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Phytotoxicity of Alantolactone*

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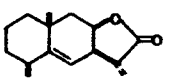
Alantolactone is a member of the eudesmane class of sesquiterpenes and its occurrence in several species of Compositae has been reported.[1] Its structure was elucidated as I shown below by Marshall and Cohen[2] in 1964. It contains, as one of structural features, the α-methylene-γ-butyrolactone moiety which has been known to exhibit a broad range of physiological properties including carcinogenic[3] antitumor[4], antibacterial[5], allergenic[6], and growth promoting[7] and inhibitory[8] activities. As a matter of fact, alantolactone shows bactericidal activity to several species of bacteria[1c] (but its inhibitory effect on the growth of *E. coli* and *St. aureus* is hardly shown[8]), and inhibits the growth of several pathogenic fungi[8b] and some species of yeasts.[9] On the proliferations of HeLa S3, it shows sever inhibition of the cell growth, however, it seems to be no inhibitory effect on the permeability of cell membrane.[9]

Anti-helminthic actions to *Ascaris lumbricoides*[10] and *Fasciola hepatica*[11] were also reported. It has practically been used as a vermifuge and is still available commercially as helenin which consists of alantolactone and isoalantolactone[12](II) in approximately a 2 : 3 ratio. The allergenicity for sensitized individuals has also been known.[6] In our institute, biological activity of alantolactone has been investigated for several years. We will review the effect of alantolactone on the growth and the respiration of plants in this paper.

Effect on the Growth of *Phaseolus vulgaris*[13]—On the application of alantolactone to



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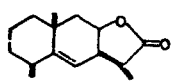

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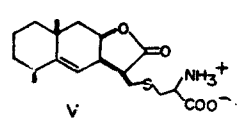

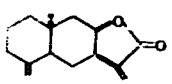

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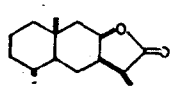

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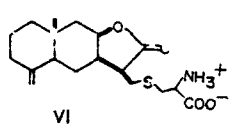

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P. vulgaris inhibitory symptoms are observed but restricted on the applied zone. It inhibits the elongation of cells in immature shoot and the growth of young leaves. In mature stems, it inhibits the meristematic growth and the differentiations of cambium to the vessels and phloem tissues. It also inhibits the formation of root system in water culture, and the formation of adventitous roots in rooting test of seedlings. The inclusion of leaves is completely disappeared after 3 days of the folia application of alantolactone in mature leaves. In culture media, it inhibits the growth of whole plant, but gibberellin can restore the growth of stems with a certain extent (Table I).

Table I—Effects of alantolactone and gibberellin on the growth of *P. vulgaris*.

Addition	Conc. M.	Height of internodes, mm			Growth of leaf
		1st	2nd	3rd	
None	—	52	14	0.5	Normal
Alantolactone	2×10^{-4}	18	0.3	—	Repressed
Gibberellin	1.2×10^{-6}	80	36	14	Normal
Alantolactone and Gibberellin	2×10^{-4} & 1.2×10^{-6}	41	11	0.3	Repressed

The seedlings germinated in petri dishes in the dark room were transferred to the 500 ml beaker containing Hoagland medium[14] and cultured in the light for 15hr and in the dark for 9 hr alternatively until second internodes grow to 1.5~2.0 cm long. Then each plant was transferred to 1000 ml beaker containing the medium with or without lactone and/or gibberellin. All values are means of 10 determinations after 5 day treatment.

Effect on the Germination of *Phaseolus mungo* Seed[15]—Alantolactone is not only able to inhibit the germination of mung seeds but also causes a significant reduction in the growth

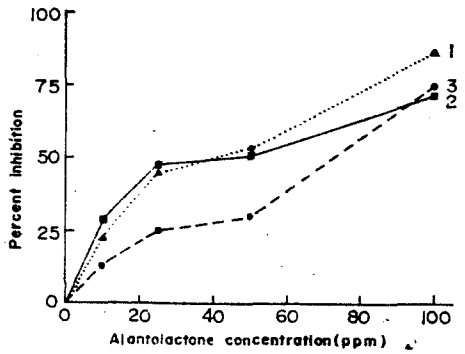

Fig. 1—Effect of alantolactone on shoot length (1), root length (2), and germination (3) of mung seeds. 100 seeds were germinated in each of the petri plates, lined with 2 sheets of filter paper and containing an aqueous solution of alantolactone and 0.01% Trition B-1956 as a surfactant for 120 hr in the dark at 30°(Dalvi, *et al.*)[15]

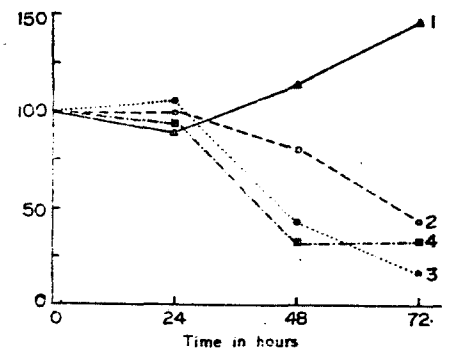

Fig. 2—Effect of alantolactone on the contents of starch (1), amino acids (2), reducing sugars (3) and the rate of respiration (4) of germinating mung beans. Incubation conditions were same as those described in Fig. 1. Concentration of the lactone was 100 ppm. (Dalvi, *et al.*)[15]

of root and shoot in the germinated seeds (Fig. 1). Partial reversal of inhibition of germination by alantolactone is also achieved when gibberellin is added to the medium(Table II).

An analysis of seed treated with alantolactone indicates that this treatment significantly diminishes the contents of reducing sugars and amino acids. While starch in the control seeds decreases with time, in the treated seeds it remains unchanged. The diminshed reducing sugars and unchanged starch contents of the treated seeds are likely to be associated with the inhibition of rate of respiration (Fig. 2).

Table II—Effect of gibberellin on alantolactone-induced inhibition of mung bean germination.

Addition	Germination %	Root length cm	Shoot length cm
Control	91.66	1.80	3.80
Alantolactone	19.15	0.34	0.80
Alantolactone+10^{-5}g GA_3	34.16	0.40	0.83
Alantolactone+10^{-6}g GA_3	40.00	0.39	0.83
Alantolactone+10^{-7}g GA_3	40.83	0.41	0.97
Alantolactone+10^{-8}g GA_3	36.66	0.40	0.84

Incubation conditions were same as those described in Fig. 1. Concentration of the lactone was 100 ppm (Dalvi, *et al.*)[16]

The reduction in reducing sugars, with a concomitant reduction in amino acids in germinating seeds, suggests that alantolactone inhibits the degradation of starch and protein, which is essential for the supply of energy and for building up new proteins for germination and growth.

Table III—Effect of alantolactone on the respiration of potato slices.

Slice	Alantolactone μg/ml	Oxygen consumption, μl/10mg dry wt. 1	2	3hr
Fresh slice	None	1.4	4.0	7.0
	10	1.2	3.8	6.6
	25	1.6	4.2	7.1
	50	2.1	4.6	6.7
	100	2.1	3.4	6.2
Aged slice	None	9.4	19.0	29.5
	10	8.2	19.0	31.4
	25	5.2	14.7	34.1
	50	3.7	13.4	36.0
	100	5.7	19.8	40.1

Five pieces of slices (6mm in a diameter) were incubated in each vessels in 0.2N phosphate buffer, pH 7.0 containing various amounts of the lactone, at 25°. Aged-slices were prepared by the aeration of fresh slices in 10^{-4}M $CaSO_4$ solution for 15 hr.

Effect on the Respiration of Potato Slices[16]—In potato tuber slices, alantolactone shows marked increase of oxygen consumption in aged slices, but not in the fresh ones(Table III). It is well known that the respiratory system especially electron transport system of aged slices is quite different from the fresh.[17] Thus, it is obvious that the stimulation of respiratory activity by the lactone seems not to be a general but a specific phenomenon depending upon the kinds of respiratory system which cells possess. The activation of respiratory activity in the aging processes of slices is severely inhibited by long treatment with the lactone (Table IV). This fact also indicates that alantolactone inhibits some metabolic processes essential for aging.

Table VI—Effect of alantolactone on the activation of respiratory activity during the aging process in potato tuber slices.

Alantolactone μg/ml	Oxygen consumption, μl/10 mg dry wt.		
	1	2	3 hr
None	6.6	14.5	21.5
10	1.6	5.3	12.3
25	1.0	4.4	9.8
50	1.4	2.9	5.8
100	0.6	1.0	2.2

Fresh slices were aerated in the presence of various amounts of the lactone for 15 hr. Oxygen consumption of the slices was measured without addition of the lactones into the mixture. Other conditions were the same as those described in Table III.

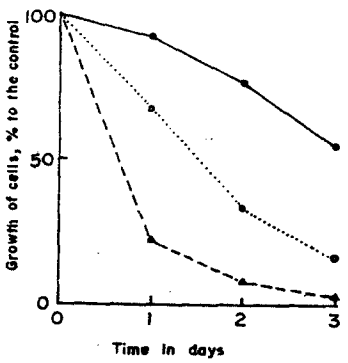

Fig. 3.—Cells were cultured in 100 ml flask containing 30 ml of inorganic medium (Devlin and Galloway[19]) in the light at 25°. The growth rate of the cells was calculated by the cell count hemacytometer. Concentration of the lactone:
▲—▲, 25×10^{-5}M
○—○, 2.5×10^{-5}M
●—●, 5×10^{-6}M

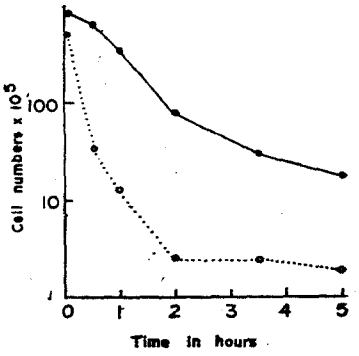

Fig. 4—Effect of alantolactone on the viability of *Chlorella* and reversal by cysteine. Cell suspension (1.4×10^{-6} cells/ml) in phosphate buffer (pH 7.2) containing the lactone (2.5×10^{-4}M) was incubated at 25°. At the time indicated cells were collected and cultured in the absence of the lactone for 3 days as described in Fig. 3. ●—●, pretreatment of cysteine (7.5×10^{-4}M) for 1 hr; ○—○, no pretreatment.

Effect on the Growth of *Chlorella pyrenoidosa*[16,18]—Alantolactone has also an inhibitory action on the growth of *C. pyrenoidosa* (Fig. 3, Table V). The viability of the cells are greatly reduced by 2 hr treatment with the lactone (Fig. 4) and the inhibitory action is not reversible.

It inhibits the conversion of ^{14}C-glucose to alcohol insoluble fraction, but the production of $^{14}CO_2$ from labelled glucose, the consumption of oxygen, and the incorporation of ^{14}C-glucose into alcohol soluble fractions are enhanced (Table VI). Thus it is believed that alantolactone can inhibit some biosynthetic processes in *Chlorella*.

Table V—Effect of alantolactone on the growth of *Chlorella*.

Alantolactone μg/ml	Cell number, x10^6 cells/ml		
	I	II	III
none	85.25	13.80	67.01
1	38.25	3.20	43.10
5	8.43	2.05	19.28
10	1.62	1.15	7.35
100	1.62	1.03	1.90

Initial cell numbers were 2.37×10^6 cells/ml for I and II, and 5.02×10^6 cells/ml for III. Culture conditions were: proteose pepton plus glucose medium for I and II; basal medium for III; I and II were cultivated in the light at 25°, II was in the dark at 30° for 3 days.

Table VI—Effect of alantolactone on the incorporation of glucose into CO_2 and cell materials in *Chlorella*.

Alantolactone x 10^{-4}M	Fractions, cpm/mg protein			Oxygen consumption, O_2 μl/mg cell protein
	CO_2	EtOH sol.	EtOH insol.	
None	10344	517	12596	40.6
2.5	13966	529	10316	42.2
5.0	15503	537	10356	46.8

Two hr after incubation of cell suspension (12.3 mg as protein) in 2.2ml of phosphate buffer solution(pH 7.2) of glucose-U-^{14}C(4 μCi/20 μmoles/ml), at 25°, reaction mixture was centrifuged at 1000 x g and washed twice with medium containing nonlabelled substrate. Cells were then extracted twice with hot EtOH.

It is noted that oxygen consumption of cells is not only increased by alantolactone, but the production of carbon dioxide is also promoted when acetate is used as a substrate (Table VII).

And the generation of $^{14}CO_2$ is increased to the same extent by alantolactone when either glucose-1-^{14}C or glucose-6-^{14}C was supplied as a substrate (Table VIII).

It is therefore postulated that TCA cycle in *Chlorella* is activated by alantolactone, but neither EMP nor HMP is affected. The inhibitory action of alantolactone may be compared with that of uncouplers, such as 2, 4-dinitrophenol and 2, 4-dichlorophenoxyacetic acid, which have been reported to inhibit plant growth and to stimulate tissue respiration (Table IX).

Table VII—Effect of cysteine on the stimulatory action of alantolctone in the respiration of *Chlorella*.

Addition	Glucose-U-^{14}C		Acetate-U-^{14}C	
	O_2 μl/mg protein	cpm/mg protein	O_2 μl/mg protein	cpm/mg protein
None	34.5	3127	41.6	9428
Alantolactone	39.2	4263	48.6	12665
Cysteine	33.6	3008	42.0	9807
Cysteine+lactone	36.3	3567	44.1	11078

Incubation conditions were the same as those described in Table VI except that glucose (0.5 μCi/20 μmoles) or acetate (0.3 μCi/50 μmoles) was used. Cysteine (1.5×10^{-4}M) was add 1hr before the addition of the lactone (5×10^{-5}M).

Table VIII—Utilization of glucose in the respiration of *Chlorella* in the presence of alantolactone.

Substrate	Alantolactone x10^{-6}M	Oxygen consumption O_2 μl/mg cell protein	Radioactivity in CO_2 cpm/mg cell protein
Glucose-1-^{14}C	None	14.2	3484
	0.5	14.1	3628
	5.0	16.4	3760
	50.0	17.6	4682
Glucose-6-^{14}C	None		3172
	0.5		3486
	5.0		4214
	50.0		4336

Incubation conditions were the same as those described in Table VI except that G-1-^{14}C or G-6-^{14}C (0.5 μCi/20 μmoles) was used and incubation time was 1hr.

Table IX—Effects of several uncouplers on the respiration of various plants.

Chemical	Concentration	Material	Accerelation of respiration	Reference
DNP	5×10^{-5}M	Potato, aged	74%	(20)
DNP	5×10^{-5}M	Potato, fresh	114%	(20)
DNP	1.5×10^{-4}M	*Chlorella*	400%	(21)
DNP	1.0×10^{-4}M	Pea, internode	30%	(22)
2, 4-D	2 μg/ml	Bean, hypocotyl	20%	(23)
2, 4-D	0.1μg/ml	Corn, seedling	24%	(24)
2, 4-D	250~2000μg/ml	Carrot, slices	positive	(25)
Alantolactone	10~100μg/ml	Potato, aged	5~25%	(16)
Alantolactone	10~100μg/ml	*Chlorella*	5~30%	(16)

Structure Requirement for Activity—Alantolactone and isoalantolactone reduce the growth of *Chlorella*, while di-, and tetrahydroalantolactones(**III** and **IV**) show no effect on the

growth, and it is observed that amino acids having no SH group do not show any effect, while cysteine reduces the effect of alantolactone and isoalantolactone (Table X)[18].

Table X—Effect of alantolactone and its derivatives on the growth of *Chlorella* and effect of several amino acids on lactone-induced inhibition.

Addition	Cell number, $\times 10^7$ cells/ml			
	Conc. of lactones($\times 10^{-6}$M)			
	0.5	5.0	25.0	50.0
Alantolactone	74.02	35.07	13.11	0.96
Isolantolactone	70.54	33.25	12.42	1.43
Dihydroalantolactone	—	72.16	—	73.53
Tetrahydroalantolactone	—	74.06	—	72.86
Alantolactone+cysteine	72.51	49.87	38.36	9.44
Isoalantolactone+cysteine	71.78	51.26	36.23	10.47
Alantolactone+tryptophan	—	30.97	—	0.85
Alantolactone+histidine	—	37.45	—	1.08
Cysteine	74.28	71.88	75.22	75.80
Tryptophan	—	75.40	—	74.28

The growth conditions were the same as those described in Fig. 3. Initial number of cells in the cultures was 2.46×10^6 cells/ml. The concentration of amino acids was maintained 3 times higher than that of the lactone. Cell number in control tube was 73.42×10^7 cells/ml.

Table XI shows that alantolactone and isoalantolactone stimulate the respiration of *Chlorella*, while di-, and tetrahydroalantolactones exhibit no effect, and cysteine acts as an antagonist on the respiratory stimulation of alantolactone when glucose or acetate is used as a substrate (see Table VII)[18].

Table XI—The respiration of *Chlorella* in the presence of alantolactone, its derivatives and cysteine.

Addition	Oxygen consumption, O_2 μl/mg protein		
	1	2	3 hr
none	15.3	38.7	60.2
Alantolactone	16.0	41.5	73.4
Isoalantolactone	15.2	43.6	71.3
Dihydroalantolactone	15.8	37.2	58.3
Tetrahydroalantolactone	15.0	38.1	61.6
Alantolactone+cysteine	15.2	40.3	64.6
Isolantolactone+cysteine	14.5	38.2	63.4
Cysteine	16.0	37.4	58.9

Incutation conditions were the same as those described in Table VI except that unlabelled glucose was used as a substrate. The concentrations of the lactone and cysteine were 5×10^{-5} M and 1.5×10^{-4} M, respectively.

Moreover, di- and tetrahydroalantolactones show no effect on the elongation of *Avena* coleoptiles (Fig. 5) and on the formation of adventitious roots of *P. vulgaris* (Fig. 6)[9]. These results indicate that structure requirement for biological activity of alantolactone is the presence of a conjugated lactone group, and support the view that the reactions of α-methylene-γ-lactones with sulphydryl groups may play a significant role in the mechanism of its phytotoxicity.

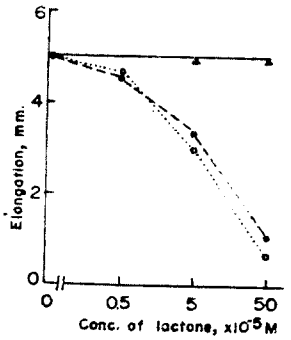

Fig. 5—Effect of alantolactone and its derivatives on the elongation of *Avena* coleoptiles. The seeds of *Avena sativa* were germinated in the dark, then coleoptiles over 4~5 cm long were collected, and removed the 4 mm part of apex and then 6 mm coleoptiles sections were floated on the 10 ml of H_2O containing 1 mg of IAA and the lactones. After incubation of the sections for 18 hrs exactly, the longitudinal growth was measured. ●—●, alantolactone; ○—○, isolantolactone; ▲—▲, di- or tetrahydroalantolactones.

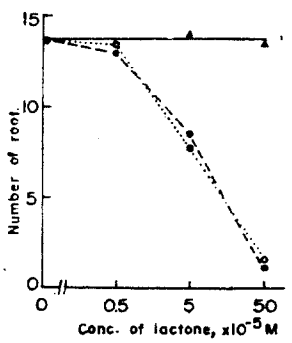

Fig. 6—Effect of alantolactone and its derivatives on the formation of advantitious roots of *Phaseolus*. When the first leaves were almost fully spread, distal sections of root of *Phaseolus* seedlings was, together with endosperm, removed at the point of 2 cm from the basal part. The seedling was treated with the lactones for 24hr and transferred into water. Six days after cultivation, numbers of adventitous root were counted. ●—●, alantolactone; ○—○, isoalantolactone; ▲—▲, di- or tetrahydroalantolactones.

Reaction of Alantolactone with Thiols *in vitro*—It is well known that α-unsaturated-γ-lactones react with nucleophiles, such as thiols[26] and amines[27]. Alantolactone and isoalantolactone react with cysteine rapidly at physiological pH (Fig. 7), to give adducts, **V**, mp 200~202°, and **VI**, mp 208~209°, while di-, and tetrahydroalantolactones are proved to have no reactivity. Reduced glutathione also reacts with alantolactone, as cysteine does, but other amino acids, cystine, histidine, tryptophan, or glycine are inactive under the same conditions[18]. It has been known that alantolactone can also undergo the addition with the imidazole group of histidine, the ε-amino group of lysine, and α-amino groups of tryptophan, methionine, and serine[29]. However, at the physiological hydrogen ion concentration, the reactivity of thiol is very higher than those of other groups[30].

The biological activity of alantolactone is greatly reduced under the presence of cysteine out of amino acids tested. It indicates that alantolactone might react with thiols in the cells. It is demonstrated that thiols in the homogenates of *Chlorella* and rat liver react with alantolactone(Fig. 8)[18]. However, only a small fraction of thiol groups in cell homogenates reacts with it. It might be explained by the assumption that the reactivities of SH group

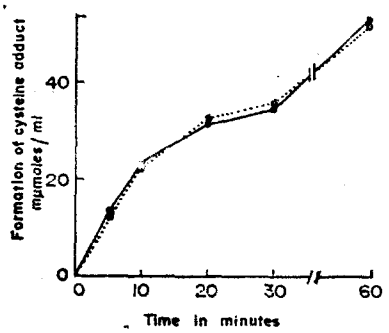

Fig. 7—Formation of cysteine adduct with alantolactone and isoalantolactone. One tenth ml of alantolactone(10^{-2}M) in tetrahydrofuran was mixed with 4.9 ml of cysteine (10^{-4}M) in phosphate buffer(pH 7.2) and incubated at 30°. After an appropriate reaction time, SH content was measured by the method of Grassetti and Murray.[28] ●—●, alantolactone, ○—○, isoalantolactone.

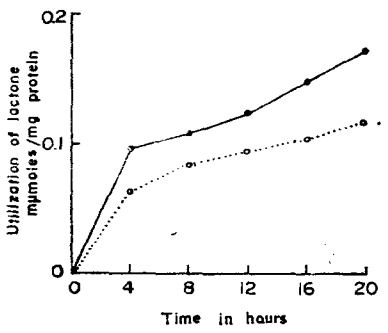

Fig. 8—Reactivity of alantolactone with thiols in the cell free homogenates. *Chlorella* cells harvested from the culture and tissue from rat liver were homogenized in cold phosphate buffer (pH 7.2) and heated in water bath at 90° for 5min, then cooled rapidly. Homogenates containing lactone(2.5×10^{-4}M) were incubated at 40°. ●—●, *Chlorella*; ○—○ Rat liver.

in free cysteine solution and those in a protein solution (in protoplasm) could be different. It is obvious that some of thiols in protein can be protected by certain molecules or ions, or tertiary structure of the proteins. However, when once one of thiols in protein reacts with the lactone, the activity of the protein might be profoundly changed.

Several cytotoxic and antitumor sesquiterpene lactones, such as elephantopin, euparotin acetate, and vernolepin, which posses α-methylene-γ-lactone moiety, have been demonstrated and ascribed to a rapid Michael type addition reaction of the biological nucleophiles, such as cysteine or sulfhydryl-bearing enzymes, *e.g.*, phosphofructokinase[31] and glycogen synthetase[32]. It is, therefore, suggested that alantolactone and isoalantolactone may act *via* alkylation of biologically important sulfhydryl groups.

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