4,9-Dihydroxyphenazine-1,6-dicarboxylic Acid Dimethylester and the 'Missing Link' in Phenazine Biosynthesis

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4,9-Dihydroxyphenazine-1,6-dicarboxylic acid dimethylester, the ester form of a proposed 'missing link' in the biosynthesis of phenazines, has been isolated from a strain of *Pseudomonas cepacia*.

INTRODUCTION

Morris & Roberts (1959) isolated a phenazine pigment from a bacterium able to synthesize poly- β -hydroxybutyric acid, but did not elucidate its structure. Later this pigment-producing bacterium was classified as *Pseudomonas multivorans* by Stanier, Palleroni & Doudoroff (1966), but is now known as *Pseudomonas cepacia* (Ballard *et al.*, 1970).

From a strain of *P. multivorans* (= *P. cepacia*) we have isolated probably the same pigment and found that its structure seems to be related to that of the proposed 'missing link' in phenazine biosynthesis (Podojil & Gerber, 1970). During the biosynthesis of the phenazines pyocyanin and iodinin, two molecules of shikimic acid give rise to the phenazine ring (Hollstein & Marshall, 1972; Flood, Herbert & Holliman, 1972; Hollstein & McCamey, 1973; Herbert, Holliman & Sheridan, 1974, 1976) with the branch point at chorismic acid (Calhoun, Carson & Jensen, 1972; Longley *et al.*, 1972; Byng & Turner, 1976). However, the nature of any steps between chorismic acid and the phenazine ring was unknown. We report here the isolation and structural elucidation of a phenazine pigment which fits in with the common precursor hypothesis of Podojil & Gerber (1970).

METHODS

Strain. The pigment was isolated from strain ATCC 17460 of *P. multivorans* (Stanier *et al.*, 1966), now named *P. cepacia* (Ballard *et al.*, 1970); in contrast to the findings of Stanier *et al.* (1966), it did not utilize saccharose or xylose as sole carbon source.

Medium. The medium, used in solid form, contained (g l⁻¹): KH₂PO₄, 4; MgSO₄·7H₂O, 0·5; (NH₄)₂SO₄, 5; FeSO₄·4H₂O, 0·05; glucose, 15; Bacto-agar (Difco), 18; the pH was adjusted to 7·4 with KOH. This medium (800 ml) was mixed with 200 ml DST agar (Oxoid) before pouring into plates.

Culture conditions. After inoculation the plates were incubated at 30 °C for 24 h and subsequently at room temperature for 3 d.

Isolation of the pigment. Organisms were scraped off plates, suspended in acetone, stirred for 1 h, allowed to stand and then the supernatant was removed by decanting. Treatment with acetone and decantation was repeated twice. The cell material was washed with methanol and stirred for 30 min with methanol/11·6 M-HCl (19:1, v/v); 1 vol. chloroform and 4 vol. water were then added. The chloroform phase was concentrated to one-tenth of its original volume and extracted with 2 vol. 3 M-HCl. The aqueous phase was

	Compound	R ¹	R ²	R ³	R ⁴
I	OH	COOCH ₃	COOCH ₃	OH
	II	OCH ₃	COOCH ₃	COOCH ₃	OCH ₃
	III	OCH ₃	COOH	COOH	OCH ₃
	IV	OCH ₃	H	H	OCH ₃
	V	OH	CH ₃	CH ₃	OH
	VIII	OCH ₃	OCH ₃	H	H
	IX	OH	OH	COOCH ₃	COOCH ₃
	VI	R = H			
	VII	R = CH ₃

	Structure A			Chemical shift (δ)	
				8.60 p.p.m.	
				7.28 p.p.m.	

Fig. 1. Chemical structure of the phenazine pigment of *Pseudomonas cepacia* and related derivatives.

diluted with water (4 vol.) and extracted twice with chloroform. The washed and dried chloroform phase yielded the pigment (I, Fig. 1) after evaporation.

Analytical procedures. Infrared (i.r.) spectra of KBr discs were recorded using a Perkin-Elmer 720 spectrometer and nuclear magnetic resonance (n.m.r.) spectra were obtained using a Varian EM 390 instrument. A Varian MAT 731 mass spectrometer was used to obtain mass spectra and for precise mass determinations. Melting points could not be determined due to sublimation prior to liquefaction.

Preparation of derivatives of the pigment.

4,9-Dimethoxyphenazine-1,6-dicarboxylic acid dimethylester (II, Fig. 1). To the pigment (I) dissolved in benzene, an ether solution of diazomethane was added. Evaporation to dryness and sublimation (133 Pa, 200 °C) yielded pure II.

4,9-Dihydroxy-1,6-dimethylphenazine (V, Fig. 1). Pigment I was treated for 2 h with LiAlH₄ in boiling tetrahydrofuran then 2 M-HCl was added, followed by extraction with ether and sublimation (133 Pa, 150 °C).

4,9-Dimethoxyphenazine-1,6-dicarboxylic acid (III, Fig. 1). Compound II was treated overnight with 10% (w/v) NaOH (60 °C), washed twice with chloroform and acidified with 2 M-HCl. The red precipitate was collected and washed with chloroform and methanol.

4,9-Dimethoxyphenazine (1,6-Dimethoxyphenazine) (IV, Fig. 1). Compound III was dissolved in diphenyl ether, mixed with Cu powder and heated overnight at 250 °C. The mixture was filtered through silica gel. Chromatography on silica gel using petroleum ether eluted the diphenyl ether. Subsequent elution with chloroform yielded IV.

5,10-Dihydro-4,9-dimethoxy-5,10-dimethylphenazine-1,6-dicarboxylic acid dimethylester (VII, Fig. 1). This derivative was obtained by hydrogenation (PtO₂) of II in ethanol for 2 h. The dihydro derivative (VI) was dissolved in dimethylformamide and methylated with NaH/CH₃I.

Table 1. Summary of data on the pigment (I) and its derivatives

Compound	Number	Precise mass (<i>m/e</i>)		Empirical formula	U.v. absorption maxima in methanol (nm)
		Found	Calculated		
4,9-Dihydroxyphenazine-1,6-dicarboxylic acid dimethylester	I	328.0702	328.0695	C ₁₆ H ₁₂ N ₂ O ₆	450, 365, 350
4,9-Dimethoxyphenazine-1,6-dicarboxylic acid dimethylester	II	358.1166*	358.1176	C ₁₈ H ₁₈ N ₂ O ₆	430, 362
4,9-Dimethoxyphenazine-1,6-dicarboxylic acid	III	330*			
1,6-Dimethoxyphenazine	IV	240.0899	240.0898	C ₁₄ H ₁₂ N ₂ O ₂	
4,9-Dihydroxy-1,6-dimethylphenazine	V	240.0899	240.0899	C ₁₄ H ₁₂ N ₂ O ₂	
5,10-Dihydro-4,9-dimethoxy-5,10-dimethylphenazine-1,6-dicarboxylic acid dimethylester	VII	386.1473	386.1477	C ₂₀ H ₂₂ N ₂ O ₆	

* Hydrogenation in the source (Budzikiewicz, 1970).

Table 2. Features of n.m.r. spectra of the phenazine pigment of *Pseudomonas cepacia* and its derivatives

Spectra were obtained at 90 MHz using samples dissolved in CDCl₃ or, for compound III, CF₃-COOD. Results show chemical shifts (δ -values, in p.p.m.) and, where appropriate, coupling constants (*J*, in Hz) and multiplicity (s, singlet; m, multiplet).

Compound	H-2,7	H-3,8	H-4,9	O-CH ₃	COOCH ₃	-CH ₃	N-CH ₃
I	8.60 <i>J</i> 8.4	7.28 <i>J</i> 8.4	—	—	4.04 s	—	—
II	8.58 <i>J</i> 8.4	7.14 <i>J</i> 8.4	—	4.24 s	4.08 s	—	—
III	9.45 m	7.93 m	—	4.57 s	—	—	—
IV	7.15 <i>J</i> 7.5; 1.5	7.80 <i>J</i> 8.0; 7.5	8.08 <i>J</i> 9.0; 1.5	4.20 s	—	—	—
V	7.63 <i>J</i> 8.4	7.02 <i>J</i> 8.4	—	—	—	2.83 s	—
VII	8.50 <i>J</i> 8.4	7.30 <i>J</i> 8.4	—	4.27 s	4.05 s	—	2.77 broad s

RESULTS

The pigment (I) was extracted from harvested organisms as described in Methods. The properties of various derivatives prepared from it are summarized in Table 1, and features of their n.m.r. spectra are shown in Table 2. Figure 1 shows the basic chemical structure of the isolated pigment together with eight theoretically possible derivatives (II to IX).

The elemental composition of the isolated pigment, C₁₆H₁₂N₂O₆, was determined by exact mass measurement. D₂O exchange proved the presence of two acidic protons. Infrared absorption at 3300 to 3500 cm⁻¹ and formation of a dimethyl ether (*vide infra*) showed that these protons belonged to two hydroxyl groups. The remaining four oxygen atoms were accounted for by two aromatic carbomethoxyl groups (i.r., strong absorptions at 1715 cm⁻¹; n.m.r., singlet at δ = 4.04 p.p.m., 6H, disappearing after reduction with LiAlH₄). The remaining four hydrogen atoms were aromatic ones (n.m.r., two doublets at δ = 8.60 and 7.28 p.p.m.), the coupling constant (*J* = 8.4 Hz) showing them to be *ortho* to each other. The large difference in chemical shift ($\Delta\delta$ = 1.32 p.p.m.) could be explained only by opposing substituent effects (Pretsch *et al.*, 1976) (de-shielding by the COOCH₃, and

shielding by the OH function). Correspondingly, in the dimethyl derivative (V) the low-field protons were found at $\delta = 7.63$ p.p.m. All these data were consistent with the partial structure A (Fig. 1). The shielding effect involving *para*-H in phenol is only of minor importance in phenazines (cf. IV, and Sigg & Toth, 1967; Weigele & Leimgruber, 1967) supporting the identification of the proton giving a signal at $\delta = 7.28$ p.p.m. as *ortho* to the hydroxyl group. Analogous arrangement of the substituents and identical spectral behaviour have been reported for lomofungin (1-carbomethoxy-5-formyl-4,6,8-trihydroxyphenazine; Tipton, Kenneth & Rinehart, 1970). The observation of the coincidence of all n.m.r. signals by pairs with reference to partial structure A was compatible both with structure I (C_{2v} symmetry) and structure IX (C_{2v} symmetry). It was possible to distinguish between these because the pigment could be degraded to 1,6-dimethoxyphenazine (IV) which was identical with an authentic sample (thin-layer chromatography, mass spectrometry, n.m.r., i.r.). Further evidence for structure I has been provided by the *N,N*-dimethyl-*N,N*-dihydro derivative, VII (obtained by hydrogenation and methylation). The n.m.r. spectrum of this derivative exhibited only one *N*-methyl signal (slightly broadened by the quadrupole moment of nitrogen) while for the analogous product from IX two signals would have been expected. The pigment isolated from *P. cepacia* was thus shown to be the same as compound I (partial structure A).

DISCUSSION

4,9-Dihydroxyphenazine-1,6-dicarboxylic acid was suggested by Podojil & Gerber (1970) to be an intermediate in the biosynthesis of phenazines. Byng & Turner (1976, 1977) presented evidence that, in *Pseudomonas phenazinum*, phenazine-1,6-dicarboxylic acid acts as a branch point compound for the biosynthesis of 1,6-dihydroxyphenazine (and iodinin) and for phenazine-1-carboxylic acid and related compounds. This would explain why all naturally occurring phenazines possess C-substituents only on C₁ or C₄ and C₆ (Tipton *et al.*, 1970; Nakamura, Maeda & Umezawa, 1964; Nakamura *et al.*, 1959; Aguilar-Santos, 1970; Gerber, 1969; Olson & Richards, 1967). The intermediates between chorismic acid and phenazine-1,6-dicarboxylic acid are not known. Byng & Turner (1976) suppose that the number of steps between chorismic acid and 'the hypothetical key intermediate common to all natural phenazines' may be large. It may well be that 4,9-dihydroxyphenazine-1,6-dicarboxylic acid dimethylester is one of these intermediates. Hollstein, Krisov & Mock (1976) reported that phenazine-1,6-dicarboxylic acid dimethylester is metabolized by *Pseudomonas aureofaciens*. We have found that a mutant of a strain of *P. aureofaciens* excretes in addition to phenazine-1-carboxylic acid, 2-hydroxyphenazine-1-carboxylic acid and 2-hydroxyphenazine-1,6-dicarboxylic acid (strain 13cR, unpublished observation).

In preliminary experiments with *P. aureofaciens*, degradation of the pigment to phenazine-1,6-dicarboxylic acid or phenazine-1-carboxylic acid was not observed. A possible explanation is that the molecule did not penetrate the cell wall (Byng & Turner, 1976).

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