Identification of Tyrosinase Inhibitors in Cultures of *Pityrosporum*

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Lipid fractions capable of inhibiting the dopa-tyrosinase reaction in vitro were isolated by thin-layer chromatography from submerged cultures of *Pityrosporum* supplemented with oleic acid or vaccenic acid. Analysis of these fractions by gas chromatography-mass spectrometry revealed the presence mainly of C9 and C11 dicarboxylic acids. Standard dicarboxylic acids from C8 to C13 were capable of inhibiting tyrosinase *in vitro* to varying extents. Enzymatic kinetic studies showed that they act as competitive inhibitors of tyrosinase. These observations suggest that dicarboxylic acids could be used in the treatment of people with hyperpigmentary disorders.

Although pityriasis versicolor has been recognized since 1846, the depigmentation that occurs in the skin after colonization by *Pityrosporum orbiculare* is still not understood. Recent ultrastructural studies [1–4] have shown that melanocytes in the affected skin produce poorly melanized and abnormal melanosomes, and exhibit specific pathological features ranging from mitochondrial vacuolation to degeneration. We have reported earlier, a general inflammatory, or possibly immunologic cellular reaction in the dermis. These observations suggest that *P. orbiculare* produces substances that directly inhibit the normal mechanism of epidermal pigmentation.

This report, a sequel to and an extension of previous studies on skin surface lipids in pityriasis versicolor, and on the lipid metabolism of *P. orbiculare*, deals with the identification of tyrosinase inhibitors in cultures of *Pityrosporum*.

**MATERIALS AND METHODS**

**Organism**

Strain 4709 of *P. orbiculare* from our collection was used. The cultures were grown aerobically in 500-ml Erlenmeyer flasks on a gyrorotary shaker at 30°C for 30 days. The initial cell density of cultures was adjusted to 20 to 30 mg (dry weight) of cells/ml.

**Growth Medium**

A prepared synthetic medium contained the following: 1 liter of distilled water, 20 gm glucose, 4 gm (NH4)2SO4, 1 gr KH2PO4, 500 mg MgSO4·7H2O, 100 mg NaCl, 100 mg CaCl2, 1 gm FeCl3, 2 mg ZnSO4·7H2O, 2 mg MnSO4·H2O, 0.05 mg CuSO4·5H2O, 1 mg H2BO3, 1 mg KJ, 1 gr l-asparagine, 10 gr 1-histidine HCl, 10 gr 1-methionine, 20 mg l-tryptophan, 5 μg biotin, 1 mg calcium pantothenate, 0.2 mg p-aminobenzoic acid, 0.5 gr thiamine HCl, 2 mg inositol, 5 mg folic acid, 500 mg cholesterol, 300 mg cholesterol stearate, and 500 gm glycerol monostearate. Cholesterol and cholesterol esters were added because of cholesterol effects on cell membranes, and because they occur in the scaly patches of pityriasis versicolor [5]. In the experiments, oleic acid (C18:1, Δ9), vaccenic acid (C18:1, Δ11), and petroselinic acid (C18:1, Δ9) were added to culture medium as lipid supplements. Vaccenic acid was emulsified in Triton X 100.

**Extraction of Lipids**

Dry weight was evaluated by filtration of the liquid culture through a Büchner filter. Whole cells were triturated in a mortar, mixed with quartz sand, and extracted with 50 v/v vol of chloroform-methanol (2:1 v/v) for 3 hr at room temperature. Two further extractions were performed with 20 vol of chloroform-methanol (2:1 v/v). The filtrate of the culture was evaporated under reduced pressure on a rotary evaporator, and the residue was extracted 3 times with chloroform-methanol (2:1 v/v). Pooled lipid extract of the cells and of filtrate was dried over anhydrous Na2SO4, and the lipids were recovered by evaporation of the solvents under reduced pressure on a rotary evaporator at less than 30°C.

**Saponification of Total Lipids**

The lipids were transferred into a saponification flask, mixed with 100 ml of a 2 M KOH solution in ethanol, and boiled under reflux for 3 hr. The solution was then transferred into a 500-ml separatory funnel to which were added 200 ml distilled water.

* A. *Nonsaponifiable fractions.* Nonsaponified material was removed through 3 extractions with peroxide free diethyl ether. The combined ether extracts were simultaneously washed and vigorously shaken 4 times, the first and third times with 20 ml distilled water and the second and fourth times with 20 ml 0.5 N KOH. The ether extracts were then repeatedly washed with 20-ml fractions of distilled water until the water was alkaline. The combined ether fractions, dehydrated with Na2SO4, were then evaporated to dryness.

Three extracts (A, B, and C) were obtained from the nonsaponifiable fractions. Which extract was obtained depended on the fatty acid added as the lipid supplement (oleic acid, vaccenic acid, and petroselinic acid, respectively) (Table).

* B. *Saponifiable fractions.* The aqueous solution with the saponified material was acidified with 1 M HCl to a pH of 2 to 2.5, and extracted with 3 successive 50-ml portions of peroxide free diethyl ether. The combined ether fractions were washed 3 times with 20 ml of distilled water, dehydrated with Na2SO4, and evaporated to dryness.

Three extracts (A, B, and C) were obtained from the saponifiable fractions. Which extract was obtained depended on the fatty acid added as the lipid supplement (oleic acid, vaccenic acid, and petroselinic acid, respectively) (Table).

Besides the pooled lipid extracts, we also separately studied the lipid content of the cells (nonsaponifiable fractions: extracts A, B, and C); saponifiable fractions: extracts A, B, and C); and the lipid content of the filtrate culture medium (nonsaponifiable fractions: extracts A, B, and C); saponifiable fractions: extracts A, B, and C) (Table).

**Thin-Layer Chromatography**

Standard 20 × 20 cm thin-layer plates (E. Merck, Darmstadt, art. 5721) coated with 0.25-mm-thick layer of silica gel G, were developed in ether for the removal of lipid contamination, and then were heat-extracted (130°C for 20 min).

The extracts were dissolved separately in ether and deposited as bands 2 cm in length near one edge of each plate. After the plates were developed in petroleum ether (30–50°C): diethyl ether: acetic acid (70:30:2), they were air-dried and sprayed with a bromocresol green solution [6]. The 10 lipid fractions were separately scraped off from the plates into a small glass column, from which the lipids were eluted with 5 washings of peroxide-free diethyl ether.

**Gas Chromatography-Mass Spectrometry**

Gas chromatography-mass spectrometry (GS-MS) was used for the identification of components of the individual fractions. Methyl esterification of the fractions were performed according to the method of Boniforti et al [6]. Mass spectra were obtained on an LKB model 9000 S combination GC-MS machine. The gas chromatograph inlet system was equipped with a 2-m coiled glass column packed with 3% OV-101 on chromosorb W HP 80/100 mesh. Column temperature ranged from 100°C to 250°C/min; the carrier gas was He (25 ml/min); and the temperature of the He separator was 250°C. The ion source was
Tyrosinase inhibitory activity of lipid extracts from Pityrosporum cultures on the 30th day of growth with different fatty acids as lipid supplement

<table>
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<th>Lipid extracts</th>
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<th>Filtrate medium lipids</th>
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<td>Lipid fractions</td>
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<td>Non sapon A3</td>
<td>Sapon A4</td>
<td>Non sapon A5</td>
<td>Sapon A6</td>
<td>Non sapon B1</td>
<td>Sapon B2</td>
<td>Non sapon B3</td>
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<td>Tyrosinase inhibition in vitro</td>
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<td>GC-MS</td>
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* C6, dicarboxylic acid (azelaic acid).
* C6, dicarboxylic acid (undecandioic acid).
* C6, dicarboxylic acid (adipic acid).

Abbreviations: TLC = thin layer chromatography; GC-MS = gas-liquid chromatography-mass spectrometry.

maintained at 270°C; ionizing potential was 70 eV, and the ionizing current was 60 μA. All spectra were recorded on the apex of the gas chromatographic peak.

**Spectrophotometric Assays and Enzyme Kinetics**

Spectrophotometric studies and enzyme kinetics were carried out on 1 cm² cells in a Beckman Acta VI spectrophotometer with mushroom tyrosinase (grade III, Sigma). The initial rate of linear increase in optical density, caused by formation of dopachrome, was measured at 475 nm with a substrate of L-dopa (L-3, 4-dihydroxyphenylalanine) in 0.1 m phosphate buffer at pH 6.8.

**RESULTS**

**Lipid Extracts**

The Table shows that in *Pityrosporum* cultures containing a lipid supplement of either oleic acid or vaccenic acid, on the 30th day, the saponifiable fractions of the pooled lipid extracts A2 and B2 (from both the cells and the filtrate) inhibited the dopa-tyrosinase reaction. The lipid extracts from cultures with petroselinic acid (C5) were ineffective.

When we examined the lipids of the cells and the filtrate separately, about 80% of the substance or substances inhibiting the dopa-tyrosinase reaction was present in the filtered culture medium.

**Thin-Layer Chromatography**

We found 10 principal fractions: one of these, with an Rf = 0.12, strongly inhibited the dopa-tyrosinase reaction in vitro. The saponifiable fraction of the pooled lipid extract from cultures with petroselinic acid (C5) gave a fraction with the same Rf on the plate; this fraction had no effect on the tyrosinase activity.

**Gas Chromatography-Mass Spectrometry**

The GC-MS analysis of the fractions with an Rf of 0.12 on thin-layer chromatography plates showed the presence of about 10 components.

In extract A2 (pooled lipid extract obtained from cultures with oleic acid), we found a series of dicarboxylic acids. Glutaric acid (C5) and pimelic acid (C6) were present in small quantities, but there was a greater amount of azelaic acid (C6) (Fig 1).

In extract B2, obtained from cultures with vaccenic acid, we found small quantities of both azelaic acid (C6) and pimelic acid (C6) with undecandioic acid (C11) as the main component (Fig. 2).

The analysis of the corresponding fraction of extract C5 obtained from cultures with petroselinic acid, revealed a small quantity of adipic acid (C6).

**Enzymatic Kinetic Studies**

The inhibition of tyrosinase by dicarboxylic acids was competitive, and the values ranged from 7.40 × 10⁻⁴ M for C6 to 0.98 × 10⁻⁴ M for C11 dicarboxylic acid (Fig 3).

Sodium or potassium salts of dicarboxylic acids gave the same types and degrees of inhibition as their corresponding acids. Methyl esters, however, were ineffective.

Traumatic acid (1 decene, 1-10 dicarboxylic acid) had about the same inhibitory activity as undecandioic acid.
the tyrosinase inhibitory effect of culture extracts, and in fact commercially pure standard dicarboxylic acids (C_6 to C_{12}), are capable of inhibiting tyrosinase activity, to varying extents, in vitro. Furthermore, our studies have shown that these dicarboxylic acids are competitive inhibitors of tyrosinase with different K_i values (Fig 3).

As it has been possible to demonstrate a tyrosinase inhibitory activity in the saponified and then acidified fractions of the lipid extract of the filtrate it is likely that azelaic and undecanedioic acids may occur in the filtrate in a polar bond form, perhaps as glycolipids, [but the problem requires further investigation]. Although dicarboxylic acids with chain lengths over C_6 displayed tyrosinase inhibitory activity in vitro as free acids, or as sodium or potassium salts, their methyl or ethyl esters were ineffective. The only unsaturated dicarboxylic acid we have tested, tricarboxylic acid (1 decene, 1-10 dicarboxylic acid), a plant hormone, showed the same antityrosinase activity as azelainic acid. Because the unsaturation of the molecule probably does not alter the tyrosinase inhibitory activity of dicarboxylic acids, the presence in the molecule of 2 carboxylic groups in the α,ω position probably is essential to the inhibition of tyrosinase. In fact, monocarboxylic acids such as caprilic acid (C_{9}), pelargonic acid (C_{8}), and capric acid (C_{10}), do not inhibit tyrosinase.

The origin of dicarboxylic acids in the cultures of *Pityrosporum* is inferred on the basis of current knowledge about the oxidation of unsaturated fatty acids. This is shown by our experiments with monounsaturated fatty acids with the same chain length (C_{16}) but with double bonds at different positions. Oleic acid (C_{16:1}, w_9) gave rise mainly to C_9 dicarboxylic acid, vaccenic acid (C_{16:1}, w_7) to C_{12} dicarboxylic acid, and metalsilic acid (C_{18:1}, w_9) to C_{6} dicarboxylic acid. We studied the position of the double-bond linkages of monounsaturated fatty acids of human skin lipids by using a high resolution GC-MS system, and found that, although in the fatty acids with a chain of 14, 15, and 16 carbon atoms, the double-bond is mainly present at the Δ6 position, in the fatty acids with a chain length of 17, 18, 19, and 20 carbon atoms, most of the double bonds are present at the Δ7, Δ8, Δ9, Δ10, Δ11, and Δ12 position [9]. Therefore, we suggest that the hypopigmentation in pityriasis versicolor is due to dicarboxylic acids formed through the oxidation, by a *Pityrosporum* enzyme system, of the double bond linkages at the Δ9, Δ11, and probably Δ8 and Δ10 positions of the unsaturated fatty acids normally present in skin surface lipids.

The demonstration that dicarboxylic acids are competitive inhibitors of tyrosinase in vitro, their presence in cultures of *Pityrosporum*, and evidence of damage to melanocytes in skin areas affected by pityriasis versicolor suggest that these substances might be of therapeutic value in the treatment of people with pigmentary disorders [10]. We have had encouraging results [11,12] with the application of a cream containing azelaic acid on hypopigmentary conditions such as chloasma and lentigo maligna. The application of the cream to normal skin affected the melanocytes in a manner similar to that seen in naturally occurring pityriasis versicolor (Breathnach, unpublished data). These results further suggest that, aside from a purely inhibitory effect, dicarboxylic acids may have a specific cytotoxic effect on melanocytes. We are investigating the implications of this phenomenon in *in vitro* and *in vivo* experiments in human and nonhuman tissues.

**REFERENCES**


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**Announcement**

March 2-4, 1979, Postgraduate Seminar in Dermatology, Carillon Hotel, Miami Beach, Florida.

Registration Fee: $200

March 2-4, 1979, Fourth Annual Conference in Skin Disorders for the Nurse, Carillon Hotel, Miami Beach, Florida

Registration Fee: $100

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