ACTA CHEMICA SCANDINAVICA 20 (1966) 151–158

On the Role of 6-Methylsalicylic Acid in the Biosynthesis of Fungal Benzoquinones

GÖSTA PETTERSSON

Institute of Biochemistry, University of Lund, Lund, Sweden

6-Methylsalicylic acid (I), biogenetically ^{14}C-labelled from acetate, has been tested as a precursor of the quinonoid pigments produced by *Lentinus degener*, *Gliocladium roseum*, *Aspergillus fumigatus*, and *Penicillium spinulosum*. The radioactive phenolic acid was not incorporated into any of the pigments (*e.g.* fumigatin; V) formed by *A. fumigatus* and *P. spinulosum*, and labelled aurantiogliocladin (IV) from *G. roseum* to a very small extent, only. In *L. degener*, however, 6-methylsalicylic acid was excellently utilized for pigment formation, and chemical degradation of radioactive 4-methoxy-6-hydroxy-2,5-toluquinone (VI; the major pigment in *L. degener*) derived from specifically ^{14}C-labelled 6-methylsalicylic acid established that the precursor was incorporated as a unit (above the acetate level). The biogenetic role of 6-methylsalicylic acid in the formation of fungal benzoquinones is discussed.

The benzoquinonoid pigments isolated from the moulds *Lentinus degener*,[1] *Gliocladium roseum*,[2] and *Aspergillus fumigatus*[3] have been shown to be biosynthesized by the acetate-polymalonate pathway, a process which might involve the intermediate formation of aromatic C_8 compounds such as 6-methylsalicylic acid (I) or orsellinic acid (II); the latter phenolic acids are known to be immediate products of acetate-polymalonate condensation reactions.[4] Theoretically, one would expect that 6-methylsalicylic acid functions as a precursor of the pigments produced by *L. degener*, which lack an oxygen function in position 3 of the 2,5-toluquinonoid nucleus, while orsellinic acid (which contains one hydroxyl group more than 6-methylsalicylic acid) would be the likely precursor of the quinones formed by *G. roseum* and *A. fumigatus* (*cf.* Fig. 1). Confirmatively, the present author has shown that orsellinic acid can be utilized for pigment formation in *A. fumigatus*,[5] and Birch in a preliminary report has stated that radioactive 6-methylsalicylic acid was incorporated as a unit into 4-methoxy-2,5-toluquinone (III) from *L. degener*.[6] However, Birch also found that 6-methylsalicylic acid (in contrast to orsellinic acid) was directly converted into aurantiogliocladin (IV) from *G. roseum*.[6] Furthermore, Packter and Steward recently reported of the incorporation of

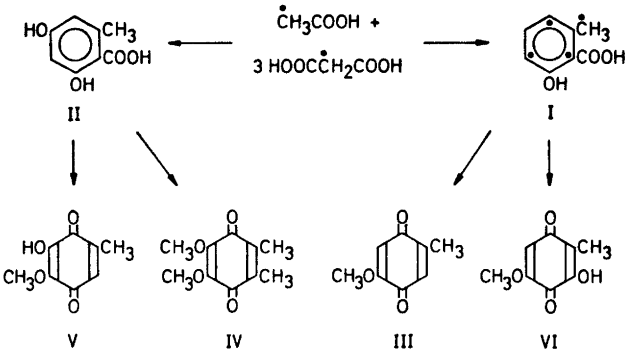

Fig. 1. Probable biogenetic relationships of the quinonoid pigments produced by *Aspergillus fumigatus* (V), *Gliocladium roseum* (IV), and *Lentinus degener* (III and VI).

6-methylsalicylic acid into fumigatin (V) from *A. fumigatus*.[7] As these observations might indicate that 6-methylsalicylic acid functions as a common natural precursor of the above quinones, and since they do not completely agree with the results obtained by the present author,[2,5] it was considered to be of importance to reinvestigate the biogenetic role of 6-methylsalicylic acid in the formation of fungal benzoquinones. The present work was, therefore, concerned with testing the possible precursor activity of radioactive 6-methylsalicylic acid (biosynthetically ^{14}C-labelled from acetate) in quinone producing strains of *L. degener*, *G. roseum*, *A. fumigatus*, and *Penicillium spinulosum;* the latter mould is closely related to *A. fumigatus* with respect to the formation of secondary metabolites.[8] The incorporation patterns were determined by scanning paper chromatograms of ethereal extracts of the cultures in a strip-counter.

The investigations showed that 6-methylsalicylic acid was excellently utilized for pigment formation in *L. degener*. The experiment was, therefore, repeated with the latter mould, using specifically ^{14}C-labelled 6-methylsalicylic acid as the precursor. The distribution of the radioactivity incorporated into the major pigment, 4-methoxy-6-hydroxy-2,5-toluquinone (VI), was then determined by chemical degradation, and was compared with the pattern of incorporation of radioactive acetate. Specifically labelled 6-methylsalicylic acid was obtained by biosynthesis from 2-^{14}C-malonate using *Penicillium urticae* (*cf.* Ref. 9); 2-^{14}C-malonate is known to label the phenolic acid at the carbon atoms in position 1, 3, and 5, whereas the "starter" C_2 unit (the methyl group and adjacent ring carbon atom) of the acetate-polymalonate derived molecule remains non-radioactive (see Fig. 1).[10]

EXPERIMENTAL

Culture conditions. The culture conditions used for the different quinone producing moulds have previously been described in detail. *Lentinus degener*, MNHN 1027,[1] and *Gliocladium roseum*, CMI 93065,[2] were cultivated submerged in shaken Erlenmeyer flasks on a Raulin-Thom solution, whereas *Aspergillus fumigatus*, LSHTM A 46,[3] and

Penicillium spinulosum, CMI 91950,[8] were grown as surface cultures on Raulin-Thom and Czapek-Dox medium, respectively.

Preparation and chemical degradation of radioactive 6-methylsalicylic acid. 6-Methylsalicylic acid, biosynthetically labelled from 1-^{14}C-acetate (1.0 mC), was obtained from *Penicillium urticae* Bainer, CBS R 909, as described by Gatenbeck and Lönnroth.[11] Specifically labelled 6-methylsalicylic acid was prepared in a similar way from 0.2 mC of diethyl 2-^{14}C-malonate (*cf.* Ref. 10). For determination of the distribution of radioactivity 5 mg of the isolated malonate-labelled 6-methylsalicylic acid were diluted with 55 mg of carrier 6-methylsalicylic acid, and the whole was recrystallized twice from water and finally sublimed (140°) at reduced pressure (1 mm Hg). The total activity of the compound was determined by the wet combustion technique of van Slyke and Folch.[12] The activity of the carboxyl group was obtained by decarboxylation of the acid (15 mg) in freshly distilled quinoline (5 ml) at 180° in the presence of copper chromite (50 mg); the carbon dioxide evolved was collected as barium carbonate. Kuhn-Roth oxidations of 6-methylsalicylic acid (30 mg) were carried out according to the method of Eisenbraun *et al.*,[13] and yielded quantitatively carbon dioxide and acetic acid. The carbon dioxide was collected as barium carbonate, and the acetic acid was recovered by steam distillation, the fractions being titrated with carbonate free sodium hydroxide to pH 8.7. After evaporation of the water the activity of the residual sodium acetate was determined by total combustion (see above). All determinations of radioactivity in this work were performed in a liquid scintillation counter on barium carbonate samples (20–40 mg) suspended in 10 ml of a 0.5 % solution of diphenyloxazole in toluene with the aid of 400 mg of Cab-O-Sil gel. The results of the chemical degradation of malonate-labelled 6-methylsalicylic acid are given in Table 1.

Table 1. Distribution of radioactivity in 6-methylsalicylic acid derived from 2-^{14}C-malonate.

Degradation reaction	Number of carbon atoms	Specific activity *	Total activity	Relative total activity
Total combustion	8	720	5760	1.00
Decarboxylation	1	10	10	0.00
Kuhn-Roth oxidation; carbon dioxide	6	915	5490	0.95
Kuhn-Roth oxidation; acetic acid	2	105	210	0.036

Administration of radioactive precursors. 6-Methylsalicylic acid, biologically ^{14}C-labelled from acetate (11 mg; 2 μC), was added to the medium of 4 days old cultures of *L. degener*, *G. roseum*, and *A. fumigatus*, and to a 7 days old culture of *P. spinulosum*. In each case growth was allowed to continue for 2 days in the presence of the labelled precursor. The cultures were then filtered and the filtrates thoroughly extracted with ether at pH 1. The concentrated ethereal extracts were chromatographed on Whatman No. 1 paper strips, using the solvent systems "B", "D", and "E" of Reio,[14] as well as propanol-butanol-2 M ammonium hydroxide (6:1:3 by vol.); the latter system is particularly suitable for the separation of benzoquinone derivatives.[15] For detection of radioactive compounds the air-dried chromatograms were scanned in a strip-counter.

Malonate-labelled 6-methylsalicylic acid (15 mg; 3 μC), 1-^{14}C-acetate (0.1 mC), and 2-^{14}C-acetate (0.1 mC), which were used as precursors of 4-methoxy-6-hydroxy-2,5-toluquinone, were administered to shaken-flask cultures of *L. degener* on the 4th day of growth, and radioactive 4-methoxy-6-hydroxy-2,5-toluquinone was isolated 2 days later.

* Counts per min and mg $BaCO_3$.

Identification of radioactive products. Only one labelled compound, chromatographically identified as unchanged 6-methylsalicylic acid, could be detected in the ethereal extracts of *P. spinulosum* cultures that had been grown in the presence of radioactive 6-methylsalicylic acid. The identity was further established by reisolation of the added 6-methylsalicylic acid (6 mg were obtained after purification by sublimation); the specific activity of the reisolated 6-methylsalicylic acid corresponded well to that of the added sample. Unchanged 6-methylsalicylic acid was, similarly, identified as the major labelled compound in the extracts of *A. fumigatus* and *G. roseum*.

Radioactive 6-methylsalicylic acid was not incorporated into any one of the toluquinonoid pigments produced by *A. fumigatus*, but significantly labelled aurantiogliocladin from *G. roseum*. The radioactive aurantiogliocladin was eluted from the chromatograms with acetone, and 25 mg of carrier aurantiogliocladin were added. After removal of most of the solvent aurantiogliocladin crystallized in a pure form; the specific activity (15 cpm/mg) remained constant on recrystallization from petroleum ether (b.p. 40–60°). Alkaline demethylation [16] of the labelled aurantiogliocladin yielded a radioactive product that was chromatographically identical with 2-methoxy-3-hydroxy-5,6-dimethyl-1,4-benzoquinone in the solvent systems given above. No detectable counts were present in the methyl iodide (isolated as tetramethylammonium iodide) obtained on Zeisel demethylation of the radioactive aurantiogliocladin; the latter reaction was carried out as described elsewhere.[17]

In *L. degener* 6-methylsalicylic acid was found to be metabolized almost completely, mainly being decarboxylated to *m*-cresol. The latter compound was identified by its chromatographic behaviour in the solvent systems given above, and by its colour reactions with the phenolic reagents described by Reio.[14] All of the minor labelled products of 6-methylsalicylic acid in *L. degener* could be chromatographically identified as pigments. The identities were established by elution of the pigments from the preparative chromatograms, followed by rechromatography in the different solvent systems described above. In each case radioactivity was found to appear at the same R_F-values as the pigment eluted.

Table 2. Distribution of radioactivity in 4-methoxy-6-hydroxy-2,5-toluquinone derived from malonate-labelled 6-methylsalicylic acid (MSA), 1-^{14}C-acetate (1-Ac), and 2-^{14}C-acetate (2-Ac).

Material	Number of carbon atoms	Specific activity *	Total activity	Relative total activity (%)
Precursor: MSA				
4-Methoxy-6-hydroxy-2,5-toluquinone	8	192	1536	100
Kuhn-Roth acetic acid	2	34	68	4.4
Tetramethylammonium iodide	4	2	8	0.5
Precursor: 1-Ac				
4-Methoxy-6-hydroxy-2,5-toluquinone	8	576	4608	100
Kuhn-Roth acetic acid	2	690	1380	30
Precursor: 2-Ac				
4-Methoxy-6-hydroxy-2,5-toluquinone	8	830	6640	100
Kuhn-Roth acetic acid	2	805	1610	24

* Counts per min and mg $BaCO_3$.

Isolation and chemical degradation of radioactive 4-methoxy-6-hydroxy-2,5-toluquinone. The radioactive 4-methoxy-6-hydroxy-2,5-toluquinone derived from malonate-labelled 6-methylsalicylic acid and from 1- and 2-^{14}C-acetate was isolated by ether extraction and purified by paper chromatography as described previously.[1] After dilution with 40—50 mg of carrier 4-methoxy-6-hydroxy-2,5-toluquinone the pigment was recrystallized from acetic acid to constant specific activity, and was finally sublimed at 130° in vacuum (1 mm Hg). The total activity of the pigment was determined by the wet combustion technique of van Slyke and Folch.[12] The activity of the methyl group and adjacent ring carbon atom was determined by total combustion of the acetic acid obtained on Kuhn-Roth oxidation, which was carried out as described above for 6-methylsalicylic acid. The activity of the methoxyl carbon was obtained by Zeisel demethylation of the pigment, as described above for aurantioglioclad in. The results of these radioactivity determinations are listed in Table 2.

RESULTS AND DISCUSSION

The addition of radioactive 6-methylsalicylic acid (I) to *L. degener* was found to result in the production of labelled *m*-cresol (25 % incorporation of activity), 4-methoxy-6-hydroxy-2,5-toluquinone (VI; 6.5 %), and 4-methoxy-2,5-toluquinone (III; 0.8 %). Small amounts of activity were also found to be associated with two minor, un-characterized, pigments that normally are formed by the mould, but no further labelled compounds could be detected on chromatographic examination of the culture extracts. There is no doubt that the radioactive *m*-cresol was formed by a direct decarboxylation of the added precursor; *m*-cresol is not present in detectable amounts in *L. degener* under the ordinary culture conditions.[1] Furthermore, quinone producing moulds are known to be capable of removing the carboxyl group from phenolic acids such as orsellinic acid (II) and 2,4-dihydroxy-5,6-dimethylbenzoic acid,[5,8,18] and a similar decarboxylase system (acting on 6-methylsalicylic acid) may be expected to be present in *L. degener;* a step of decarboxylation is, obviously, involved in the biosynthesis of the acetate-polymalonate derived pigments produced by the mould.[6] On the other hand, it is not evident that the added radioactive 6-methylsalicylic acid was directly incorporated into the different pigments, since the latter compounds might have been labelled *via* the acetate pool or the C_1-pool, after primary degradation of the radioactive precursor. In order to distinguish between these possibilities the experiment had to be repeated, using specifically labelled 6-methylsalicylic acid as the precursor.

As shown in Table 1, the results obtained on chemical degradation of the radioactive 6-methylsalicylic acid prepared from 2-^{14}C-malonate confirmed that isotope was located as expected (see above). Only a minor fraction (3.6 %) of the total activity was present in the methyl group and adjacent ring carbon atom, isolated as acetic acid after Kuhn-Roth oxidation; the k_m-value [9] calculated was 0.11. Administration of this malonate-labelled 6-methylsalicylic acid to *L. degener* resulted in the formation of radioactive 4-methoxy-6-hydroxy-2,5-toluquinone with a corresponding comparative lack of activity in the methyl group and adjacent ring carbon atom, which were found to contain 4.4 % of the total activity incorporated; no activity was present in the methoxyl carbon (see Table 2). On the other hand, Kuhn-Roth oxidation of radioactive 4-methoxy-6-hydroxy-2,5-toluquinone derived from 1-^{14}C-

acetate (2-^{14}C-acetate) yielded acetic acid containing approximately one-third (one-fourth) of the total activity, confirming that the pigment is synthesized by the acetate-polymalonate pathway. It may, for these reasons, be concluded that the malonate-labelled 6-methylsalicylic acid was incorporated as a unit into 4-methoxy-6-hydroxy-2,5-toluquinone, without primary degradation to labelled acetate or C_1 units.

The above results are in consistence with those obtained by Birch,[6] who reported that 6-methylsalicylic acid was incorporated intact into 4-methoxy-2,5-toluquinone (the first quinonoid metabolite to be isolated from *L. degener*). For these reasons, and considering the fact that 6-methylsalicylic acid can be synthesized by the mould (it was recently isolated from a non-pigmented strain of *L. degener* [1]), it seems likely that 6-methylsalicylic acid functions as the natural precursor of the toluquinones produced by *L. degener*.

The radioactive 6-methylsalicylic acid added to *A. fumigatus*, *P. spinulosum*, and *G. roseum* remained essentially unmetabolized by the moulds, and could be recovered in almost quantitative yields after two days of incubation. Paper chromatographic examinations of the culture filtrates gave no evidence for the presence of any further labelled compounds in *P. spinulosum*, whereas 6-methylsalicylic acid was found to be converted into two minor labelled products (less than 2 % incorporation of activity) in *A. fumigatus*, as well as in *G. roseum*. The two radioactive products formed by *A. fumigatus* have not been identified, but were chromatographically distinct from the pigments isolated; also previous attempts to incorporate 6-methylsalicylic acid into the pigments (fumigatin (V) and related toluquinones) formed by *A. fumigatus* and *P. spinulosum* have been unsuccessful.[5]

The above results with *A. fumigatus* are in contrast to those obtained by Packter and Steward,[7] who found that 6-methylsalicylic acid was effectively incorporated into fumigatin (1.3 % incorporation of activity; 6740 cpm/mg) and to a much lesser extent into fumigatin hydroquinone (85 cpm/mg); orsellinic acid, which is known to be formed by the mould,[5] was also found to be labelled. The results were claimed to show that 6-methylsalicylic acid can be directly converted into orsellinic acid and fumigatin in *A. fumigatus*, and 6-methylsalicylic acid was further proposed to inhibit the synthesis of fumigatin hydroquinone, in order to explain the comparative lack of incorporation of activity into this metabolite. The observation that cultures supplemented with 6-methylsalicylic acid produced only one-sixth of the normal yield of fumigatin hydroquinone, whereas the fumigatin content remained unchanged, was taken as evidence in this direction.

However, it seems unlikely that fumigatin and the corresponding hydroquinone can be differentially synthesized and labelled by the mould. Biogenetically, the two compounds must be regarded as different oxidation states of the same metabolite, being in immediate and reversible dynamic equilibrium with each others, and with other redox systems present in the mould culture.[19] Variations of the relative amounts of the oxidized and the reduced form of the pigment are thus a result of corresponding changes in the r_H-value of the culture medium (which is affected by factors such as culture conditions, availability of oxygen from the air, age and growth of the culture), and do not indicate that the two compounds are formed at different rates, inde-

pendently of each others. It has, in fact, been shown that fumigatin hydroquinone must be considered as the primary metabolic product, being converted into fumigatin by a non-enzymatic air oxidation process.[20] Normally, this process takes place at a very late stage in the development of the mould, but the addition of 6-methylsalicylic acid (in the presence of which *A. fumigatus* was observed to grow poorly) would be expected to increase the rate of fumigatin formation (increased availability of oxygen) and to decrease the total yield of pigments (poor growth), as described by Packter and Steward. It may, therefore, be concluded that the observed difference in labelling of fumigatin and its hydroquinone cannot be due to an inhibition of the synthesis of the hydroquinone form of the pigment; a more likely explanation is that the fumigatin isolated might have contained small amounts of some closely related, highly labelled, impurity. Consequently, it appears that the observed fairly low incorporation of activity into fumigatin hydroquinone gives a measure of the actual utilization of 6-methylsalicylic acid for pigment formation in *A. fumigatus*.

It appears, in fact, well established that orsellinic acid functions as the natural precursor of the pigments produced by *A. fumigatus* and *P. spinulosum*. Orsellinic acid is known to be formed by the moulds, and has been found to be incorporated as a unit into fumigatin.[5,8] Furthermore, short-term studies of the utilization of radioactive acetate for quinone synthesis in *A. fumigatus* have provided strong evidence for the intermediate formation of orsellinic acid.[3,5] In view of these results it seems most unlikely that 6-methylsalicylic acid is involved in the biosynthesis of toluquinones in *A. fumigatus* and *P. spinulosum*.

The major (about 1 % incorporation of label) conversion product of radioactive 6-methylsalicylic acid in *G. roseum* has not been characterized; it was found to be chromatographically distinct from *m*-cresol, as well as from all of the secondary metabolites that normally are formed by the mould.[2] The minor labelled product was, however, identified as aurantiogliocladin (IV), and contained 0.04 % of the activity added. Zeisel demethylation of the radioactive aurantiogliocladin isolated showed that no significant amounts of activity were present in the two methoxyl carbons, which might indicate that the precursor was incorporated above the acetate level; the methoxyl carbons are derived from the C_1-pool, which is known to be labelled from radioactive acetate to an unusual large extent in *G. roseum*.[6,17,21] The same results were obtained by Birch, who concluded that 6-methylsalicylic acid can be directly converted into aurantiogliocladin by the mould; the detailed experimental data have not been published.[6] In the present work, however, the specific activity of the isolated aurantiogliocladin was very low (15 cpm/mg). Radioactivity determinations after chemical degradation were, therefore, ambiguous and it cannot be definitely excluded that aurantiogliocladin was labelled after primary degradation of the radioactive 6-methylsalicylic acid to acetate.

Orsellinic acid has, similarly, been shown to be poorly incorporated (0.07 %) into aurantiogliocladin, without labelling the methoxyl carbons of the pigment.[2] However, while 6-methylsalicylic acid remained essentially unmetabolized by the mould during the two days of incubation, orsellinic acid was

found to be rapidly decarboxylated to orcinol. This might indicate that orsellinic acid is the natural precursor of aurantiogliocladin, since a similar decarboxylation of the added precursor takes place in *A. fumigatus* [5] and *P. spinulosum* [6] (orsellinic acid), as well as in *L. degener* (6-methylsalicylic acid; see above). Orcinol has, in fact, been observed to be produced along with aurantiogliocladin in *G. roseum*,[21] supporting the idea that the latter pigment is formed *via* orsellinic acid. Anyhow, there is for the present no conclusive evidence for the participation of 6-methylsalicylic acid in the biosynthesis of quinones in *G. roseum*.

To summarize the above results, it seems most likely that 6-methylsalicylic acid functions as the natural precursor of the toluquinones produced by *L. degener*. 6-Methylsalicylic acid can also possibly be directly converted into pigments in *G. roseum*, as well as in the certain strain of *A. fumigatus* studied by Packter and Steward, but appears not to be a natural intermediate in the biogenetic pathways leading to toluquinones with an oxygen function in position 3 (*A. fumigatus*, *P. spinulosum*, and *G. roseum*).

REFERENCES

1. Pettersson, G. *Acta Chem. Scand.* **20** (1966) 45.
2. Pettersson, G. *Acta Chem. Scand.* **19** (1965) 1827.
3. Pettersson, G. *Acta Chem. Scand.* **19** (1965) 543.
4. Glover, J. *Biochemistry of Quinones*, Academic Press, London and New York 1965, p. 207.
5. Pettersson, G. *Acta Chem. Scand.* **18** (1964) 1202.
6. Birch, A. J. *CIBA Foundation Symposium on Quinones in Electron Transport*, London 1961, p. 233.
7. Packter, N. M. and Steward, M. W. *Biochem. J.* **95** (1965) 4P.
8. Pettersson, G. *Acta Chem. Scand.* **19** (1965) 1016.
9. Pettersson, G. *Acta Chem. Scand.* **18** (1964) 335.
10. Bu'Lock, J. D., Smalley, H. M. and Smith, G. N. *J. Biol. Chem.* **237** (1962) 1778.
11. Gatenbeck, S. and Lönnroth, I. *Acta Chem. Scand.* **16** (1962) 2298.
12. van Slyke, D. D. and Folch, J. *J. Biol. Chem.* **136** (1940) 509.
13. Eisenbraun, E. J., McElvain, S. M. and Aycock, B. F. *J. Am. Chem. Soc.* **76** (1954) 607.
14. Reio, L. *J. Chromatog.* **1** (1958) 338.
15. Pettersson, G. *J. Chromatog.* **12** (1963) 352.
16. Pettersson, G. *Acta Chem. Scand.* **18** (1964) 2309.
17. Bentley, R. and Lavate, W. V. *J. Biol. Chem.* **240** (1965) 532.
18. Pettersson, G. *Acta Chem. Scand.* **19** (1965) 2013.
19. Pettersson, G. *Acta Chem. Scand.* **18** (1964) 1428.
20. Pettersson, G. *Acta Chem. Scand.* **18** (1964) 1839.
21. Pettersson, G. *Acta Chem. Scand.* **19** (1965) 414.

Received October 9, 1965.