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# BLUE PIGMENTS OF *PENICILLIUM HERQUEI*

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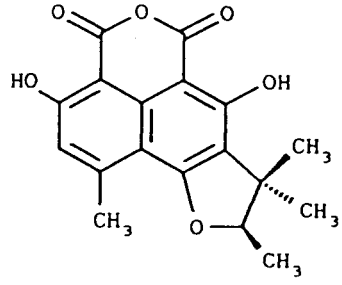
ABSTRACT.—A blue pigment produced by *Penicillium herquei*, previously well-studied but of unknown structure, has been isolated and demonstrated to be a phenalenone derivative. As isolated, the major blue pigment is a zinc complex of elemental formula $C_{76}H_{64}O_{20}N_2Zn$ in which two anions of the dimeric phenalenone **2** act as the tridentate ligands. Compound **2** and the zinc complex have been identified previously as secondary metabolites of two other Ascomycetes. In addition, strong evidence was obtained for the presence in the $CH_2Cl_2$ extract of *P. herquei* of minor amounts of a novel compound, having a structure identical with that of the major blue pigment, but with nickel as the coordinating metal.

The pigments of *Penicillium herquei* Bainier and Sartory (Moniliaceae) have been studied extensively, and a number of yellow-red compounds based on the phenalenone structure have been reported (1–6). In their original description of *P. herquei*, Bainier and Sartory (7) observed that yellow pigments were produced on a variety of media; however, they also noted that in media rich in peptone a deep blue pigment was formed. Since this initial observation, a number of other workers have reported the presence of blue or green pigments in cultures of *P. herquei*. Stodola *et al.* (8) confirmed the dependence on medium composition, commenting that "on a medium rich in peptone the pigment was a beautiful blue." Neill and Raistrick (9) noted the presence of $CHCl_3$-extractable dark green pigments in *P. herquei* and *Penicillium atrovenetum*. More detailed chemical characteristics were provided by Narasimhachari and Vining (4) who isolated a small amount of a green pigment, which they reported to be an amorphous solid of no distinct melting point and with a mol wt of 1090. Insufficient material was available for carbon and hydrogen analyses, but the molecule was believed to contain one atom of nitrogen. Repeated reduction with zinc in HOAc, followed by slow oxidation in air of the green pigment, caused a change in color of the oxidized form to bluer shades, with a corresponding decrease in λ max from 612 to 604 nm. It was suggested by the authors that the pigment could be formed by a ninhydrin-like reaction between atrovenetinone and amino acids.

Despite the evident interest in the identities of these blue and green pigments of *P. herquei*, no unambiguous structural determination has been published, to the best of our knowledge. The following describes the elucidation of the structure of the blue pigment.

Flash chromatography on Si gel of the $CH_2Cl_2$-extract of lyophilized mycelia produced a blue-green fraction. Further purification of this fraction by reversed-phase hplc afforded the blue pigment and two known compounds, physcion and 4,7-dihydroxy-2,3,3,9-tetramethyl-2,3-dihydronaphtho[1,2-*b*]furan-5,6-dicarboxylic anhydride [**1**], identified by



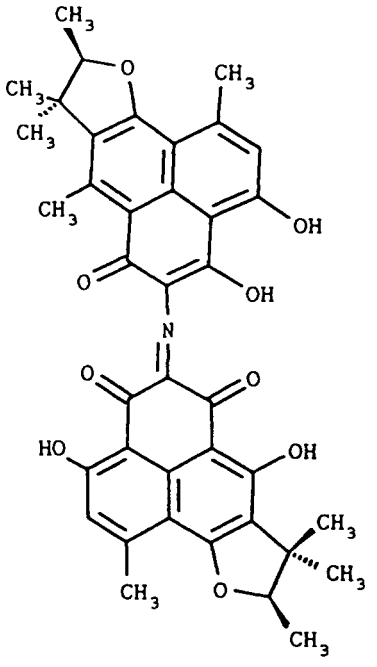

**1**

comparison of their spectral characteristics with data in the literature (4, 10–12).

The blue pigment proved to be unstable and repeated purifications were necessary during acquisition of full spectral data. It was noted that a later-eluting, overlapping peak formed during the period of purification and eventually became dominant; small differences were seen in the uv/vis spectra of the two peaks, i.e., λ max 226, 264, 366, 404, and 610 nm becoming λ max 226, 266, 368, 400 (sh), and 608 nm, as determined by the photodiode array detector during hplc. During the final purification, a minor blue compound occurring as a shoulder on the trailing edge of the peak corresponding to the major blue pigment was collected separately.

One of the decomposition products of the blue pigment was shown to be **1** by spectral comparison and co-chromatography; this is consistent with previously reported work (11), in which a close relationship between **1** and the green pigments of *Roesleria* and *Penicillium* spp. was postulated. Compound **1** or its enantiomer has been isolated previously, together with a blue pigment, from the Ascomycete *Gremeniella abietina* (13) and *Roesleria hypogea* (14). The blue pigment of *G. abietina* was reported (13) to be **2**; whereas, although the authors believed **2** to be the major form (≥92%) of the pigment in *R. hypogea*, the color of the pigment changed from green to blue-green during purification, and it was isolated as a zinc complex, in which two anions of **2** are formulated as the ligands, with zinc coordinated to the nitrogen and two of its vicinal oxygens in each ligand. The authors suggested that zinc was introduced during repeated chromatography on Si gel and that the complex probably exists as a mixture of diastereoisomers.

The $^1$H-nmr, ir, and uv/vis data of the major blue pigment isolated from *P. herquei* in the present work match closely those reported for *G. abietina* (13) and *R. hypogea* (14). Analysis by fabms with 3-nitrobenzyl alcohol as matrix produced an intense cluster of ions centered on *m/z* 1390, indicating that the pigment from *P. herquei* was isolated as the zinc complex ($^{12}C_{76}{}^{1}H_{64}{}^{16}O_{20}{}^{14}N_2{}^{64}Zn$ = 1388.3343 daltons). A daughter ion spectrum of the *m/z* 1390 peak resulted in fragment ions at *m/z* 663 (loss of Zn to give **2**), a cluster at *m/z* 728 (single molecule of **2** coordinated to Zn), and a cluster at *m/z* 1064 (loss of $C_{19}H_{18}O_5$ from cleavage of C-N bond). The highest mass ion observed in the eims was *m/z* 326 corresponding with the $C_{19}H_{18}O_5$ fragment (cf. 13), while in $NH_3$ desorption cims the equivalent protonated fragment was seen at *m/z* 327. Ions at *m/z* 341 and 342 may represent a similar fragmentation but with charge retention by the nitrogen-bearing moiety.



**2**

Fabms of the minor blue pigment revealed that the high mass cluster of ions was shifted compared with that of the major pigment, that is, *m/z* 1383 was the most intense ion and the profile differed. It was suspected that the difference between the two compounds could

be the replacement of $Zn^{2+}$ by $Ni^{2+}$ in the minor pigment. Thus, the two compounds were submitted to analysis by inductively coupled plasma ms (icpms). The distribution of the metal content of the major pigment (Figure 1) indicates the expected dominance by zinc; however, the predominant metal in the minor pigment is nickel, supporting the postulated $Ni^{2+}$ complex (the relatively high content of zinc is not unexpected as the minor pigment was isolated as a shoulder in the chromatogram and probably contains a significant content of the major zinc-containing pigment). From the elemental formula $C_{76}H_{64}O_{20}N_2Zn$, a zinc content of 47 mg/g pigment would be expected. The icpms analysis resulted in a figure of $44 \pm 3$ mg/g, with iron, cobalt, nickel, and copper accounting for a further $12 \pm 1$ mg/g. Quantitative results cannot be presented for the minor pigment, as there was insufficient mass for accurate measurement.

Hence, the major blue pigment of *P. herquei* is based on **2** as postulated previously (13). Although the present data do not allow unambiguous proof that the zinc-complexed form exists in the organism, the presence of abundant blue pigment in the initial mycelial extract lends credence to this view (cf. 14). The fact that **1** is produced by *P. herquei*, *G. abietina* (13), *R. hypogea* (14), and *Aspergillus silvaticus* (12) and that **2** or its zinc complex has been shown to be produced by the first three organisms is noteworthy, as these are taxonomically relatively divergent Ascomycetes. In addition to the $Zn^{2+}$ and novel $Ni^{2+}$ complexes, similar octahedral complexes of two anions of **2** to $Fe^{2+}$, $Co^{2+}$, and $Cu^{2+}$ are feasible based on the icpms data and known coordination chemistry.

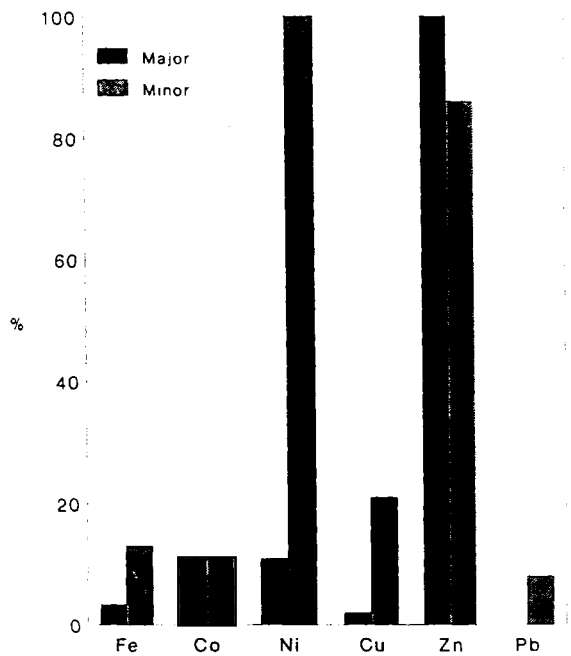



FIGURE 1. Distribution of metals in major (solid bars) and minor (hatched bars) blue pigments of *Penicillium herquei*, as determined by inductively coupled plasma ms. The concentration of metals other than those shown is <1% that of zinc in the major pigment and <7% that of nickel in the minor pigment.

## EXPERIMENTAL

INSTRUMENTATION.—Analytical hplc was performed on a Waters 600E/990 Photodiode Array Detector system, while a Waters Prep 3000 system was used for preparative hplc. A Perkin-Elmer Lamda 17 spectrophotometer and a Nicolet 5PC FT-IR were used to acquire uv/vis and ir data, respectively. $^1$H-nmr spectra were recorded on a Bruker AM 300 MHz spectrometer. Ms analyses were made on a VG Trio 3 instrument, and metal content was determined on a VG Plasmaquad II icpms.

FERMENTATION CONDITIONS.—*P. herquei* (IMI culture collection number 89376) submerged culture inoculum was prepared by suspending the biomass of a mature potato dextrose agar slope culture in 5 ml 10% aqueous glycerol. An aliquot (1.5 ml) of the suspension was transferred to a 2-ml cryovial and stored at −135°. After recovery from storage, 0.25 ml suspension was inoculated into 10 ml GGSM-1JT (glycerol 1.5%, glucose 10%, soya bean peptone 1.5%, malt extract 0.5%, NaCl 0.3%, $CaCO_3$ 0.1%, Tween 80 0.1%) in a test tube (200 × 25 mm) and shaken at 240 rpm, inclined to 60°, for 4 days at 25°, ensuring that the biomass remained submerged throughout. This culture was then transferred to 300 ml of the same medium in a 2 liter conical flask and incubated for 4 days (240 rpm, 25°). The whole seed culture was then transferred, via a sterile 500-ml aspirator, to a 14-liter Chemap stirred vessel containing 10 liters CASMO-NP3T (molasses 6.04%, casein enzymatic hydrolysate 0.339%, sodium phytate 0.006%, $CaCO_3$ 0.1%, Tween 80 0.1%). This production culture was incubated for 7 days with impeller speed 500 rpm, air flow rate 2.5 standard liters per min, temperature 25°, and on-line silicone anti-foam control. The biomass of the culture was separated from the liquor by centrifugation.

ISOLATION OF MAJOR BLUE PIGMENT.—The $CH_2Cl_2$ extract of lyophilized mycelia was chromatographed on flash Si gel, eluting with $CHCl_3$ to afford a blue-green fraction. The blue pigment was separated from physcion and **1** by chromatography on a 47 × 300 mm Delta-Pak $C_{18}$ radially compressed cartridge (Waters) eluted isocratically with MeCN-$CH_2Cl_2$ (55:45) at 60 ml/min. Purity checks were made on an 8 × 100 mm Nova-Pak $C_{18}$ cartridge (Waters) eluted with the above mobile phase at 2 ml/min. Uv ($CH_2Cl_2$) λ max (ε) 227 (64000), 265 (68000), 367 (53000), 404 (43000), 612 (50000) nm; ir (KBr diffuse reflectance) 2922, 2856, 2360, 1745, *1608*, 1550, 1460, 1455, 1442, 1394, 1386, 1384, 1354, 1332, 1306, 1280, *1206*, *1184*, 1104, 1062, 1034, 970, 956, 932, 870, 818, 778, 704 cm$^{-1}$; $^1$H nmr ($CDCl_3$) δ 1.28 (bm, alkyl $CH_3$ groups), 2.81 (bs, aryl $CH_3$ groups), 4.62 (q, methine H of dihydrofuran), 6.66 (bs, aryl H), 6.88 (bs, aryl H), 13.5 and 14.5 (m, strongly associated OH groups).

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