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On the Biosynthesis of Aurantiogliocladin

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The biogenetic role of possible aromatic intermediates in the formation of the acetate-polymalonate derived quinone aurantiogliocladin (I), which is produced along with two related pigments (X, XI) by a certain strain of *Gliocladium roseum*, has been investigated. The quinonoid pigments appear to be formed by autoxidation of the corresponding hydroquinones, in which form the pigments were found to be present during the larger part of the production phase; the major pigment, aurantiogliocladin, could be isolated in the hydroquinone form (II) from young cultures of the mould. Radioactive orsellinic acid (IV) and 2,4-dihydroxy-5,6-dimethylbenzoic acid (IX) were (in contrast to their decarboxylated derivatives V and VIII) found to be incorporated as units into aurantiogliocladin, giving support to the biogenetic pathway proposed in Fig. 1. Attempts to incorporate biologically ^{14}C-labelled 2,3-dihydroxy-5-methyl-1,4-benzoquinone, 2-hydroxy-3-methoxy-5-methyl-1,4-benzoquinone, 2-methoxy-3-hydroxy-5-methyl-1,4-benzoquinone(III), 2,3-dihydroxy-5,6-dimethyl-1,4-benzoquinone (X), 2-hydroxy-3-methoxy-5,6-dimethyl-1,4-benzoquinone (XI), and the corresponding hydroquinones into aurantiogliocladin were unsuccessful. No detectable amounts of radioactive products were formed when the mould was cultivated in the presence of carboxyl labelled orsellinic acid and 2,4-dihydroxy-5,6-dimethylbenzoic acid.

In 1951 Brian *et al.* isolated three new antibacterial materials, which were named aurantiogliocladin, rubrogliocladin, and gliorosein, from a certain strain of *Gliocladium roseum.*[1] Aurantiogliocladin (I) has been shown to be 2,3-dimethoxy-5,6-dimethyl-1,4-benzoquinone;[1,2] this structure was confirmed by chemical synthesis.[3] Rubrogliocladin is the quinhydrone corresponding to aurantiogliocladin, while gliorosein is one of the two possible dihydroaurantiogliocladins.[1,2]

The isolation of rubrogliocladin from the culture medium of *G. roseum* indicates that aurantiogliocladin is present partly in the quinone form, and partly in the hydroquinone form; quinhydrone complexes are known to be largely dissociated in dilute aqueous solution,[4] and rubrogliocladin may be considered as an artifact obtained in the isolation procedures rather than as a true metabolic product. The present author, in fact, observed that aurantiogliocladin was present exclusively in a reduced form during the first 10—20

days of cultivation; r_H-value determinations and iodometric titrations [5] of the pale-yellow filtrates from young *G. roseum* cultures gave no evidence for the presence of quinones, and extraction with petroleum ether at pH 12 yielded only trace amounts of aurantiogliocladin (extractions were carried out quickly and in the absence of air). However, when the culture medium was shaken for a few moments with air or oxygen at pH 8 the colour changed to deep yellow with simultaneous consumption of oxygen, indicating the formation of aurantiogliocladin. Confirmatively, the aerated medium showed a quinone concentration of 1—3 mM on iodometric titration, and corresponding amounts of aurantiogliocladin could be extracted with petroleum ether at pH 12. After treatment with a slight excess of sodium dithionite the aerated medium exhibited an ultraviolet absorption spectrum which was indistinguishable from that of the original culture filtrate. Aurantiogliocladin thus appeared to be present in the hydroquinone form in young cultures of the mould, and not in the form of a dihydro derivative such as gliorosein (see above). This was confirmed by the isolation of aurantiogliocladin hydroquinone (II) from freshly separated filtrates of 2 weeks old *G. roseum* cultures. No indications on the presence of gliorosein as a metabolic product have been obtained during this and other recent works with *G. roseum*.[6-8]

Further studies showed that aurantiogliocladin hydroquinone was successively converted into aurantiogliocladin after 10—20 days of cultivation; ether extractions of 2 weeks old *G. roseum* cultures usually yielded a mixture of aurantiogliocladin and rubrogliocladin. This conversion process was found to be dependent on the availability of oxygen from the air; when the mycelium of young cultures was removed from the medium by filtration aurantiogliocladin hydroquinone was quantitatively oxidized within a few hours, and the oxidation rate could be further increased by passing a stream of oxygen gas or air through the solution. Aeration of the culture filtrates containing aurantiogliocladin hydroquinone at pH-values above pH 6 led to an immediate and quantitative formation of aurantiogliocladin, while the rate of quinone formation was negligible below pH 1.5 (also when oxygen was supplied to the solution by aeration). The oxidation rate at pH 4 and 20° was not affected by preincubation of the culture filtrates at pH 0 and 100° for 10 min (in the absence of air). All of these observations clearly indicate that aurantiogliocladin is formed from the corresponding hydroquinone by a non-enzymatic air oxidation process.[9] Confirmatively, the rate of autoxidation of a 2 mM phosphate buffer solution of aurantiogliocladin hydroquinone corresponded well to the rate of quinone formation in freshly separated filtrates of young *G. roseum* cultures.

The reduction-oxidation potential of a quinone-hydroquinone system is known to be linearly related to the energy of the lowest unoccupied molecular orbital (ELUMO) of the quinone, and to the delocalisation energy change (ΔDE) on the conversion into the corresponding hydroquinone.[5] A calculation of these molecular orbital data for aurantiogliocladin, 2,3-dihydroxy-5,6-dimethyl-1,4-benzoquinone (X), and 2-hydroxy-3-methoxy-5,6-dimethyl-1,4-benzoquinone (XI) showed that the redox potential of each of the latter two quinones, which recently were isolated as minor secondary metabolites of aurantiogliocladin producing cultures of *G. roseum*,[6] was closely agreeing with

Table 1. Molecular orbital data for the quinone-hydroquinone systems present in *G. roseum.*

Derivative of 5,6-dimethyl-1,4-benzoquinone	$\frac{\text{ELUMO}-\alpha}{-\beta}$	$\frac{\Delta\text{DE}}{-\beta}$	$\frac{\text{EHOMO}-\alpha}{\beta}$
I 2,3-Dimethoxy- *	0.154	0.450	0.730
X 2-Hydroxy-3-methoxy-	0.152	0.448	0.734
XI 2,3-Dihydroxy-	0.150	0.438	0.744

* Aurantiogliocladin.

that of aurantiogliocladin; the differences were estimated to be lower than 0.02 V. It may, therefore, be concluded that also the two minor pigments are present in the hydroquinone form during the larger part of the production phase. Furthermore, the hydroquinone forms of the three pigments produced by *G. roseum* appear to be autoxidized at comparable rates; closely agreeing values were obtained for the three hydroquinones on compution of the energy of the highest occupied molecular orbital (EHOMO), which is known to be related to the autoxidation rate constant.[9] The results of the above molecular orbital calculations are given in Table 1.

Investigations on the biosynthesis of the toluquinones produced by *Aspergillus fumigatus* [10] have given analogous results; the latter pigments were found to be present in the hydroquinone form during the entire production phase, a non-enzymatic conversion into the corresponding hydroquinones taking place at a late stage in the development of the mould (immediately before autolysis begins).[5,9] For these reasons, there seems to be little doubt that autoxidation of the corresponding hydroquinones also is the final step in the biosynthesis of aurantiogliocladin and the related pigments produced by *G. roseum.*

The results of chemical degradations of aurantiogliocladin, biosynthesized in the presence of ^{14}C-labelled acetate, malonate, and formate, were consistent with the formation of a C_8 carbon precursor by the acetate-polymalonate pathway, followed by the loss of one carboxyl carbon and the introduction of one C-methyl group and two O-methyl groups from the C_1-pool (see Fig. 1).[8,11] Aurantiogliocladin is thus biogenetically related to fumigatin (III) from *A. fumigatus* (*cf.* Fig. 4).[12,13] In the latter mould quinone production was found to be accompanied by the formation of orsellinic acid (IV) and its decarboxylated product, orcinol (V); it appears well established that orsellinic acid, which was shown to be incorporated as a unit into fumigatin, functions as the natural precursor of the latter pigment.[14] However, attempts to detect orsellinic acid in the culture fluids of *G. roseum* have been unsuccessful.[7,8] Furthermore, Birch in a preliminary report has stated that ^{14}C-labelled 2-hydroxy-6-methylbenzoic acid (VI) was incorporated as a unit into aurantiogliocladin, while radioactive orsellinic acid was not so incorporated.[15] 2-Hydroxy-6-methylbenzoic acid (which contains one hydroxyl group less than orsellinic acid) has also been shown to be a likely C_8 carbon precursor of the acetate-polymalonate derived 2-methoxy-5-methyl-1,4-benzoquinone (VII) from *Lentinus degener.*[15] On the other hand, orsellinic acid must be regarded

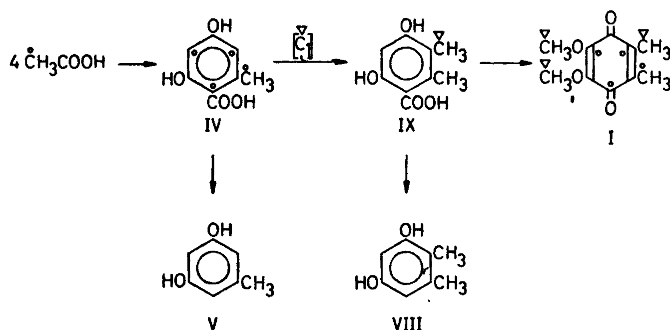

Fig. 1. Proposed pathway for the formation of aurantiogliocladin (I), the major secondary metabolite in *G. roseum.* Orcinol (V), 1,3-dihydroxy-4,5-dimethylbenzene (VIII), and 2,4-dihydroxy-5,6-dimethylbenzoic acid (IX) have been isolated as minor metabolic products of the mould.

as a more likely natural precursor of aurantiogliocladin than 2-hydroxy-6-methylbenzoic acid; as pointed out by Bentley and Lavate [8] it is difficult to understand why a hydroxyl group should be removed to form 2-hydroxy-6-methylbenzoic acid, and then reintroduced for aurantiogliocladin synthesis. A recent investigation gave, in fact, the first indication of a possible role for orsellinic acid in aurantiogliocladin biosynthesis, when orcinol (the decarboxylated product of orsellinic acid) was isolated from quinone producing cultures of *G. roseum;* a fairly strong decarboxylase activity was found to be present in the mould, which might explain the failure to detect orsellinic acid in the culture fluids.[7] Furthermore, 1,3-dihydroxy-4,5-dimethylbenzene (VIII) and 2,4-dihydroxy-5,6-dimethylbenzoic acid (IX) were isolated from the mould, and it was suggested that also the latter phenolic acid functions as a precursor of aurantiogliocladin.[7] The proposed biogenetic pathway, which is shown

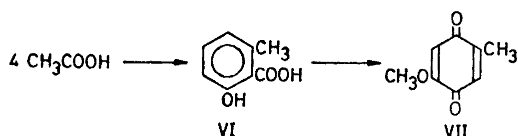

Fig. 2. Probable pathway for the formation of 2-methoxy-5-methyl-1,4-benzoquinone (VII) in *Lentinus degener.* 2-Hydroxy-6-methylbenzoic acid (VI) has also been claimed to be incorporated as a unit into aurantiogliocladin.

in Fig. 1, postulates the initial formation of orsellinic acid by an acetate-polymalonate condensation process, followed by the introduction of a nuclear methyl group from the C_1-pool to yield 2,4-dihydroxy-5,6-dimethylbenzoic acid. The latter compound is then converted into aurantiogliocladin hydroquinone by a sequence of reactions involving the removal of a carboxyl group and the introduction of two nuclear hydroxyl groups and two O-methyl groups.

This pathway is consistent with the results obtained in previous tracer studies on the biosynthesis of aurantiogliocladin (see above), and receives some support from recent experiments with flavipin producing strains of *Aspergillus flavipes*, which provided strong evidence for the intermediate formation of orsellinic acid and 2,4-dihydroxy-5,6-dimethylbenzoic acid in the biosynthesis of flavipin (1,2-diformyl-3,4,5-trihydroxy-6-methylbenzene).[16] The biogenetic pathway outlined in Fig. 1 has now been tested by radioactive tracer experiments, in which aurantiogliocladin was biosynthesized in the presence of ^{14}C-labelled orcinol, 1,3-dihydroxy-4,5-dimethylbenzene, orsellinic acid, and 2,4-dihydroxy-5,6-dimethylbenzoic acid, respectively.

Radioactive orcinol was found not to be metabolized by *G. roseum;* it could be recovered in almost quantitative amounts two days after the addition to shaken-flask cultures of the mould. The aurantiogliocladin isolated at this time was non-radioactive, and besides unchanged orcinol no labelled compounds could be detected on paper chromatographic examination of ethereal extracts of the cultures. Similarly, radioactive 1,3-dihydroxy-4,5-dimethylbenzene failed to be incorporated into aurantiogliocladin, or into any of the other secondary metabolites isolated from the mould. On the other hand, the latter precursor appeared to be metabolized by the mould, giving rise to one (not identified) radioactive product; this product was not formed when 1,3-dihydroxy-4,5-dimethylbenzene was added to culture filtrates of the mould.

Table 2. Chemical degradation of aurantiogliocladin derived from biologically ^{14}C-labelled orsellinic acid (0.07 % incorporation of label).

Material	Number of carbon atoms	Specific activity *	Total activity	Relative total activity
Aurantiogliocladin	10	7660	76 600	1.00
Tetramethylammonium iodide	4	80	320	0.00

* Counts per min and g $BaCO_3$.

Orsellinic acid, biologically ^{14}C-labelled from acetate, was mainly converted into orcinol (64 % incorporation yield) when added to *G. roseum* cultures; less than 0.005 % of the activity added was incorporated into 1,3-dihydroxy-4,5-dimethylbenzene. However, in contrast to the results obtained by Birch *et al.*[15] orsellinic acid was found to label aurantiogliocladin to a small, but significant, extent (0.07 % incorporation of label). Chemical degradation of the radioactive aurantiogliocladin obtained (see Table 2) established that essentially no activity was present in the methoxyl carbons, which are known to be derived from the C_1-pool. This observation strongly indicates that the precursor was directly (without primary degradation to labelled acetate) converted into aurantiogliocladin, since radioactive acetate has been shown to label the C_1-pool to an unusual large extent in *G. roseum.*[7,8,11]

Table 3. Chemical degradation of aurantiogliocladin derived from 2,4-dihydroxy-5,6-dimethylbenzoic acid, specifically ^{14}C-labelled at the methyl group in position 5 (0.9 % incorporation of label).

Material	Carbon atoms isolated	Specific activity *	Total activity	Relative total activity
Aurantiogliocladin	all	190	1900	1.00
Kuhn-Roth acetic acid, methyl group	7,8	890	1780	0.94
Kuhn-Roth acetic acid, carboxyl group	5,6	5	10	0.01

* Counts per min and mg $BaCO_3$.

2,4-Dihydroxy-5,6-dimethylbenzoic acid, synthetically ^{14}C-labelled at the methyl group in position 5, was similarly found to yield 1,3-dihydroxy-4,5-dimethylbenzene as the major product (32 % incorporation of activity). No label was incorporated into orcinol, whereas aurantiogliocladin contained 0.9 % of the activity added. Chemical degradation of the radioactive aurantiogliocladin obtained provided clear evidence that the precursor was incorporated as a unit; isotope was found to be located almost exclusively at the nuclear methyl groups of the symmetrical compound aurantiogliocladin (see Table 3).

The results of the above tracer experiments are, obviously, consistent with and give support to the biogenetic pathway shown in Fig. 1. In the hope of providing further evidence for this pathway, *G. roseum* was cultivated in the presence of orsellinic acid, synthetically ^{14}C-labelled at the carboxyl group; the precursor was labelled in this way in order to decrease the number of radioactive products and thus to facilitate the detection of products containing a carboxyl group (*e.g.* 2,4-dihydroxy-5,6-dimethylbenzoic acid). Samples of the culture were then withdrawn at fixed intervals until the orsellinic acid added had been metabolized completely, and were examined by paper chromatography with respect to the presence of labelled conversion products of the precursor. Unfortunately, it was not possible to detect any significant amounts of radioactive 2,4-dihydroxy-5,6-dimethylbenzoic acid (or of any labelled compounds besides unchanged orsellinic acid) in this experiment. A similar experiment, in which carboxyl labelled 2,4-dihydroxy-5,6-dimethylbenzoic acid was tested as a precursor, gave the same negative result; no evidence was obtained for the presence of detectable amounts of carboxyl group containing intermediates in the conversion of the latter phenolic acid into aurantiogliocladin hydroquinone.

The fact that 2,3-dihydroxy-5,6-dimethyl-1,4-benzoquinone (X) and its monomethyl ether (XI) are formed as minor metabolic products of *G. roseum* [6] indicates that the two O-methyl groups of aurantiogliocladin are introduced at a late biosynthetic stage, and suggests that the two minor pigments function as precursors of aurantiogliocladin. The O-methylation process would then be

expected to involve the hydroquinone forms of the pigments (see above), as shown in Fig. 3. (The possibility that the two hydroxyquinones are formed by a non-enzymatic demethylation of aurantiogliocladin [17] can be definitely excluded, since they remained non-radioactive when the mould was cultivated on a medium containing ^{14}C-labelled aurantiogliocladin or aurantiogliocladin

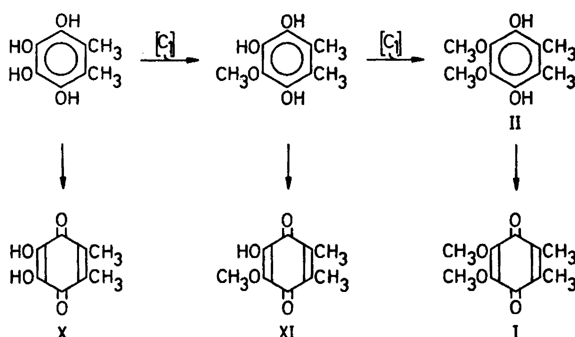

Fig. 3. Possible interrelationship of the different pigments produced by *G. roseum,*

hydroquinone). The reaction sequence indicated in Fig. 3 is analogous to the proposed pathway (Fig. 4) for the formation of fumigatin (III) by O-methylation of 2,3-dihydroxy-5-methyl-1,4-benzoquinone (XII),[18] and received some added support from determinations of the pigment concentrations in *G. roseum* cultures during the growth of the mould (see Table 4). Aurantiogliocladin hydroquinone was thus found to be formed at an almost linear rate for more than two weeks, whereas the concentration of the two hydroxyhydroquinones reached a maximum value within a few days of cultivation; preliminary experiments showed that the reduced forms of the different pigments were equally stable in the culture medium. 2,3-Dihydroxy-5,6-dimethyl-1,4-benzoquinone, 2-hydroxy-3-methoxy-5,6-dimethyl-1,4-benzoquinone, and the corresponding hydroquinones (biologically ^{14}C-labelled from acetate) were, therefore, tested as precursors of aurantiogliocladin in submerged as well as surface cultures of *G. roseum.* These experiments failed to provide any evidence for the proposed reaction sequence; the radioactive pigments added were

Table 4. Pigment concentration (μM) in the medium of shaken-flask cultures of *G. roseum* at different stages in the development of the mould.

	Derivative of 5,6-dimethyl-1,4-benzoquinone	Age of culture in days 4	6	8	10	15	20
I	2,3-Dimethoxy- *	0	100	300	650	1550	2300
X	2-Hydroxy-3-methoxy-	0	35	40	40	45	50
XI	2,3-Dihydroxy-	0	20	25	25	20	15

* Aurantiogliocladin.

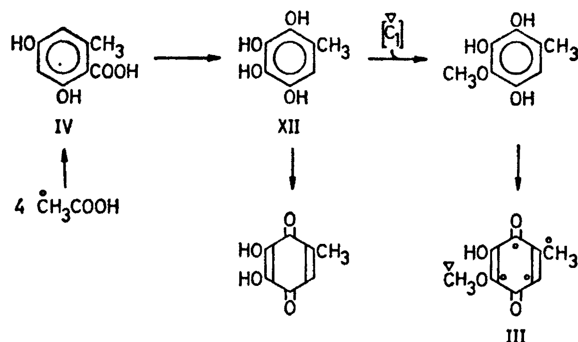

Fig. 4. Possible interrelationship of the two major toluquinonoid pigments produced by *Aspergillus fumigatus*. The figure also shows the established biogenetic origin of the different carbon atoms of fumigatin (III).

found not to be metabolized by the mould. This might, however, be due to technical difficulties with permeability, and does not exclude that the pigments are interrelated as shown in Fig. 3.

Biologically ^{14}C-labelled 2,3-dihydroxy-5-methyl-1,4-benzoquinone, 2-hydroxy-3-methoxy-5-methyl-1,4-benzoquinone, 2-methoxy-3-hydroxy-5-methyl-1,4-benzoquinone (III), and the corresponding hydroquinones (aurantiogliocladin can be formally derived from these metabolic products of *A. fumigatus*) also failed to be incorporated into aurantiogliocladin; the latter experiments were undertaken before 2,4-dihydroxy-5,6-dimethylbenzoic acid was recognized as a probable precursor of aurantiogliocladin.

The present investigation appears to have provided strong evidence for the formation of aurantiogliocladin by autoxidation of the corresponding hydroquinone, in its turn being derived from acetate-polymalonate by a sequence of reactions involving the intermediate formation of 2,4-dihydroxy-5,6-dimethylbenzoic acid; the latter compound is known to be produced by the mould,[7] and was shown to be incorporated as a unit into aurantiogliocladin. Considering that orcinol has been isolated as a metabolic product of *G. roseum*,[7] it further seems likely that orsellinic acid (which was found to be directly converted into aurantiogliocladin) functions as the immediate precursor of 2,4-dihydroxy-5,6-dimethylbenzoic acid. The established presence of a phenolic acid decarboxylase activity in the mould [7] might, in fact, explain the difficulties to provide evidence for the intermediate formation of benzoic acid derivatives in the biosynthesis of aurantiogliocladin, and also gives a probable explanation for the fact that not more than 0.07 % of the biologically ^{14}C-labelled orsellinic acid added was incorporated into aurantiogliocladin. On the other hand, it has not been possible to establish by which sequence of reactions 2,4-dihydroxy-5,6-dimethylbenzoic acid is converted into aurantiogliocladin hydroquinone. Further experiments, in which the enzymatic activities of *G. roseum* are studied on the cell-free level, are in progress and may give more detailed information about the latter biogenetic processes.

EXPERIMENTAL

Culture conditions. Gliocladium roseum, A. C. C. 650, was used throughout this work. The mould was cultivated as described previously,[6,7] with the exception of a minor change (omission of $FeSO_4$, $7H_2O$) in the composition of the Raulin-Thom medium used; this change was found to result in a more regular production of aurantiogliocladin when the mould was grown as submerged cultures. The isolation, paper chromatographic separation, and quantitative analysis (spectrophotometry) of the different secondary metabolites of the mould have been described elsewhere.[6,17,9]

Hydroquinone forms of the pigments. The techniques used in order to establish in which form the pigments are present during the growth of *G. roseum* (iodometric titrations and determinations of the r_H-value of the culture medium) have been described previously, in relation to similar studies on the biosynthesis of toluquinones in *Aspergillus fumigatus.*[5] When *G. roseum* was grown as surface cultures, or as submerged cultures in aerated fermenter tanks, aurantiogliocladin was found to be present exclusively in the hydroquinone form during the first 2—3 weeks of cultivation. Subsequently, a fairly slow conversion into the quinone form took place, and was completed after a further 3—6 days of growth. This oxidation process usually started at an earlier stage (after 6—10 days of cultivation) in shaken-flask cultures, where the mould grew slowly and often in a pellet form; it was a general experience that a weak growth of the mycelium led to a rapid formation of aurantiogliocladin.

Isolation of aurantiogliocladin hydroquinone. The filtered medium (3.4 l) from a 2 weeks old submerged aerated culture of *G. roseum* was strongly acidified by the addition of conc. hydrochloric acid (100 ml), and was extracted twice with an equal volume of ether. Removal of the ether in vacuum yielded on oily, pale-yellow residue, which was crystallized from hot water (5 ml) and recrystallized from 1 M hydrochloric acid (5 ml) to give aurantiogliocladin hydroquinone as colourless needles (260 mg) of m.p. 82—83°. The mixed m.p. with an authentic sample of aurantiogliocladin hydroquinone (m.p. 84°), prepared by reduction of aurantiogliocladin with sodium dithionite,[2] was 84°. 50 mg of the colourless crystals isolated were dissolved in 50 ml of an aqueous phosphate buffer solution, pH 8. The solution was vigorously aerated for 5 min, when it became orange in colour, and was extracted with petroleum ether (b.p. 40—60°). On removal of most of the solvent aurantiogliocladin crystallized as orange needles, m.p. 63—64°, not depressed on admixture with an authentic sample of the quinone.

Autoxidation of aurantiogliocladin hydroquinone. The rate of autoxidation of aurantiogliocladin hydroquinone in phosphate buffer solutions, and in the culture medium of *G. roseum*, was determined manometrically and spectrophotometrically, as previously described in investigations on the autoxidation of the toluhydroquinones produced by *Aspergillus fumigatus.*[9] No indications were obtained on the participation of enzymes in the hydroquinone oxidation in culture filtrates of *G. roseum*, where the availability of oxygen and the pH of the solution were found to determine the oxidation rate in the same manner as in phosphate buffer solutions of aurantiogliocladin hydroquinone (*cf.* Ref. 9).

Molecular orbital calculations. Molecular orbital calculations were performed on the undissociated forms of 2,3-dihydroxy-5,6-dimethyl-1,4-benzoquinone, 2-hydroxy-3-methoxy-5,6-dimethyl-1,4-benzoquinone, 2,3-dimethoxy-5,6-dimethyl-1,4-benzoquinone, and the corresponding hydroquinones. A detailed description of the methods used (parameter values *etc.*) has been given elsewhere.[5] Inspection of Table 1, where the results of the above calculations are listed, shows that the molecular orbital data of the two minor pigments produced by *G. roseum* correspond well to those of aurantiogliocladin, which strongly indicates that the three pigments have closely agreeing redox potentials [5] and reaction rate constants for the autoxidation of the hydroquinone forms.[9] The difference of 0.002 β (0.004 β) in the ELUMO values of aurantiogliocladin and 2-hydroxy-3-methoxy-5,6-dimethyl-1,4-benzoquinone (2,3-dihydroxy-5,6-dimethyl-1,4-benzoquinone) can, for example, be estimated to correspond to a difference of 0.008 V (0.016 V) in the redox potentials of the quinones, by using the regression line of redox potentials on ELUMO obtained in previous investigations.[5] It, therefore, appears that also the two minor pigments are present predominantly in the hydroquinone form in young *G. roseum* cultures; quinones-hydroquinones are known to form readily reversible redox systems, and the two minor pigments must, consequently, be in redox equilibrium

with aurantiogliocladin hydroquinone. Similarly, considering the established relationship between EHOMO and autoxidation rate constants [5] a comparison of the EHOMO values calculated for the hydroquinone forms of the pigments shows that the two hydroxy-hydroquinones are autoxidized almost equally rapidly as aurantiogliocladin hydroquinone; no enzymes are required for their conversion into the corresponding quinones.

Preparation of ^{14}C-labelled precursors. Radioactive 2,3-dihydroxy-5-methyl-1,4-benzoquinone, 2-hydroxy-3-methoxy-5-methyl-1,4-benzoquinone, and 2-methoxy-3-hydroxy-5-methyl-1,4-benzoquinone were prepared biosynthetically from 1-^{14}C-acetate, using *Aspergillus fumigatus*, L.S.H.T.M. A 46.[12,13] Biologically ^{14}C-labelled aurantiogliocladin was, similarly, obtained from *G. roseum*,[8] and was partly submitted to alkaline demethylation to give radioactive 2-hydroxy-3-methoxy-5,6-dimethyl-1,4-benzoquinone and 2,3-dihydroxy-5,6-dimethyl-1,4-benzoquinone.[17] The above quinones were converted into the corresponding hydroquinones by treatment with a slight excess of aqueous sodium dithionite, and the ^{14}C-labelled hydroquinones obtained were administered to *G. roseum* immediately after their preparation. The above radioactive pigments were also tested as precursors in the quinone form. 2,4-Dihydroxy-5,6-dimethylbenzoic acid (1,3-dihydroxy-4,5-dimethylbenzene), specifically ^{14}C-labelled at the methyl group in position 5 (4), carboxyl labelled orsellinic acid, and carboxyl labelled 2,4-dihydroxy-5,6-dimethylbenzoic acid were prepared by chemical synthesis, as described previously.[16] Biologically ^{14}C-labelled orsellinic acid was obtained from 1-^{14}C-acetate, using *Penicillium baarnense*.[12] A part of this orsellinic acid was submitted to decarboxylation to yield radioactive orcinol.[14]

Administration of the labelled precursors. In preliminary experiments about 1 μC of each of the ^{14}C-labelled precursors (see above) was added to 10 days old surface and shaken-flask cultures of *G. roseum*. Growth was continued for a further 2 days, when the cultures (medium and mycelium) were extracted with ether at pH 1. The ethereal extracts were separated on paper chromatograms,[7] and the incorporation of radioactivity into the different secondary metabolites of the mould was determined in a paper chromatogram scanner. Only two of the precursors tested (orsellinic acid and 2,4-dihydroxy-5,6-dimethylbenzoic acid) were found to be significantly incorporated into aurantiogliocladin. The experiments were then repeated with biologically ^{14}C-labelled orsellinic acid (10 mg; 3 μC) and 2,4-dihydroxy-5,6-dimethylbenzoic acid, specifically ^{14}C-labelled at the methyl group in position 5 (18 mg; 10 μC), using 10 days old shaken-flask cultures of *G. roseum*. Two days later radioactive aurantiogliocladin was isolated and degraded as described below.

Isolation and chemical degradation of radioactive aurantiogliocladin. The cultures of *G. roseum* that had been grown in the presence of radioactive orsellinic acid and 2,4-dihydroxy-5,6-dimethylbenzoic acid were filtered, and the pH of the filtrates was adjusted to pH 10 by the addition of dilute sodium hydroxide. The culture filtrates were then aerated for a few moments to oxidize aurantiogliocladin hydroquinone, and the resulting quinone was extracted with petroleum ether (b.p. 40—60°). After paper chromatographic purification [7] the radioactive aurantiogliocladin isolated (20—30 mg) was eluted from the chromatograms with petroleum ether, and was diluted with 30 mg of carrier aurantiogliocladin. On removal of most of the petroleum ether the quinone separated in a pure, crystalline form, m.p. 63°. The specific activity of the radioactive aurantiogliocladin obtained in this way remained constant on recrystallization from petroleum ether.

The numbering system used to refer to individual carbon atoms of aurantiogliocladin is shown in the degradation scheme outlined in Fig. 5; the degradation reactions were carried out as described by Bentley and Lavate.[8] The radioactive aurantiogliocladin

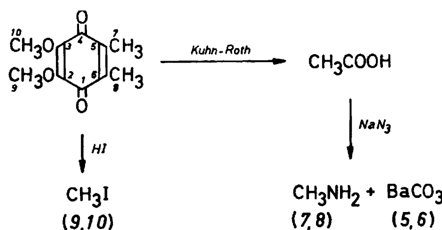

Fig. 5. Chemical degradation of aurantiogliocladin. Numbers within brackets indicate carbon atoms isolated by the degradation reactions.

derived from 2,4-dihydroxy-5,6-dimethylbenzoic acid (specifically ^{14}C-labelled at the methyl group in position 5) was thus submitted to Kuhn-Roth oxidation to give acetic acid, which was decarboxylated by the Schmidt reaction. Total combustion of the resulting methylamine showed that almost the whole of the radioactivity incorporated into aurantiogliocladin was located at the two nuclear methyl groups (C-7 and C-8). The results of this experiment, which are listed in Table 3, are obviously consistent with a direct conversion of the specifically labelled 2,4-dihydroxy-5,6-dimethylbenzoic acid into the symmetrical compound aurantiogliocladin.

As shown in Table 2, Zeisel demethylation of the radioactive aurantiogliocladin derived from biologically ^{14}C-labelled orsellinic acid established that less than 1 % of the total activity incorporated into aurantiogliocladin from the latter precursor was present in the two methoxyl carbons (C-9 and C-10). This observation strongly indicates that the precursor was incorporated as a unit (without primary degradation to labelled acetate), since radioactive acetate has been shown to yield labelled aurantiogliocladin in which each methoxyl group contains about 5 % of the total activity of the quinone.[8,11]

All determinations of radioactivity were made in a liquid scintillation counter on barium carbonate samples (40—100 mg) suspended in 10 ml of 0.5 % diphenyloxazole in toluene with the aid of 400 mg of Cab-O-Sil gel.

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