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# New Phenolic Metabolites from *Gliocladium roseum*

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Orcinol (I) and 1,3-dihydroxy-4,5-dimethylbenzene (II) have been isolated from a certain strain of *Gliocladium roseum*, known to produce aurantiogliocladin (V) and related quinones. 1,3-Dihydroxy-4,5-dimethylbenzene, which has not previously been reported as a mould metabolic product, was found to be biosynthesized by the acetate-polymalonate pathway, with introduction of a methyl group from the $C_1$-pool. The two phenols isolated seem to be derived from orsellinic acid (III) and 2,4-dihydroxy-5,6-dimethylbenzoic acid (IV), respectively, which were found to be rapidly metabolized in the mould, mainly by decarboxylation. Neither of the phenolic acids could, therefore, normally be detected in *G. roseum* cultures, but occasionally 2,4-dihydroxy-5,6-dimethylbenzoic acid was identified as a metabolic product of the mould.

In relation to investigations on the quinonoid metabolites of a certain strain of *Gliocladium roseum*,[1] culture filtrates of the mould were routinely examined with respect to the presence of phenolic metabolic products. These studies showed that quinone production was accompanied by the formation of two diazotisable compounds, which have now been isolated and identified as 1,3-dihydroxy-5-methylbenzene (orcinol: I) and 1,3-dihydroxy-4,5-dimethylbenzene (II).

There seems, for structural reasons, to be little doubt that the two phenols are biosynthesized by the acetate-polymalonate pathway, the methyl group in position 5 being derived from the methyl group of acetate. The biogenetic origin of the second methyl group in 1,3-dihydroxy-4,5-dimethylbenzene is, however, more uncertain. It could, for instance, be derived from the $C_1$-pool or from acetate (malonate), in the latter case being formed by total reduction of the terminal carboxyl group in the acetate-polymalonate condensation product; these pathways toward the formation of nuclear methyl groups are both illustrated by the example of barnol, a dimethylethylpyrogallol isolated from *Penicillium baarnense*.[2]

*G. roseum* was, therefore, cultivated in the presence of 1-$^{14}$C-acetate and $^{14}CH_3$-L-methionine, respectively, and the distribution of activity incorporated into 1,3-dihydroxy-4,5-dimethylbenzene was determined by chemical degradation. The results of these experiments, listed in Tables 1–2, seem to exclude

*Table 1.* $^{14}C$-Distribution in 1,3-dihydroxy-4,5-dimethylbenzene derived from 1-$^{14}C$-acetate (0.08 % incorporation).

| Material | Number of carbon atoms | Specific activity* | Total activity | Relative total activity Found | Calc. |
|---|---|---|---|---|---|
| Total combustion | 8 | 4520 | 36 160 | 3.18 | 3 |
| Kuhn-Roth oxidation, carbon dioxide | 4 | 5490 | 21 960 | 1.93 | 2 |
| Kuhn-Roth oxidation, methyl group of acetic acid | 2 | 990 | 1 980 | 0.17 | 0 |
| Kuhn-Roth oxidation, carboxyl group of acetic acid | 2 | 5690 | 11 380 | 1.00 | 1 |

* counts per min and mg $BaCO_3$

*Table 2.* $^{14}C$-Distribution in 1,3-dihydroxy-4,5-dimethylbenzene derived from $^{14}CH_3$-L-methionine (0.05 % incorporation).

| Material | Number of carbon atoms | Specific activity* | Total activity | Relative total activity Found | Calc. |
|---|---|---|---|---|---|
| Total combustion | 8 | 290 | 2320 | 1.00 | 1 |
| Kuhn-Roth oxidation, carbon dioxide | 4 | 45 | 180 | 0.08 | 0 |
| Kuhn-Roth oxidation, methyl group of acetic acid | 2 | 1100 | 2200 | 0.95 | 1 |
| Kuhn-Roth oxidation, carboxyl group of acetic acid | 2 | 0 | 0 | 0.00 | 0 |

* counts per min and mg $BaCO_3$

that either of the methyl groups of the phenol are derived from the carboxyl group of acetate (the slight incorporation of activity from 1-$^{14}C$-acetate into the methyl groups will be explained later in this paper). They are consistent with the formation of 1,3-dihydroxy-4,5-dimethylbenzene (II) by head-to-tail linkage of four acetate units (probably by the acetate-polymalonate pathway), with decarboxylation of the terminal carboxyl group and introduction of a methyl group from the $C_1$-pool (compare Fig. 1). The degradation reactions used did not distinguish between the two methyl groups, both of which were isolated as methylamine after Schmidt decarboxylation of acetic acid obtained on Kuhn-Roth oxidation of the phenol. It seems, however, only reasonable that the methyl group in position 4 is derived from the $C_1$-pool, as shown in Fig. 1; the hydroxyl groups must be expected to be located at carbon atoms derived from the carboxyl group of acetate, and thus the methyl group in position 5 of the phenol should be derived from the methyl group of acetate.

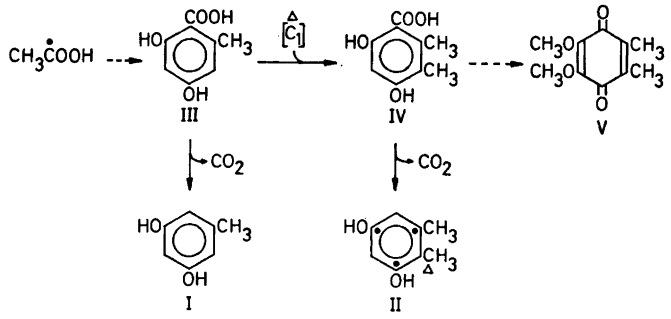

*Fig. 1.* Probable biogenetic relationships of the metabolic products isolated from *G. roseum*. Orsellinic acid (III) has not been detected in the mould.

1,3-Dihydroxy-4,5-dimethylbenzene has not previously been recognized as a mould metabolic product. Orcinol, on the other hand, has been identified in cultures of *Penicillium griseo-fulvum*,[3] and isolated from quinone producing strains of *Aspergillus fumigatus*.[4] In both of the moulds orcinol was formed along with orsellinic acid (III), which was shown to be directly converted into orcinol in *A. fumigatus*. Theoretically, it seems likely that orcinol has the same origin in *G. roseum*, and similarly 1,3-dihydroxy-4,5-dimethylbenzene might be formed by decarboxylation of 2,4-dihydroxy-5,6-dimethylbenzoic acid (IV). The latter compound has, in fact, been found to be produced along with orsellinic acid in mutant strains of *Aspergillus terreus;* the two acids were isolated by Hassall *et al.*, who suggested that 2,4-dihydroxy-5,6-dimethylbenzoic acid (which was labelled from $^{14}CH_3$-L-methionine) was formed by C-methylation of orsellinic acid.[5]

Neither of the above phenolic acids was found to be regularly present in the medium of *G. roseum* cultures. However, small amounts (in the order of 0.1 mg/l) of a diazotisable compound, that seemed to be identical with 2,4-dihydroxy-5,6-dimethylbenzoic acid (see experimental section), were frequently detected in submerged cultures of the mould, generally in cultures where quinones were produced at a decreased rate and the yield of phenols was unusually high. Occasionally, the concentration of 2,4-dihydroxy-5,6-dimethylbenzoic acid exceeded 1 mg/l medium, making it possible to isolate it in the crystalline form. Attempts to favour or control the production of this metabolite by variations of the culture conditions were unsuccessful. For this reason, insufficient material was available for a conclusive characterization of the phenolic acid by chemical analysis.

The failure to detect orsellinic acid does not exclude that it functions as a biosynthetic intermediate in the mould. When added to the culture medium it was found to be rapidly metabolized (see Table 3), and examination of the phenol fraction established that orcinol was the major product obtained. 50 mg or orsellinic acid, added to one culture flask, were thus completely metabolized after 3 days, at which time 32 mg of orcinol could be isolated from the culture; the normal yield of orcinol never exceeded 5 mg/flask. Apparently, a strong decarboxylase activity is present in *G. roseum* cultures, for which reason

*Table 3.* Disappearance of orsellinic acid, added to shaken-flask cultures of *G. roseum*.

| Incubation time | Concentration (mM) of orsellinic acid | | |
|---|---|---|---|
| Start | 0.20 | 0.40 | 2.00 |
| 10 h | 0.02 | 0.12 | 1.48 |
| 24 h | 0.00 | 0.00 | 1.04 |
| 72 h | 0.00 | 0.00 | 0.03 |
| Increase in concentration (mM) of orcinol after 72 h | 0.16 | 0.35 | 1.80 |

orsellinic acid could not be expected to be present in significant amounts, even if it were formed by the mould. Similarly, 2,4-dihydroxy-5,6-dimethylbenzoic acid was found to be rapidly metabolized (mainly by decarboxylation), which explains why it generally cannot be detected in the culture fluids. On the other hand, the decarboxylase system seemed to be rather specific with respect to the above phenolic acids. 2,4-Dihydroxybenzoic acid and 2-hydroxy-6-methylbenzoic acid could, for instance, be recovered in almost quantitative amounts 3 days after the addition to the culture medium of *G. roseum*. Further studies on the decarboxylase system, using cell-free preparations of the mould, are in progress.

## EXPERIMENTAL

*Culture conditions.* The culture conditions have previously been described in detail.[1] *Gliocladium roseum*, C.M.I. 93065, was used throughout this work; it was found to give a better yield of phenolic metabolites than *G. roseum*, A.C.C. 650. For larger-scale isolation of the phenols the mould was grown as submerged cultures in aerated (2 l/min) fermenters (Model FS – 307; New Brunswick Scientific Co., New Brunswick, N.J., U.S.A.), operated at a working volume of 5 l of medium. Agitation was at 150 r.p.m. and fermentations were carried out at 25° for 3 weeks. Other experiments described below all involved shaken-flask cultures (150 ml portions of medium in 500 ml conical flasks on a rotary shaker operated at 300 r.p.m. with a stroke of 1″). Radioactive precursors (0.5 mC of sodium 1-$^{14}$C-acetate; 0.05 mC of $^{14}CH_3$-L-methionine) were supplied to selected flasks on the seventh day of growth, labelled 1,3-dihydroxy-4,5-dimethylbenzene being isolated after a further seven days of cultivation.

*Chromatographic methods.* For routine examination of the production of secondary metabolites in *G. roseum*, the filtered culture fluids were strongly acidified with conc. hydrochloric acid, and extracted with ether. After removal of the 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.[6] This system is particularly suitable for analysis of the quinonoid metabolites of the mould, which appear as intensely coloured, self-indicating, spots (aurantiogliocladin, $R_F$ 0.95; 2-hydroxy-3-methoxy-5,6-dimethyl-1,4-benzoquinone, $R_F$ 0.72; 2,3-dihydroxy-5,6-dimethyl-1,4-benzoquinone, $R_F$ 0.53). For detection of phenolic metabolites the chromatograms were sprayed with a freshly prepared 0.1 % alcoholic solution of 4-benzoylamino-2,5-dimethoxyaniline diazotate, followed by exposure to ammonia vapour to promote the coupling reaction. With this reagent authentic samples of orsellinic acid ($R_F$ 0.58), 2,4-dihydroxy-5,6-dimethylbenzoic acid ($R_F$ 0.69), and the corresponding decarboxylation products ($R_F$ 0.90 – 0.95)were readily detected in amounts of 1 – 3 μg. The phenolic acids were found to be stable in the solvent system used.

For examination of the metabolites, that moved with or near the solvent front in the above system, a part of the chromatogram ($R_F$ 0.80—1.00) was eluted with ethanol. The eluate was rechromatographed on paper, using chloroform-methanol-formic acid-water (prepared as described by Reio [3]) as the solvent, when orcinol ($R_F$ 0.38), 1,3-dihydroxy-4,5-dimethylbenzene ($R_F$ 0.59), and aurantiogliocladin ($R_F$ 0.90—1.00) became well separated; the phenols were detected with the above diazonium reagent. The latter solvent system was also used for separation of the phenols in the preparative isolation procedures.

For chromatographic identification of the phenolic components isolated from the mould, the solvent systems (6 in all) and spraying reagents described by Reio were employed.[3]

*Isolation and identification of phenols.* The filtrate (3.8 l) from a 3 weeks old submerged aerated culture of *G. roseum* was extracted at pH 4 with half the volume of ether. After concentration the ethereal extract was chromatographed on Whatman No. 3 MM paper (see above). Orcinol and 1,3-dihydroxy-4,5-dimethylbenzene were detected by spraying a part of the chromatogram with the diazonium reagent, and the corresponding zones were cut out of the paper. The phenols were then eluted with ethanol and sublimed at 140° in vacuum (1 mm Hg) after removal of the solvent. They were further purified by crystallization from chloroform-petroleum ether (40—60°), followed by resublimation in vacuum at 120°.

In this way 12 mg of orcinol, m.p. 108—109°, were obtained. (Found: C 67.7; H 6.5. Calc. for $C_7H_8O_2$: C 67.7: H 6.4). The m.p. was unchanged by admixture with an authentic sample of anhydrous orcinol. The ultra-violet and infra-red absorption spectra, and paper chromatograms in the systems given above, were identical with those of authentic material.

Sublimation of the eluates of 1,3-dihydroxy-4,5-dimethylbenzene yielded 28 mg of white micro-crystals, m.p. 134°. After recrystallization the m.p. was 136°, unchanged on resublimation. (Found: C 69.7: H 7.2. Calc. for $C_8H_{10}O_2$: C 69.5: H 7.3). The mixed m.p. with an authentic sample (m.p. 136°), prepared according to the method of Strating and Backer,[7] was 136°. The ultra-violet and infra-red absorption spectra, and the chromatographic behaviour, were identical with those of authentic material.

Paper chromatography studies showed that the above phenols were regularly present in all quinone producing cultures of *G. roseum*. The total amount of phenols formed was found to vary considerably from batch to batch, ranging from 0.4 to 10 mg/l medium (spectrophotometric estimation); occasionally up to 10 mg of phenolic metabolites were present in one single culture (150 ml of medium). The concentration of 1,3-dihydroxy-4,5-dimethylbenzene was generally 2—5 times higher than that of orcinol. Those cultures that formed large amounts of phenols were usually found to give a poor yield of quinonoid products. On the other hand, there was no significant production order in time between the different secondary metabolites of the mould.

*Isolation and identification of 2,4-dihydroxy-5,6-dimethylbenzoic acid.* The filtered medium (4.2 l) of an aerated submerged *G. roseum* culture, that in routine examinations was found to form 2,4-dihydroxy-5,6-dimethylbenzoic acid, was strongly acidified by the addition of conc. hydrochloric acid and extracted with ether. The ethereal extract was shaken with a small volume of phosphate buffer solution (pH 4.0), which removed the phenolic acid. After acidification of the buffer solution 2,4-dihydroxy-5,6-dimethylbenzoic acid was reextracted with ether, purified by paper chromatography (propanol-butanol-ammonium hydroxide), and eluted from the chromatograms with ethanol. Removal of the solvent gave a semi-solid material (6 mg), which was extracted with 0.1 mM sodium hydroxide (0.2 ml). Acidification of this solution gave 2,4-dihydroxy-5,6-dimethylbenzoic acid as a white, microcrystalline precipitate, m.p. 158—160° (decomp.); the mixed m.p. with an authentic sample (m.p. 163°), prepared according to the method of Robertson and Whalley,[8] was 163°. In ethanolic solution the natural product showed two absorption maxima in the ultra-violet (I, 263 mμ; II, 306 mμ); the first maximum exhibited a significant bathochromic shift (10 mμ) with alkali. The spectra obtained were indistinguishable from those of authentic material. The chromatographic behaviour (in the systems given above) of the natural product was identical with that of authentic sample.

*Degradation of labelled 1,3-dihydroxy-4,5-dimethylbenzene.* The radioactive 1,3-dihydroxy-4,5-dimethylbenzene recovered from the preparative paper chromatograms was diluted with carrier 1,3-dihydroxy-4,5-dimethylbenzene (60 mg), and the whole was

recrystallized from chloroform-petroleum ether (40—60°), 2—3 recrystallizations being necessary to obtain a constant specific activity of the material. The total radioactivity of the phenol was determined by the wet combustion technique of van Slyke and Folch.[9] Kuhn-Roth oxidations (on 25 mg portions of the phenol) were carried out according to the method of Eisenbraun *et al.*;[10] acetic acid was recovered by steam distillation, and was further degraded by the Schmidt reaction as described by Phares.[11] All determinations of radioactivity were made in a liquid scintillation counter on barium carbonate samples (about 20 mg), suspended in 10 ml of 0.5 % diphenyloxazole in toluene with the aid of 400 mg of Cab-O-Sil gel.

*Decarboxylase activity.* In preliminary experiments 20 mg portions of different phenolic acids, dissolved in the minimal volume of ethanol, were added to seven days old shaken-flask cultures of *G. roseum*. Three days later the culture filtrates were acidified and thoroughly extracted with ether. The extracts were chromatographed on paper, using chloroform-methanol-formic acid-water as the solvent. After elution with ethanol the concentration of unchanged phenolic acid was measured spectrophotometrically (at the wavelength of maximum absorption in the ultra-violet). These studies showed that orsellinic acid and 2,4-dihydroxy-5,6-dimethylbenzoic acid had been metabolized completely, while 2,4-dihydroxybenzoic acid and 2-hydroxy-6-methylbenzoic acid were recovered in a yield of 91 and 96 %, respectively. The disappearance of the former two acids was found to be accompanied by a considerable increase in the concentration of the corresponding decarboxylation product.

More detailed studies on the disappearance of orsellinic acid, and the appearance of orcinol, were performed in a similar manner; at different times after the addition of definite quantities (5—100 mg) of orsellinic acid, 10 ml portions of the culture medium were withdrawn and analyzed as described above. Results of these experiments are listed in Table 3.

## DISCUSSION

Inspection of Table 1 shows that a small (5.3 %), but significant, fraction of the activity incorporated into 1,3-dihydroxy-4,5-dimethylbenzene from 1-$^{14}$C-acetate was present in the methyl groups. This could be explained as a result of processes by which the latter precursor labels the methyl group of acetate or the $C_1$-pool. The corresponding "conversion factors" ($k_1$ and $k_3$, respectively) may conveniently be used for a discussion of the quantitative effect of such processes; $k_1$ has been defined previously,[12] and analogously $k_3$ is defined as the activity incorporated from 1-$^{14}$C-acetate into a carbon atom derived from the $C_1$-pool, divided by the activity incorporated into a carbon atom derived from the carboxyl group of acetate. The results obtained on degradation of Kuhn-Roth acetic acid from 1,3-dihydroxy-4,5-dimethylbenzene, biosynthesized in the presence of 1-$^{14}$C-acetate, then give the following relation (compare Table 1 and Fig. 1):

$$\frac{k_1 + k_3}{1 + k_3} = \frac{0.17}{1.00} \qquad (1)$$

Results from previous radioactive tracer studies on acetate-polymalonate derived mould products show that $k_1$ and $k_3$ generally are negligible (0.00—0.05). However, in an investigation on the biosynthesis of aurantiogliocladin (V), the major secondary product in *G. roseum*, Birch *et al.* found that (in this organism) the $C_1$-pool was labelled to an unusually large extent from 1-$^{14}$C-acetate;[13] calculations on the experimental figures obtained give $k_1 = 0.02$ and $k_3 = 0.17$. This was confirmed by Bentley *et al.* ($k_3 = 0.18$) in their studies on the same mould product.[14] Obviously, labelling of the $C_1$-pool quantitatively

explains the incorporation of activity from 1-$^{14}$C-acetate into the methyl groups of 1,3-dihydroxy-4,5-dimethylbenzene; insertion of $k_1 = 0.02$ into eqn. (1) yields $k_3 = 0.15$, in excellent agreement with the values obtained on degradation of aurantiogliocladin.

The tracer experiments described seem to establish that the methyl group in position 4 of 1,3-dihydroxy-4,5-dimethylbenzene is derived from the $C_1$-pool, but do not indicate at which biosynthetic stage it is introduced. However, the observations that small amounts of 2,4-dihydroxy-5,6-dimethylbenzoic acid occasionally were present in cultures of *G. roseum*, and that the phenolic acid was submitted to decarboxylation when added to the culture medium, strongly indicates that it is also the natural immediate precursor of 1,3-dihydroxy-4,5-dimethylbenzene. It further seems likely that orsellinic acid functions as the immediate precursor of 2,4-dihydroxy-5,6-dimethylbenzoic acid, as suggested by Hassall *et al.* (see above).[5] The established presence of a strong and specific decarboxylase activity in the mould would then explain the failure to detect orsellinic acid, as well as the formation of orcinol, which was regularly present as a metabolic product of the mould.

As mentioned above, orsellinic acid has been found to be directly converted into orcinol in quinone producing strains of *A. fumigatus*. It was further shown that orsellinic acid functions as an intermediate in the quinone biosynthesis, whereas orcinol seemed to be a metabolic end-product.[4,15] In view of these results, the presence of orcinol and 1,3-dihydroxy-4,5-dimethylbenzene in quinone producing cultures of *G. roseum* might indicate that orsellinic acid and 2,4-dihydroxy-5,6-dimethylbenzoic acid are formed as intermediates in the biosynthesis of aurantiogliocladin. The probable biogenetic relationships of the different metabolic products isolated from *G. roseum* are shown in Fig. 1.

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