Sesquiterpenoid Phytoalexins From Fruits of Eggplants

E. W. B. Ward, C. H. Unwin, J. Hill, and A. Stoessl

Research Institute, Agriculture Canada, University Sub Post Office, London, Ontario N6A 5B7. Part 16 in a series titled "Postinfectional inhibitors from plants".

The valuable technical assistance of E. Zmeko and G. Rock is gratefully acknowledged.

We thank R. S. Burden and R. F. Curtis for informing us of their results prior to publication.

Accepted for publication 10 March 1975.

ABSTRACT

The production of phytoalexins in eggplants (*Solanum melongena*) was investigated using *Monilinia fructicola* routinely as the inducing fungus, but also *Penicillium frequentans*, *Aspergillus fumigatus*, *Botrytis cinerea*, and *Fusarium oxysporum* f. *vasinfectum* for comparison. Five sesquiterpenes in varying proportions and with varying degrees of antifungal activity were induced by each of the fungi. These included lubimin, formerly described from potatoes, and a biogenetically related bicyclic enone. Two additional sesquiterpenes, probably artifacts, were also isolated. The results indicate that the major diffusable ether-soluble stress compounds in eggplant are sesquiterpenes as in other Solanaceae, and that while there are biogenetic relationships both between the compounds demonstrated in eggplants, and to phytoalexins produced in other species, phytoalexin production itself appears to be nonspecific.

Phytopathology 65:859-863

The production of the sesquiterpenoid phytoalexin, capsidiol, by peppers in response to inoculation with a variety of fungi has been described in detail previously (3, 14). Rishitin (16), phytuberin (17), and lubimin (8) are all sesquiterpenoid phytoalexins from potatoes, and rishitin is also produced in tomatoes (10). Recently, two additional sesquiterpenes have been described from potato tubers inoculated with *Phytophthora infestans* or *Erwinia carotovora* (5), capsidiol has now been described from tobacco following infection with tobacco necrosis virus (2), and a new sesquiterpene, glutinosone, has been described from *Nicotiana glutinosa*, after similar treatment (4). Whether these also are instances of phytoalexin production has yet to be established, but it seems probable. A preliminary report of the extension of our studies of phytoalexin production in the Solanaceae to eggplant (*Solanum melongena*) and *Datura stramonium* has indicated that in these two species the phytoalexins also are sesquiterpenes (13). These results have served to satisfy one aim of these investigations, which was to provide additional information on the extent to which sesquiterpenes, almost certainly related biogenetically, are characteristically the phytoalexins of the Solanaceae, just as the pterocarpans are typical of the Leguminoseae.

This paper describes the induction and isolation of phytoalexins in fruits of eggplant. Details, additional to those already published (13) of the chemical and physical characterization and structure elucidation will be published elsewhere.

MATERIALS AND METHODS.—Eggplants (*Solanum melongena* 'Black Beauty') were grown locally in field plots; the fruits were harvested in late August or September, prior to ripening, when the flesh was still firm. They were immediately stored in the dark at 10 C and used as rapidly as possible after harvest, usually within 24 hours.

Phytoalexins were induced routinely with spore suspensions of *Monilinia fructicola* (Wint.) Honey, but in certain experiments the following fungi were also used: *Aspergillus fumigatus* Fres., *Botrytis cinerea* Pers., *Fusarium oxysporum* f. *vasinfectum* (Atkinson) Synder and Hansen, and *Pencillium frequentans* Westling. *Monilinia fructicola* and *Phytophthora infestans* (Mont) deBary were used to assay fungitoxicity. All fungi except *P. infestans* were maintained routinely at 15 C on slants of potato-dextrose agar (PDA). *P. infestans* was maintained at 18 C on plugs of autoclaved potato tuber tissue. Sterile distilled water suspensions of conidia of these fungi were obtained by washing the surface of sporulating colonies

TABLE 1. Percentage inhibition of spore germination of *Monilinia fructicola* by diffusates and diffusate extracts, from fruits of eggplant inoculated with *M. fructicola*

Exp.	Control	Inoculated 0[b]	1:1	1:3	1:7
Exp. 1[a]					
Diffusate	0	95	56	27	0
Exp. 2					
Diffusate	0	79	31	9	1
Ether Extract	0	69	24	11	0
Extracted Diffusate	0	0	0	0	0

[a]Experiments 1 and 2 refer to two preliminary experiments. In experiment 2 the diffusates were partitioned with ether. In experiment 1, four fruits were incubated 48 hours with sterile distilled water for controls (354 ml diffusate collected), seven fruits were incubated with a spore suspension of *M. fructicola* (5×10^5 spores per ml, 889 ml diffusate collected). In experiment 2, six fruits were used for controls (280 ml of diffusate collected), 38 fruits were incubated with spore suspension (1,450 ml of diffusate collected).

[b]Diffusates and fractions from *M. fructicola* inoculated fruit were tested either undiluted (0) or after dilution as indicated, for antifungal activity in standard slide spore-germination assays against *M. fructicola*. Control diffusates and fractions were tested undiluted.

grown on PDA- or V8-juice agar-plates at 25 C. For suspensions of *P. infestans* zoospores, cultures on agar plates (V8 juice) were incubated 12-14 days at 18 C in the dark, sporangia were removed under aseptic conditions by washing with sterile distilled water chilled to 12 C, and separated from mycelial fragments by filtration through sterile gauze. The sporangia were then incubated in the dark, in a shallow layer of sterile distilled water at 12 C for 1-3 hours to allow release of zoospores. The zoospore suspension was filtered through sterile filter paper (Whatman No. 54) to remove empty and nongerminated sporangia. Spore suspensions were adjusted to a standard concentration, usually 5×10^5/ml using a haemocytometer.

Fruits were scrubbed under running tap water and then dipped in 95% ethanol and the excess drained off. They were cut into halves longitudinally and the pithy tissue hollowed out from the cut surface down to the seed-bearing layer. Spore suspensions (or sterile distilled water in controls) were run into the cavities to cover as much of the exposed surface as possible (10-50 ml depending on fruit size). The fruits were then incubated 48 hours at room temperature in trays covered with aluminum foil to reduce evaporation. The diffusates were filtered through a double layer of gauze to remove debris and immature

TABLE 2. Silica gel column fractionation of ether extract from diffusate from eggplant fruits inoculated with *Monilinia fructicola*[a], and distribution of antifungal activity

Fraction	Eluant[b] (PE:E)	Residue[c] (mg)	Components[d]	Activity[e] (percent inhibition at μg/ml) 400	200	100	50
1	100:0	31	several un.	not tested			
2	95:5	21	several un.	0	0	0	0
3	90:10						
4	85:15	41	II; I; four un.	71	36	10	0
5	85:15						
6	85:15						
7	85:15	56	III; I; II; three un.	88	56	18	13
8	85:15						
9	80:20						
10	80:20	12	II; I; III; three un.	92	42	17	8
11	80:20						
12	80:20	12	II; V; IV; VII three un.	100	37	7	0
13	80:20						
14	70:30	151	VII; V; IV; VI; II (trace); several un.	100	52	10	2
15	70:30						
16	70:30						
17	70:30	94[f]	VI; VII (trace); several un.	20	0	0	0
18	70:30						
19	50:50						
20	50:50	39[f]	VI; several un.	10	0	0	0
Total residue (mg)		457					
Crude			Crude extract	100	61	0	0

[a]Residue (427 mg) from ether extract of diffusate from fruits challenged with spore suspensions of *M. fructicola* (5×10^5 spores/ml) applied to top of column of silica gel (400 g, 60-120-mesh, column internal diameter 4.75 cm), eluted with increasing concentrations of ether in petroleum ether.

[b]Proportions of petroleum ether (PE): ether (E) in fractions each of 5 liter volume.

[c]Residues of fractions of similar composition, as judged by TLC, are combined.

[d]Components of individual or combined fractions listed in order of decreasing spot intensity on TLC irrigated with methanol:chloroform (5:95, v/v) and developed with phosphomolybdic acid reagent. The Roman numerals refer to the identified compounds illustrated in Fig. 1. un. = unidentified, the number of these are minima presumably more compounds would be detected if higher concentrations were used.

[e]Antifungal activity of individual or combined fractions determined in standard slide spore-germination assays with *M. fructicola*.

[f]This material also contained solvent-derived impurities.

seeds, and centrifuged at 12,000 *g* for 5 minutes to remove spores. An aliquot was taken for measurement of fungitoxicity. The remainder, together with a water rinse of each fruit was extracted three times with half-volumes of ether. The extracts were combined, dried with anhydrous Na_2SO_4, filtered, evaporated in vacuo below 40 C, re-evaporated from ethanol to remove traces of water, and stored at −10 C until assayed or fractionated.

Fungitoxicity of diffusates, extracts, fractions, and purified compounds was determined by inhibition of spore germination, using the standard slide spore-germination assay (1).

Fractionation of extracts was done by column- and thin-layer chromatography (TLC). Details of column chromatography are given below. For TLC, silica gel (Camag DF5) was routinely employed with three solvent systems: (A) ether:petroleum ether (1:1, v/v); (B) methanol:chloroform (5:95, v/v); and (C) *sec*-butanol:ethyl acetate (5:95, v/v). Plates were developed by spraying with a 5% solution of phosphomolybdic acid in ethanol or 0.5% vanillin in 80% sulfuric acid in ethanol, followed by heating for a few minutes at 110 C. Elution after TLC was with methanol:methylene-chloride (1:9, v/v). Petroleum ether was the fraction boiling at 37-60 C; all solvents were of reagent grade and were redistilled before use.

RESULTS.—In several preliminary experiments diffusates from fruits inoculated with spore suspensions of *M. fructicola* were strongly inhibitory to spore germination of this fungus, while water controls were inactive (Table 1). Almost all of the activity (about 90%) was recovered in ether extracts of the diffusate, and no activity was detected in the diffusate after extraction.

Fractionation of ether extract of diffusates by column chromatography.—In a typical run, the major aliquot (427 mg) of an ether extract (437 mg), obtained from the diffusate (6,067 ml) from about 100 fruits, was applied to the top of a column of silica gel (British Drug Houses 400 g, 60- to 120-mesh, column internal diameter 4.75 cm) packed in petroleum ether. The column was eluted with increasing concentrations of ether in petroleum ether as specified in Table 2. Fractions of 5 liters volume each, were collected and monitored by TLC. Fractions, recombined as justified by similar composition, were evaporated under reduced pressure, the residues weighed and small aliquots tested for antifungal activity. Solutions for this purpose were prepared by injecting solutions of fractions 3 through 20 in ethanol into water to a concentration of 5% ethanol. Because of solubility problems acetone was used similarly for fraction 2, and fraction 1 was not tested. Ethanol and acetone (5%) were included in controls. Table 2 records the activity of the fractions together with the activity of the extract prior to fractionation. The fractions were complex. Apart from the first two, all contained one or more major components together with several minor ones. No attempt was made to identify the latter. Antifungal activity was widely distributed throughout the fractions, indicating that it resided in more than one compound.

Isolation and antifungal activity of pure compounds.—Individual components (Fig. 1) were isolated from fractions 3 to 15 by preparative TLC. Only major bands, located by ultraviolet (UV) light or by spraying a small portion of the chromatogram with

TABLE 3. Isolation by preparative thin-layer chromatography of purified compounds, after fractionation by column chromatography of the ether extract of the diffusate from eggplant fruits inoculated with *Monilinia fructicola*

	Amount isolated[b]		R_f value[c]		
Compound[a]	(mg)	percent of extract	A	B	C
I	8	1.9	.49	.60	.65
II	12	2.8	.44	.53	.65
III	17	4.0	.26	.52	.65
IV	9	2.1		.35	.57
V	32	7.5		.32	.48
VI	22	5.2		.35	.42
VII	33	7.7		.25	.56
Total amount	133	31.2			

[a]Roman numerals refer to the identified compounds illustrated in Fig. 1.

[b]Weight of essentially pure compound obtained in the particular run described.

[c]TLC on silica, Camag DF5, 300 μ thick in glass tanks lined with filter paper and humidified with solvent. Solvent systems were: (A) ether: petroleum ether (1:1, v/v); (B) methanol:chloroform (5:95, v/v); (C) *sec*-butanol:ethyl acetate (5:95, v/v).

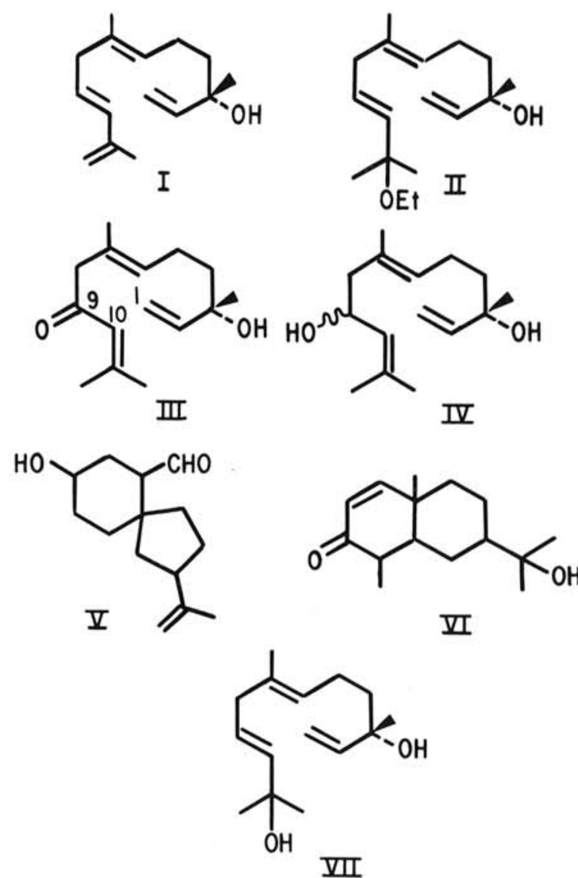

Fig. 1. Structural formulae of compounds from eggplant diffusates, induced by *Monilinia fructicola*. (In structures II and VII stereochemistries at C-9 are not implied).

TABLE 4. Inhibition of spore germination of *Monilinia fructicola* and *Phytophthora infestans* by eggplant phytoalexins

Compound[a]	$ED_{50}(M \times 10^3)$[b]	
	Monilinia fructicola	*Phytophthora infestans*
I	0.76	0.09
II	1.69	0.34
III	1.10	0.59
IV	>1.70	0.89
V	0.49	0.36
VI	>1.70	>1.70
VII	>1.70	1.14

[a]Roman numerals refer to identified compounds illustrated in Fig. 1.

[b]Determined in standard slide spore-germination assays from dose-inhibition curves.

phosphomolybdic acid, were eluted by thorough extraction of the adsorbent with methanol in methylene chloride (1:9, v/v) at room temperature. Adequate purification of the products usually required repetition of the TLC, sometimes with change of solvent system. Table 3 lists weights and R_f values for the purified compounds obtained. Fractions 16 to 19 and 20 were found by ^1H-nuclear magnetic resonance spectroscopy to contain a large excess of an impurity derived from the large volumes of solvent used. The recombined fractions 16 to 19 were readily purified by further chromatography on a second column of silica (Camag DF5, 100 g) in solvent system B. Fractions 11 to 13 from this column (fraction volumes 10 ml) furnished almost pure compound VI (22 mg). A final purification was effected by preparative TLC in pure ether.

Inhibition of spore germination of both *M. fructicola* and *P. infestans* was determined for each of the seven compounds, and data for active compounds are presented as ED_{50} values (Table 4). The two most active compounds were I and lubimin (V) although with the exception of VI, all compounds had appreciable activity against *P. infestans*.

Induction of compounds by additional fungi.—A comparison was made by TLC of the compounds in ether extracts of diffusates induced by several different fungi (Table 5). Although compounds III - VII were induced by each of the fungi, the relative proportions varied, and some compounds were detected only in trace amounts. As discussed below, compounds I and II are almost certainly artifacts. Traces of compounds III, IV, and VII were also detected in extracts of water controls.

DISCUSSION.—It is clear that phytoalexins, produced by fruits of eggplants are sesquiterpenoid in nature, thus conforming to the pattern demonstrated for other members of the Solanaceae (2, 4, 5, 7, 8, 10, 13, 14, 16, 17). The isolation of lubimin indicates that eggplants share a common biosynthetic pathway with potatoes, and also with *Datura stramonium* (13). In fact, lubimin can be linked in a common biogenetic scheme with rishitin from potatoes and tomatoes, capsidiol from peppers and tobacco, α-keto-cyperone from tobacco (9), and compound VI from eggplants (13). Phytuberin, a revised structure for which is soon to be published (R. F. Curtis, *private communication*), also fits into this scheme, as do the two recently reported vetispiranes from potatoes (5), and glutinosone from tobacco (4).

The experiments described here, using large volumes of spore suspensions and diffusates, were designed to provide sufficient material to study the broad spectrum of response, rather than to attempt to precisely duplicate a disease reaction under natural conditions. The result was that instead of demonstrating a single major compound that would be regarded as the phytoalexin, a complex mixture was obtained, several components of which have antifungal activity. The ether extract of the diffusate was in fact very complex. Fractionation required different solvent systems. The need for repeated chromatography is evident from the overlapping R_f values of the pure compounds (Table 3, 5). Nevertheless, seven compounds were isolated which accounted together for about 30% of the extract. In addition to lubimin, for which the revised structure we reported recently (13) is illustrated (Fig. 1, V), these consisted of 9-oxonerolidol reported by Hiroi and Takaoka (6) from camphor leaf oil (III), its 9-hydroxyanalogue (IV) and allyl isomer (VII) and the bicyclic compound (VI). Compounds I and II are almost certainly artifacts since they were not detected in fresh crude extracts. Compound I would easily be formed by dehydration of compound IV during fractionation. Similarly compound II could be formed from compound IV by allylic substitution of ethanol. It is also possible that compound VII could be formed from compound IV by a similar allylic rearrangement (addition of −OH to C-11, elimination from C-9). In addition to these identified compounds, the extract contained also additional components as indicated in part by Table 2. Thus there was a large number of unidentified compounds present only in very small amounts, there were two highly lipophilic fractions [fractions 1 and 2, (Table 2)] and presumably there were additional amounts of the identified compounds which were unavoidably lost

TABLE 5. Relative amounts of five sesquiterpene phytoalexins in ether extracts of diffusates from eggplant inoculated with five different fungi as indicated from TLC[a]

Compound	*Monilinia fructicola*	*Penicillium frequentans*	*Aspergillus fumigatus*	*Botrytis cinerea*	*Fusarium oxysporum*
III	+++[b]	+++	++	++	+
IV	++++	+++	++	+++	tr.
V	++++	++	+	+++	++++
VI	++++	+	tr.	++++	++++
VII	+++	+++	++	++++	++

[a]Combined results using two solvent systems: methanol:chloroform (5:95, v/v) and *sec*-butanol:ethyl acetate (5:95, v/v).

[b]Quantities estimated visually (tr = trace, increasing amounts + → ++++) after developing plates with 0.5% vanillin in 80% sulfuric acid in ethanol.

during the isolation procedure.

From reports originating from a number of sources, evidence is accumulating that potato tubers similarly produce several compounds in response to infection (5, 7, 8, 16, 17). It is unlikely, therefore, that phytoalexin production, at least in potato and eggplant, can be regarded as a discrete biochemical response to a unique stimulus provided by a challenging fungus. A similar nonspecific response involving a spectrum of compounds has also been described for legumes (11). Lack of specificity in phytoalexin induction has been discussed elsewhere (e.g., 11, 12), but is further emphasized here by probable differences in the biosynthetic origins of the compounds. Thus the nerolidol derivatives (III, IV, VII) on the one hand, and the eudesmane (VI) and the vetispirane, lubimin on the other, while ultimately derived from the same precursors, nevertheless are products lying on divergent biosynthetic routes (13).

The observation that all the fungi tested induced all of the compounds, but in differing amounts, also suggests that the process is nonspecific. While degradation cannot be ruled out as an explanation for lower concentrations of some of the compounds, as occurs with the related phytoalexin, capsidiol, in peppers (15, 18), it would be unreasonable to suggest that each fungus has discrete induction mechanisms for each of the compounds described and indeed for all the minor components not individually isolated. It seems more probable that each fungus causes a disturbance of cell metabolism which in itself will be likely to lead to an accumulation of compounds not found in healthy tissue. The spectrum of compounds appearing in the diffusate may be influenced by minor differences in the biochemical environment created by the fungi and presumably by differences in the rate at which the fungi interfere with host cell metabolism.

Finally it should be emphasized that while these investigations followed only compounds that were extracted by ether from water diffusates, other changes undoubtedly took place in the fruits. In a consideration of the specificity of the interaction between host and pathogen, these may be of equal importance, and should not be overlooked.

LITERATURE CITED

1. AMERICAN PHYTOPATHOLOGICAL SOCIETY, Committee on Standardization of Fungicidal Tests, 1947: Test tube dilution technique for use with slide germination method of evaluating protectant fungicides. Phytopathology 37:354-356.
2. BAILEY, J. A., R. S. BURDEN, and G. G. VINCENT. 1975. Capsidiol: an antifungal compound produced in Nicotiana tabacum and Nicotiana clevelandii following infection with tobacco necrosis virus. Phytochemistry 14: (In press).
3. BIRNBUM, G. I., A. STOESSL, S. H. GROVER, and J. B. STOTHERS. 1974. The complete structure of capsidiol. X-ray analysis and ^{13}C nuclear magnetic resonance of eremophilane derivatives having trans-vicinal methyl groups. Can. J. Chem. 52:993-1005.
4. BURDEN, R. S., J. A. BAILEY, and G. G. VINCENT. 1975. Glutinosone, a new antifungal sesquiterpene from Nicotiana glutinosa infected with tobacco mosaic virus. Phytochemistry 14:221-224.
5. COXON, D. T., K. R. PRICE, B. HOWARD, S. F. OSMAN, E. B. KALAN, and R. M. ZACHARIUS. 1974. Two new vetispirane derivatives: stress metabolites from potato (Solanum tuberosum) tubers. Tetrahedron Lett. 2921-2924.
6. HIROI, M., and D. TAKAOKA. 1974. Non-volatile sesquiterpenoids in the leaves of camphor tree (Cinnamomum camphora). Nippon Kagaku Kaishi 762-765.
7. KATSUI, N., A. MATSUNAJA, K. IMAIZUME, T. MASAMUNE, and K. TOMIYAMA. 1971. The structure and synthesis of rishitinol, a new sesquiterpene alcohol from diseased potato tubers. Tetrahedron Lett. 83-86.
8. METLITSKII, L. V., O. L. OZERETSKOVSKAYA, N. S. VUL'FSON, and L. I. CHALOVA. 1971. Chemical nature of lubimin a new potato phytoalexin. Doklady Akad. Nauk. SSSR 200:1470-1472.
9. ROBERTS, D. L. 1972. The structure of a new sesquiterpene isolated from tobacco. Phytochemistry 11:2077-2080.
10. SATO, N., K. TOMIYAMA, N. KATSUI, and T. MASAMUNE. 1968. Isolation or rishitin from tomato plants. Ann. Phytopathol. Soc. Jap. 34:344-345.
11. SMITH, I. M. 1971. The induction of antifungal inhibitors in pods of tropical legumes. Physiol. Plant Pathol. 1:85-94.
12. STOESSL, A. 1970. Antifungal compounds produced by higher plants. Rec. Adv. Phytochem. 3:143-180.
13. STOESSL, A., J. B. STOTHERS, and E. W. B. WARD. 1974. Lubimin: a phytoalexin of several Solanaceae. Structure revision and biogenetic relationships. J. Chem. Soc., Chem. Commun. 709-710.
14. STOESSL, A., C. H. UNWIN, and E. W. B. WARD. 1972. Postinfectional inhibitors from plants. I. Capsidiol an antifungal compound from Capsicum frutescens. Phytopathol. Z. 74:141-152.
15. STOESSL, A., C. H. UNWIN, and E. W. B. WARD. 1973. Postinfectional inhibitors from plants: fungal oxidation of capsidiol in pepper fruit. Phytopathology 63:1225-1231.
16. TOMIYAMA, K., T. SAKUMA, N. ISHIZAKA, N. SATO, N. KATSUI, M. TAKASUGI, and T. MASAMUNE. 1968. A new antifungal substance isolated from resistant potato tuber tissue infected by pathogens. Phytopathology 58:115-116.
17. VARNS, J. L., J. KUĆ, and E. B. WILLIAMS. 1971. Terpenoid accumulation as a biochemical response of the potato tuber to Phytophthora infestans. Phytopathology 61:174-177.
18. WARD, E. W. B., and A. STOESSL. 1972. Postinfectional inhibitors from plants. III. Detoxification of capsidiol, an antifungal compound from peppers. Phytopathology 62:1186-1187.