Occurrence and Distribution of Phenoxazinone Pigments in the Genus *Pycnoporus*

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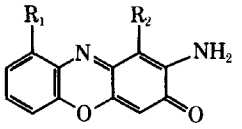
Abstract □ A TLC procedure was developed for the separation of phenoxazinone-type pigments. By employing silica gel G as the adsorbent and a solvent system of benzene–ethyl acetate–glacial acetic acid–formic acid (12:6:1:1), the separation of the following reference compounds was accomplished: cinnabarinic acid (R_f 0.35), cinnabarin (0.47), 2-amino-3-oxo-3*H*-phenoxazine (0.57), tramesanguin (0.67), and phenoxazine (0.99). Examination of the individual acetone extracts of *Pycnoporus cinnabarinus* (Jacq. ex Fr.) Karst., *P. coccineus* (Fr.) Bond. and Sing., and *P. sanguineus* (L. ex Fr.) Murr. by this technique revealed that all three species contained the same pigments. In addition to cinnabarinic acid, cinnabarin, and tramesanguin, two additional phenoxazinone-type pigments (R_f 0.53 and 0.90) were found in the respective carpophores.

Keyphrases □ *Pycnoporus* genus—occurrence, distribution of phenoxazinone pigments □ Phenoxazinone pigments—separation, identification from *Pycnoporus* genus □ Spectrophotometry, visible—analysis □ TLC—analysis, identification

Nobles and Frew (1) presented genetical and morphological evidence which supports the validity of three *Pycnoporus* species: *P. cinnabarinus* (Jacq. ex Fr.) Karst., which occurs in the North Temperate Zone; *P. coccineus* (Fr.) Bond. and Sing., which occurs in the South Temperate Zone and in countries bordering on the Indian and Pacific Oceans; and *P. sanguineus* (L. ex Fr.) Murr., found in tropical and subtropical regions of the Northern and Southern Hemispheres.

Three phenoxazinone-type pigments were isolated from *Pycnoporus* species and their structures elucidated by Gripenberg *et al.* (2–5) and Cavill *et al.* (6, 7). The chemical structures for cinnabarinic acid (2-amino-3-oxo-3*H*-phenoxazine-1,9-dicarboxylic acid) (I), cinnabarin (2-amino-9-hydroxymethyl-3-oxo-3*H*-phenoxazine-1-carboxylic acid) (II), and tramesanguin (2-amino-1-formyl-3-oxo-3*H*-phenoxazine-9-carboxylic acid) (III) are illustrated here.

Gripenberg (5) stated that three different types of phenoxazinone-producing fungi appeared to exist: one producing only cinnabarin, *Trametes cinnabarina* (Jacq.) Fr. (= *P. cinnabarinus*); one producing cinnabarin and cinnabarinic acid, *Polystictus sanguineus* L. (= *P. sanguineus*); and one producing cinnabarin and tramesanguin, *T. cinnabarina* var. *sanguinea* (L.) Pilat (= *P. sanguineus*). Lemberg (8) reported the isolation and characterization of polystictin (cinnabarin) from carpophores of *Coriolus sanguineus* (Fr.) Cunningham (= *P. sanguineus*) and also the presence of an uncharacterized pigment, polystictinin. Cavill *et al.* (6) worked with both carpophores and cultures of *C. sanguineus* and only cinnabarin was reported to be present.



I: R_1 = COOH, R_2 = COOH
II: R_1 = CH_2OH, R_2 = COOH
III: R_1 = COOH, R_2 = CHO

In 1962, Madhosingh (9) worked with cultures of all three species of *Pycnoporus*. By utilization of paper chromatographic techniques, eight different pigments were observed, one of which was thought to be identical with cinnabarin. The remaining seven pigments were not characterized or identified. Consequently, the chromophore of these compounds and their relationship to the phenoxazinone-type pigments are unknown.

Prior to the present study, cinnabarin, cinnabarinic acid, and tramesanguin were reported to be present in carpophores of *P. sanguineus*, whereas only cinnabarin was reported in *P. cinnabarinus*. No report was found concerning the pigment content of carpophores of *P. coccineus*. Therefore, the present study represents an effort to establish the occurrence and distribution of phenoxazinone-type pigments as they exist in the carpophores of the three *Pycnoporus* species.

EXPERIMENTAL

Several investigators (10–12) reported the successful separation of phenoxazinone-type pigments by paper chromatography; Gerber (13) used TLC for the separation and purification of several phenoxazinone-type pigments. None of these techniques was found satisfactory for this investigation.

Table I—Collection Data and Weight of Powdered Carpophores

Pycnoporus Species	Collection Site	Collection Date	Weight, g.	Source
P. cinnabarinus	Long Branch, N. Y.	Oct. 27, 1915	1.82	—[a]
P. cinnabarinus	Washington, D. C.	Aug. 19, 1912	1.44	—[a]
P. cinnabarinus	Ontario, Canada	Sept. 22, 1955	1.84	DAOM 31954[b]
P. coccineus	Bougainville, Solomon Islands	Nov. 6, 1959	1.76	DAOM 72107[b]
P. sanguineus	Westland, New Zealand	April 1963	1.75	DAOM 99448[b]
P. sanguineus	Kingston, Jamaica	Jan. 18, 1966	0.62	—[c]

[a] Obtained from Dr. J. L. Lowe, Department of Botany, New York State College of Forestry, Syracuse, N. Y. No herbarium number given. [b] Obtained from Mycological Herbarium, Plant Research Institute, Department of Agriculture, Ottawa, Canada. [c] Obtained from Dr. C. J. Alexopoulos, Department of Botany, University of Texas at Austin, Austin, Tex. No herbarium number given.

Preparation of TLC Plates—TLC plates were prepared by placing 20 g. of silica gel G[1] in a 250-ml. conical flask with 40 ml. of distilled water and shaking vigorously for 1 min. A uniform 200-μ layer was applied to five 20 × 20-cm. glass plates. The plates were allowed to stand for 30 min. and activated at 110° for 30 min.

Developing Solvent—Solvents used were: benzene, chloroform, methanol, ethanol, and a number of solvent mixtures including ethanol–water (1:1), methanol–water (1:1), methanol–10% HCl (1:1), chloroform–ethyl acetate (9:1), chloroform–glacial acetic acid (9:1), chloroform–ethanol (99:1), chloroform–acetone (95:5), ethyl acetate–glacial acetic acid (9:1), butanol–glacial acetic acid–water (4:1:1), toluene–ethanol–water (4:17:1), benzene–ethyl acetate–diethylamine (6:3:1), benzene–ethyl acetate–glacial acetic acid (6:3:1), benzene–ethyl acetate–ethyl formate (6:3:1), benzene–ethyl acetate–formic acid (6:3:1), and benzene–ethyl acetate–glacial acetic acid–formic acid (60:30:1:1 and 12:6:1:1). Excellent separation of compounds was obtained when plates were developed with a solvent system composed of benzene–ethyl acetate–glacial acetic acid–formic acid (12:6:1:1).

Reference Compounds—Cinnabarin[2], tramesanguin[2], cinnabarinic acid[3], 2-amino-3-oxo-3*H*-phenoxazine[3], and phenoxazine[4] were dissolved in minimal amounts of methanol–pyridine (3:1) and were employed as standard spotting solutions.

Extraction of Carpophores—Individual carpophores were ground to a 20-mesh powder in a Wiley-laboratory mill, placed in a continuous extraction apparatus, and exhaustively extracted with acetone. Each acetone extract was evaporated to dryness under reduced pressure, and the residual pigments were dissolved in a methanol–pyridine solution.

Species designation, collection data, and weights of powdered carpophores are presented in Table I.

Detection—Reference compounds and pigments isolated from the carpophores were detected after TLC separation by visual inspection. Less than 0.02 mcg. could be detected in this manner. Iodine vapors intensified the colors.

Preparative TLC—The methanol–pyridine solution containing the extracted pigments was streaked in a continuous band 2 cm. from the lower edges of the plates, prepared as described previously. These plates were developed in a solvent system composed of benzene–ethyl acetate–glacial acetic acid–formic acid (12:6:1:1) for a distance of 15 cm., removed from the developing chamber, and allowed to air dry. The corresponding individual bands on each plate were removed, combined, placed in a continuous extraction apparatus, and exhaustively extracted with acetone. Each acetone extract was taken to dryness under reduced pressure and dissolved in ethanol.

Visible Spectra—The visible spectral characteristics of the reference compounds and the respective pigments obtained from the preparative TLC bands were determined with a spectrophotometer[5]. Ethanol was employed as the solvent in all spectrophotometric determinations.

RESULTS AND DISCUSSION

Satisfactory separation of the reference compounds into discrete spots was accomplished using silica gel G as the adsorbent and any of the following solvent systems: benzene–ethyl acetate–ethyl formate (6:3:1), benzene–ethyl acetate–formic acid (6:3:1), and benzene–ethyl acetate–glacial acetic acid–formic acid (60:30:1:1). Excellent separation of cinnabarinic acid (R_f 0.35), cinnabarin (R_f 0.47), 2-amino-3-oxo-3*H*-phenoxazine (R_f 0.57), tramesanguin (R_f 0.67), and phenoxazine (R_f 0.99) resulted when a solvent system of benzene–ethyl acetate–glacial acetic acid–formic acid (12:6:1:1) was employed. Chromatographic systems and R_f values are listed in Table II.

Examination of the acetone extracts of all three *Pycnoporus* species by preparative TLC resulted in the formation of seven distinct colored bands: bands 1 (origin), 2 (R_f 0.35), 3 (R_f 0.47), 4 (R_f 0.53), 5 (R_f 0.67), 6 (R_f 0.90), and 7 (R_f 0.99). The pigments eluted from bands 2 through 6 each exhibited identical visible spectral characteristics, with maximum absorption at 430 nm., and were identical to the spectra obtained with the reference phenoxazinone-type compounds. Since the pigments obtained from the origin and at R_f 0.99 did not exhibit absorbance in this region, they were of no further interest in this investigation. The pigments isolated from bands 2, 3, and 5 were chromatographically indistinguishable from cinnabarinic acid, cinnabarin, and tramesanguin, respectively. Since the pigments eluted from bands 4 (R_f 0.53) and 6 (R_f 0.90) exhibited spectral characteristics identical to the reference compounds, they were assumed to be phenoxazinone derivatives. Due to the limited supply of these compounds, no further characterization was attempted.

It is apparent from this study that carpophores of all three *Pycnoporus* species contain identical pigments and that species differentiation cannot be supported by the presence of these secondary metabolites.

Table II—Chromatographic Separation of Reference Compounds

Compound	Chromatographic System[a] and R_f Values A	B	C	D	E
Cinnabarinic acid	0.01	0.44	0.28	0.26	0.35
Cinnabarin	0.27	0.54	0.48	0.42	0.47
2-Amino-3-oxo-3*H*-phenoxazine	0.51	0.43	0.78	0.68	0.57
Tramesanguin	0.37	0.79	0.73	0.66	0.67
Phenoxazine	0.95	0.97	0.97	0.98	0.99

[a] Silica gel G thin layer. System A: benzene–ethyl acetate–ethyl formate (6:3:1); System B: benzene–ethyl acetate–formic acid (6:3:1); System C: benzene–ethyl acetate–glacial acetic acid (6:3:1); System D: benzene–ethyl acetate–glacial acetic acid–formic acid (60:30:1:1); and System E: benzene–ethyl acetate–glacial acetic acid–formic acid (12:6:1:1).

SUMMARY

Cinnabarinic acid, cinnabarin, tramesanguin, and two additional phenoxazinone-type pigments, which are as yet uncharacterized, were detected by TLC in the carpophores of *P. cinnabarinus*, *P. coccineus*, and *P. sanguineus*.

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[1] E. Merck AG, Darmstadt, Germany.
[2] Obtained from Dr. Jarl Gripenberg, Department of Chemistry, Technical University Finland, Otaniemi, Finland.
[3] Obtained from Dr. L. C. Vining, Atlantic Regional Laboratory, National Research Council of Canada, Halifax, Nova Scotia, Canada.
[4] Obtained from K & K Laboratories of California, Inc., Hollywood, Calif.
[5] Beckman model DB.