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CERCOSPORIN FROM *CERCOSPORA HAYII*

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Key Word Index—*Cercospora hayii*; *Cercospora kikuchii*; Fungi Imperfecti; cercosporin; red pigment; isolation and structural determination; influence of N source on biosynthesis.

Abstract—The red pigment, cercosporin ($C_{29}H_{26}O_{10}$) has been isolated from cultures of a banana pathogen *Cercospora hayii*. Spectroscopic and chromatographic investigations suggest the structure is 1,12-(2-hydroxypropyl)-2,11-dimethoxy-4,9-dihydroxy-6,7-methylenedioxyperylene-3,10-quinone. The structure of two related compounds are proposed. Nitrogen sources influencing pigment formation have been determined for *C. hayii* and *C. kikuchii*.

INTRODUCTION

Cercospora hayii, the causal organism of brown spot of banana fruit, produces a red pigment when grown on chemically defined or undefined media. The typical symptom of the disease, a dark discolored lesion in the fruit peel, suggests involvement of this pigment which could not, however, be isolated from diseased fruit.[1] A closely related fungus *Cercospera kikuchii* also produces a red pigment, cercosporin, which is responsible for the purple pigmentation associated with purple stain disease of soybean. Lukezic has shown that the red pigment isolated from *C. hayii* is similar to cercosporin from *C. kikuchii*.[2] Previous investigators have proposed that cercosporin is a derivative of 4,9-dihydroxyperylene-3,10-quinone (I).[3,4] Recently Lousberg *et al.* proposed the structure (II) for cercosporin.[5] Cercosporin has also been isolated from *C. beticola*[6] and *C. personata*.[7] We have independently isolated the red pigment from cultures of *C. hayii*, determined its structure and that of related compounds and shown the red pigment to be identical to cercosporin. In addition, nutrient nitrogen sources influencing pigment formation have been determined for *C. kikuchii* and *C. hayii*.

RESULTS AND DISCUSSION

Two red pigments, II, III, were isolated from ethanolic extracts of *C. hayii* by chromatography. Table 1 presents the thin-layer and paper chromatographic properties of II, III and IV (produced by sulfuric acid treatment of II). The most abundant red pigment II

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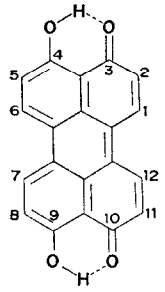
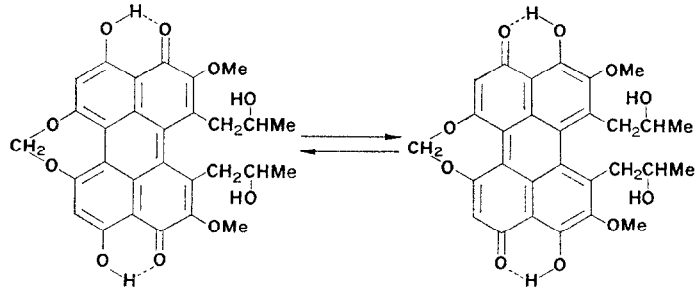
[1] LUKEZIC, F. L. unpublished results.
[2] LUKEZIC, F. L. (1970) *Phytopathol.* **60**, 576.
[3] KUYAMA, S. and TAMURA, J. (1957) *J. Am. Chem. Soc.* **79**, 5725; *ibid.* (1957) **79**, 5726.
[4] KUYAMA, S. (1962) *J. Org. Chem.* **27**, 939.
[5] LOUSBERG, R. J. J. CH., WEISS, U., ARNONE, A., MERLINI, L. and NASINI, G. (1971) *Chem. Commun.* 1463.
[6] BALIS, C. and PAYNE, M. G. (1971) *Phytopathol.* **61**, 1477.
[7] VENKATARAMANI, K. (1967) *Phytopathol. Z.* **58**, 379.

($C_{29}H_{26}O_{10}$) is spectroscopically identical[2] to that of cercosporin, a red pigment isolated from *C. kikuchii*.[3,4] The less abundant pigment III is an isomer of cercosporin and corresponds to the reported isocercosporin.[3,4]



(I)



(II)

Heating crystalline II or III at 245° or in refluxing toluene converts them into a mixture of approx. 50% II and III, demonstrating the interconvertibility of the two pigments. Extracts of the fungus, following workup, always contained the two pigments, with III being present in smaller amounts. However, when the mycelium from *C. hayii* or *C. kikuchii* was immersed in chloroform and this chloroform solution immediately chromatographed (PC or TLC) only pigment II was present. Thus pigment III is an artifact of the isolation procedure. However, as ethanolic extraction yielded a greater amount of II than ethyl ether or chloroform extraction, it was used routinely.

TABLE 1. CHROMATOGRAPHIC PROPERTIES OF CERCOSPORIN, ISOCERCOSPORIN AND BISNORANHYDROCERCOSPORIN

Compound	R_f TLC *A*	TLC *B*	PC *C*	PC *D*
Cercosporin (II)	0·7	0·51	0·59	0·37
Isocercosporin (III)	0·87	0·70	0·87	0·64
Bisnoranhydrocercosporin (IV)		0·86		

A—C_6H_6–MeOH; 1:1, v/v, Supelcosil 12A.
B—$CHCl_3$–MeOH; 18:1, v/v, Chromagram Sheet.
C—$CHCl_3$–HOAc–H_2O; 2:1:1, v/v, Whatman No. 1 (upper phase).
D—C_6H_6 (sat. with H_2O), Whatman No. 1 (upper phase).

The MS of II and III are identical and mass measurements (see Experimental) indicate the MW of II and III to be 534·1466 ± 0·01 corresponding to $C_{29}H_{26}O_{10}$, in contrast to the previously reported $C_{30}H_{28}O_{10}$.[3,4]

The NMR spectra contains eight different proton absorptions equivalent to thirteen protons (Table 2). Therefore, the results of the MS require each of these NMR absorptions to correspond to two equivalent protons, emphasizing the symmetry of the molecule. To explain the presence of the odd number of carbon atoms and the symmetry, a methylenedioxy group is placed at position C-6 and C-7, possessing equivalent proton absorptions in the NMR spectra (5·74 δ in II, 5·71 δ in III and 5·72 δ in IV). From the MS and NMR spectra

and from correlation of spectroscopic analyses of somewhat similar fungal pigments[8,9] we propose that II and III possess the same basic structure. The assignments of the alkyl groups at C-1 and C-12 and the methoxy groups at C-2 and C-11 are consistent with the formation of the hydrocarbon resembling benzo[ghi]perylene in zinc dust distillation of II and the formation of a hydrocarbon resembling benzo[e]pyrene in zinc dust distillation of the permanganate oxidation product of pentamethyl norcercosporin.[4]

TABLE 2. NMR SPECTRA

Absorption	Relative intensity	δ II Cercosporin	III Isocercosporin	IV Bisnoranhydrocercosporin
Aromatic H	2	7·05(*s*)	6·96(*s*)	7·01(*s*)
OCH_3	6	4·20(*s*)	4·24(*s*)	Absent
$-O\diagdown CH_2\diagup O-$	2	5·74(*s*)	5·71(*s*)	5·72(*s*)
$-CH_2-$	4	3·48(*m*)	3·50(*m*)	3·7(*m*)
$-CH\diagup\diagdown$	2	2·92(*m*)	2·90(*m*)	3·0(*m*)
$-CH_3$	6	0·62(*d*) (*J* 5·9 Hz)	0·94(*d*) (*J* 6·3 Hz)	1·62(*d*) (*J* 6·3 Hz)
–OH (periphenolic)	2	14·09	14·88	
–OH	2	1·8	1·72	

The parent mass spectral ion (*m/e* 534) of II and III is present in great abundance, and indicates considerable stability of the polynuclear system. All the major ions arise from fragmentation of the alkyl ($-C_2H_4O$ or $-C_3H_7O$), methoxy groups (–OMe), or dehydration ($-H_2O$) which results in a less sterically hindered structure, for example: *m/e* 516 = P–H_2O, 503 = P–OMe, 490 = P–C_2H_4O, 475 = P–C_3H_7O, 459 = P–OMe–C_2H_4O, 445 = P–C_2H_4O–C_2H_5O and 431 (base) = P–C_2H_4O–C_3H_7O. The benzylic cleavage of $-C_2H_4O$ (*m/e* 490) rather than $-C_2H_5O$ (*m/e* 491) suggests that the hydroxyl hydrogen is hydrogen bonded to the methoxy oxygen and remains with the oxygen upon cleavage. This cleavage also supports the assigned close proximity of the methoxy and alkyl groups. The base peak probably arises first by benzylic cleavage of $-C_2H_4O$, followed by the loss $-C_3H_7O$, resulting in the formation of a new ring (*m/e* 431). Similar fragmentations are common with aporphine alkaloids.[10]

Although II and III have identical MS they have slightly different UV absorptions indicating the basic chromophores are different. Also, II and III show differences in the NMR spectra, particularly in the terminal methyl absorptions. In II the methyl absorption (0·62 δ) is more shielded than in III (0·94 δ) suggesting the methyls in II are above the plane

[8] LOUSBERG, R. J. J. CH., SALEMINK, C. A. and WEISS, U. (1970) *J. Chem. Soc.* **C**, 2159.
[9] LOUSBERG, R. J. J. CH., PAOLILLO, L., KON, H., WEISS, U. and SALEMINK, C. A. (1970) *J. Chem. Soc.* **C**, 2154.
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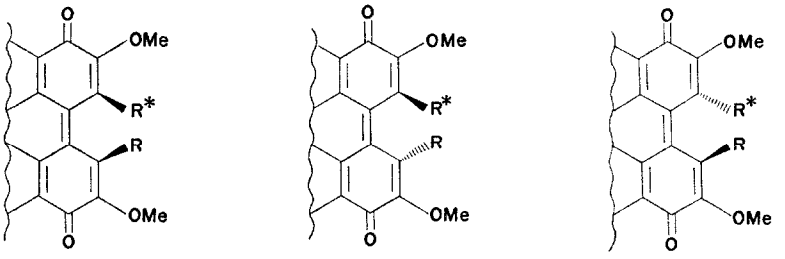
of the aromatic ring relative to the methyls in III. The NMR absorptions of the methylene protons of the propyl chains show an *ABX*-type absorption indicating nonequivalent protons.



Cis-trans ISOMERISM OF THE C-1 AND C-12 ALKYL GROUPS.

Any proposed structure of II and III must account for the observed difference in optical rotation[3] of II ($[\alpha]^{20}_{7000} + 470°$) and III ($[\alpha]^{20}_{7000} - 826°$) and requires at least one center of asymmetry. Tautomerism between the two possible quinoid forms (II) is generally recognized to be rapid, especially in perihydroxyquinones. If the alkyl groups at C-1 and C-12, because of their steric hinderance, were forced out of the plane of the rings a center of asymmetry would be created. The alkyl groups could then exist in two different *trans* isomers or one *cis* isomer. However, the *cis* isomer would be thermodynamically unfavored. We, therefore, propose that the thermal isomerization of II to III consist of the flipping of the two *trans* alkyl groups giving rise to diasteroisomers. Thus, the observed optical rotation of II and III requires the asymmetric carbons of the propyl chains to be either *R,R* or *S,S*.

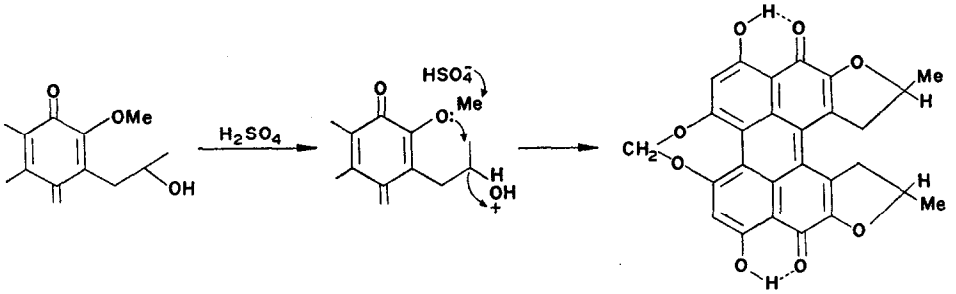
Since the alkyl groups possess asymmetric carbon atoms they will assume two different geometric positions in the two possible *trans* isomers. In both cases the primary difference will be the position of the methyl groups relative to the plane of the aromatic rings as the Dreiding models of the two *trans* isomers shows. In the one *trans* isomer the methyl groups will be above the plane of the polynuclear ring (II) and in the other *trans* isomer they will be somewhat in the plane of the ring (III). Interestingly, the fungi produce only the one isomer.

Bisnoranhydrocercosporin (IV), previously referred to as noranhydrocercosporin, formed by treating cercosporin with concentrated sulfuric acid, was also analyzed spectroscopically. The mass measurements of the molecular ion of IV indicate the molecular formula to be $C_{27}H_{18}O_8$, so that IV differs from II by $-2(CH_2 + H_2O)$. The NMR spectrum of IV (Table 2) indicates the methoxy groups have been removed but the methylenedioxy, methyl, methylene and methine protons are present. MS and NMR are consistent with bisnoranhydrocercosporin possessing structure IV and this structure is probably formed as shown see (iv) p. 921.

The parent MS ion (*m/e* 470) of IV is also the base ion. All the major ions arise from loss of $-H_2$, -Me, -CO, or -COH, for example: *m/e* 468 = P–2H, 455 = P–Me, 453 = P–2H–Me, 441 = P–COH, 427 = P–Me–CO, 413 = P–COH–CO, and 370 = P–COH–2CO. A small ion evidently due to an impurity was observed at *m/e* 484.

The influence of the nitrogen source on the biosynthesis of II in *C. kikuchii* and *C. hayii* is presented in Table 3. The formation of II by the fungi does not appear obligatory, especially for *C. hayii*, and is nutrient controlled. *C. hayii* does not produce this pigment

when it grows on the banana fruit. Since there is a striking difference between the amount of pigment present when *C. kikuchii* and *C. hayii* are grown on similar media this suggests that perhaps the biosynthetic pathways are different. Of the constituents tested with *C. hayii*



(IV)
Bisnoranhydrocercosporin

only media containing potato-dextrose broth or β-alanine produce favorable conditions for red pigment formation. It is possible that β-alanine is a needed nutrient factor for the synthesis of II in *C. hayii*, at least in the particular isolate used. Additional experiments are being performed to determine the metabolic pathway for pigment biosynthesis.

TABLE 3. INFLUENCE OF NITROGEN SOURCE ON MYCELIUM AND PIGMENT

Source	*Cercospora kikuchii* Mycelium*	*Cercospora kikuchii* Pigment†	*Cercospora hayii* Mycelium	*Cercospora hayii* Pigment
KNO_3	0·11†	47·1	0·18	1·4
KNO_2	0·01	0	0·01	0
NH_4Cl	0·11	16·8	0·15	1·4
Acetamide (0·1 M)	0·08‡	81·3	0·23	1·9
α-Alanine (0·1 M)	0·11	12·3	0·21	0
β-Alanine (0·1 M)	0·36	11·3	0·05	15·0
Phenylalanine (0·1 M)	0·01	22·3	0·04	0
Glycine (0·1 M)	0·07	36·7	0·13	2·4
Diethylamine (0·1 M)	0·01	0	0·03	0
PDB§	0·23	2·3	0·27	19·6
No nitrogen	0·02	0·5	0·01	0·7

* g dry wt. † mg/g mycelium. ‡ Means of 3 determinations. § Potato-dextrose broth.

EXPERIMENTAL

Growth and extraction of organism. Cercospora hayii (isolate No. 90) was streaked on potato-dextrose agar and incubated at 25°. Approximately 17 days later the agar was chopped into a beaker containing 20 ml of 95% EtOH per plate. The residue was washed with additional 95% EtOH until no more red pigment was extracted. The filtrate was evaporated to near dryness under reduced pressure. The residue was washed with $CHCl_3$ which dissolved the pigment. The $CHCl_3$ solution was washed with H_2O, dried over anh. Na_2SO_4 and evaporated to dryness (*ca.* 250 mg). *C. kikuchii* (isolate No. 100) was grown and extracted in the same manner.

Influence of nitrogen source. Preliminary experiments with a modified Czapek solution showed that the presence of nitrogen containing compounds and the C/N ratio governed the production of the pigment. In order to determine possible precursors for pigment production a defined glucose–inorganic salt basal medium

was used consisting of the following: glucose, 20 g; KH_2PO_4, 1 g; $MgSO_4.7H_2O$, 1 g; and $FeCl_3.6H_2O$, 0·24 mg. Enough glass-distilled H_2O was added to make 1 l. The following test compounds, in amounts producing a final concentration of 0·1 M, were added to the nutrient solution before heat sterilization; KNO_3, KNO_2, NH_4Cl, while acetamide, α-alanine, β-alanine, phenylalanine, glycine and diethylamine were sterilized by membrane filtration and subsequently added. Potato-dextrose broth (H_2O in which 200 g of unpeeled white potatoes had been autoclaved, made up to 1 l., plus 20 g glucose) and the basal inorganic salt medium without a nitrogen source were used as controls. The flasks were inoculated with 0·5 ml of a suspension of mycelium that had been grown in the basic media plus N for 2 weeks, then washed 3× with sterile distilled H_2O. The cultures were incubated for 2 weeks on a rotatory shaker, timed to shake 5 min every 30 min. At the end of this period the mycelium was harvested by filtration onto tared papers, dried overnight at 80°, weighed and then extracted with absolute EtOH for 30 min in a Soxhlet extractor. The ethanolic extracts were taken to near dryness, the residues reextracted with 5 ml of $CHCl_3$ and the amount of pigment was determined photometrically at 464 nm.

Isolation of pigments. The pigments were separated by silicic acid column chromatography and a typical separation is described. Acid treated florisil (ATF, 100/200 mesh, Supelco, Inc.) was placed in a small column as an acetone slurry (10 × 1 cm). The adsorbant was properly conditioned by percolation with 25 ml Me_2CO, 50 ml 0·25% HOAc in absolute MeOH, and 50 ml of $CHCl_3$ respectively. The pigment extract (120 mg) was placed on the column in a minimum volume of $CHCl_3$ and the column eluted with $CHCl_3$. The following fractions were taken: 1, 8 ml; 2, 20 ml; 3, 10 ml; 4, 30 ml; 5, 40 ml; 6, 30 ml; 7, 30 ml ($CHCl_3$–MeOH; 9:1). Fraction 4 contained relatively pure III. Fraction 6 and 7 contained pure II. Fraction 4 was rechromatographed in order to eliminate any traces of triglycerides. Upon evaporation of fractions 6 and 7 crystalline pigment was obtained. II, m.p. 235°, λ_{max}^{EtOH} 223, 260, 271, 275, 470 nm. III, λ_{max}^{EtOH} 223, 260, 267, 329, 470, 475 nm. Major MS ions of II and III are *m/e* 534·1466 ≅ $C_{29}H_{26}O_{10}$, 516·1380 ≅ $C_{29}H_{23}O_9$, 503·1359 ≅ $C_{29}H_{23}O_9$, 490·1230 ≅ $C_{27}H_{22}O_9$, 475·1030 ≅ $C_{26}H_{19}O_9$, 471·1033 ≅ $C_{27}H_{19}O_8$, 459·1093 ≅ $C_{26}H_{19}O_8$, 445·0880 ≃ $C_{25}H_{17}O_8$, 431·0777 ≅ $C_{24}H_{15}O_8$, 415·0854 ≅ $C_{24}H_{15}O_7$, 401, 387, 371, 360, 344, 330 and 313.

Chromatographic and spectroscopic techniques. All solvents were redistilled. Two thin-layer adsorbants, Supelcosil 12A (Supelco, Inc.) and Chromagram Sheet (Distillation Products, Inc.) and two solvent systems: C_6H_6–MeOH; 1:1 (v/v) and $CHCl_3$–MeOH; 18:1 (v/v) were used. Two PC solvent systems were used: the upper layers of a mixture of C_6H_6 saturated with H_2O and of C_6H_6–HOAc–H_2O; 2:1:1 (v/v/v). TLC was employed to follow all chromatographic separations and chemical reactions.

Preparation of bisnoranhydrocercosporin. II (20 mg) was left in concentrated H_2SO_4 (1 ml) for 30 min. Water was then added and the purple suspension allowed to stand overnight. The precipitate was filtered, washed with H_2O, and dried in vacuum. λ_{max}^{EtOH} 274, 325, 522 and 526 nm. Major mass spectral ions are *m/e* 470·0980 ≅ $C_{27}H_{18}O_8$, 468·0809 ≅ $C_{27}H_{16}O_8$, 455, 553, 441, 427, 413, 400, 385, 370 and 355.

Isomerication of II *to* III. II (40 mg) was placed in 10 ml toluene and refluxed for 15 min. Chromatographic analysis showed the presence of *ca.* a 1:1 mixture of II and III. Also, pure II was heated to 245° giving a mixture of II and III. Pigment III upon heating was also converted into a mixture of II and III.

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