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MONOLINOLEIN AS A SELECTIVE FUNGUS INHIBITOR FROM CYMBIDIUM, ORCHIDACEAE

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Abstract

An antifungal factor isolated from extracts of *Cymbidium* (Orchidaceae) roots and infected pseudobulbs was identified as monolinolein.

Introduction

Phytoalexins were first discovered (5) and studied in orchids (for reviews see Arditti, (2) and Nüesch (16)). Three phytoalexins, all phenanthrenes, have been isolated from orchids since the initial discovery: orchinol (2,4-dimethoxy-7-hydroxy-9-10-dihydrophenanthrene) from *Orchis militaris* as well as hircinol (2,5-dihydroxy-4-methoxy-9,10-dihydrophenanthrene) and loroglossol (5-hydroxy-2,4-dimethoxy-9,10-dihydrophenanthrene) from *Loroglossum hircinum* (8) (for a review see Arditti, (2)). There is substantial evidence that these phytoalexins function as postinfectional fungal (including mycorrhizal) growth regulators (6, 8, 9, 10, 20, 21). To determine whether functionally or chemically similar compounds are produced by other orchids an investigation was initiated using hybrid *Cymbidium* roots and pseudobulbs. Several sterols were isolated during that study (4). Antifungal factors observed in extracts were not isolated and characterized at the time.

In continuation of this work, we have now isolated an inhibitor which was unambiguously identified as monolinolein (I). It does not appear to be identical with any of the inhibitors observed in the earlier work (4), since it seems to differ considerably in R_f value. Nevertheless, it accounted for most of the activity of the extract and has an antifungal spectrum which may be of some interest in itself.

Materials and methods

Plant Material

Roots of *Cymbidium* hybrids grown in non-sterile fir bark in a lath house were obtained from commercial growers in Santa Barbara, California. Pseudobulbs removed from the same plants were infected with *Rhizoctonia repens* M32 (kindly supplied by Dr. J. Nüesch, Institut für Spezielle Botanik, Eidgenössische Technische Hochschule, Zürich, Switzerland) as described previously (4). *Monilinia fructicola*, *Phytophthora infestans*, and *Cladosporium cucumerinum* were from the permanent collection maintained at the Research Institute in London, Ontario, Canada.

Extraction, Purification, and Chemical Determination

After two weeks of incubation with the fungus, tissues were extracted with three changes of acetone at room temperature. A portion (1.24 g) of the residue (3.6 g) left on evaporation of the filtered extract was chromatographed (25 ml fractions) over silica gel (British Drug Houses, 60–120 mesh, 125 g) with increasing concentrations of methanol in methylene chloride as eluant. Each fraction (45 were collected) was evaporated and the residue examined by (1) bioassay with *P. infestans* and *M. fructicola*, (2) UV spectrophotometry (1 mg in 100 ml ethanol, 1 cm cell, Beckman DK1 spectrophotometer), and (3) TLC (5 ml of 1 % solutions applied to silica gel, Gamag DF5; solvent systems were A, ether; B, *sec*-butanol:ethyl acetate 5 : 95; C, methanol:chloroform 2 : 98; D, benzene:acetone 9 : 1; E, benzene:dioxane:acetic acid, 90 : 25 : 4). Spots were visualized with UV illumination (sensitive test for hydroxy- and methoxy-substituted phenanthrenes and dihydrophenanthrenes), and sulphuric acid, phosphomolybdic acid, and vanillin/phosphoric acid as chromogenic reagents.

Fractions 31–43 were rechromatographed over a column of silica gel (Camag DF5, not activated; 470 g) in solvent B. The ^{1}Hmr spectrum of the highly purified material was determined in deuteriochloroform at 100 MHz with a Varian XL-100 instrument. IR spectrum was measured in 2 % carbon tetrachloride with a Beckman Acculab 4 spectrophotometer. Further verification was obtained chemically.

Alkaline hydrolysis (0.2N NaOH in 80 % methanol; 2h reflux) furnished glycerol (TLC) which was rigorously characterized as triacetin: the hydrolyzate was extracted with ether, the aqueous phase was evaporated to dryness and the residue was extracted with ethanol.

Enzymatic hydrolysis (15) with pancreatic lipase (1 mg; Calbiochem, La Jolla, CA) in M-Tris buffer (pH 8,1 ml) and calcium chloride solution (2.2 %; 0.1 ml) containing a trace of digitonin, for 10 min at 40 °C. The sole ether-extractable product was assayed by TLC and was methylated (diazomethane in ether/methanol). Its identity was confirmed by gas chromatography [Hewlett-Packard 5750 gas chromatograph fitted with a glass column, 6' × 2 mm, packed with 3 % OV-1 on Chromosorb W High Performance resin and operating at 180 °C with inlet at 185 °C and detector (flame ionization at 210 °C)] in a direct comparison with both authentic linoleic acid methyl ester and the product obtained similarly from authentic monolinolein.

Oxidation of the extract and authentic monolinolein with sodium periodate-potassium permanganate (19) was carried out at room temperature in aqueous *t*-butanol. Identification of the products obtained from hydrolysis of the purified extract was by direct comparison with authentic acids by TLC (several systems) and after methylation (ethereal diazomethane), by gas chromatography (as above except for column temperatures of 70 °C for caproic and 140 °C for azelaic acid, with the inlet at 170 °C and detector at 190 °C).

Bioassays

To determine activity against *Cladosporium cucumerinum*, the cymbidium factor and authentic monolinolein were applied as spots (5 of 1 % solutions) to silica gel (Camaul DF5) plates and developed in solvent A and in isopropanol ethyl acetate 5 : 95. After drying at room temperature overnight, plates were sprayed with a heavy spore suspension of the fungus and incubated in the dark in a moist atmosphere for 6 days. Bioassays on spore germination of *Monilinia fruticola* (Wint.) Honey, and *Phytophthora infestans* (Mont.) de Bary were carried out by the Standard Slide Germination Method (1).

The effects of monolinolein on *Rhizoctonia repens* M32 were estimated by growing the fungus on modified Knudson C medium (17) and determining the dry weight of mycelial mats after 21 days of culture.

Results

None of the fractions exhibited either the pronounced activity (21) towards *M. fructicola* or the ultraviolet spectra (8, 11, 14, 20) expected of the oxy-substituted phenanthrenes or dihydrophenanthrenes.

Fractions 13–21, eluted by methylene chloride, were only weakly active and consisted mainly of sterols, crystallizing from alcohol, which were not further examined.

In a duplicate separation on the same scale, the corresponding fractions were partitioned between ether and aqueous sodium hydrogen carbonate (5 %). Acidification of the aqueous layer with dilute hydrochloric acid and extraction with ether gave a mixture of acids. The principal component of this mixture was indistinguishable from linoleic acid by R_f and color reaction, with 50 % sulfuric acid at 110 °, by TLC (system D, and ether:light petrol: acetic acid, 50 : 50 : 1; v/v/v). The ^{1}Hmr spectrum of the mixture in deuteriochloroform showed all the characteristic bands of linoleic acid as prominent features.

On elution with 5–10 % methanol, fractions 31–41 (413 mg), were strongly active towards *P. infestans* and contained practically homogenous monolinolein (125 mg). Considerable quantities of the same compound were present in adjacent fractions in a mixture with other, probably structurally related compounds, as indicated by TLC.

The ^{1}Hmr spectrum of the highly purified material was essentially identical with that obtained from authentic monolinolein (Sigma Chem. Co., St. Louis, MO.), the small differences being clearly attributable to (different) trace impurities in both samples. The results of bioassays (Table 1) were also identical within experimental error. Evaporation of the ethanol extract of the aqueous phase of the NaOH hydrolyzate gave pure glycerol which was acetylated (acetic anhydride/pyridine at room temperature), the product being indistinguishable from authentic triacetin by TLC and mass spectrum (Varian MAT 311A spectrometer).

Gas chromatographic comparisons established the identity of the product of enzymatic hydrolysis as linoleic

Table 1. Antifungal activity of monolinolein-inhibition of spore germination.

Fungus	Sample	Percent inhibition* (concentration, ppm) 100	50	25	12.5	6.25	3.125
Monilinia fructicola	*Cymbidium*	0	0	0	0	0	0
Monilinia fructicola	authentic**	0	0	0	0	0	0
Phytophthora infestans	*Cymbidium*	96	89	69	56	28	0
Phytophthora infestans	authentic	100	100	78	46	31	0

* Determined at London, Ontario, Canada, on one and the same occasion
** Purchased from Sigma Chemical Co., St. Louis, MO.

acid. This was confirmed by the sodium periodate-potassium permanganae oxidation which produced caproic and azelaic acids as sole acidic products. Identical results were obtained with monolinolein.

In the bioassays with *Cladosporium cucumerinum* both the purified extract and authentic monolinolein showed clear inhibitory zones of identical R_f values: 0.21 and 0.44 in solvent A and in isopropanol:ethyl acetate (5 : 95; v/v) respectively.

Both the purified factor from *Cymbidium* and authentic monolinolein were inactive against *Monilina fructicola* but highly inhibitory towards *Phytophthora infestans* in spore germination tests (Table 1). Monolinolein was inactive against *Rhizoctonia repens* growing on potato dextrose broth (Table 2). When the fungus was cultured in modified Knudson C medium (17) monolinolein was inhibitory at 1.25 ppm and at 250 and 500 ppm, but stimulated growth at intermediate concentrations.

Discussion

Isolation of monolinolein was accomplished by careful column chromatography, monitored by bioassays and UV spectroscopy. Phenanthrenes, dihydrophenanthrenes, or stilbenes, any of which would have been readily detected by means of their strong and characteristic UV absorptions, appeared to be entirely absent. This absence is not surprising since this genus belongs to the tribe Kerosphaeroideae of the Orchidaceae (18), and is therefore taxonomi-

Table 2. Antifungal activity of monolinolein-effect on mycelial growth of *Rhizoctonia repens*.

Concentration (mg/l)	Modified Knudson C medium weight, mg	% of water control	% of solvent control	Potato-dextrose agar weight, mg	% of water control	% of solvent control
0 control*	85	106	121	64	91	114
Water control**	80	100	114	58	110	104
Solvent control***	70	88	100	56	114	100
1.25	38	46	54	53	91	95
2.5	68	85	98	62	107	111
5	78	96	111	62	107	111
12.5	104	103	149	59.5	103	106
50	71	89	101	52.5	91	94
125	74	93	106	52	90	93
250	39	49	49	54	93	96
500	43	54	61	77	85	88

* Medium used as is; no liquid was added to compensate for the addition of monolinolein stock solution.
** Water (1 ml/20 ml medium) was added to compensate for the dilution by the monolinolein stock solution.
*** Solvent used to dissolve monolinolein (1 ml 95 % ethanol plus few drops Twen 80 diluted to 10 ml with water) was added at the rate of 1 ml/20 ml medium.

cally distant from *Orchis militaris* and *Logoglossum hircinum* (tribe Ophryoideae).

The antimicrobial activity of fatty acids and glycerides is well known (12, 13). Hence the effects of monolinolein on *Cladosporium cucumerinum*, *Phytophthora infestans*, and *Rhizoctonia repens* are fully in line with previous reports regarding these chemicals. The apparent differential activity (no inhibition of *Monilinia fructicola* and of *R. repens* in potato dextrose broth) may be of general interest and deserves further exploration.

Fractions containing sterols were eluted before monolinolein and were only weakly antifungally active. This may confirm a previous report that increases in the production of antifungal substance(s) and sterols may coincide (4). On the other hand, it should be noted that these fractions were present in each of the four extracts examined, but only one contained appreciable amounts of monolinolein.

The weak activity of the sterol-containing fractions was probably due to the presence of linoleic acid, as demonstrated by TLC and ^{1}Hmr spectroscopy.

A generally, even if tacitly, accepted view seems to be that phytoalexins are produced (as their names imply) to ward off fungi which in the great majority of cases may be pathogenic. Our findings with orchids and those by others (2, 16) suggest that antifungal compounds may have an additional and no less important biological role – the regulation of mycorrhyzae. And, in orchids (the largest flowering plant family in existence) these compounds may be diverse enough to be of chemo-taxonomic value.

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