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BIOSYNTHESIS OF BRYONOLIC ACID IN CULTURED CELLS OF WATERMELON

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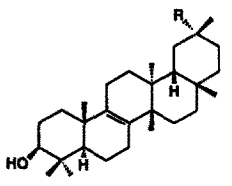
Key Word Index—*Citrullus lanatus*; Cucurbitaceae; watermelon; biosynthetic pathway; bryonolic acid; isomultiflorenol; mevalonic acid; cultured cell.

Abstract—Bryonolic acid, a pentacyclic triterpene having an anti-allergic activity, was biosynthesized from *R*-[2-^{14}C]mevalonate or [2-^{14}C]acetate administered to cell cultures of watermelon (*Citrullus lanatus*). The radioactivity of the labelled mevalonate added to cell-free extracts in the presence of ATP, Mg^{2+} and NADPH was also incorporated into bryonolic acid as well as into the possible intermediates, isomultiflorenol, bryonolol and bryonolal. Furthermore, [^{14}C]2,3-oxidosqualene added to the microsomal solution of the same cell-free extracts was converted enzymatically into isomultiflorenol, which is considered to be the first cyclization product in the biosynthesis of bryonolic acid. These results indicate that bryonolic acid is biosynthesized from 2,3-oxidosqualene via isomultiflorenol and subsequent oxidations of the methyl group at C-29.

INTRODUCTION

Bryonolic acid (**1**, Chart 1), D:C-friedoolean-8-en-3β-ol-29-oic acid, was first isolated from a cucurbitaceous plant *Bryonia dioica* [1] and its absolute structure and conformation were elucidated by X-ray analysis and ^{13}C NMR spectroscopy [2]. This compound proved to be active against various types of allergy in animal experiments [3]. Interestingly, **1**, which is found only in the roots of certain cucurbitaceous plants [4], can be produced in greater quantities by cell suspension cultures of luffa (*Luffa cylindrica*) [5] and of watermelon (*Citrullus lanatus*) [4]. Cell fractionation and electron microscopic studies of cultured cells suggested that **1** is probably synthesized in the rough endoplasmic reticulum (rER) and transported by minute vesicles originated from the terminal ends of rER to the cell wall where it is accumulated [5].

Little is known about the biosynthetic reaction steps leading to **1**, although it is supposed to be synthesized by the mevalonic acid pathway. Cattel *et al.* [6] postulated that isomultiflorenol (**2**), which they isolated from the seedlings of *B. dioica*, might be the precursor of **1**, but this hypothesis has not yet been verified experimentally. The present study was undertaken to elucidate the biosynthetic steps leading to **1** from squalene by tracer experiments using cell suspension cultures of watermelon, which are capable of producing *ca* 200–300 mg l^{-1} of **1** in 15 days.



1 : R=COOH Bryonolic acid
2 : R=CH_3 Isomultiflorenol
3 : R=CH_2OH Bryonolol
4 : R=CHO Bryonolal

Chart 1. Structures of bryonolic acid and its possible precursors.

RESULTS AND DISCUSSION

Triterpenes and phytosterols in cultured cells of watermelon

GC-MS analysis showed that **1** was the main component of triterpenes found in the $CHCl_3$–MeOH (2:1) extract of watermelon cell cultures (Table 1). In addition,

Table 1. GC-MS analysis of triterpenes and phytosterols extracted from cultured cells (15-day-old) of watermelon

Compound	Content (mg g^{-1} dry wt)
Triterpenes	
bryonolic acid	15.2
D:C-friedoolean-7,9(11)-dien-3β-ol-29-oic acid	0.5
triterpene alcohol (unidentified, M_r 426)	0.2
triterpene alcohol (unidentified, M_r 426)	0.1
Phytosterols	
stigmasta-7,22-dien-3β-ol	0.3
stigmasta-7,25-dien-3β-ol	0.5
stigmasta-7,22,25-trien-3β-ol	0.4

small amounts of D:C-friedoolean-7,9(11)-dien-3β-ol-29-oic acid (a diene form of **1**), Δ^7-phytosterols characteristic of cucurbitaceous plants, and two unidentified triterpene alcohols were detected. However, none of the possible biosynthetic intermediates such as squalene, 2,3-oxidosqualene and **2** was detectable in the extract. Examination of the acid hydrolysis or saponification products of the extract suggested that **1** does not exist in the form of a glycoside in the cells, but is accumulated as a water-insoluble free compound.

Tracer experiments using cultured cells

Ten-day-old cell suspension cultures (300 mg fresh wt) of watermelon actively producing **1** were administered with [2-^{14}C]acetate (37 kBq) and incubated at 25° for 24 hr, and the $CHCl_3$–MeOH (2:1) extract of the cells was subjected to radio-TLC with the developing solvent, cyclohexane–EtOAc–HOAc (75:25:2). The analysis showed that the radioactivity of [2-^{14}C]acetate was mostly incorporated into **1** (incorporation rate: 4.3%) and partly into squalene and steryl esters (Fig. 1). A similar pattern was observed when *R*-[2-^{14}C]mevalonate was administered to suspension cultures. No labelled intermediates except for squalene and **1** could be detected during the whole period of incubation between 5 min and 24 hr. These results suggested that the precursors were rapidly converted into **1** via squalene, presumably by such a multienzyme complex as reported for the biosynthesis of dhurrin from tyrosine in sorghum tissues [7].

Tracer experiments using cell-free extracts

In order to investigate the metabolism of mevalonate in cell-free extracts of watermelon cell cultures, *R*-[2-^{14}C]-mevalonate was added, together with ATP, Mg^{2+} and NADPH, to the 10000 *g* supernatant prepared from 10-day-old cell cultures. The reaction mixture was incubated at 30° for 60 min before the products were analysed by radio-TLC. Interestingly, the radiochromatogram of the Et_2O extract from the reaction mixture revealed not only the formation of labelled **1**, but also the occurrence of a series of possible intermediates, i.e. squalene, 2,3-oxidosqualene, **2**, bryonolol (**3**) and bryonolal (**4**, Fig. 2). These intermediate compounds were identified by radio-TLC using a different developing solvent system (Fig. 3), gas chromatography equipped with a radio gas counter and by recrystallizations with authentic carrier standards. In addition to the intermediates, *trans,trans*-farnesol, which is not directly related to bryonolic acid biosynthesis and undetectable in cultured cells, was also formed in a small quantity, probably due to hydrolysis of farnesyl pyrophosphate during incubation. The formation of *trans,trans*-farnesol from mevalonate has been reported for the cell-free systems of rat liver [8] and tobacco callus [9].

Although some plants are known to contain a group of triterpenoids whose methyl groups are oxidized in sequence to form alcohol, aldehyde and acid [10–12], such

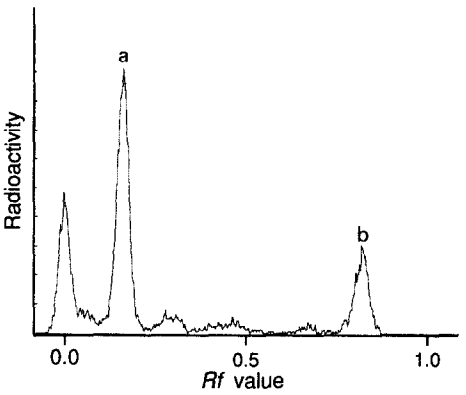


Fig. 1. Radiochromatogram of the $CHCl_3$–MeOH (2:1) extract of watermelon cell suspension cultures incubated with [2-^{14}C]acetate (37 kBq) for 24 hr. The extract was developed on TLC plate (silica gel) with cyclohexane–EtOAc–HOAc (75:25:2) as the solvent system. (a) Bryonolic acid, (b) a mixture of squalene and steryl esters.

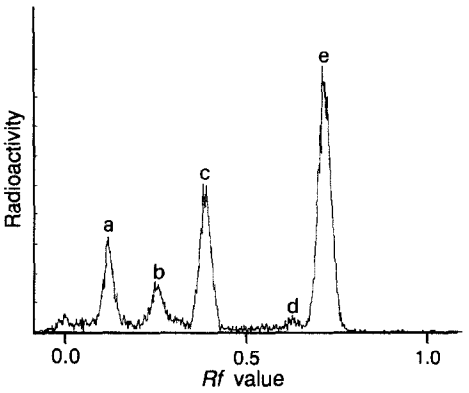


Fig. 2. Radiochromatogram of the Et_2O extract of the cell homogenate (10000 *g* supernatant) incubated with *R*-[2-^{14}C] mevalonate (3.7 kBq) for 1 hr at 30° in the presence of ATP, NADPH and $MgCl_2$. The extract was developed on TLC plate (silica gel) with cyclohexane–EtOAc–HOAc (75:25:2) as the solvent system. (a) A mixture of bryonolol and bryonolic acid (3:2), (b) a mixture of bryonolal and *trans,trans*-farnesol (3:1), (c) isomultiflorenol, (d) 2,3-oxidosqualene, (e) squalene.

intermediates in watermelon were detectable only when labelled mevalonate was administered to the cell-free preparation, not to the intact cells. The reason for the appearance of the intermediates only in the *in vitro* system is not clear, but it might be due to disorganization of a hypothetical complex of oxidative enzymes by cell homogenization, which would cause retardation of the oxidative reaction steps leading to **1**.

The results obtained from the tracer experiments mentioned above suggest that all the enzymes involved in the biosynthesis of **1** from mevalonic acid remained active in the cell-free extract prepared from cultured cells of watermelon. It has been reported that cell-free extracts of the rat liver, *Candida* and the latex of *Euphorbia* are capable of converting mevalonic acid to cholesterol [13], ergosterol [14], and triterpenol [15], respectively. Unlike

Euphorbia and watermelon, the cell-free system of tobacco was unable to convert mevalonic acid to triterpenoids or phytosterols, but accumulated squalene and 2,3-oxidosqualene [16].

Co-factors required for enzymatic synthesis of 1

The enzymatic synthesis of **1** from labelled mevalonate using the 10000 *g* supernatant prepared from cultured cells of watermelon required the addition of ATP, NADPH and Mg^{2+} to the reaction mixture (Table 2). Neither **1** nor intermediate compounds appeared in the absence of ATP and Mg^{2+}, which are essential to the phosphorylation of mevalonic acid to form isopentenyl pyrophosphate [17]. Mg^{2+} is also known to participate in many reaction steps from isopentenyl pyrophosphate to presqualene pyrophosphate [18]. When NADPH, which is required for both squalene synthesis from presqualene pyrophosphate [19] and squalene epoxidation [20], was not added to the reaction mixture, the amounts of detectable intermediate compounds after presqualene pyrophosphate were greatly decreased. Under anaerobic incubation conditions where oxygen in the air was absorbed into an alkaline solution of pyrogallol, the intermediates including 2,3-oxidosqualene markedly decreased while squalene accumulated. Epoxidation of squalene is known to be dependent on molecular oxygen and NADPH [21], although it is not clear whether molecular oxygen is necessary also in the oxidation of **2** to **1**.

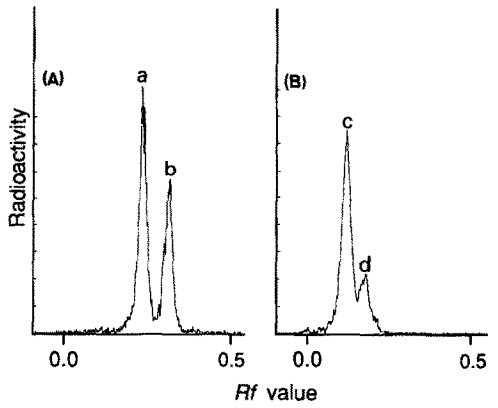


Fig. 3. Radiochromatograms of eluates from the bands a and b in Fig. 2. Each eluate was redeveloped on TLC plate with cyclohexane–EtOAc–HOAc (60:12:4.4), (A) twice and benzene–EtOAc (20:1), (B) twice. (a) Bryonolol, (b) bryonolic acid, (c) bryonolal, (d) *trans,trans*-farnesol.

Effect of enzyme inhibitors on biosynthesis

Lauryl dimethylamine *N*-oxide (LDAO), known to inhibit 2,3-oxidosqualene cyclase because of its electrochemical similarity to 2,3-oxidosqualene [22], dose-dependently blocked the cyclization process in the *in vitro* experiment, causing an accumulation of 2,3-oxidosqualene and a marked reduction in its metabolites (Table 3). Similar effects were observed also *in vivo* when LDAO (100 μM) was administered to 11-day-old cell suspension cultures together with [2-^{14}C] acetate and incubated for 4 hr; 2,3-oxidosqualene accumulated as a main labelled product, while radioactive **1** was no longer detectable (Fig. 4). Tolnaftate, which is used clinically as an antifungal agent inhibiting fungal squalene epoxidase [23], also caused an accumulation of squalene by inhibiting the formation of its metabolites in watermelon cells (Table 4).

The effect of CO on triterpenoid biosynthesis was investigated in order to find whether or not any cytochrome P-450 dependent oxidation reaction might be involved in the conversion of **2** to **1**. Incubation of the cell-free system (10000 *g* supernatant) with *R*-[2-^{14}C]mevalonate under a mixture of CO and O_2 (8:2) in the dark failed to inhibit the oxidation of the C-29 methyl group of **2**, giving an incorporation pattern similar to that of the control incubated in the air (data not shown). Addition (10–100 μM) of ancymidol or KSCN, known as cytochrome P-450 inhibitors [24], to the incubation mixture also did not affect the incorporation of the labelled mevalonate into **1** (Table 5). However, another cytochrome P-450 inhibitor ketoconazole (10–100 μM) partially inhibited the incorporation of

Table 2. Requirements for the incorporation of *R*-[2-^{14}C]mevalonate into bryonolic acid (BA) and its precursors in cell-free preparations of watermelon

	Incorporation (%) of *R*-[2-^{14}C]mevalonate				
Assay system	Squalene	2,3-Oxidosqualene	Isomultiflorenol	Bryonolal + farnesol	Bryonolol + BA
Complete*	25.8	3.1	17.9	5.0	13.3
−ATP	0	0	0	0	0
$-Mg^{2+}$	0	0	0	0	0
−NADPH	11.7	0	1.3	3.1	0
$-O_2$	59.0	0	3.4	3.3	0

*Complete reaction mixture (0.35 ml) consisted of 3.7 kBq sodium *R*-[2-^{14}C]mevalonate, 3 mM ATP, 5 mM $MgCl_2$, 1 mM NADPH and 0.3 ml of the 10000 *g* supernatant of 10-day-old cell cultures (150 mg) homogenized in 0.1 M phosphate buffer (pH 7.4) containing 0.4 M sucrose, 10 mM dithiothreitol, and polyvinylpolypyrrolidone (6% of cell, w/w). The reaction was carried out at 30° for 1 hr.

Table 3. Effect of LDAO on incorporation of *R*-[2-^{14}C]mevalonate into bryonolic acid (BA) and its precursors in cell-free preparation of watermelon

	Incorporation (%) of *R*-[2-^{14}C]mevalonate				
LDAO (μM)	Squalene	2,3-Oxidosqualene	Isomultiflorenol	Bryonolal + farnesol	Bryonolol + BA
0	12.9	1.3	12.6	3.7	5.6
1	14.3	10.6	5.2	2.3	2.7
2	14.7	14.1	4.0	2.3	2.3
5	16.5	17.3	2.0	1.9	1.1
10	13.0	16.3	1.1	1.6	0.6

Standard incubation mixture (0.35 ml) containing cell-free extract, ATP, Mg^{2+} and NADPH was preincubated with LDAO for 20 min before 3.7 kBq *R*-[2-^{14}C]mevalonate was administered to the mixture. Then, the reaction mixture was incubated for 60 min at 30°.

Table 4. Effect of tolnaftate on the incorporation of *R*-[2-^{14}C]mevalonate into bryonolic acid (BA) and its precursors

	Incorporation (%) of *R*-[2-^{14}C]mevalonate				
Tolnaftate (μM)	Squalene	2,3-Oxidosqualene	Isomultiflorenol	Bryonolal + farnesol	Bryonolol + BA
0	26.1	1.2	19.3	8.6	13.7
1	52.3	0.8	4.4	2.9	3.1
10	62.9	0.5	1.7	1.3	1.3

Standard incubation mixture (0.35 ml) containing cell-free extract, ATP, Mg^{2+} and NADPH was preincubated with tolnaftate for 20 min prior to the administration of 3.7 kBq *R*-[2-^{14}C]mevalonate. Incubation for 60 min at 30°.

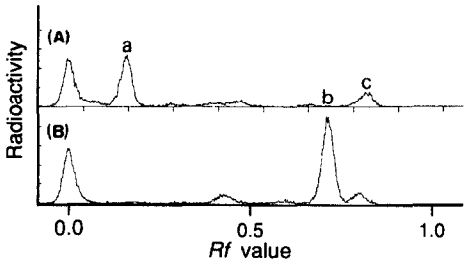


Fig. 4. Radiochromatograms of $CHCl_3$–MeOH (2:1) extracts of watermelon cell suspension cultures incubated with [2-^{14}C]acetate (37 kBq) for 4 hr without (A) or with (B) LDAO. The extracts were developed on TLC plate (silica gel) with cyclohexane–EtOAc–HOAc (75:25:2) as the solvent system. (a) Bryonolic acid, (b) 2,3-oxidosqualene, (c) squalene.

mevalonate into the intermediate compounds subsequent to **2**, although its effect on oxidations can not be critically interpreted as it appeared to suppress the cyclization step as well, causing a reduction in the quantity of **2**. Nevertheless, the results described above seem to suggest that the oxidation reactions subsequent to the formation of **2** may not be dependent on cytochrome P-450, as reported for the oxidative demethylation of lanosterol in cholesterol biosynthesis [25], in spite of many mixed function oxidation reactions known to require cytochrome P-450 [26, 27]. This possibility must be ascertained by further experiments using labelled **2** as a substrate.

Cyclization of [^{14}C]2,3-*oxidosqualene to* **2**

[^{14}C]2,3-Oxidosqualene prepared enzymatically from [2-^{14}C]mevalonate was converted into labelled **2** with a conversion rate of 7% when incubated with a microsomal preparation (100000 *g*) from watermelon cell-free extracts at 30° for 1 hr. The reaction did not require any exogenous cofactors and **3**, **4** and **1** were not formed in this system even when NADPH was added to the reaction mixture. The enzymatic synthesis of **2** was confirmed by GC-MS analysis of the product in the incubation mixture consisting of a 10000 *g* supernatant solution (2 ml) of cell-free extracts and non-labelled 2,3-oxidosqualene (0.2 mg) dispersed in Triton X-100. The results of this experiment indicate that **2**, which is formed by the cyclization of 2,3-oxidosqualene, is the first triterpenoid precursor in the biosynthesis of **1**. It has been reported that the reaction steps involved in the cyclization of 2,3-oxidosqualene proceed in a fully concerted manner, without forming any stabilized intermediates [28]. In the formation of **2**, 2,3-oxidosqualene would presumably be converted to a protosterol cation, followed sequentially by D-ring enlargement, E-ring formation, a series of 1,2-shifts and formation of a double bond through deprivation of a proton at the C-9 position (Scheme 1).

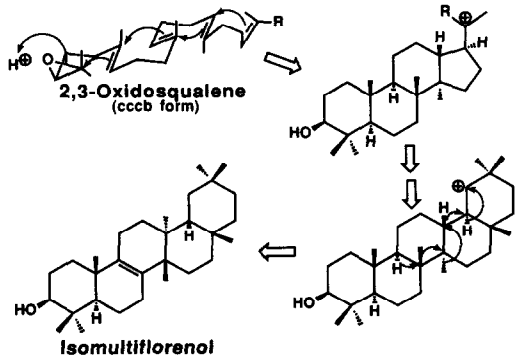
Time course of triterpenoid synthesis in vitro

In an attempt to elucidate the sequence of events preceding the formation of **1**, cell-free extracts were

Table 5. Effect of cytochrome P-450 inhibitors on incorporation of *R*-[2-^{14}C]mevalonate into bryonolic acid (BA) and its precursors in cell-free preparations of watermelon

Inhibitors	Concn (μM)	Incorporation (%) of *R*-[2-^{14}C]mevalonate				
		Squalene	2,3-Oxidosqualene	Isomultiflorenol	Bryonolal + farnesol	Bryonolol + BA
Control		14.9	0.7	9.5	4.2	4.9
Ancymidol	10	13.1	0.5	5.8	3.8	4.6
	100	19.6	0.8	9.0	5.2	3.2
Ketoconazole	10	12.8	2.9	5.8	3.4	3.3
	100	15.8	6.0	3.2	1.6	0.8
KSCN	10	16.5	1.1	9.9	4.4	5.4
	100	17.9	1.8	7.5	4.3	4.1

Standard incubation solution (0.35 ml) containing cell-free extract, ATP, Mg^{2+} and NADPH was first incubated with each inhibitor for 20 min, and then administered with 3.7 kBq *R*-[2-^{14}C]mevalonate. Incubation was carried out at 30° for 60 min.



Scheme 1. A hypothetical scheme for the formation of isomultiflorenol from 2,3-oxidosqualene.

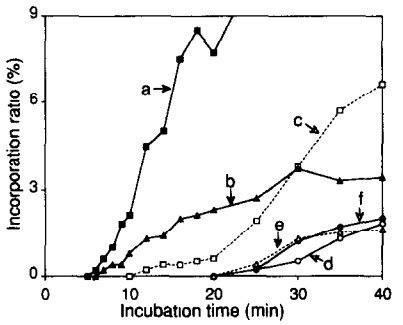


Fig. 5. Time course of incorporation of *R*-[2-^{14}C]mevalonate into bryonolic acid via intermediate metabolites in the watermelon cell-free extract incubated at 15° for the first 20 min and then at 25° for another 20 min. (a) Squalene, (b) 2,3-oxidosqualene, (c) isomultiflorenol, (d) bryonolol, (e) bryonolal, (f) bryonolic acid.

incubated with *R*-[2-^{14}C]mevalonate and the time course of triterpenoid formation was studied during a period of 40 min after incubation. At a temperature of 30°, biosynthetic reactions in the cell-free system were carried out so rapidly that radioactive intermediates and **1** were formed almost simultaneously as early as 5 min after incubation. This made it impossible to determine the order of events in sequence. However, this difficulty could be overcome considerably by lowering the incubation temperature to 15° for the first 20 min, before the temperature was raised to 25° during the following period of 20 min. At 15°, a sharp increase of squalene that began 6 min after the administration of mevalonate was immediately followed by a gradual increase of 2,3-oxidosqualene at 7 min and by that of **2** at 12 min (Fig. 5). In contrast, the delayed formation of **3**, **4** and **1** was observed when the temperature was raised to 25° to accelerate the enzymatic reactions. It is interesting that the two series of biosynthetic activities could be differentiated by the temperature control. It appears that the enzymatic oxidation reactions leading to **1** from **2** require higher thermal energy than do the preceding reactions.

The results obtained from the time-course study strongly support the validity of the assumption that **1** is biosynthesized from mevalonic acid via squalene, 2,3-oxidosqualene, **2**, **3** and **4**, in that order.

EXPERIMENTAL

Plant and cell culture. Cell suspension cultures of watermelon (*C. lanatus* Matsum. et Nakai cv Zuisho), which were originally derived from the seedling, have been maintained in Linsmaier–Skoog medium (30 ml) [29] containing 10^{-5} M naphthaleneacetic acid in 100-ml conical flasks over a period of 40 months by subculturing at an interval of 15 days [4]. The culture flasks were agitated on a reciprocating shaker (100 rpm) at 25° in the dark. Ten-day-old cells at the linear growth stage actively producing **1** were used for biosynthetic studies.

Radiochemicals. Na[2-^{14}C]acetate (1.96 GBq mmol^{-1}) and *R*-[2-^{14}C]mevalonic acid lactone in toluene (1.98 GBq mmol^{-1}) were purchased from Amersham. *R*-[2-^{14}C]mevalonic acid lactone was converted into its Na salt by addition of an equivalent amount of aq. $NaHCO_3$ after evapn of toluene [30]. [^{14}C]2,3-oxidosqualene was prepared enzymatically from Na *R*-[2-^{14}C]mevalonate using the cell-free extracts of watermelon cell culture by addition of 10 μM LDAO to the reaction mixt. to block the cyclization of 2,3-oxidosqualene.

Chemicals. Compound **1** was isolated by recrystallization from the $CHCl_3$–MeOH extracts of watermelon cell

cultures. Compound **3** was synthesized from **1** by $LiAlH_4$ reduction after methylation with diazomethane. Compound **4** was prepared by the Cr_2O_3–pyridine complex oxidation of C-29 alcohol radical which was obtained by the partial hydrolysis of bryonolol diacetate. Compound **2** was a gift from Dr R. Tanaka of Osaka Pharmaceutical College; it was also synthesized from **4** by Wolff–Kishner reduction by us. Ketoconazole and tolnaftate were purchased from Sigma. LDAO, mevalonic acid lactone and *trans,trans*-farnesol were purchased from Serva, Nakalai, and Aldrich, respectively. The other chemicals used in this study were purchased from Wako.

Analysis of triterpenes and phytosterols. Hexane extracts from dried cultured cells (15-day-old) of watermelon were saponified by heating for 2 hr under reflux with 10% KOH in 80% EtOH for the analysis of triterpenes and phytosterols. The non-saponifiable lipids were methylated and subjected to GC using a capillary column (0.53 mm i.d. × 30 m, 1 μm film thickness) of MPS-50 (Quadrex Co., equivalent to OV-17) at the temp. of 275°. For the quantitative analysis of **1**, a mixt. of $CHCl_3$–MeOH (2:1) was used for its extraction from dried cultured cells. Methyl esters of **1** and D:C-friedoolean-7,9(11)-dien-3β-ol-29-oic acid, stigmasta-7,22-dien-3β-ol, stigmasta-7,25-dien-3β-ol, and stigmasta-7,22,25-trien-3β-ol were identified by GC-MS in comparison with the corresponding authentic samples.

Tracer experiments in vivo. Na [2-^{14}C]acetate (37 kBq) or Na *R*-[2-^{14}C]mevalonate (37 kBq) was added to 2 ml of cell culture (equivalent to *ca* 300 mg of fresh cells) and incubated at 25° in the dark for 24 hr. Cells harvested were freeze-dried and extracted with 2 ml of hot $CHCl_3$–MeOH (2:1). An aliquot of the extract was used for the measurement of total radioactivity by a liquid scintillation counter (Aloka LSC-9000). The remaining was applied to silica gel TLC using cyclohexane–EtOAc–HOAc (75:25:2, system 1) as the developing solvent and subjected to analysis by radiochromanizer (Aloka JTC-501).

Preparation of cell-free extracts. Cultured cells of watermelon were homogenized with 1.5 vol. of 0.1 M KPi buffer (pH 7.4) containing 0.4 M sucrose, 10 mM dithiothreitol and PVPP (6% of cell, w/w) using a Potter-Elvehjem homogenizer. The homogenate was filtered through Miracloth and the filtrate was centrifuged at 10000 *g* for 20 min. The 10000 *g* supernatant soln was used as enzyme after gel chromatography using a column of PD-10 (Sephadex G-25M, Pharmacia). All the procedures were carried out at 4°. The final cell-free extract soln was stable for several weeks when stored at −85°.

Administration of Na R-[2-^{14}C]*mevalonate* in vitro. The standard incubation mixt. (0.35 ml) contained 0.3 ml cell-free extract, 3 mM ATP, 1 mM NADPH and 5 mM $MgCl_2$. The assay mixt. was incubated at 30° for 1 hr after addition of 3.7 kBq of Na *R*-[2-^{14}C]mevalonate, and then extracted with 0.8 ml Et_2O, twice. After measurement of the total radioactivity of the Et_2O extract, the remaining extract was applied to radio-TLC with authentic standards using the developing solvent system 1.

Identification of radioactive compounds. Five main peaks (a–e) appeared in the radiochromatogram (TLC) of the Et_2O extract developed with the solvent system 1. The most polar peak, a, was sepd into 2 components when developed twice with cyclohexane–EtOAc–HOAc (60:12:4.4) which were identified as **1** and **3**. Peak b was also separable into 2 compounds with the solvent system of benzene–EtOAc (20:1) and identified as a mixt. of **4** and *trans,trans*-farnesol. Peak c was identified as **2** by cochromatography with an authentic sample using the system 1, benzene–EtOAc (5:1), and benzene–EtOH (10:1) as the developing solvents. Peaks d and e showed the same R_f values as 2,3-oxidosqualene and squalene, respectively, on TLC with the system 1 as well as with hexane–EtOAc (97:3). Each compound was also identified by GC equipped with a radio counter (Aloka RGC-212) after the methylation and acetylation of the Et_2O extract. The column of 3% OV-17 (3 mm i.d. × 1 m) was used at the programmed temperature of 160–290° (8° min^{-1}). Compounds **1**–**4** were reconfirmed by successive recrystallizations with authentic carrier compounds using the indicated solvents until they showed a constant specific radioactivity. Changes in the specific radioactivity (dpm mg^{-1}) during successive recrystallizations were as follows: **1** (benzene–MeOH, 4:1): 1170, 1100, 1010, 1040, 1010; **2** (MeOH): 4700, 3510, 3590, 3620; **3** (EtOH): 2520, 2060, 1930, 1780, 1810; **4** (80% MeOH): 2740, 2820, 3100, 2800.

Inhibitor experiments. LDAO and KSCN were dissolved in H_2O. Tolnaftate was dissolved in acetone; ancymidol and ketoconazole were dissolved in EtOH. Each soln was added to an incubation mixt. at a concn of 2% (v/v). Inhibitors were pre-incubated for 20 min with the enzyme soln prior to the addition of the substrate, while controls were treated with an equal amount of appropriate solvent. For setting up an anaerobic condition, an open vial containing the incubation mixt. was placed inside a larger vial containing a filter paper moistened with 3 M pyrogallol. The larger vial was sealed with a silicone rubber cap and the air inside was replaced with N_2 by repeated evacuation and flushing. A trace amount of O_2 was further removed by injecting 10% NaOH soln with a syringe into the pyrogallol soln around the open vial [31].

Measurement of 2,3-oxidosqualene cyclase activity. [^{14}C]2,3-oxidosqualene was prepared from Na *R*-[2-^{14}C]mevalonate (370 kBq) using watermelon cell-free extracts to which 10 μM LDAO was added. The labelled 2,3-oxidosqualene (12000 dpm, 8 nmol) dispersed in Triton X-100 (the final concn in incubation mixt.: 0.1%) was administered to 1 ml microsomal suspension (3.2 mg protein ml^{-1}) of the cell-free extract and incubated for 60 min. Compound **2** appeared on the radio-TLC of the Et_2O extract of incubation mixt. with the solvent system 1 and it was confirmed as **2** by TLC using another solvent system, benzene–EtOAc (5:1). The cyclization product (**2**) of 2,3-oxidosqualene was identified by the following experiments using a nonlabelled substrate. 2,3-Oxidosqualene (200 μg), synthesized from squalene and

N-bromosuccinimide by the method of ref. [32], was dispersed with Triton X-100 (2 mg) and administered to the 10000 *g* supernatant (2 ml) prepared from watermelon cell-free extracts. After incubation for 60 min at 30°, the reaction mixt. was extracted with 5 ml Et_2O, twice, and the extract was subjected to prep. TLC on a silica gel plate (benzene–EtOAc, 3:1). Compound **2** was identified by GC analysis of the eluate from the band corresponding to a standard sample of isomultiflorenol on TLC. A capillary column (0.53 mm i.d. × 30 m, NB 5, GL Science) was used at a constant column temp. of 280°. The main product of the enzymatic reaction was further confirmed as **2** by comparison with an authentic sample by GC-MS. GC-EIMS 75 eV *m/z* (rel. int.): 426 (78), 411 (48), 259 (100), 247 (97), 241 (54), 229 (72), 218 (48), 205 (88). In addition to **2**, cycloartenol was detected in the reaction mixt.

Time course study. Na *R*-[2-^{14}C]mevalonate (74 kBq), ATP, NADPH and $MgCl_2$ were added to the 10000 *g* supernatant (5 ml) that had been passed through a column of PD 10 (Pharmacia). The mixt. was incubated at 15° for the first 20 min and then at 25° thereafter to chase changes in the pattern of intermediate products. A sample (300 μl) was collected from the reaction mixt. at intervals of 1–5 min, and extracted immediately with cold Et_2O (3 ml). The extracts were subjected to radio-TLC, as described above.

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