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An Orsellinic Acid Decarboxylase Isolated from *Gliocladium roseum*

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A decarboxylase with the ability of removing the carboxyl group from orsellinic acid and 2,4-dihydroxy-5,6-dimethylbenzoic acid has been isolated from *Gliocladium roseum* and purified 40-fold. The enzyme appeared not to require any metal ions or cofactors for activity, but was considerably activated in the presence of 0.01 M sodium azide. A few salicylic acid derivatives were found to inhibit the decarboxylation of orsellinic acid competitively. The general properties of the enzyme have been studied, and some kinetic data are reported.

Although decarboxylation is an extremely common reaction in biochemistry, the non-oxidative removal of a carboxyl group directly attached to an aromatic ring has been demonstrated only in a few cases (see Table 1).[1-8] Among phenolic acids the decarboxylation of 2,3-dihydroxybenzoic acid has been studied with cell free preparations obtained from a certain strain of

Table 1. Previous reports on the non-oxidative enzymatic removal of a carboxyl group directly attached to an aromatic ring. The decarboxylations of 2,4- and 3,4-dihydroxybenzoic acid have not been studied on the cell free level.

Substrate	Product	Organism	Ref.
2,3-Dihydroxybenzoic acid	Catechol	*Aspergillus niger*	1
2,4-Dihydroxybenzoic acid	Resorcinol	*Aspergillus spec.*	2
3,4-Dihydroxybenzoic acid	Catechol	Moulds	3
p-Aminobenzoic acid Anthranilic acid	Aniline	*Escherichia coli*	4
Stipitatonic acid Puberulonic acid	Stipitatic acid Puberulic acid	*Penicillium stipitatum*	5
2-Methyl-3-hydroxypyridine-4,5-dicarboxylic acid	2-Methyl-3-hydroxypyridine-5-carboxylic acid	*Pseudomonas*	6
Orotidine-5′-phosphate	Uridine-5′-phosphate	Yeast	7
Quinolinic acid	Nicotinic acid N-ribotide	Rat liver	8

Aspergillus niger,[1] and the conversion of 2,4-dihydroxybenzoic acid into resorcinol was recently shown with intact cultures of an *Aspergillus* species.[2] Similarly, 3,4-dihydroxybenzoic acid (formed by the shikimic acid pathway) has been found to be converted into catechol by several moulds.[3] On the other hand, phenolic acids that are formed by acetate-polymalonate condensations (*e.g.* 2-hydroxy-6-methylbenzoic acid and orsellinic acid) seem not to be substrates for any of the decarboxylase systems described, even though such acids may function as precursors of a large number of acetate-polymalonate derived compounds, in the formation of which a step of decarboxylation is inferred from structural evidence or from the results of radioactive tracer studies. A cell free enzyme system carrying out the decarboxylation of orsellinic acid has now been obtained from *Gliocladium roseum* mycelium. The preparation, partial purification, and general properties of this decarboxylase are described in the present report.

EXPERIMENTAL

Culture conditions. Gliocladium roseum, C.M.I. 93065, was obtained from the Commonwealth Mycological Institute, Kew, Surrey, England. The mould was grown as aerated submerged cultures on a Raulin-Thom solution in 5 l fermenter tanks; the culture conditions have previously been described in detail.[9] After 10—14 days of growth the cultures were harvested and the mycelium removed by filtration. The mycelium could be stored at −15° for several months without losing its enzymatic activity.

Isolation of the enzyme. The mycelium was washed twice with distilled water and then carefully dried by pressing between filter papers. The cell mass (6 g dry weight) was frozen to −15°, and was desintegrated in the frozen state by means of a BIOX X-press.[10] After thawing the homogenate was extracted twice with 100 ml of 0.05 M phosphate buffer solution, pH 5.5, and unbroken cells and cell debris were eliminated by centrifugation for 10 min at 10 000 *g*. The supernatant solution was then filtered through a coarse paper to remove a small lipid layer. To 200 ml of the pale yellow filtrate were added 65 g of ammonium sulphate (52 % saturation) and the solution was allowed to stand overnight at room temperature. The precipitate was removed through centrifugation at 20 000 *g* for 15 min, and the supernatant solution was brought to about 80 % saturation by the addition of 45 g of ammonium sulphate at 4°. After 15 min at 4° the solution was centrifuged at 10 000 *g* for 10 min, and the supernatant fraction was discarded. The precipitate, which contained the enzymatic activity, was dissolved in 20 ml of distilled water. In the next step this enzyme solution was treated with DEAE-cellulose in order to remove a part of the enzymatically inactive protein material; attempts to adsorb the decarboxylase on calcium phosphate gel, CM-cellulose, or DEAE-cellulose (at pH values ranging from pH 4 to pH 9) were unsuccessful. To each ml of the enzyme solution were slowly added with stirring 50 mg (dry weight) of DEAE-cellulose, and the pH of the mixture was adjusted to pH 8.0 by the addition of dilute sodium hydroxide. After 10 min the cellulose was removed by filtration and discarded, and the filtrate was buffered with sodium phosphate to a final concentration of 0.05 M, pH 5.5. To 30 ml of the buffered enzyme solution 10 ml of acetone (25 %) were added at 20°, followed by centrifugation at 5000 *g* for 10 min. The precipitate was discarded, and the addition of a further 10 ml of acetone (40 %) to the supernatant solution at 4° yielded a precipitate carrying the enzyme activity. This precipitate was removed from the solution by centrifugation at 5000 *g* for 10 min, and was dissolved in 10 ml of 0.05 M phosphate buffer solution, pH 5.2. The latter solution was finally purified by allowing it to stand at room temperature for 4 days. A precipitate which formed was removed by centrifugation at 5000 *g* for 10 min. The clear supernatant obtained contained 3—6 mg of protein per ml, and the specific activity was usually higher than 0.15 units/mg; one enzyme unit (U) is defined as the amount that catalyzes the decarboxylation of 1 μmole of orsellinic acid per min under the specified assay conditions. The enzyme solution prepared in this way lost little activity when

stored at −15° for several months, and was used as a source of protein throughout the study. The typical fractionation scheme given in Table 2 shows that the over-all purification achieved was about 40-fold.

Table 2. Purification of orsellinic acid decarboxylase from *G. roseum*.

Procedure	Vol. ml	Conc. U/ml	Protein mg/ml	Spec. act. U/mg	Yield %	Purification
Initial extract	200	0.064	13.3	4.8	100	1.0
52–80 % ammonium sulphate ppt.	20	0.52	24.2	21.5	81	4.4
After DEAE-cellulose	40	0.25	5.3	47.2	78	9.8
25–40 % acetone ppt.	10	0.83	6.6	125.8	65	26.2
After aging	10	0.81	4.1	196.8	63	41.0

Assay method. Orsellinic acid decarboxylase was assayed manometrically by measurement of carbon dioxide evolution from orsellinic acid at 25°. Despite a pH-optimum of 6.0 sufficient activity was retained at pH 5.2 to permit assays at this lower pH value, with only a small error due to carbon dioxide retention. Enzyme solution (generally about 1 mg of protein) and appropriate buffer solution (generally 0.05 M phosphate buffer, pH 5.2) were placed in the main chamber of the Warburg vessels; the total liquid volume in the main chamber was 3.0 ml. The substrate solution (placed in the side arm) contained 10–15 μmoles of orsellinic acid dissolved in 0.5 ml of 0.05 M phosphate buffer, pH 5.2. The orsellinic acid concentrations of 3–5 mM were adequate to saturate the enzyme. After 15 min of temperature equilibration the substrate was tipped in to start the reaction. Carbon dioxide production began immediately, and an example of a typical assay is shown in Fig. 1.

The pH-dependence of the reaction was tested over a range from pH 3 to pH 9 using various buffer systems (pH 3.0–5.0, 0.05 M acetate; pH 5.0–7.5, 0.05 M phosphate; pH 7.5–9.0, 0.05 M Tris). Each flask contained 0.3 ml of enzyme solution (1.4 mg of protein) and 2.5 ml of appropriate buffer solution in the main chamber; 0.5 ml of a 23.2 mM aqueous solution of orsellinic acid was in one side arm and 0.2 ml of 5 M sulphuric acid in the second side arm. Enzyme and substrate solutions were adjusted to appropriate pH values by the addition of dilute sulphuric acid or sodium hydroxide. One blank without substrate and one without enzyme was run at each pH value. After incubation for 20 min at 25° the sulphuric acid was tipped in to stop the reaction, and to release bound carbon dioxide. As shown in Fig. 4 the pH-optimum was found to be about pH 6.

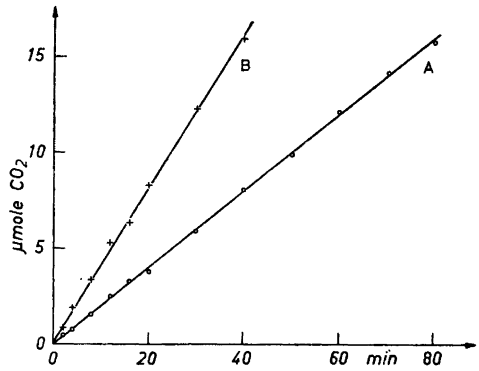

Fig. 1. Enzymatic (1.42 mg of protein; 1.40 U/mg) decarboxylation of orsellinic acid under standard assay conditions (curve A) and in the presence of 0.01 M sodium azide (curve B).

Stoichiometry of the reaction. Orsellinic acid (25 μmoles) was decarboxylated under the standard assay conditions. The reaction was stopped by the addition of 0.5 ml of 5 M sulphuric acid and the reaction mixtures were thoroughly extracted with ether. The extracts were chromatographed on Whatman No. 1 paper, using propanol-butanol-2 M ammonium hydroxide (6:1:3 by vol.) as the solvent. Orsellinic acid (R_F 0.55) and orcinol (R_F 0.90) were then eluted from the chromatograms with ethanol and their concentration determined spectrophotometrically. The results of these studies are given in Table 3.

Table 3. Stoichiometry in the decarboxylation of orsellinic acid.

Substrate and products	Amount (μmoles) after 0.5 h	2 h	6 h
Orsellinic acid decomposed	4.3	16.3	23.8
Carbon dioxide produced	4.1	15.9	23.9
Orcinol produced	3.9	15.8	22.5

Reverse reaction. Acetone precipitated enzyme (1.82 U; 0.11 U/mg) dissolved in 6 ml of 0.05 M phosphate buffer solution, pH 5.2, was mixed with 6 ml of 0.05 M phosphate buffer, pH 8.0, containing 0.13 mg of $NaH^{14}CO_3$ (0.1 mC). After addition of 20 mg of orcinol the pH of the mixture was adjusted to pH 6.0, followed by incubation at 25° for 1 h. The reaction was stopped by the addition of 1 ml of 5 M sulphuric acid, and 50 mg of non-radioactive orsellinic acid were added. The reaction mixture was then extracted with ether and the ethereal extract was shaken with sodium bicarbonate solution, from which orsellinic acid separated on acidification. After recrystallization from water the orsellinic acid was found to contain less than 0.01 % (168 cpm/mg) of the activity added; after further purification by paper chromatography (in the solvent system given above) there were no detectable counts in the sample of orsellinic acid.

RESULTS AND DISCUSSION

The enzymatic decarboxylation of orsellinic acid was studied using preparations that had been purified at least 30-fold. The enzyme solutions showed a linear carbon dioxide production for more than 60 min (see Fig. 1). The reaction rate was strictly proportional to the enzyme concentration (Fig. 2). K_m for orsellinic acid was found to be 2.6×10^{-4} M (at pH 5.2 and 25°) by using the Lineweaver-Burk plot (Fig. 3, curve A). The stoichiometry of the reaction is shown in Table 2; there was a good molar equivalence between the amount of orsellinic acid disappearing and the amounts of orcinol and carbon dioxide formed. The enzyme seemed not to be capable of performing the reverse reaction, that is the carboxylation of orcinol (see experimental section).

The orsellinic acid decarboxylase was active over a broad pH range, with a maximum occurring at pH 6 (Fig. 4, curve A). Since the maximum was not sharp, manometric assays were possible at pH 5.2, where activity was still high and where carbon dioxide retention was minimal. The effect of pH on the stability of the enzyme was tested by exposing the enzyme to a range of pH values (pH 3—9) for 30 min, and then measuring the activity after readjusting the pH to 5.2. The activity/pH curve obtained, which is shown in Fig. 4

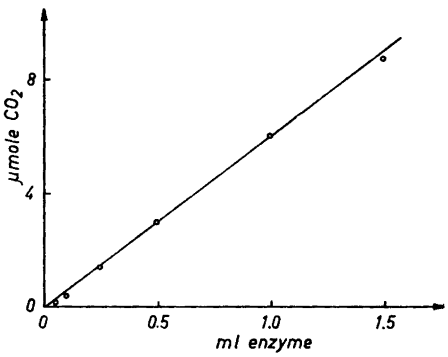

Fig. 2. Effect of protein concentration on the rate of decarboxylation of orsellinic acid. The enzyme preparation used contained 2.0 mg of protein per ml (0.19 U/mg). Reaction conditions: 13.2 µmoles of orsellinic acid in 0.5 ml of 0.05 M phosphate buffer solution, pH 5.2, and the respective ml of enzyme solution from the graph diluted to a volume of 3.0 ml with 0.05 M phosphate buffer, pH 5.2. Incubated for 15 min at 25°.

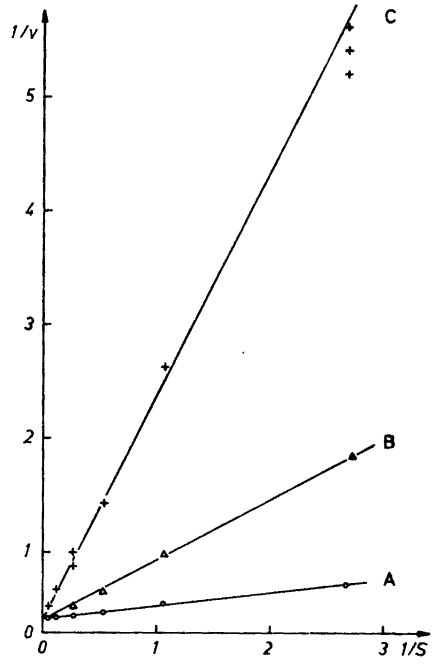

Fig. 3. Rate (v µmoles CO_2/10 min) of decarboxylation of orsellinic acid at different substrate concentrations (S mM). Plot of $1/v$ against $1/S$ according to the method of Lineweaver and Burk. The enzyme preparation used contained 5.0 mg of protein per ml (0.16 U/mg). Reaction conditions: Appropriate amounts of orsellinic acid in 0.5 ml of 0.05 M phosphate buffer, pH 5.2, 0.3 ml of enzyme solution, and 2.7 ml of phosphate buffer, pH 5.2. Incubated at 25° in the absence of inhibitors (curve A), in the presence of 18.6 mM 2,4-dihydroxybenzoic acid (curve B), and in the presence of 18.8 mM 2-hydroxy-6-methylbenzoic acid (curve C).

(curve B), indicates that the fall in enzymatic activity on the acid side of the pH-optimum is due to destruction of the enzyme.

The enzyme seemed to be fairly stable and could be kept at room temperature for two weeks or stored in the frozen state (−15°) for more than 3 months, with only a slight decrease in activity. It did not show any loss of activity by 10 min treatment at different temperatures up to 55° prior to incubations; at temperatures above 65° the enzyme was rapidly inactivated (see Fig. 5). The effect of temperature on the reaction rate was studied by carrying out incubations at temperatures between 25° and 50°. Within this interval the

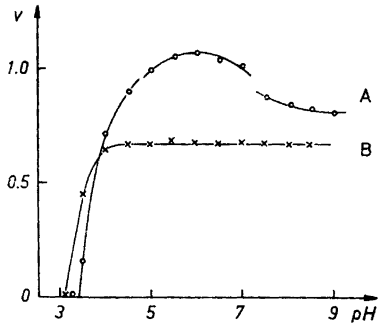

Fig. 4. Effect of pH on orsellinic acid decarboxylase. For curve A the activity (v μmoles CO_2/10 min) was tested at the pH values given; for curve B the enzyme was exposed for 30 min to the pH values given and the activity then tested at pH 5.2. Curve A was determined by the use of three different buffer systems (see experimental section).

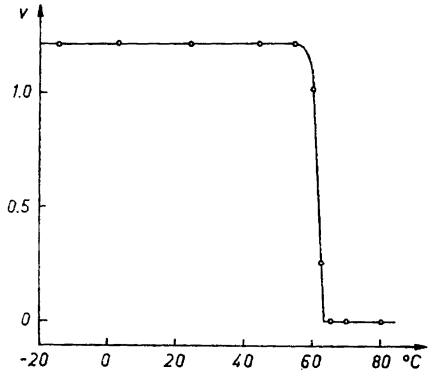

Fig. 5. Temperature inactivation of orsellinic acid decarboxylase. The enzyme solution was treated for 10 min at the temperatures given before the activity was tested under the usual assay conditions. The reaction rate (v) is given in μmoles CO_2 per 10 min.

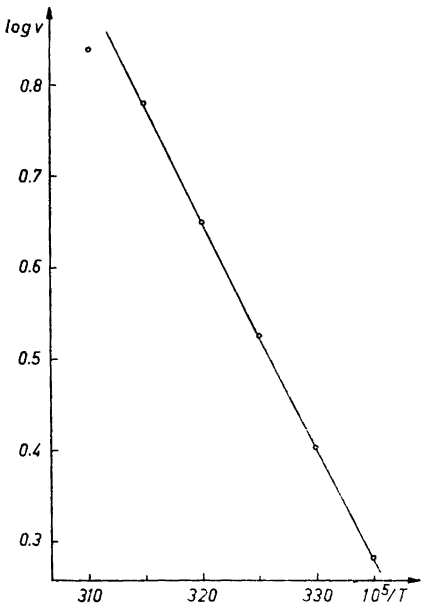

Fig. 6. Effect of temperature (T °K) on the rate (v μmoles CO_2/10 min) of decarboxylation of orsellinic acid. Plot of log v against $1/T$ according to the method of Arrhenius.

logarithm of the reaction rate was linearly related to the inverse value of the incubation temperature (°K), as shown by the Arrhenius plot in Fig 6. The temperature coefficient Q_{10} (the factor by which the velocity is increased on raising the temperature by 10°) was found to be 1.79, which corresponds to an activation energy of 10.3 kcal/mole.

The enzyme preparations also decarboxylated 2,4-dihydroxy-5,6-dimethylbenzoic acid, but at a considerably lower rate than orsellinic acid. The relative rates of decarboxylation with the two substrates remained essentially unchanged at the different stages of purification of the enzyme (see Table 4).

Table 4. Rate of decarboxylation of orsellinic acid (I) and 2,4-dihydroxy-5,6-dimethylbenzoic acid (II) during enzyme purification; the substrate concentrations were 3.4 mM.

Preparation	Enzyme activity (μl CO_2/10 min)		Ratio
	I	II	
Initial extract	42	11	3.8
Ammonium sulphate ppt.	38	9	4.2
After DEAE-cellulose	38	10	3.8
Acetone ppt.	22	5	4.4
After aging	20	5	4.0

Benzoic acid, 2-, 3-, and 4-hydroxybenzoic acid, 2- and 4-hydroxy-6-methylbenzoic acid, 2,3-, 2,4-, 2,5-, 2,6-, and 3,4-dihydroxybenzoic acid, 2,4-dihydroxy-3,6-dimethylbenzoic acid, 2,3,4- and 3,4,5-trihydroxybenzoic acid were not substrates for the decarboxylase. On the other hand, five of the above acids (see Table 5) were inhibitors when used in substrate amounts. A Lineweaver-Burk plot of the rate of decarboxylation of orsellinic acid in the presence of either of these acids (*cf.* Fig. 4, curve B and C) provided evidence that the inhibition was competitive; the K_i-values obtained are listed in Table 5. The following compounds were neither substrates nor inhibitors, even when used in substrate amounts: Benzene-1,2-dicarboxylic acid, 3-hydroxybenzene-1,2-dicarboxylic acid, benzene-1,3-dicarboxylic acid, 2- and 4-hydroxybenzene-1,3-dicarboxylic acid, benzene-1,4-dicarboxylic acid, benzene-1,2,3-tricarboxylic acid, benzene-1,3,5-tricarboxylic acid, benzene -1,2,4,5-tetracarboxylic acid, phenylacetic acid, 3-hydroxyphenylacetic acid, and 2,5-dihydroxyphenylacetic acid.

Table 5. Competitive inhibition of orsellinic acid decarboxylase by salicylic acid derivatives.

Inhibitor	K_i (mM)
2-Hydroxybenzoic acid	4.6
2-Hydroxy-6-methylbenzoic acid	1.0
2,3-Dihydroxybenzoic acid	1.5
2,4-Dihydroxybenzoic acid	5.2
2,5-Dihydroxybenzoic acid	1.1

Table 6. Metal ion inhibition of orsellinic acid decarboxylase.

Metal ion concentration	Inhibition in %		
	Hg^{2+}	Fe^{2+}	Ag^{+}
10^{-2} M	74	40	68
10^{-3} M	36	3	2
10^{-4} M	18	0	0
10^{-5} M	0	0	0

The enzyme did not exhibit any cofactor or metal ion requirement. No activity was lost when the enzyme preparations were dialyzed for 4 h against repeated changes of 0.05 M phosphate buffer, pH 5.5, or when the enzyme solutions were treated with EDTA (ethylenediaminetetraacetate) to a final concentration of 0.01 M at pH 8.0, followed by dialysis for 2 h against 0.05 M phosphate buffer, pH 7.0. The addition of 0.01 M EDTA to the reaction mixtures had no effect on the rate of decarboxylation of orsellinic acid. Investigation of the ion effects on dialyzed enzyme preparations showed that the following ions did not affect the enzyme activity even at a concentration of 0.01 M: Mn^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+}, Sn^{2+}, Pb^{2+}, K^{+}, Na^{+}, and CN^{-}. On the other hand, a pronounced inhibition was obtained with Hg^{2+}, Fe^{2+}, and Ag^{+} (see Table 6). In the presence of sodium azide the decarboxylation rate was increased by the same factor at all stages of enzyme purification (Table 7); the activation of the enzyme was most efficient at a concentration of 0.01 M

Table 7. Activation of orsellinic acid decarboxylase by 0.01 M sodium azide during enzyme purification.

Preparation	Enzyme activity (µl CO_2/10 min) without NaN_3	with NaN_3	Ratio
Initial extract	12	23	1.9
Ammonium sulphate ppt.	62	127	2.0
After DEAE-cellulose	48	94	2.0
Acetone ppt.	83	158	1.9

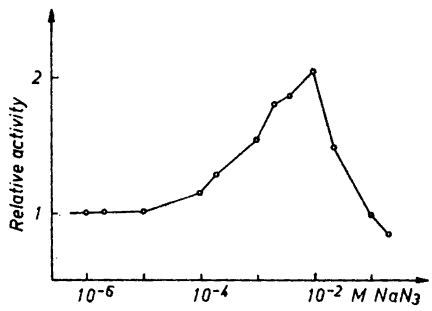

Fig. 7. Activation of orsellinic acid decarboxylase by sodium azide as a function of the concentration of the activator.

sodium azide (Fig. 7). The enzyme activity was not inhibited by preincubation with SH-reagents such as *p*-chloromercuribenzoate (10^{-4} M), iodoacetamide (10^{-3} M), or *o*-iodobenzoic acid (10^{-4} M), and incubations in the presence of glutathione (0.01 M) did not increase the enzyme activity. The addition of thiamine pyrophosphate (10 mg/ml) or pyridoxal phosphate (10 mg/ml) to the reaction mixtures had no effect on the decarboxylation rate.

The orsellinic acid decarboxylase present in *G. roseum* thus appears to be similar to the 2,3-dihydroxybenzoic acid decarboxylase obtained from *A. niger*,[1] and to the stipitatonic acid decarboxylase isolated from *Penicillium stipitatum*;[5] these enzyme systems are all highly specific and seem not to require any easily dissociable prosthetic group for activity.

Orcinol and 1,3-dihydroxy-4,5-dimethylbenzene have been isolated as minor metabolic products of the certain strain of *G. roseum* used in the present work;[9] the major secondary metabolite of this strain is 2,3-dimethoxy-5,6-dimethyl-1,4-benzoquinone (aurantiogliocladin).[11,12] The demonstrated existence of an orsellinic acid and 2,4-dihydroxy-5,6-dimethylbenzoic acid decarboxylase system in the mould provides strong evidence that the above two phenolic acids function as the natural precursors of orcinol and 1,3-dihydroxy-4,5-dimethylbenzene, and lends weight to the hypothesis,[13] that the two phenolic acids are formed as intermediates in the biosynthesis of aurantiogliocladin.

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