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J. Nat. Prod., **1993**, 56 (2), 165-174• DOI: 10.1021/np50092a001 • Publication Date (Web): 01 July 2004

Journal of Natural Products
Vol. 56, No. 2, pp. 165-174, February 1993

PRODUCTION OF AN ANTI-ALLERGIC TRITERPENE, BRYONOLIC ACID, BY PLANT CELL CULTURES[1]

M. TABATA,* S. TANAKA, H.J. CHO, C. UNO, J. SHIMAKURA, M. ITO,

Faculty of Pharmaceutical Sciences, Kyoto University, Yoshida, Kyoto 606, Japan

W. KAMISAKO, and C. HONDA

Faculty of Pharmaceutical Sciences, Mukogawa Women's University, Edagawa-cho 4-16, Nishinomiya 663, Japan

ABSTRACT.—Cell suspension cultures of *Luffa cylindrica, Citrullus lanatus,* and related cucurbitaceous plants accumulate large quantities of bryonolic acid (3β-hydroxy-D:C-friedo-olean-8-en-29-oic acid) [**1**], an acidic, pentacyclic triterpene found exclusively in the roots of the intact plants. This compound could readily be isolated from cultured cells with $CHCl_3$ and purified simply by recrystallization. Pharmacological tests using mice demonstrated that bryonolic acid or its derivative is active against at least three types of allergies and that its activity could be increased significantly by preparing synthetic derivatives, in particular a potassium salt of its succinate ester. The biosynthesis of bryonolic acid from mevalonic acid via isomultiflorenol has been elucidated by tracer and enzymological experiments using cultured cells of watermelon both in vitro and in vivo. Furthermore, cell fractionation and electron microscopic studies on subcellular structures of luffa cells suggested that minute vesicles originating from elongated, rough endoplasmic reticulum probably play an important role in the transport of bryonolic acid which largely accumulates in the cell wall of cultured cells. The results obtained from the present study indicate that plant cell culture would be useful not only as a biological system for elucidating biosynthetic mechanisms but also as a potential source of new pharmacologically active compounds.

Attempts to utilize the potential biosynthetic ability of plant cell cultures for the production of useful compounds have primarily been focused on well-known natural products that have already been established as medicines (1,2). However, plant cell cultures may produce hitherto unknown biologically active compounds which might be developed into new drugs. We will illustrate such a possibility with an anti-allergic compound, bryonolic acid [**1**], produced by cell suspension cultures of *Luffa cylindrica*. In this paper, our studies on the biosynthesis and pharmacology of this compound as a potential source of a new drug will be discussed.

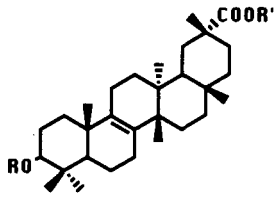
Luffa cylindrica Roem. (Cucurbitaceae) is an annual vine native to the Old World tropics and is cultivated around the world. In Malaysia, luffa fruits are used as a purgative; in Java juice from the leaves is given for amenorrhea and in India for snake-bite (3). In China, various parts of this plant, including flowers, fruits, seeds, leaves, and roots, are used against such inflammations as pharyngitis, rhinitis, mastitis, edema, swellings, burns, and hemorrhoids (4). Secondary metabolites of *L. cylindrica*, α-spinasterol and oleanolic acid glycoside, were isolated from the seeds (5). Takemoto and co-workers (6,7) isolated, in addition to ginsenosides-Re and -Rg_1, new oleanane saponins named lucyosides A–H from the aerial parts, and nine oleanane saponins including four new lucyosides J–M from the roots of *L. cylindrica*.

In 1984, Kamisako *et al.* (8) reported that cell suspension cultures of *L. cylindrica*, grown in Linsmaier-Skoog (LS) medium (9) containing 1 μM 2,4-dichlorophenoxyacetic acid (2,4-D) as a growth hormone, produced bryonolic aicd (3β-hydroxy-D:C-friedoolean-8-en-29-oic acid), which was first isolated from the roots of *Bryonia dioica* (Cucurbitaceae) by Biglino *et al.* (10). The content of **1**, which readily crystallized as

[1]Presented as a plenary lecture at the "Applications of Biotechnology" Symposium of the 33rd Annual Meeting of the American Society of Pharmacognosy, Williamsburg, Virginia, July 26–31, 1992.

colorless needles when extracted from dried, cultured luffa cells with $CHCl_3$, was nearly 1% of dry wt. The absolute structure and conformations of **1** and its derivatives were elucidated by X-ray analysis and ^1H, ^{13}C-nmr spectroscopy, respectively (11–13). This friedooleanane-type triterpene is characterized by the presence of a carboxyl group at C-29, having a planar structure similar to that of glycyrrhetinic acid [**2**], the aglycone of glycyrrhizin that is clinically used for the treatment of hepatitis and allergy in Japan.

Taking the structural similarity with glycyrrhetinic acid into consideration, we carried out pharmacological tests on **1** on rats and mice to find that it shows not only a stronger anti-allergic activity but much fewer side effects or acute toxicity than glycyrrhetinic acid (14,15). Compound **1** did not inhibit the activity of 3-ketosteroid 5β-reductase of the rat liver that inactivates endogenous aldosterone both in vivo and in vitro whereas the enzyme activity was strongly inhibited by glycyrrhetinic acid [**2**], having a ketone group at C-11 and a double bond at C-12 (16,17). It is likely that **1**, lacking the C-11 ketone, would not bring about such undesirable side effects as the pseudoaldosterone syndrome (18), which was reported for patients taking an overdose of glycyrrhetinic acid or its derivatives (19,20).



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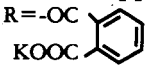
7 R=-OC  , R′=K
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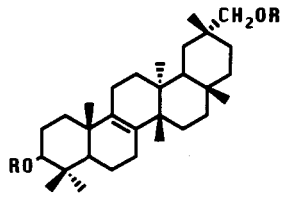

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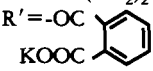
8 R=R′=-OC 
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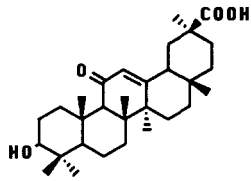

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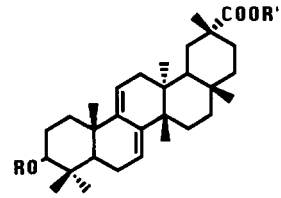

10 R=-CO(CH$_2$)$_2$COOK, R′=K

CAPABILITY OF CUCURBITS AND THEIR CALLUS CULTURES TO PRODUCE BRYONOLIC ACID [**1**].—Gc analysis of mature cucurbitaceous plants of *L. cylindrica, Citrullus lanatus, Cucumis sativus, Cucumis melo, Benincasa cerifera,* and *Lagenaria siceraria* showed that **1** is not detected in the aerial parts but only in the roots of these species (21). The content of **1** in the roots varied widely from 0.02 to 0.19% of dry wt. On the other hand, all of callus cultures derived from the cotyledons, hypocotyls, and radicles of nine cucurbitaceous species were capable of producing **1** (0.02–0.45%; average 0.2% of dry wt) irrespective of the original organs used for callus induction, when grown on LS agar medium containing 1μM 2,4-D and 1μM kinetin. Furthermore, cultured cells as well as excised radicles of both luffa and watermelon converted exoge-

nously administered sodium [2-^{14}C]acetate into **1**, whereas excised cotyledons and hypocotyls failed to do so (Figure 1), showing that the radicle is the sole site of the biosynthesis of **1**. It is therefore interesting that the biosynthetic activity specific to the roots is fully expressed in the cultured cells that do not synthesize any other known saponins. However, no significant correlation was found between the content of **1** in radicles and that in callus cultures. Time course of the production of **1** in luffa cell suspension cultures grown in LS medium containing 0.1 μM 1-naphthaleneacetic acid

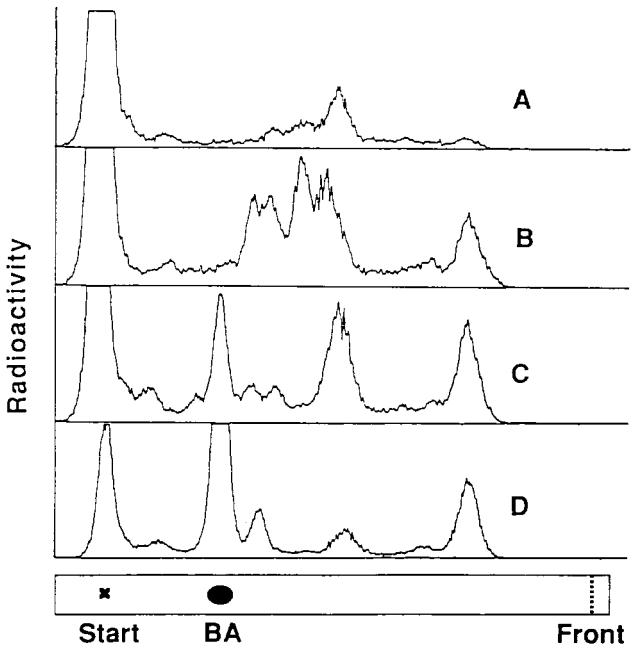

FIGURE 1. Radiochromatograms of $CHCl_3$/MeOH extracts from excised cotyledons (A), hypocotyls (B), and radicles (C) of 10-day-old seedlings and cultured cells (D) of watermelon. The extracts were incubated with sodium [2-^{14}C]acetate for 20 h at 25° and then developed on a Si gel plate with cyclohexane-EtOAc-HOAc (70:27:3). BA: bryonolic acid.

(NAA) at 25° in the dark is shown in Figure 2. The content of **1** in luffa cell suspension cultures has recently been increased up to 8% of dry wt through repeated selections of cell clones and cultivation of selected cell lines on LS medium containing NAA instead of 2,4-D (unpublished data).

BIOSYNTHESIS OF BRYONOLIC ACID [**1**].—The biosynthesis of **1** was studied by using watermelon cell suspension cultures, which grew faster than luffa cells to reach the stationary phase in 12 days after inoculation and produced on the average ca. 200 mg of **1** per liter of medium. Administration of ^{14}C-labeled mevalonic acid to the cell cultures on day 10 showed that the radioactivity was largely incorporated into **1** after 10 min, but radioactive intermediates were hardly detectable. Therefore, tracer experiments were carried out by using crude enzyme extracts prepared from 10-day-old cultured cells. The 10,000 × *g* supernatant obtained by centrifugation from the cell homogenate in 0.1 M tris-HCl buffer (pH 7.5) containing 0.4 M sucrose, 10 mM glutathione, and 3% polyviny polypyrrolidone was incubated with *R*-[2-^{14}C] mevalonate with 3mM ATP, 1 mM NADPH, and 5 mM $MgCl_2$ at 30° for 60 min. Radio-tlc analysis of the ethereal extract of the reaction mixture using two different solvent sys-

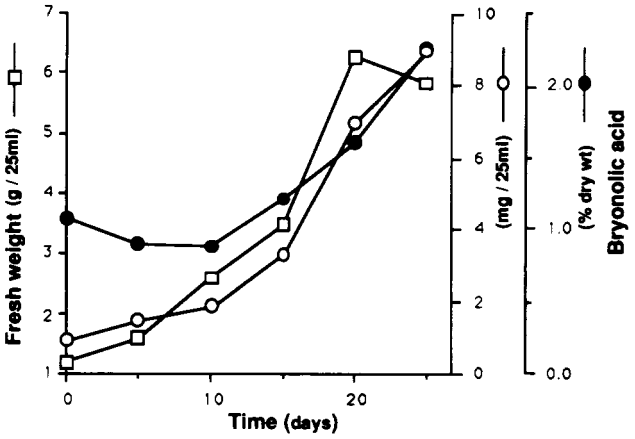

FIGURE 2. Time course of cell growth and bryonolic acid [**1**] production in cell suspension cultures of *Luffa cylindrica*.

tems revealed radioactive spots corresponding to a series of presumable intermediates: squalene, 2,3-oxidosqualene, isomultiflorenol, an alcohol derivative bryonolol, and a new aldehyde named bryonolal, in addition to the radioactive spot of **1** (Figure 3). These compounds separated by tlc were identified with authentic samples by gc and dilution tests.

In vitro experiments showed that the conversion of labeled mevalonic acid into **1** requires ATP, Mg^{2+}, NADPH, and O_2. In the absence of ATP and Mg^{2+}, none of the possible intermediates mentioned above, including squalene, was synthesized, since these co-factors are known to be essential for the biosynthesis of squalene from mevalonic acid (22). Removal of NADPH from the reaction mixture resulted in a notable accumulation of squalene and a marked reduction in quantities of the subsequent terpenoid products, suggesting that NADPH, not NADP, is required for the oxidation of squalene to yield 2,3-oxidosqualene. Similarly, under anerobic conditions in N_2, a large amount of squalene accumulated without formation of **1**, probably because the

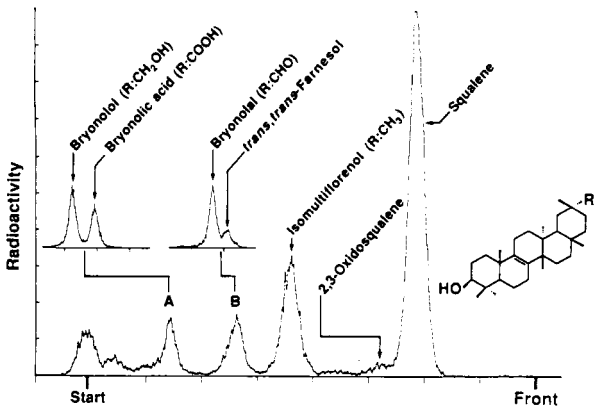

FIGURE 3. Radiochromatograms of an ethereal extract of the cell homogenate (10,000 × *g* supernatant) incubated with *R*-[2-^{14}C]mevalonate for 60 min at 30° in the presence of ATP, NADPH, and $MgCl_2$. The extract was developed on tlc plate (Si gel) with cyclohexane-EtOAc-HOAc (75:25:2) as the solvent system. The eluates of peaks A and B were re-chromatographed with cyclohexane-EtOAc-HOAc (60:12:4.4) and C_6H_6-EtOAc (20:1), respectively, for further separation.

epoxidation of squalene to give 2,3-oxidosqualene was blocked for lack of oxygen. Addition of an inhibitor of 2,3-oxidosqualene cyclase, lauryl dimethyl amine-*N*-oxide (1–10 μM) (23,24), to the enzyme solution caused a remarkable increase of 2,3-oxidosqualene, while the amounts of isomultiflorenol, bryonolol, bryonolal, and **1** decreased drastically depending on the concentration of the inhibitor. Although co-factors that might be required for oxidation reactions involving the C-29 methyl group of isomultiflorenol remain to be clarified, a series of enzymatic reactions from mevalonic acid to **1** did not seem to be dependent on cytochrome P-450, as they were not inhibited by either CO or P-450 inhibitors such as ancymidol and ketoconazole.

Isomultiflorenol was first isolated from *Bryonia dioica* seedlings by Cattel *et al.* (25), who suggested that this triterpene might be a precursor of **1**. To confirm that isomultiflorenol is directly synthesized from 2,3-oxidosqualene, [U-^{14}C]2,3-oxidosqualene suspended in Triton-X was administered to a microsomal fraction prepared from cultured cells of watermelon. The results showed that 7% of the total radioactivity was incorporated into isomultiflorenol. Similarly, non-labeled 2,3-oxidosqualene was converted into isomultiflorenol, which was identified by gc-ms. Thus, isomultiflorenol proved to be the first pentacyclic triterpene resulting from the cyclization of 2,3-oxidosqualene. Interestingly, the cyclization process did not require addition of any co-factors. The cyclization process was catalyzed by a specific membrane-bound cyclase located in the 100,000 × *g* microsome; the cyclase activity began to increase sharply at the logarithmic growth stage before the onset of the biosynthesis of **1** at the linear growth stage. Presumably, isomultiflorenol would be formed via a tetracyclic carbonium ion occurring by ring-opening of the epoxide, followed by intermediary reaction steps including the Wagner-Meerwein shifts and the formation of a double bond due to deprivation of a proton at C-9.

The sequence of enzymatic reactions leading from squalene to **1** was investigated by pursuing the time course of each reaction product after the administration of ^{14}C-labeled mevalonate to a crude enzyme solution prepared from watermelon cell cultures. In order to slow down the speed of the whole enzymatic reaction by which **1** is formed (less than 5 min at 30°), the reaction mixture was incubated at 15° for the first 20 min and then at 25° for another 20 min. As shown in Figure 4, the appearance of radioactive

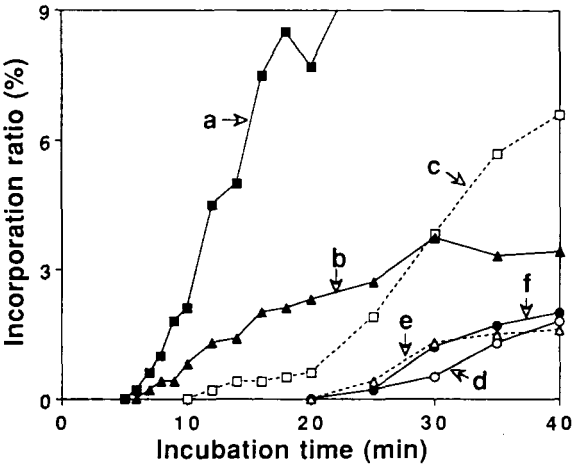

FIGURE 4. Time course of incorporation of *R*-[2-^{14}C]mevalonate into bryonolic acid [**1**] via intermediate metabolites in watermelon cell-free extract incubated at 15° for the first 20 min and then at 25° for another 20 min. a: squalene, b: 2,3-oxidosqualene, c: isomultiflorenol, d: bryonolol, e: bryonolal, f: bryonolic acid.

squalene was first detected 6 min after incubation, which was immediately followed by that of 2,3-oxidosqualene. Isomultiflorenol began to occur 12 min after incubation, whereas it took 25 min for bryonolol, bryonolal, and **1** to appear almost simultaneously. These observations strongly support the validity of our postulation that **1** is biosynthesized via squalene, 2,3-oxidosqualene, isomultiflorenol, bryonolol, and bryonolal, in that order.

INTRACELLULAR LOCALIZATION OF BRYONOLIC ACID [**1**].—Fractionation of cultured luffa cells at the stationary growth phase (day 30) by centrifugation showed that ca. 70% of the total content of **1** was recovered from the cell wall fraction (Table 1)

TABLE 1. Distribution of Bryonolic Acid [**1**] in Cellular Fractions of Cultured Cells of *Luffa cylindrica*.

Fraction	Weight (mg)	Content of bryonolic acid [**1**]		Cellulose (% of total)
		(mg)	(% of total)	
Crude cell wall	500	11.93	69	99.2
1000 × *g* upper layer	80	3.79	21	0.7
1000 × *g* starch grains	58	0.11	1	<0.1
10000 × *g* pellet	58	0.66	4	<0.1
Supernatant	—	0.88	5	—

(26). The rest of **1** (ca. 20%) was found in the 1000 × *g* fraction containing broken nuclei, plastids, etc. Compound **1** that accumulated in the cell wall could be extracted from living cells with "artificial blood" (FC-43 Emulsion, Green Cross Co. Ltd., Japan) containing perfluorotributylamine as an oxygen carrier and Pluronic F-68 as a detergent. When cells were transferred to a culture medium containing FC-43 Emulsion in

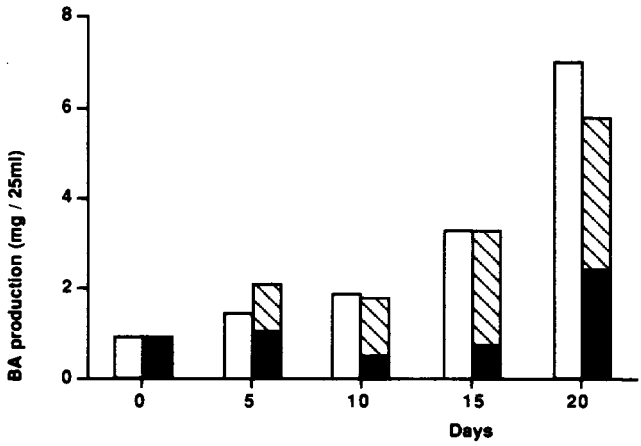

FIGURE 5. Effects of FC-43 Emulsion on the production and extracellular release of bryonolic acid [**1**] in luffa cell suspension cultures. ▭: In cells of control cultures, ▬: in cells of cultures treated with FC-43 Emulsion (25% v/v), and ▨: in the medium of cultures treated with FC-43 Emulsion. No bryonolic acid was released into the medium of control cultures. Three replicates.

place of H_2O, the amount of **1** released into the medium increased logarithmically with time, amounting to two-thirds of the total yield after 48 h. When sodium [2-^{14}C]acetate was administered to such a medium, radioactive **1** was detected in the medium after 10 min, and the radioactivity of **1** in the medium became 1.5 times as high as that in the cells after 45 min. In the absence of FC-43 Emulsion, no radioactive **1** was extracted into the medium but **1** did accumulate in the cells. Apparently, H_2O-insoluble **1** accumulating in the cell wall was extracted into the medium by FC-43 Emulsion. When cells were cultured for a long term (3 weeks) in the presence of FC-43 Emulsion (25%), the amount of **1** extracted into the medium increased with time, while cells continued to grow normally during the whole period of culture (Figure 5).

Electron microscopic examination of cultured luffa cells producing **1** at the linear growth stage revealed a remarkable development of multiple layers of elongated, rough endoplasmic reticulum (rER). Furthermore, there occurred characteristic swellings at both ends of rER, which appeared to release membraneous vesicles into the cytoplasm (Figure 6). These vesicles (ca. 0.1 μm in diameter) moved toward the peripheral region of the cell, occasionally coming into contact with the plasma membrane. By contrast, neither rER elongation nor vesicle formation was observed in bryonolic-acid-free cells cultured in a medium containing 2,4-D in place of NAA.

In *Lithospermum erythrorhizon* cell cultures, H_2O-insoluble shikonin derivatives produced in vesicles (0.1–0.2 μm in diameter), which originated from a number of swellings along elongated rER membranes, fused with the plasma membrane to excrete the naphthoquinone pigments through exocytosis (27). In luffa cells, however, it was not clear whether **1** was excreted into the cell wall by exocytosis or by simple diffusion. In any case, cytological and enzymological studies suggest that, as in the case of shikonin

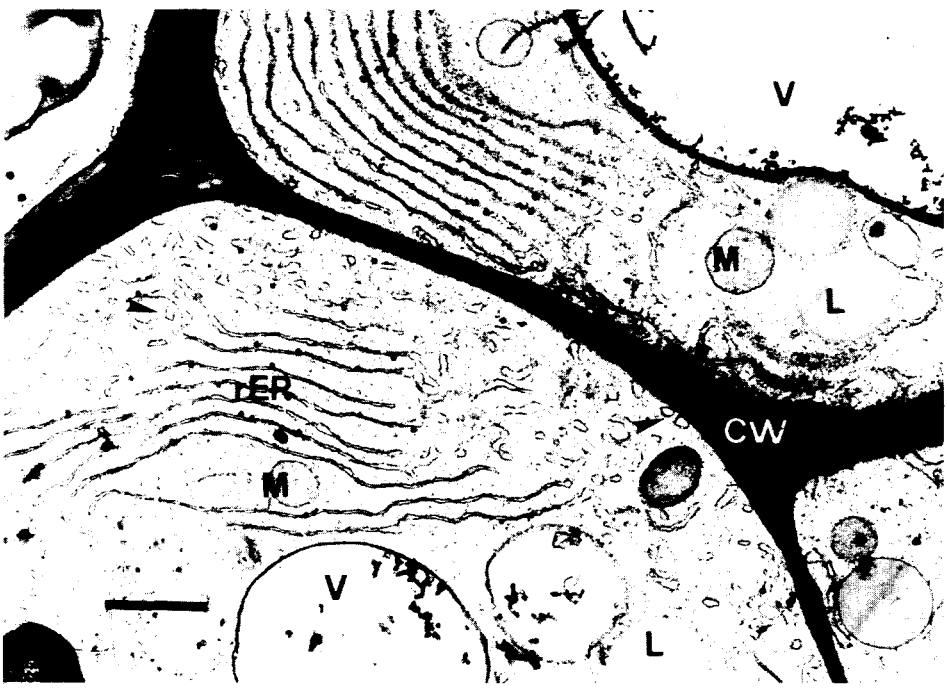

FIGURE 6. A cross section of bryonolic acid-producing cultured cells of *Luffa cylindrica* at the linear growth stage. CW: cell wall, rER: rough endoplasmic reticulum, M: mitochondria, V: vacuole, L: lipid body. Arrows indicate vesicles. Bar corresponds to 1 μm.

production, both rER and vesicles may play important roles in the biosynthesis and transport of **1**.

ANTI-ALLERGIC ACTIVITIES OF BRYONOLIC ACID [**1**] AND ITS DERIVATIVES.—Anti-allergic activities of **1** and its synthetic derivatives **3–10** were studied for three types of allergies which were artificially induced in male ddY mice.

Activity against the type-I allergy.—The type-I allergy is thought to be responsible for ailments such as atopic dermatitis, asthma, and pollinosis. Effects of **1** and its synthetic derivatives on the type-I allergy were evaluated by their capability of inhibiting passive cutaneous anaphylaxis in mice (6-week-old, 25–30 g). For that purpose, an anti-egg-albumin antiserum was injected to both ears of mice 48 h prior to an intravenous injection of egg albumin (antigen) and a marker pigment (Evans Blue). After 30 min, the amount of pigment leaked out in each ear was measured spectrophotometrically as an estimate of the anaphylactic reaction. The results are shown in Table 2. Ip administra-

TABLE 2. Effect of Intraperitoneal Administration of Bryonolic Acid [**1**] and Its Derivative on Passive Cutaneous Anaphylaxis in Mice.

Compound	ID_{50} (mg/kg)
Bryonolic acid [**1**]	376
Bryonolic acid 3-succinate-2K [**4**]	55.3
Bryonolic acid 3-phthalate-2K [**7**]	34.2
Diol [**3**]	315
Diol 3-succinate-K [**5**]	50.3
Diol 29-succinate-K [**9**]	>400
Diol 3,29-disuccinate-2K [**6**]	52.4
Diol 3,29-diphthalate-2K [**8**]	41.4
Diene 3-succinate-2K [**10**]	92.4
Tranilast[a]	152

[a]Reference drug.

tion of **1** 15 min prior to an injection of the antigen inhibited cutaneous anaphylaxis by 50% at a dose of 376 mg/kg body wt. Similarly, a diol derivative **3** of **1** gave a 50% inhibition at 315 mg/kg (ip), indicating that the reduction of the C-29 carboxyl group to an alcohol only slightly increased the activity. Interestingly, esterification of **1** and **3** with either succinic acid (producing **4–6**) or phthalic acid (producing **7** and **8**) resulted in a six- to ten-fold increase in the activity, although the esterification of C-29 alone with succinate (producing **9**) was ineffective. On the other hand, a succinate ester **10** of the diene derivative having two double bonds at C-7 and C-9 showed a 40% decrease in the activity, as compared with **4**, the succinate of **1**. In the case of oral administration 30 min prior to an injection of the antigen, the compound **4** exhibited a higher anti-anaphylactic activity than the phthalate esters **7** and **8** (Table 3). Accordingly, compound **4** was chosen as a test compound for further bioassays.

TABLE 3. Effect of Oral Administration of Bryonolic Acid Derivatives on Passive Cutaneous Anaphylaxis in Mice.

Compound	ID_{50} (mg/kg)
Bryonolic acid succinate-2K [**4**]	63.7
Bryonolic acid phthalate-2K [**7**]	172
Diol diphthalate-2K [**8**]	92.5

Activity of compound **4** *against the type-III allergy*.—The type-III allergy, which occurs through an immunological activation of complement by an immune complex, is supposed to cause chronic articular rheumatism and glomerulonephritis. Male ddY mice (4-week-old, 16–18 g) were sensitized by an intravenous injection of sheep erythrocytes 19 and 5 days before the Arthus reaction was induced by injecting sheep erythrocytes subcutaneously to their right paws. The effectiveness of a drug sample, which was administered orally 30 min prior to the induction of the Arthus reaction, was evaluated by measuring the degree of inhibition of edema (the difference in thickness between the right and the left paw). As a result, the Arthus reaction was inhibited by 35.3 and 62.4% at 300 and 600 mg/kg of **4**, respectively (Table 4).

TABLE 4. Effect of Oral Administration of Bryonolic Acid Succinate-2K [**4**] on Sheep-Erythrocytes-Induced Arthus Reaction in Mice.

Compound	Dose (mg/kg)	Inhibition (% of control)
Bryonolic acid succinate-2K [**4**] . . .	300	35.3[b]
	600	62.4[b]
Prednisolone succinate[a]	30	37.4[c]

[a]Reference drug.
[b]$P<0.01$.
[c]$P<0.001$.

Activity of compound **4** *against the type-IV allergy*.—The type-IV allergy, which is distinguished from the other types by not involving antibody production but T-lymphocytes, is considered to be responsible for both contact dermatitis and the rejection of skin graft. Contact dermatitis was induced by an external application of picryl chloride to the right ears of male ddY mice (6-week-old, 25–30 g), 7 days after they were sensitized with picryl chloride applied on the left ears. Test compounds were administered orally to the mice 3 h after the application of picryl chloride, and the thickness of the swollen right ears was measured after 24 h (Table 5). These experiments indicated that compound **4** also inhibited edema due to contact dermatitis by 34.0–53.0% at doses between 75 and 300 mg/kg.

TABLE 5. Effect of Oral Administration of Bryonolic Acid Succinate-2K [**4**] on Picryl-Chloride-Induced Contact Dermatitis in Mice.

Compound	Dose (mg/kg)	Inhibition (% of control)
BA succinate-2K [**4**]	75	34.0
	150	40.7
	300	53.0[b]
Prednisolone succinate[a]	30	41.0[b]

[a]Reference drug.
[b]$P<0.05$.

The results obtained from the above experiments clearly demonstrated that compound **4** is effective against three different types of allergies, showing a wide spectrum of activity. Furthermore, this compound showed very low acute toxicity, exhibiting no lethal effect on mice even at an oral dose of 4 g/kg. We consider that bryonolic acid is an interesting material for developing a new anti-allergic remedy, because of its unique pharmacological property, its high content in luffa cell cultures, the readiness of extrac-

tion and purification, and the easy chemical conversion to potent derivatives, such as **4**. Further studies on the pharmacological action mechanisms of **4**, as well as a large-scale production of **1** by high-producing luffa cell lines, are in progress. The present work has shown that plant cell cultures could be useful not only for the elucidation of secondary metabolism but also as a potential source of novel pharmacologically active compounds.

ACKNOWLEDGMENTS

The present work was supported by a grant-in-aid (No. 01470138) from Ministry of Education, Science and Culture, Japan. We are grateful to Prof. H. Matsunaga and Dr. R. Tanaka (Osaka University of Pharmaceutical Sciences) for a gift of isomultiflorenol.

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