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 postinfectious 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 Rp 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 1Hmr 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, 1 ml) and calcium chloride solution (2.2%; 0.1 ml) containing a trace of digitoxin, for 10 min at 40 °C. The sole ether-extractable product was assayed by TLC and was methylated (diazomethane in ether/methanol).

Identification of the products obtained from hydrolysis was confirmed by gas chromatography [Hewlett-Packard 5750 gas chromatograph fitted with a glass column, 6′ x 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 (etheral 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 phenan-threnes or dihydrophenanthenes.

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 Rf 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 1Hmr 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 1Hmr 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.

This was confirmed by the sodium periodate-potassium permanganate 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 Rf 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 Knudsen 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 Kerosphaerideae 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 Knudsen 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 proportion of triglycerides by a semimicro technique for use with the slide germination method of evaluating protectant fungicides. Phytopathology 37: 354–356.

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