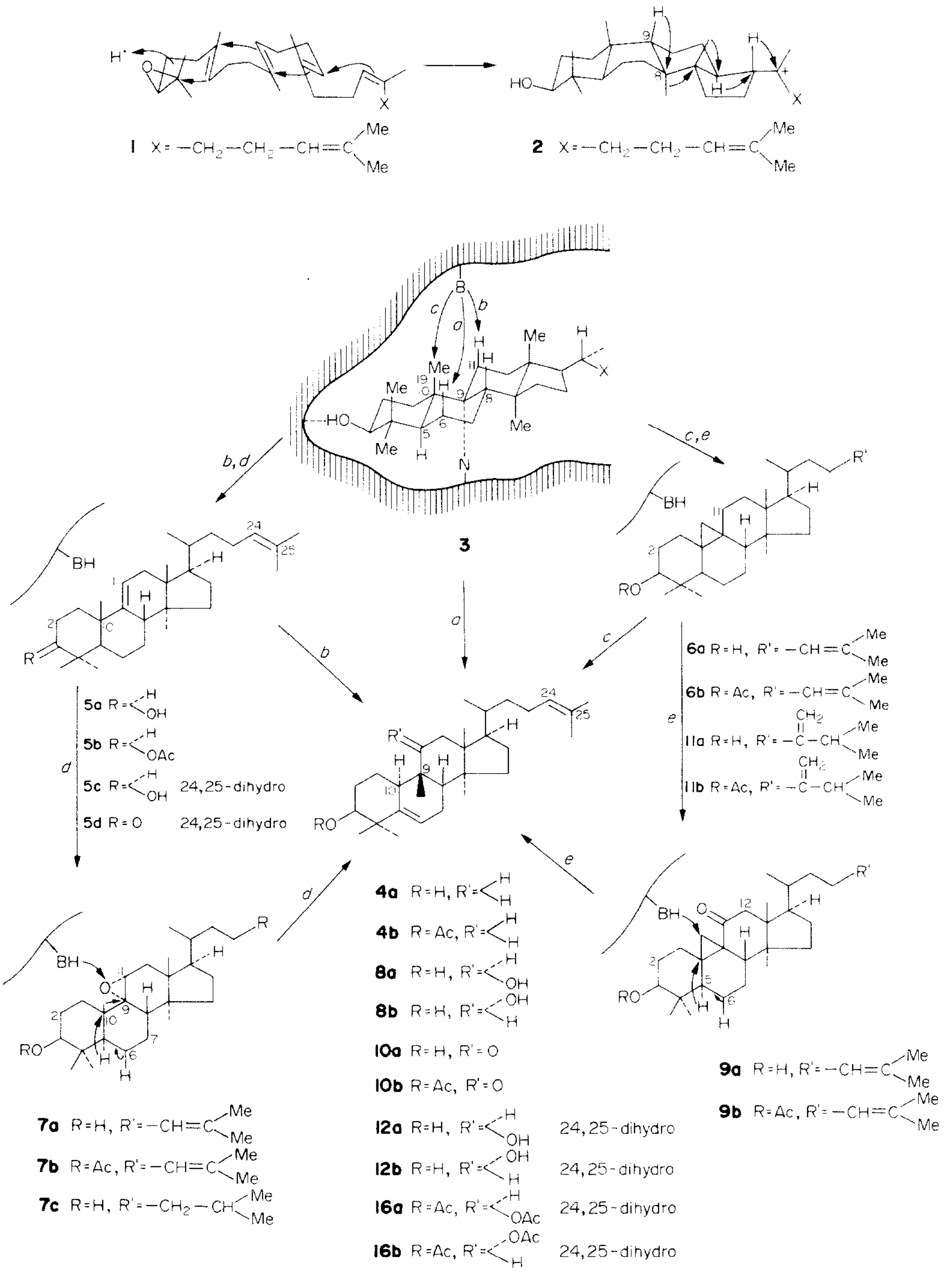
Nomenclature. 10α-Cucurbita-5,24-dien-3β-ol (10α-cucurbitadienol): 19(10 → 9β)-abeo-10α-lanosta-5,24-dien-3β-ol (**4a**); parkeol: lanosta-9(11),24-dien-3β-ol (**5a**); cycloartenol: 4,4,14α-trimethyl-9β,19-cyclo-5α-cholest-24-en-3β-ol (**6a**); 24-methylenecycloartanol: 4,4,14α-trimethyl-9β,19-cyclo-5α-ergost 24(28)-en-3β-ol (**11a**); cucurbitacin B: 25-acetoxy-2α,16α,20β-trihydroxy-19 (10 → 9β)-abeo-10α-lanosta-5, 23-diene-2, 11, 22-trione; cucurbitacin C: 25-acetoxy-3β,16α,19,20β-tetrahydroxy-19(10 → 9β)-abeo-10α-lanosta-5,23-diene-11,22-dione; α-amyrin: urs-12-en-3β-ol; β-amyrin: olean-12-en-3β-ol; litsomentol: 19 (10 → 9β)-abeo-10α-lanost-24-ene-5α,3β-diol (**13**); bryogenin: 3β-hydroxy-19(10 → 9β)-abeo-10α-lanost-5-ene-11,24-dione (**14**); cycloartenyl acetate dibromide: 24,25-dibromo-4,4,14α-trimethyl-9β,19-cyclo-5α-cholestan-3β-yl acetate (**19**); 11-keto-cycloartenone: 4,4,14α-trimethyl-9β,19-cyclo-5α-cholestan-24-en-3,11-dione (**21**); glutinone: glutin-5-en-3-one.



Scheme 1.

squalene-2,3-epoxide (**1**) into 10α-cucurbitadienol (**4a**, route *a*), apparently exclude the intermediacy of cycloartenol (**6a**), parkeol (**5a**) and the oxygenated derivatives **9a** or **7a** (routes *b–e*) for cucurbitacin biosynthesis.

However, the described conversion of 10α-cucurbitadienol (**4a**) into cucurbitacin C does not provide conclusive evidence, because of the possibility that plants may utilize non-specific substrates [9]. In addition, it could also be possible that a compartmentalization effect, common in higher plants [10], may prevent enzymic oxidation of the administered precursors **6a** or **5a**, utilized for the *in vivo* experiments [8]. Considering this, we thought it to be of biogenetic interest: (a) to study the cyclization of squalene-2,3-epoxide by microsomal fraction of Cucurbitaceae; and (b) to determine the possible skeletal rearrangement, under the same conditions, of parkeol (**5a**), cycloartenol (**6a**), 9α,11α-epoxy-24,25-dihydroparkeol (**7c**) and 11-ketocycloartenol (**9a**) to the postulated cucurbitane derivatives.

RESULTS

In preliminary work [8] a selection of different Cucurbitaceae species enabled us to establish the period of maximum cucurbitacin production. In addition, the ability of the individual seedlings of some Cucurbitaceae to incorporate both [2-^{14}C]acetate and [2-^{14}C]mevalonic acid into cucurbitacins had already been verified [3, 11, 12]. In the present work, first we tested the ability of microsomal fractions from different Cucurbitaceae tissues to synthesize triterpenoids from squalene-2,3-epoxide as a function of seedling age. These studies led us to use 3–5-day-old seedlings of *Cucurbita maxima* var. True Hubbard as starting material for the *in vitro* incubation of squalene-2,3-epoxide and the other postulated cucurbitacin precursors. It should be noted that *Cucurbita maxima* has been recommended as a source of cucurbitacin B [13] and it has been utilized in the biosynthetic conversion of [(4*R*)-4-^{3}H$_1$, 2-^{14}C]mevalonic acid into cucurbitacin B [3].

Cyclization of [3-^{3}H]*squalene-2,3-epoxide* (**1**) *by Hubbard squash microsomes*

Microsomes from *Cucurbita maxima* seedlings were incubated at 31° for 4 hr in the presence of [3-^{3}H]squalene-2,3-epoxide. Extraction and chromatography yielded a 4,4-dimethyl sterol fraction, whose constituents were analysed by acetylation followed by $AgNO_3$–Si gel TLC separation. Under these conditions, radioactive pentacyclic triterpene acetates were separated either from cycloartenyl acetate (**6b**) or a labelled fraction A, co-chromatographing with both 10α-cucurbitadienyl acetate (**4b**) and 24-methylenecycloartanyl acetate (**11b**) (Table 1).

The labelled **6b** was transformed into the corresponding epoxy acetate, which was crystallized to constant specific radioactivity.

The pentacyclic triterpene acetate fraction, which had previously been found to be a mixture of α- and β-amyrin acetates [14], was subjected to a multiple HPLC fractionation, giving the labelled α- and β-amyrin acetates in a ratio of 1 : 2.

The radiolabelled fraction A, after dilution with carrier **4b**, was epoxidized and the 10α-cucurbitadienylepoxy acetate, after TLC purification, was crystallized to constant specific activity. To confirm the labelling of **4b**, the mixture of labelled **4b** and **11b**, diluted with the corresponding carriers, was separated by prep. HPLC and the purity of the isolated **4b** and **11b** was tested by GC and analytical HPLC.

The purified 10α-cucurbitadienyl acetate (**4b**) was diluted with more carrier material and crystallized to constant specific radioactivity (see Experimental).

The control experiments with an inactivated enzyme preparation gave no cyclization products, the substrate being recovered unaltered.

Comparative incubation of [2,12-^{3}H]11-*ketocycloartenol* (**9a**), [2-^{3}H]24,25-*dihydro*-9α,11α-*epoxyparkeol* (**7c**), [2-^{3}H]*cycloartenol* (**6a**) *and* [2-^{3}H]*parkeol* (**5a**) *in microsomes from* Cucurbita maxima *seedlings*

The parallel incubation of **9a**, **7c**, **5a** and **6a** with microsomes from Hubbard squash seedlings was performed utilizing a standard procedure (see Experimental). In order to check the enzymatic integrity of the microsomes, labelled squalene-2,3-epoxide was added under the same conditions used for these incubations: in each case it yielded the cyclization products described in the previous section. The extracts from different incubations were analysed using several chromatographic techniques. In the case of experiments with **9a** and **7c**, the absence of radioactivity in **10a** and **12b** was established by TLC radioscanning. Acetylation and HPLC purification of the recovered labelled substrates **9a** and **7c**, also excluded the presence of any other transformation products.

In the case of incubations with labelled **6a** and **5a**, the 4,4-dimethyl sterol fraction obtained from TLC purification of the petrol extract, was subjected to acetylation. $AgNO_3$–Si gel TLC and HPLC separation of the 4,4-dimethylsteryl acetates, showed the absence of radioactivity both in cucurbitadienyl acetate (**4b**) and in any other transformation compounds.

DISCUSSION

Following incubation of squalene-2,3-epoxide with microsomal fractions from *Cucurbita maxima* seedlings,

Table 1. Cyclization of [3-^{3}H]squalene-2,3-epoxide in microsomes from *Cucurbita maxima* seedlings.

Cyclization products	Radioactivity recovered in the acetates (dpm × 10^{-6})	Substrate transformation*		
		Membrane protein (nmol/mg)	nmol/100 g seedlings	Substrate converted† (%)
Cycloartenol	0.845	1.37	18.52	9.38
α-Amyrin	0.086	0.14	1.9	0.96
β-Amyrin	0.174	0.28	3.8	1.92
24-Methylene cycloartanol	0.078‡	0.12	1.71	0.87
10α-Cucurbita-5,24-dien-3β-ol	0.117‡	0.19	2.56	1.30

Microsomes (3.6 ml), corresponding to 10.8 mg of protein, were incubated under the described conditions (see text).

*[3-^{3}H]Squalene-2,3-epoxide, 9 × 10^{6} dpm, substrate concentration 31.5 μM.

†Taking into account only the enantiomer (3*S*)-2,3-oxysqualene.

‡After HPLC purification of the acetates.

10α-cucurbita-5,24-dien-3β-ol (**4a**), together with cycloartenol (**6a**), 24-methylenecycloartanol (**11a**), α-and β-amyrin, were obtained. By contrast, under the same conditions, cycloartenol (**6a**), 11-ketocycloartenol (**9a**), parkeol (**5a**) and 24,25-dihydro-9α,11α-epoxyparkeol (**7c**) were not rearranged to the corresponding derivatives with a cucurbitane skeleton. These results are complementary to those previously obtained [8], which showed that, in *Cucumis sativus* seedlings, 10α-cucurbita-5,24-dien-3β-ol (**4a**) was transformed into cucurbitacin C, whereas neither cycloartenol (**6a**) nor parkeol (**5a**) underwent such a transformation.

These findings are in agreement with the presence, in Cucurbitaceae, of a specific enzyme, squalene-2,3-epoxide → 10α-cucurbitadienol synthetase, capable of converting squalene-2,3-epoxide directly into the simplest tetracyclic triterpene with a cucurbitane skeleton. Squalene-2,3-epoxide (**1**), folded in a chair–boat–chair–boat conformation, is cyclized to give the carbocation **2** (Scheme 1). This is then stabilized by methyl and hydrogen migrations (H-17 → H-20, H-13 → H-17, Me-14 → Me-13, Me-8 → Me-14, H-9 → H-8) yielding a lanostane C-9 carbonium ion **3**, which is bound to the enzyme by a suitable nucleophilic group. Withdrawal of the enzyme could then allow the further migration of the C-10 methyl group to C-9, according to the rules of Wagner–Meerwein rearrangements. The subsequent hydrogen migration from C-5 to C-10 and elimination of the C-6 proton by a basic site of the enzyme will give 10α-cucurbita-5,24-dien-3β-ol (**4a**).

Several examples are known of enzymatic cyclization of squalene-2,3-epoxide to triterpenoids by cell-free extracts of both animal and plant tissues [15]. In higher plants, although many types of cyclases have been postulated [16], only cycloartenol and α- and β-amyrin have been obtained from microsomal fractions [17–20]. The enzymatic cyclization of squalene-2,3-epoxide into 10α-cucurbita-5,24-dien-3β-ol (**4a**) constitutes the first description of a backbone rearrangement of the C-20 ion **2** which overcomes the intermediate state **3** to give a Δ^5-triterpenoid. Indeed glutinone, a Δ^5-pentacyclic triterpene, found in *Alnus glutinosa* [21], which has been postulated to be derived from the E-homo-lupenyl cation, the common precursor of α- and β-amyrin, by skeletal rearrangement and final formation of a C-5 glutinone ion [16, 22], was not obtained from microsomes of *Alnus glutinosa* incubated with squalene-2,3-epoxide.

In order to explain the lack of *in vitro* formation of glutinone and other triterpenoids with high potential energy [23], the requirement of activators or synergic biological factors has been suggested [24].

In our cell-free system, the squalene-2,3-epoxide → 10α-cucurbita-5,24-dien-3β-ol cyclization occurs in the absence of any added synergic cofactors, although its yield is lower than that of cycloartenol (**6a**). We believe that the difference between the yields of cycloartenol (**6a**) and 10α-cucurbita-5,24-dien-3β-ol (**4a**) (the yield of the latter, however, was comparable with those of α- and β-amyrin), should not be ascribed to the absence of an activator. Instead it merely reflects the ratio between the sterols (deriving from cycloartenol) and cucurbitacins (deriving from 10α-cucurbita-5,24-dien-3β-ol) occurring in the plant tissue.

The production, in our enzymatic system, of different triterpenoids, also reveals the presence of different cyclases, which are very similar in their mechanism. In fact, the basic group B (Scheme 1), required to remove the C-11, C-19 and C-6 protons from the transition state **3**, could be thought to occupy an identical regiospecific position at the active site of the different cyclases.

EXPERIMENTAL

General. Methods were generally as previously described [25, 26].

Cucurbita maxima var. True Hubbard seeds were obtained from Fratelli Ingegnoli (Milan, Italy).

HPLC was performed using a liquid chromatograph equipped with a RP-C-18 column (0.26 × 26 cm, 10 μm) coupled with a variable wavelength UV detector provided with an autocontrol system: mobile phase MeCN, flow rate 0.6 ml/min.

Authentic material. Litsomentol (**13**) was kindly donated by Dr. T. R. Govindachari (Ciba Research Centre, Bombay, India). Cycloartenol was supplied by Dr. A. S. Narula (Research School of Chemistry, The Australian National University, A.C.T. 2600, Australia). Parkeol (**5a**) was synthesized: (i) from pure lanosteryl acetate following the method described by Lawrie *et al.* [27]; or (ii) from cycloartenyl acetate by HCl isomerization and saponification [28, 29]. 10α-Cucurbita-5,24-dien-3β-ol (**4a**) was prepared from litsomentyl acetate (**13**) by dehydration and saponification [30]. Bryogenin was extracted from roots of *Bryonia dioica* [31]. 24-Methylenecycloartanol (**11a**) was kindly supplied by Professor P. Benveniste (Institute de Botanique, Strasbourg, France).

3β-Hydroxy-10α-cucurbita-5,24-diene-11-one (**10a**). To bryogenin (**14**) acetate (300 mg), dissolved in 15 ml CH_2Cl_2–MeOH (1:5), a soln of 20 mg $NaBH_4$ in 0.4 ml H_2O and 2 ml MeOH was added while stirring and the reaction mixture left at 0° for 1 hr. To decompose the unreacted hydride, a 20% aq. soln of NaH_2PO_4 (4 ml) was added and, after the usual work-up, the crude residue was purified by prep. TLC (Si gel 2 mm, $CHCl_3$–MeOH, 97:3) to give 3β-acetoxy-24ξ-hydroxy-10α-cucurbit-5-en-11-one (**15**, 240 mg): mp 157° (MeOH–H_2O); IR ν_{max}^{KBr} cm^{-1}: 3500, 3000, 1750, 1700, 1250; MS *m/z* (rel int): 500 $[M]^+$ (5), 440 $[M - MeCOOH]^+$ (100), 425 $[M - MeCOOH - Me]^+$ (50), 422 $[M - MeCOOH - H_2O]^+$ (30).

The alcohol, **15** (220 mg), was dehydrated in pyridine (6 ml) by dropwise addition of $POCl_3$ (1.6 ml). After 24 hr at room temp. the soln was cautiously added to 200 ml ice-cold H_2O and, after $CHCl_3$ extraction, the crude residue was purified by prep. TLC (cyclohexane–EtOAc, 70:30) to give 3β-acetoxy-10α-cucurbita-5,24-dien-11-one (**10b**, 200 mg): mp 137° (MeOH–H_2O); ^1H NMR ($CDCl_3$): δ 0.75 (3H, *s*, C-19), 0.89 (3H, *d*, C-21), 1.035 (3H, *s*), 1.06 (6H, *s*, C-30 and C-31), 1.11 (3H, *s*), 1.60 and 1.68 (6H, both *s*, C-26 and C-27), 4.70 (1H, *m*, C-3), 5.12 (1H, *m*, C-24), 5.57 (1H, *s*, C-6); IR ν_{max}^{KBr} cm^{-1}: 3000, 1750, 1700 and 1250; MS *m/z* at 482 $[M]^+$ (6), 422 $[M - MeCOOH]^+$ (100), 407 $[M - MeCOOH - Me]^+$ (55).

The acetate, **10b** (20 mg), was saponified under standard conditions giving 3β-hydroxy-10α-cucurbita-5,24-dien-11-one (**10a**): mp 121° (MeOH–H_2O); IR ν_{max}^{KBr} cm^{-1}: 3500, 3000, 1700.

10α-Cucurbit-5-ene-3β,11β-diol (**12a**) *and 10α-cucurbit-5-ene-3β,11α-diol* (**12b**). To a soln of the 11-ketone **10b** (100 mg) in dry THF (10 ml), was added $LiAlH_4$ (100 mg) and the mixture stirred under reflux for 20 hr. After the usual work-up, the residue was purified by TLC ($CHCl_3$–MeOH, 98:2), yielding two compounds identified as: (a) 10α-cucurbita-5,24-diene-3β,11β-diol (**8a**, 32 mg), mp 115° (Et_2O–petrol); ^1H NMR (60 MHz, $CDCl_3$): δ 5.67 (1H, *m*, C-6), 5.16 (1H, *m*, C-24), 3.9 (1H, *br s*, C-11α), 3.47 (1H, *m*, C-3α), 1.6, 1.17, 1.07 and 0.84 (Me); IR ν_{max}^{KBr} cm^{-1}: 3550, 4440, 1030 and 970; MS *m/z* (rel. int): 442 $[M]^+$ (10), 424 (15), 409 (6), 406 (4), 391 (4), 303 (100), 290 (80), 273 (50), 134 (100); (b) 10αcucurbita-5,24-diene-3β,11α-diol (**8b**, 29 mg), mp 135° (Et_2O–petrol); ^1H NMR (60 MHz, $CDCl_3$): δ 5.60 (1H, *m*, C-6), 5.10 (1H, *m*, C-24), 3.95 (1H, *m*, C-11β), 3.44 (1H, *m*, C-3α), 1.6, 1.17, 1.07, 0.98, 0.87 and 0.80 (Me); MS *m/z* (rel. int.): 442$[M]^+$ (8), 424 (10), 409 (6), 406 (5), 303 (95), 290 (82), 273 (50), 134 (100).

Compounds **8a** and **8b** were hydrogenated over Pd–C (10% Pd) for 2 hr with stirring at room temp. giving, after extraction and TLC purification ($CHCl_3$–MeOH, 98:2), the corresponding 24,25-dihydro derivatives **12a** and **12b** which were then acetylated. Prep. TLC (CH_2Cl_2) of the acetates gave 10α-cucurbit-5-ene-3β,11β-diol diacetate (**16a**, 17 mg) as an oil and the less polar 10α-cucurbit-5-ene-3β,11α-diol diacetate (**16b**, 15 mg), mp 90° (MeOH–$CHCl_3$). The spectral properties and the physical constants of **16a** and **16b** were identical with those previously reported [6].

Radiochemicals. ^3H_2O (0.5 ml, 10 Ci/ml) and $NaBT_4$ (100 mCi, 22 Ci/mmol) were supplied by Sorin (Saluggia, Italy). [2-^{3}H]Cycloartenol (**6a**, 30.4 mCi/mmol) and [2-^{3}H]parkeol (**5a**, 27.3 mCi/mmol) were prepared as previously described [8].

[3-^{3}H]*Squalene-2,3-epoxide* (**1**). Squalene-2,3-epoxide (**1**) and 1,1′,2-tris-nor-squalene aldehyde (**17**) were synthesized as previously reported [32, 33].

To the aldehyde (**17**) (8 mg) in MeOH (1.5 ml), $NaBT_4$ (100 mCi, 22 Ci/mmol) was added with stirring under Ar. After 45 min cold $NaBH_4$ (1.2 mg) was added and the reaction mixture stirred at room temp. for 30 min. After evaporation of the solvent under N_2, the residue was worked-up as usual and purified by TLC (cyclohexane–EtOAc, 80:20) yielding the labelled 1,1′,2-tris-nor-squalene-3-ol (**18**). This compound, dissolved in CH_2Cl_2 (0.5 ml), was treated with 20 mg CrO_3 in 0.4 ml CH_2Cl_2 and 30 μl pyridine for 25 min with stirring at room temp. After the usual work-up [34] the crude product was purified by TLC (cyclohexane–EtOAc) giving the [3-^{3}H]1,1′,2-tris-nor-squalene-3-aldehyde (**17**).

[3-^{3}H]Squalene-2,3-epoxide was synthesized from **17** by the Wittig procedure [33], utilizing the sulfurylide generated by treatment of diphenylisopropylsulfonium fluoroborate with *n*-butyllithium in THF [35, 36]. The labelled compound (4×10^9 dpm) was diluted with cold **1** (18 mg) yielding 24 mg of a product with sp. act. 32 mCi/mmol.

[2,12-^{3}H]11-*Keto-cycloartenol* (**9a**). The 24,25-dibromide of cycloartenyl acetate (**19**, 200 mg) [28] in dry C_6H_6 (13 ml) was treated with CrO_3 (270 mg) in glacial HOAc (60 ml) with stirring for 16 hr at room temp. After the usual work-up the crude 11-ketocycloartenyl acetate-24,25-dibromide (**20**) was heated under reflux for 13 hr in dry Me_2CO (20 ml) containing NaI (1 g). The reaction product was purified by TLC (cyclohexane–EtOAc, 80:20) giving 11-ketocycloartenyl acetate (**9b**), mp 130° ($CHCl_3$–MeOH), which was saponified in 3% methanolic KOH (3 hr under reflux) yielding 11-ketocycloartenol (**9a**, 25 mg), mp 180° (MeOH), IR ν_{max}^{KBr} cm^{-1}: 3500 and 1670, MS and $[\alpha]_D$ identical with those reported [37].

To **9a** (20 mg) Jones' reagent (0.3 ml) was added with stirring under N_2 at 0°. After 20 min the usual work-up gave the 11-ketocycloartenone (**21**) IR ν_{max}^{KBr} cm^{-1}: 1710 and 1670. This diketone **21** (15 mg), dissolved in a small vol. C_6H_6–cyclohexane (1:1), was applied to a column of basic alumina (10 g Merck act. I) previously deactivated with 125 μl ^3H_2O (sp. act. 10 Ci/ml) for 24 hr. The tritiated products were then eluted, first with C_6H_6–hexane (1:1) and successively with CH_2Cl_2. TLC of the mixture (cyclohexane–EtOAc, 80:20) gave the [2,12-^{3}H]11-ketocycloartenone (**21**, 12 mg), 370×10^6 dpm.

To the labelled diketone **21** in 0.75 ml MeOH–CH_2Cl_2 (1:2), 200 μl of a $NaBH_4$ soln (2 mg $NaBH_4$ + 50 μl H_2O + 250 μl MeOH) was added with stirring under Ar. After 10 min the reaction was stopped with a NaH_2PO_4 soln and the mixture worked-up as usual. The crude alcohol **9a** was then purified by TLC (cyclohexane–EtOAc, 70:30) giving the labelled [2,12-^{3}H]11-ketocycloartenol (**9a**, 5 mg), 272×10^6 dpm, sp. act. 10.8 mCi/mmol.

[2-^{3}H]24,25-*Dihydro*-9α,11α-*epoxyparkeol* (**7c**). Parkeol (**5a**, 30 mg) in cyclohexane (15 ml) was hydrogenated for 3 hr over PtO_2 (5 mg). After the removal of the catalyst by filtration and evaporation of the solvent, the product was crystallized from $CHCl_3$–MeOH to give the 24,25-dihydro derivative **5c**, mp 165–170°.

To **5c** (20 mg), dissolved in a mixture of CH_2Cl_2 (4 ml) and Me_2CO (15 ml), a soln of 1.33 g CrO_3 in 1.15 ml H_2SO_4 and 3.85 ml H_2O was added dropwise with stirring at 0° under N_2. After 20 min EtOH (2 ml) was added and the mixture worked-up in the usual manner to give 25,25-dihydrolanost-9(11)-en-3-one (**5d**, 12 mg), mp 110–112° ($CHCl_3$–MeOH). The ketone **5d** (10 mg) was tritiated utilizing the method described above for the labelling of 11-ketocycloartenone (**21**). After elution of the tritiated product with C_6H_6–hexane (40 ml, 1:1) and the usual work-up, the crude labelled ketone was purified by TLC (cyclohexane–EtOAc, 80:20) to give [2-^{3}H]24,25-dihydrolanost-9(11)-en-3-one (**5d**, 8 mg), 1.2×10^9 dpm.

The radioactive ketone **5d** (8 mg) in dry THF (2 ml) was treated with $LiAlH_4$ (8 mg) at room temp. with stirring under Ar. After 50 min a satd soln of NaH_2PO_4 was added and the reaction mixture was worked-up in the usual manner. TLC fractionation (cyclohexane–EtOAc, 85:15) of the epimeric alcohols yielded the [2-^{3}H]24,25-dihydrolanost-9(11)-en-3β-ol (dihydroparkeol **5c**, 5 mg) 960×10^6 dpm.

To a soln of [2-^{3}H]dihydroparkeol (**5c**) in $CHCl_3$ (0.9 ml), *m*-chloroperbenzoic acid (18 mg) and $NaHCO_3$ (43 mg) were added while stirring under Ar at room temp.; the reaction mixture was then kept at room temp. for 2.5 hr. A 10% soln of $Na_2S_2O_3$ was then added and the resulting mixture was washed with a satd soln of NaCl, dried over Na_2SO_4 and evaporated. TLC purification ($CHCl_3$–MeOH, 98:2) of the crude epoxy alcohol yielded the expected [2-^{3}H]24,25-dihydro-9α,11α-epoxyparkeol (**7c**, 1.1 mg), 366×10^6 dpm, sp. act. 166.3 mCi/mmol. IR, NMR and MS of the above products confirmed the assigned structures. The 9α, 11α-epoxide configuration of **7c** was assigned on the basis of the ^{1}H NMR spectrum, characterized by the signal of the 11β-proton of **7c**, which appeared as a doublet centred at δ 3 [38].

Preparation of the subcellular fraction. Sterile seeds (45 g) of *Cucurbita maxima* were soaked for 12 hr in tap H_2O before planting on moist filter paper in Petri dishes and allowed to germinate under alternate lighting (12 hr light–12 hr dark) at 25°. The seedlings (118 g) were ground in a mortar at 4° with a medium containing: Tris–HCl 0.1 M, 0.3% bovine serum albumin, 10 mM mercaptoethanol, 0.5 M sucrose, 4 mM $MgCl_2$, 2 mM EDTA, final pH 7.2. In some of these expts, 5% PVP was also added to the homogenization buffer. The homogenate was squeezed through two layers of cheesecloth and centrifuged at 6000 g for 20 min. The supernatant was centrifuged at 105 000 *g* for 1 hr and the soluble supernatant was removed. The microsomal pellets were suspended in 12 ml of a medium containing 0.1 M Tris–HCl, 2 mM mercaptoethanol and 2 mM $MgCl_2$ (microsomal buffer, pH 7.2) and dispersed in a Potter–Elvehjem homogenizer. Because of the progressive acidification the pH was adjusted to 7.5 with 0.4 M Tris soln. All these operations were performed at 4°.

Enzymatic assay. The dispersed microsomal pellets (2–5 ml, 3–4 mg protein/ml) were incubated under one of the following conditions: (i) the radioactive substrate was dissolved in 70–100 μl 0.5% Tween 80–Me_2CO and transferred into the dispersed pellets; (ii) 70–100 μl 0.5% Tween 80–EtOH was added to the substrate dissolved in C_6H_6; the mixture was shaken by a Vortex apparatus and evaporated to dryness under N_2. To the residue, 0.3–0.5 ml of microsomal buffer was added and the resulting mixture was stirred on a Vortex and then transferred to the suspended microsomal pellets. The microsomal suspension, containing the appropriate substrate, was incubated at 31° for 4 hr. The reaction was terminated by the addition of 20 % KOH in EtOH (1 vol.). Each expt was carried out in parallel with a control, consisting of boiled particles plus the substrate. Protein was quantitated by the Lowry procedure [39], using BSA as standard. For a typical assay the final vol. was 3.6 ml from 20 g of seedlings, protein 3 mg/ml and Tween 80 0.1 %.

Incubation of [3-^{3}H]*squalene*-2,3-epoxide (**1**). Microsomes from *Cucurbita maxima* (3.6 ml, 3 mg protein/ml) were incubated with [3-^{3}H]squalene-2,3-epoxide (1,9 × 10^6 dpm, 32 mCi/mmol, 31.5 mM) at 31° for 4 hr. The incubation mixture was extracted × 5 with petrol (5 ml). Combined extracts were dried over Na_2SO_4 and evaporated under red. pres. The crude extract was purified by TLC (cyclohexane–EtOAc, 85:15) to give the 4,4-dimethyl sterols together with the starting precursor. The 4,4-dimethyl sterols were acetylated and separated by $AgNO_3$–Si gel TLC (EtOH free $CHCl_3$) to give three radiolabelled fractions co-chromatographing with β-amyrin acetate (fraction A, 0.26 × 10^6 dpm), cycloartenyl acetate (**6b**) (fraction B, 0.84 × 10^6 dpm) and 10α-cucurbita-5,24-dien-3β-yl acetate (**4b**) (fraction C, 0.25 × 10^6 dpm).

Fraction A (0.26 × 10^6 dpm) was separated by HPLC (see *General*) into radioactive α- and β-amyrin acetates in a ratio of 1:2.

To fraction B (0.84 × 10^6 dpm) unlabelled cycloartenyl acetate (**6b**, 2 mg) was added before epoxidation with *p*-nitroperbenzoic acid (40 mg) in dry Et_2O (1 ml). The labelled cycloartenylepoxy acetate, after TLC purification (CH_2Cl_2) and addition of unlabelled carrier (6 mg), was recrystallized in MeOH to a constant sp. act. (49.1, 43.5, 44.3 × 10^3 dpm/mg).

Fraction C (0.1 × 10^6 dpm) was diluted with cold 10α-cucurbita-5,24-dien-3β-yl acetate (**4b**, 10 mg) and epoxidized under the above conditions giving the corresponding epoxy acetate which, after TLC purification (CH_2Cl_2), was crystallized in MeOH to a constant sp. act. (6.5, 6.1, 5.9, 6.15 × 10^3 dpm/mg).

Following a different analytical procedure, fraction C (0.15 × 10^6 dpm) was diluted with both 10α-cucurbita-5,24-dien-3β-yl acetate (**4b**, 2 mg) and 24-methylenecycloartanyl acetate (**11b**, 2 mg) and fractionated by HPLC. The radioactivity was found to be associated with both **4b** (69.3 × 10^3 dpm) and **11b** (47.5 × 10^3 dpm). The purity of the separated radioactive samples **4b** and **11b** was monitored by GC, analytical HPLC and mass spectrometry. The labelled purified 10α-cucurbita-5,24-dien-3β-yl acetate (**4b**), after isotopic dilution with unlabelled carrier (8 mg), was crystallized to a constant sp. act. (5.1, 4.9, 4.7, 4.8 × 10^3 dpm/mg).

Incubation of [2-^{3}H]*cycloartenol* (**6a**), [2,12-^{3}H]11-*ketocycloartenol* (**9a**), [2-^{3}H]*parkeol* (**5a**) *and* [2-^{3}H]24,25-*dihydro*-9α,11α-*epoxyparkeol* (**7c**). Microsomes from *Cucurbita maxima* (3.6 ml, 3 mg protein/ml) were separately incubated, under the described conditions, with the following substrates: (1) [2,12-^{3}H]11-ketocycloartenol (**9a**, 10 × 10^6 dpm, 10.8 mCi/mmol, 116 μM); (2) [2-^{3}H]24,24-dihydro-9α,11α-epoxyparkeol (**7c**, 10 × 10^6 dpm, 166 mCi/mmol, 18.8 μM); (3) [2-^{3}H]cycloartenol (**6a**, 8.8 × 10^6 dpm, 30.4 mCi/mmol, 36.5 μM); (4) [2-^{3}H]parkeol (**5a**, 10 × 10^6 dpm, 27.3 mCi/mmol, 45.7 μM).

After incubation the microsomes were extracted with light petrol. All the incubations were performed carrying out, at the same time, two controls consisting of: (i) boiled microsomes with the added substrates; and (ii) microsomes incubated with [3-^{3}H]squalene-2,3-epoxide (9 × 10^6 dpm, 31.5 μM). This latter control always yielded the labelled mixture of 4,4-dimethyl sterols (1–1.3 × 10^6 dpm).

3β-Hydroxy-10α-cucurbita-5,24-diene-11-one (**10a**, 1.5 mg) was added to the petrol extract from incubation 1, whereas 10α-cucurbit-5-ene-3β,11α-diol (**12b**, 1.5 mg) was added to the extract from incubation 2. After TLC purification ($CHCl_3$–MeOH, 97:3) and radioscanning, chromatographic fractions, corresponding to **9a**, **7c** and their products **10a** and **12b**, were scraped off and counted: **9a**, R_f 0.51, 7.6 × 10^6 dpm; **10a**, R_f 0.63, 270 dpm; **7c**, R_f 0.6, 8.1 × 10^6 dpm; **12b**, R_f 0.45, 260 dpm. Radiochemical purity of each fraction was checked by HPLC.

The petrol extracts from incubations 3 and 4 were chromatographed (cyclohexane–EtOAc, 85:15) and the recovered 4,4-dimethyl sterols were separately diluted with 1.5 mg 10α-cucurbita-5,24-dien-3β-ol (**4a**). Acetylation and $AgNO_3$–Si gel TLC (EtOH free $CHCl_3$) yielded the corresponding acetates **6b** (6.3 × 10^6 dpm) and **4b** (420 dpm) from incubation 3 and **5b** (6.9 × 10^6 dpm) and **4b** (310 dpm) from incubation 4. The fractions corresponding to 10α-cucurbita-5,24-dien-3β-yl acetate (**4b**) were subjected to HPLC purification. The radioactivity of the purified fractions fell to the background level.

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