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THE BIOSYNTHESIS OF PHENOLS—XVII.[1]

SOME PHENOLIC METABOLITES OF MUTANT STRAINS OF *ASPERGILLUS REGULOSUS*

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Abstract—Three metabolites which were isolated from different mutant strains of *Aspergillus rugulosus* have been identified as 2,4-dihydroxy-6-methylbenzaldehyde (III), 2,4-dihydroxy-6-(hydroxymethyl)-benzaldehyde (IV), and 3,3′-dihydroxy-5,5′-dimethyldiphenyl ether (V).

IN AN earlier study[2] we established that *Aspergillus rugulosus* I.M.I. 84338, growing in liquid culture, produced a variety of complex phenolic compounds. Moreover, there were distinct differences in the composition of the mixtures of phenols formed by the wild-type and by particular mutants that were derived from it by u.v. irradiation or by use of the mutagen, ethyl methanesulphonate. We have undertaken an investigation of the phenolic metabolites of these mutant strains. It was to be expected that, as in a related study utilizing *A. terreus*,[3] comparison of the molecular structures of phenolic metabolites of different blocked mutants would make it possible to define stages in the biosynthesis of complex phenolic compounds by *A. rugulosus*. The elucidation of the structures of two metabolites, asperugin A, B (I, II), produced by a mutant with specific "fluffy" morphology has been described.[4,5] In what follows, investigations which led to the determination of the molecular structures of three compounds, the major phenolic metabolites of three distinct mutants of *A. rugulosus*, are outlined.

The Phenol $C_8H_8O_3$

This compound, m.p. 176–177°, was extracted with ether from the culture medium of a mutant designated *A. rugulosus*, A.R.M. 505. The purple colour with diazotized *o*-dianisidine and the u.v. spectrum (Table 1), in particular the bathochromic shift in alkali, were characteristic of a phenol. Bands at 3140 (broad) and 1632 cm^{-1} in the i.r. spectrum were attributable to the presence of a strongly hydrogen-bonded, *ortho*-hydroxy-carbonyl system.[6]

The mass spectrum was particularly informative. The existence of an intense P-1 peak (100 per cent) suggested the presence of an aldehyde group. An analysis of the metastable transitions established that the (P-1) ion lost three molecules of carbon monoxide in succession

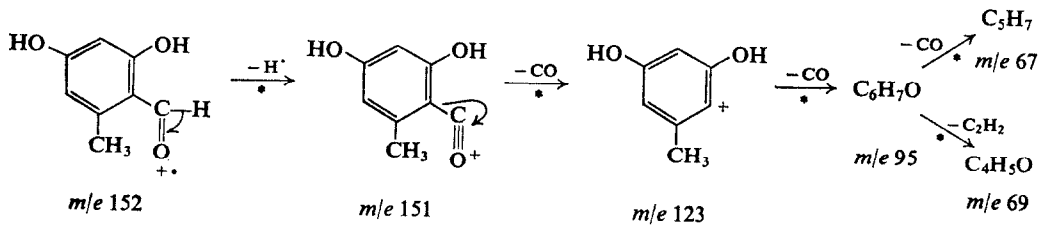
[1] Part XVI of this series. R. F. CURTIS, C. H. HASSALL and R. K. PIKE, *J. Chem. Soc.* (C), 1968, in press.
[2] C. H. HASSALL and K. LAWRENCE, *J. Gen. Microbiol.* **35**, 483 (1964).
[3] R. F. CURTIS, P. C. HARRIES, C. H. HASSALL and J. D. LEVI, *Biochem. J.* **90**, 43 (1964).
[4] J. A. BALLANTINE, C. H. HASSALL and G. JONES, *J. Chem. Soc.* 4672 (1965).
[5] J. A. BALLANTINE, C. H. HASSALL, B. D. JONES and G. JONES, *Phytochem.* **6**, 1157 (1967).
[6] D. W. CAMERON, P. M. SCOTT and LORD TODD, *J. Chem. Soc.* 42 (1964).

(Scheme I). This is a process which occurs commonly in the fragmentation of polyhydric phenols. The analysis of metastable ions also indicated that the parent ion could expel a molecule of formic acid; this suggested, on analogy with the case of salicylaldehyde, that the compound $C_8H_8O_3$ had adjacent hydroxyl and formyl groups (Scheme II).

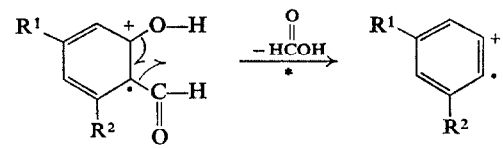
TABLE 1. ULTRAVIOLET ABSORPTION SPECTRA OF METABOLITES OF *A. rugulosus*, AND RELATED COMPOUNDS

Compound		λ_{max} nm (log ϵ_{max})
Metabolite, $C_8H_8O_3$	In ethanol	220 (4·07), 233 (3·95), 291 (4·20), 323 sh (3·92)
Benzadlehyde	In ethanol	244 (4·17), 280 (3·17), 328 (1·30)
o-Orsellinaldehyde (synthetic)	In ethanol	220 (4·09), 233 (3·98), 291 (4·25), 324 (3·94)
Metabolite, $C_8H_8O_4$	In ethanol	217 (3·75), 235 (3·60), 288 (3·81), 322 sh (3·57)
Metabolite, $C_8H_8O_3$	In ethanol–sodium hydroxide	216 (4·64), 251 sh (3·72), 337 (4·36)
o-Orsellinaldehyde (synthetic)	In ethanol–sodium hydroxide	216 (4·58), 251 sh (3·75), 337 (4·36)
Metabolite, $C_8H_8O_4$	In ethanol–sodium hydroxide	225 (4·24), 250 sh (3·66), 335 (4·03)

Electron-Induced Fragmentation of o-*Orsellinaldehyde*



SCHEME I.



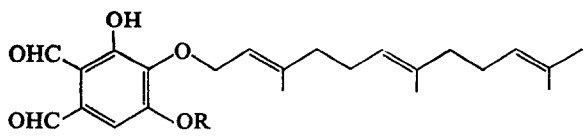
$R^1=R^2=H$ *m/e* 122 *m/e* 76

$R^1=OH, R^2=CH_3$ *m/e* 152 *m/e* 106

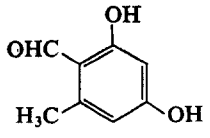
SCHEME II.

The spectroscopic evidence and the observation that both orcinol and *o*-orsellinic acid occurred in cultures of *A. rugulosus*[7] led us to compare the metabolite $C_8H_8O_3$ with a sample of synthetic *O*-orsellinaldehyde (III). They were identical in all respects.

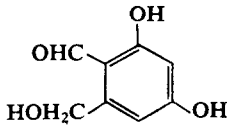
[7] G. JONES, unpublished work.



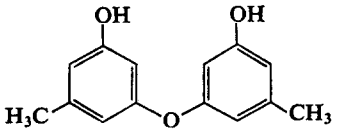
(I) R=Me
(II) R=H



(III)



(IV)



(V)

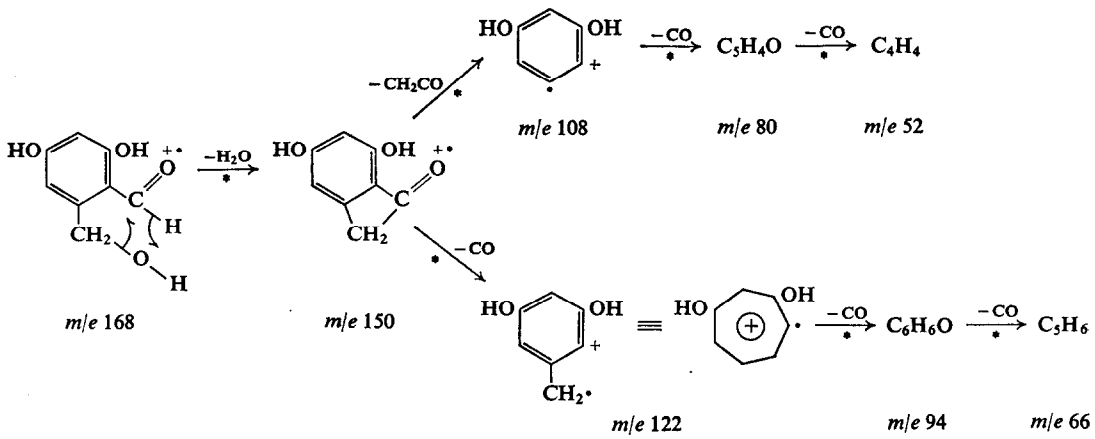
The Phenol $C_8H_8O_4$

This phenolic metabolite of the mutant designated *A. rugulosus* A.R.M. 884 was isolated from the culture fluid by extraction with ether. The crude extract was purified by chromatography on silica gel to give a solid that decomposed so rapidly in air that microanalysis by combustion did not give reproducible results. The molecular formula, $C_8H_8O_4$, was established by high resolution mass spectrometry. The u.v. spectrum was very similar to that of the previous compound $C_8H_8O_3$ (Table 1).

The PMR spectrum (Table 2) indicated that the molecule contained an unsymmetrical pair of *meta*-coupled aromatic protons, an aldehyde function, and the grouping $Ar \cdot CH_2 \cdot OH$. This, and a detailed study of the high resolution mass spectrum, led to the conclusion that the metabolite had the structure (IV).

The mass spectrum of the metabolite, $C_8H_8O_4$, was in accord with the structure (IV); in particular, it indicated that formyl and hydroxymethyl groups were attached to adjacent positions on the benzene ring (Scheme (III)).

Electron Induced Fragmentation of the Metabolite, $C_8H_8O_4$ *(IV)*



SCHEME III.

TABLE 2. PMR CHARACTERISTICS OF METABOLITE $C_8H_8O_4$, IN NaOD SOLUTION

Chemical shift (τ)	No. of protons	Assignment
−0·28 (*s*)	1	Ar·C*H*O
3·29 (*d*, *J*=2 c/s)	1	Ar·*H*
3·52 (*d*, *J*=2 c/s)	1	Ar·*H*
4·88 (*s*)	2	Ar·C*H*$_2$·OH
6·00 (*bs*)	1	·CH$_2$·O*H*

s=singlet, *d*=doublet, *b*=broad.

The Phenol, $C_{14}H_{14}O_3$

This metabolite of the mutant, *A. rugulosus* A.R.M. 333, was obtained as a colourless, viscous oil from the culture fluid. The molecular formula was determined by high resolution mass spectrometry. It was confirmed through the preparation of a tetrabromo-di-O-methyl derivative, $C_{16}H_{14}O_3Br_4$, m.p. 183°. The u.v. absorption spectrum (λ_{max} 226, 275, 282 nm; logϵ 4·26, 3·59, 3·57) was very similar to those of orcinol and 2-methylresorcinol.

The PMR spectrum provided valuable information concerning the molecular structure of this compound. It indicated that there were six aromatic protons (τ, 3·65–3·73, multiplet), two protons on phenolic hydroxyl groups (τ, 4·36, s, interchangeable with D_2O) and two methyl groups (Ar·CH$_3$, τ, 7·8, s) in the molecule. This evidence suggested that the metabolite was a dihydroxydimethyldiphenyl ether. Moreover, it was possible to deduce the most probable orientation of substituents. Since all the signals in the PMR spectrum relating to the aromatic protons were in the exceptionally high range, τ, 3·65–3·73, it followed that each of the six protons must be under the influence of two oxygen functions which were either both *ortho*, or one *ortho* and one *para* to the given proton. This was the result of the application of values of shielding constants which have already been described.[8] Only one isomer 3,3′-dihydroxy-5,5′-dimethyldiphenyl ether (V), satisfied these requirements. It was prepared through the Ullman condensation of 3-bromo-5-methoxytoluene with the potassium salt of 3-hydroxy-5-methoxytoluene. Demethylation with hydrobromic acid gave a compound which was identical with the natural product.

As far as we are aware, this is the first record of the isolation of the phenols (IV) and (V) from natural sources. The structures (III) and (IV) are, of course, closely related to *o*-orsellinic acid. It appears likely that they are derived from this well-known fungal metabolite, which has been observed in cultures of *A. rugulosus*. However, the mode of biogenesis of the diphenyl ether (V) is less obvious. We are investigating the relationship of these compounds to the more complex phenols produced by *A. rugulosus*.

EXPERIMENTAL

All melting points were determined on a Kofler hot-stage microscope. U.v. spectra were measured on a Unicam S.P. 800 spectrophotometer. I.r. spectra were obtained using Perkin–Elmer Model 257 and 137 spectrophotometers. PMR spectra were determined at 60 Mc/s with a Perkin–Elmer Model R-10 instrument. Mass spectra were measured on an A.E.I. Model MS 9 spectrometer. R_f values refer to thin-layer chromato-

[8] J. A. BALLANTINE and C. T. PILLINGER, *Tetrahedron* **23**, 1691 (1967).

grams on Kieselgel G (Merck) using either system A, benzene–dioxan–acetic acid (90:25:4 v/v)[9] or system B, benzene–methanol–acetic acid (10:2:1 v/v).[10] The chromoplates were sprayed with diazotized *o*-dianisidine solution followed by ammonia, unless otherwise stated. Light petroleum had b.p. 60–80°.

Extraction and Purification of Phenol, $C_8H_8O_3$

A yellow, fluffy, methionine-requiring mutant (A.R.M. 505), derived in three mutation stages from *Aspergillus rugulosus* I.M.I. strain 84338, was grown from a spore suspension for 7 days at 25° on a low-nitrogen medium,[2] to which methionine (10 mg/l) had been added as a growth requirement. The stationary cultures were in 800 flat-sided bottles (*ca.* 1 l. capacity) each containing 200 ml of medium. After removal of the mycelium the culture fluid was concentrated to 8 l. at 30° under reduced pressure and then extracted, continuously, with ether. Acidic material was removed by washing the ether extract with $NaHCO_3$. Evaporation of the solvent left a brown viscous residue (400 mg) which was chromatographed by gradient elution on silica gel (24 g, L. Light and Co., 200–300 mesh) when the early fractions were shown to contain almost pure metabolite (R_f 0·45, system B, purple spot). Recrystallization of the metabolite from hot water furnished analytically pure material as pale yellow needles (100 mg), m.p. 176–177°. (Found: C, 63·1; H, 5·3 per cent; M (mass spectrometry), 152. Calc. for $C_8H_8O_3$: C, 63·2; H, 5·3 per cent; M, 152.) The major peaks in the mass spectrum were as follows:

m/e	153	152	151	134	123	106	95	77	69	67	55	53	51	44	43	41	39
A%	7	70	100	2	4	7	6	7	20	8	8	5	10	13	8	10	15

with metastable peaks at *m/e* (m_2^2/m_1): 152·0 ($151^2/150$); 100·2 ($123^2/151$); 73·4 ($95^2/123$); 47·3 ($67^2/95$); 50·1 ($69^2/95$); 73·9 ($106^2/152$).

A comparison of the u.v., i.r. and mass spectra, together with evidence from a mixed melting point experiment, established that the metabolite was identical with a synthetic sample of 2,4-dihydroxy-6-methylbenzaldehyde[9] which was kindly supplied by Dr. R. K. Pike.

Extraction and Purification of the Phenol, $C_8H_8O_4$

A white, fluffy, proline-requiring mutant (A.R.M. 884), derived in two mutation steps from *A. rugulosus* I.M.I. strain 84338, was grown for 14 days as described previously on the low-nitrogen medium to which proline (10 mg/l) had been added as a growth requirement. The ether extract of the culture fluid deposited a yellow–brown solid (15·5 g from 800 bottles) upon removal of the solvent. Trituration of the solid with $CHCl_3$ (4 × 150 ml) afforded a crude solid residue (13·1 g) which was purified in small batches (1–2 g) by rapid gradient elution on silica gel (60 g). The intermediate fractions were shown to contain the pure metabolite (R_f 0·25, system A, purple spot). Evaporation of the solvent yielded the *phenol* as a pale yellow crystalline solid which decomposed in air (1·11 g total), m.p. 148–150° (d). (Found: M (mass spectrometry, 168·0423 ± 0·0008. $C_8H_8O_4$ required: M, 168·0423.) The i.r. spectrum included peaks at 3420 (OH), 3240 (chelated OH), and 1635 cm^{-1} (hydrogen bonded carbonyl). The major peaks observed in the mass spectrum were as follows:

m/e	168	150	122	121	108	94	93	81	80	69	66	65	52	51	50	39
A%	80	100	37	22	79	24	14	19	51	66	30	38	43	37	23	43

with metastable peaks at *m/e* (m_2^2/m_1); 134·0 ($150^2/168$); 99·3 ($122^2/150$); 77·8 ($108^2/150$); 72·3 ($94^2/122$); 59·2 ($80^2/108$); 46·4 ($66^2/94$) and 33·9 ($52^2/80$).

Catalytic Hydrogenation of the Phenol, $C_8H_8O_4$

The phenol (600 mg) in ethanol (75 ml) was hydrogenated over 10 per cent Pd–C (500 mg) at atmospheric pressure and temperature. After the uptake of three moles H_2, the catalyst and solvent were removed and a pale yellow oil (610 mg) was obtained. Distillation of the oil at 110°/0·8 mm resulted in the production of a solid which was recrystallized from benzene to give *the hydrogenation product* as white microcrystals (150 mg), m.p. 135°. (Found: C, 69·3; H, 7·3 per cent; M (mass spectrometry), 138. $C_8H_{10}O_2$ required: C, 69·5; H, 7·3 per cent; M, 138.) The i.r. spectrum included a peak at 3420 cm^{-1} (OH) but there were no bands due to carbonyl groups.

3,5-*Dihydroxy*-o-*xylene*

Hydrogenation of *o*-orsellinic aldehyde[11] under identical conditions to those above furnished 3,5-dihydroxy-*o*-xylene as white microcrystals, m.p. 135° (lit.[12] m.p. 136–137°) (mixed m.p. with the hydrogenation product of the phenol, $C_8H_8O_4$, 135°). (Found: C, 69·7; H, 7·3 per cent; M (mass spectrometry), 138. Calc.

[9] G. PASTUSKA, *Z. Analyt. Chem.* **179**, 355 (1961).
[10] J. D. LEVI, unpublished results.
[11] R. ADAMS and I. LEVINE, *J. Am. Chem. Soc.* **45**, 2373 (1923).
[12] O. SIMON, *Ann.* **329**, 305 (1903).

for $C_8H_{10}O_2$: C, 69·5; H, 7·3 per cent; M, 138.) A comparison of i.r., NMR and mass spectra established that this compound was identical with the catalytic hydrogenation product of the metabolite.

Extraction and Purification of the Phenol, $C_{14}H_{14}O_3$

A green mutant with normal morphology (A.R.M. 333), derived in one step from *A. rugulosus* I.M.I. strain 84338, was grown for 14 days as described previously on the low-nitrogen medium. The ether extract of the culture fluid deposited a yellow–brown viscous oil (14·3 g from 800 bottles) upon removal of the solvent. When the residue was partitioned between the two layers of the system light petroleum–methanol–water (8:10:1 v/v) (1900 ml), the fatty impurities were removed into the top layer. The bottom aqueous layer was basified with $NaHCO_3$, extracted with $CHCl_3$ (5×500 ml), the combined extracts dried ($MgSO_4$) and the solvent removed under reduced pressure to give the fraction which was insoluble in $NaHCO_3$ as a yellow–brown viscous oil (1·7 g). Gradient elution of the oil on silica gel (120 g) furnished the metabolite (R_f 0·53, system B, red spot) in the intermediate fractions. Repeated chromatography was necessary to obtain pure *phenolic metabolite* as a pale yellow viscous oil (210 mg). (Found: M (mass spectrometry), 230·0943 ± 0·0008. $C_{14}H_{14}O_3$ required: M, 230·0943.) The major peaks observed in the mass spectrum were as follows:

m/e	230	147	119	101	83	76	75	74	59	58	57	45	44	43	41
A%	11	6	7	7	6	9	16	44	68	7	9	100	12	47	17

Methylation of the Phenol, $C_{14}H_{14}O_3$

Methylation of the metabolite (224 mg) with dimethyl sulphate and anhydrous K_2CO_3 in acetone gave the *di-O-methyl ether* as a yellow oil after purification by distillation at 92°/1 mm (189 mg). (Found: C, 74·7; H, 7·4; OCH_3, 23·2 per cent; M (mass spectrometry), 258. $C_{16}H_{18}O_3$ required: C, 74·4; H, 7·0; $2OCH_3$, 24·0 per cent; M, 258.) τ ($CDCl_3$), 3·55, six proton singlet (ArH); 6·22, six proton singlet ($ArOCH_3$) and 7·70, six proton singlet ($Ar–CH_3$).

Bromination of the Dimethyl Ether of the Phenol, $C_{14}H_{14}O_3$

The di-O-methyl ether (150 mg) was treated with excess Br_2 in glacial acetic acid for 30 min at 20° and the solution poured into water. The *tetrabromo-di-O-methyl ether* separated and was recrystallized from aqueous ethanol to form colourless needles (250 mg) m.p. 183°. (Found: C, 33·2; H, 2·6. $C_{16}H_{14}O_3Br_4$ required: C, 33·5; H, 2·5 per cent.)

3,3′-*Dimethoxy*-5,5′-*Dimethyldiphenyl Ether*

3-Hydroxy-5-methoxytoluene[13] (8 g) was mixed with KOH (2·3 g A.R.) and heated to 150° in N_2 for 10 min. The temperature was raised to 180° and a mixture of 3-bromo-5-methoxytoluene[14] (11·4 g) and copper powder (200 mg precipitated) was added. The temperature was raised to 300–320° and the reaction kept at that temperature for 8 hr. After cooling to room temperature, NaOH (30 ml of 5 N) was added and the solution was extracted with $CHCl_3$ (4×150 ml) to furnish a dark residue (12 g) upon evaporation of the dried solvent. Repeated chromatography of the residue on silica gel yielded the *diphenyl ether* as a pale yellow oil (1·1 g) (R_f 0·86, system A) which was distilled at 92°/1 mm to give analytically pure material. (Found: C, 74·4; H, 7·0; OCH_3, 24·4. $C_{16}H_{18}O_3$ required: C, 74·4; H, 7·02; $2OCH_3$, 24·0 per cent.) A comparison of the u.v., i.r. and PMR spectrum established that this compound was identical to the methylation product of the phenol $C_{14}H_{14}O_3$.

This dimethoxydimethyldiphenyl ether was demethylated with HBr in refluxing acetic acid to give an oil which was identical in all respect with the phenol $C_{14}H_{14}O_3$, and was also brominated to give a solid tetrabromodimethoxy-dimethyldiphenyl ether which was identical in all respects to the derivative of the metabolite described previously.

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[13] K. AUWERS, E. BORSCHE and R. WELLER, *Ber.* **54**, 1311 (1921).
[14] F. HENRICH and G. NACHTIGALL, *Ber.* **36**, 889 (1903).