CYANOMYCIN, ITS IDENTITY WITH PYOCYANINE

MALCOLM H. VON SALTZA, JEROLD A. LAST*, PAUL G. STAPLETON, MARLENE L. RATHNUM and SAUL L. NEIDLEMAN

The Squibb Institute for Medical Research
New Brunswick, New Jersey, U.S.A.

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Pyocyanine, isolated in this laboratory from *Pseudomonas aeruginosa*, is identical to the antibiotic cyanomycin previously isolated from a *Streptomyces* species.

1. Producing Organism

An isolate, Squibb culture collection No. 8510, was identified as a strain of *Pseudomonas aeruginosa* (W. TREJO, personal communication).

2. Production and Isolation of Antibiotic

The isolated organism produced a blue antibiotic during stationary fermentation in a medium consisting of casitone (Difco) 2 g, yeast extract 1 g, and $MgCl_2$ 1 g in 1 liter of water, adjusted to pH 8.0 with NaOH. The flasks were inoculated (5 % v/v), and allowed to stand 3 days at 37°C. Antimicrobial activity was assayed by a filter paper disc method using bioautography against *Staphylococcus aureus* 209P or *Escherichia coli*. If the antibiotic was tested in the same manner against *Candida albicans*, no zones of inhibition were seen.

The active blue substance was originally extracted and purified by the procedure shown in Fig. 1. After the antibiotic's similarity to cyanomycin was recognized, the procedure outlined in Fig. 2, which is essentially that of FUNAKI *et al.*[1], was used.

3. Properties of the Isolated Antibiotic

The active material obtained by the scheme of Fig. 1, consisting mainly of bluish-black crystals, was examined by thin-layer chromatography (TLC) on silicic acid (10 % methanol : 90 % chloroform) and found to be a mixture of pigments**. A silicic acid column, developed with increasing amounts of methanol in chloroform, was used to separate these colored components. Bioassay of eluted fractions showed activity against *S. aureus* in yellow, greenish brown, and blue bands, eluted in that order. High resolution mass spectroscopic examination of the yellow crystals (m. p. 157~157.5°C), obtained from the first band by evaporation of solvent, showed a parent peak at m/e 196 and a molecular formula of $C_{12}H_8N_2O$. This compound was identified as 1-phenazinol by comparison with an authentic sample (m. p. 158°C). The mixture melting point was undepressed, and the UV spectra in absolute ethanol and metha-

* To whom all correspondence should be sent.

** This material had been kept in a desiccator for several days. Decomposition of the free base, which was later shown to occur under such conditions, probably accounted for some of the pigments seen.

Fig. 1. Purification of blue antibiotic. Scheme 1.

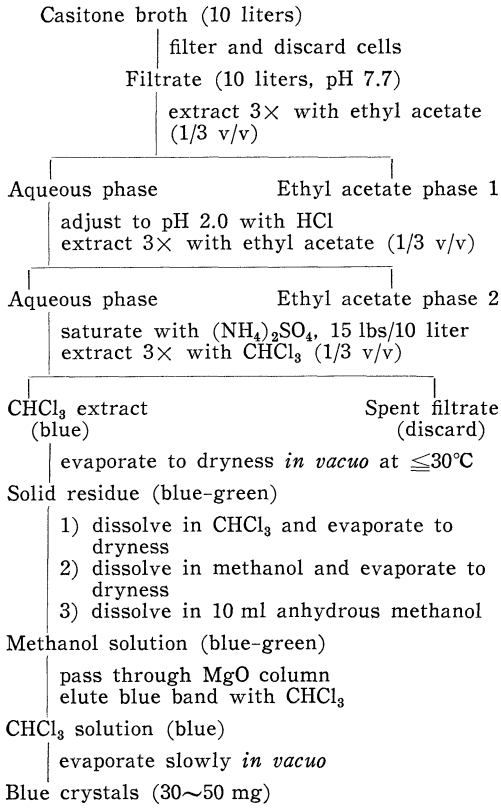
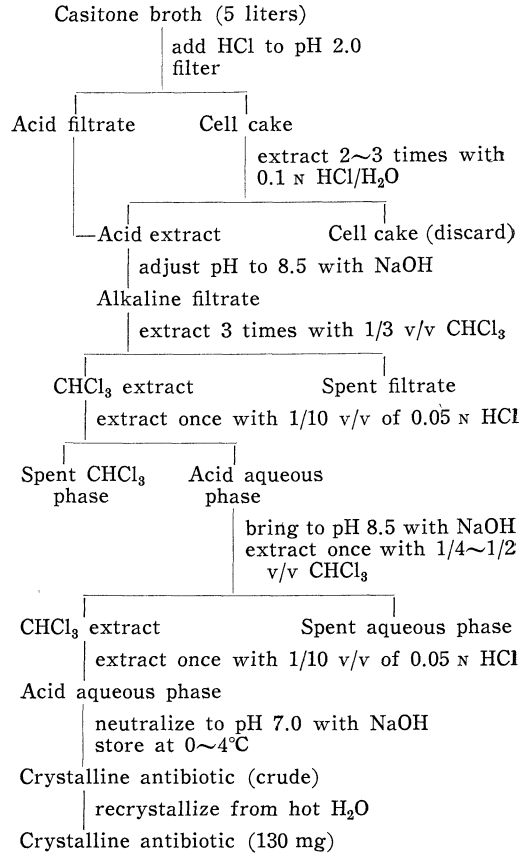


Fig. 2. Purification of blue antibiotic. Scheme 2.



nolic KOH (0.2 %) were identical; ($\lambda_{max}^{E^tOH}$ 263 mμ, 358 mμ; $\lambda_{max}^{MeOH-KOH}$ 298 mμ).* The mass spectra were very similar, with the peak at m/e 196, attributed to the molecular ion, also representing the base peak in both tracings.

The 1-phenazinol accounted for the antibiotic activity in the yellow band.

The material in the blue band was purified by TLC on silicic acid (12 % MeOH : 88 % $CHCl_3$). The blue-black crystals, obtained by elution of the bright blue band from the TLC plates with 30 % methanol - 70 % chloroform, after recrystallization from water, melted at 130~131°C.

Anal. Calc'd for $C_{13}H_{10}O_2N_2 \cdot H_2O$:**	C 68.41,	H 5.30,	N 12.27
$C_{14}H_{12}O_2N_2$:	C 69.99,	H 5.03,	N 11.66
$C_{15}H_{12}O_2N_2$:	C 71.41,	H 4.80,	N 11.11
Found :	C 70.89,	H 4.98,	N 11.83

Although the empirical formula $C_{14}H_{12}O_2N_2$ seemed most consistent with the elemental analyses, the formula $C_{15}H_{12}O_2N_2$, which had been assigned to the antibiotic cyanomycin[1], was almost as satisfactory. These aberrant analytical results were probably

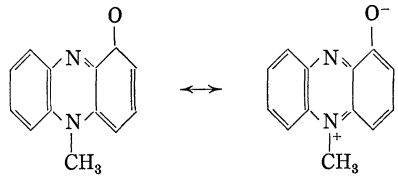
* The UV spectrum of 1-phenazinol in MeOH, in addition, shows many shoulders in the region 340~390 mμ. When petroleum ether is the solvent, the UV spectrum clearly shows six distinct peaks in this same region. This property was of value in identifying 1-phenazinol.

** It has been reported that pyocyanine recrystallized from water is obtained in a hydrated form[2].

due to the instability of the blue antibiotic in the free base form.*

To our knowledge, there have been no proposals for the structure of cyanomycin. A comparison of the reported properties of cyanomycin[1] with the properties of our blue antibiotic suggested that the compounds might be identical; however, we have not been able to obtain a sample of cyanomycin for a direct comparison. The empirical formula of our blue antibiotic, even though somewhat uncertain, was compatible with a substituted phenazine structure. The observation that decomposition of a homogeneous sample of the blue antibiotic gave rise to a yellow material, identified by its melting point and mass spectrum as 1-phenazinol, strongly supported this structural assignment.

Consideration of the properties of certain phenazines that occur as bacterial pigments[3] convinced us that the blue compound was closely related to the well-known pigment, pyocyanine, the structure of which is shown below.



4. Synthesis and Properties of Pyocyanine

We prepared synthetic pyocyanine by methylation of 1-phenazinol with dimethylsulfate[4] followed by purification by successive extractions from chloroform into aqueous 0.05 N HCl and from pH 8.5 aqueous phase into chloroform (*cf.* Fig. 2). A comparison of the physical properties of pyocyanine with those of the isolated blue compound showed them to be identical (see Table 1).

The infrared (IR) spectrum of pyocyanine in $CHCl_3$ is shown (Fig. 3). This spectrum is identical with that of the blue antibiotic isolated by us and similar to that of cyanomycin[1]. Since the solvent in which the IR spectrum of cyanomycin

Table 1. Points of similarity between cyanomycin, the antibiotic isolated from fermentations, and chemically synthesized pyocyanine

Property	Cyanomycin	Synthetic pyocyanine	Isolated antibiotic
Melting point, °C	128 (dec)	133 (dec)	130~131 (dec)
Picrate salt, m.p., °C	157.5	158~159	158~158.5
Perchlorate salt, m.p., °C	—	—	220~221**
NMR maxima, τ	—	1.1~2.4, 4.95*	1.1~2.4, 4.95
% C Found	69.22	—	70.89
% N Found	10.76	—	11.83
% H Found	5.16	—	4.98
Color	Dark blue	Dark blue	Dark blue
Antibiotic spectrum	Gram (+) (−)	Gram (+) (−)	Gram (+) (−)
Acid-base indicator	Blue-red	Blue-red	Blue-red
Crystalline form (from H_2O)	Needles	Needles	Needles

* The peaks in the aromatic region (1.1~2.4) showed an identical pattern; the ratio of aromatic to N-CH_3 protons by integration was 6.6 : 3.0 (pyocyanine) and 7.1 : 3.0 for the isolated antibiotic.
** Literature value, pyocyanine perchlorate[5], 221~223°C.

* The blue compound kept as the free base decomposed unless stored under nitrogen at 0°C in the dark. It was stable as the salt of an acid, such as hydrochloric or picric. Cyanomycin was also reported to be unstable[1].

Fig. 3. Infrared absorption spectrum of pyocyanine ($CHCl_3$).

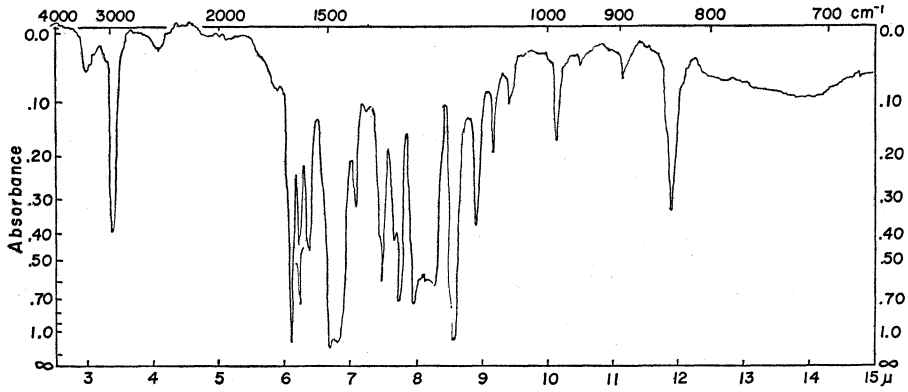
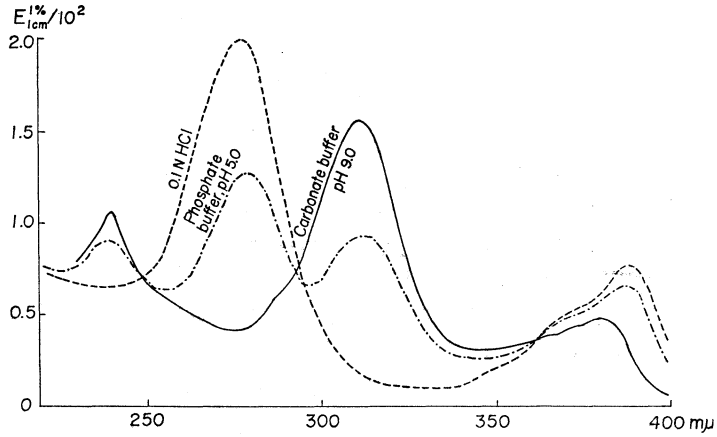


Fig. 4. Ultraviolet absorption spectra of pyocyanine in 0.1 N HCl, in pH 5 phosphate buffer, and in pH 9.0 carbonate buffer.



was measured was not described by FUNAKI *et al.*[1], an absolute comparison is not possible.

The UV spectrum of pyocyanine (Fig. 4) was determined under the conditions described for cyanomycin. Our spectral curves are essentially identical with those of FUNAKI *et al.*[1]

5. Preparation and Comparison of Derivatives

A picrate of synthetic pyocyanine was prepared in ethanol and recrystallized from absolute ethanol. The iridescent black crystals, melting point 158~159°C, were dried at 50°C (1 mm Hg, 4 hr., over P_2O_5).

Anal. Calc'd for $C_{19}H_{13}O_8N_5$:	C 51.94,	H 2.98,	N 15.94.
$C_{19}H_{13}O_8N_5 \cdot C_2H_5OH$:	C 51.96,	H 3.95,	N 14.43.
Found:	C 52.24,	H 3.47,	N 15.43.

Our elemental analyses of the picrate of pyocyanine favor the non-solvated derivative.

A picrate derivative of the blue antibiotic isolated in our laboratory, prepared in the same manner, melted at 158~158.5°C; the mixture melting point with the picrate of synthetic pyocyanine was undepressed.

Anal.: Found: C 51.81, H 2.87, N 16.02.

Moreover, the IR ($CHCl_3$) and the UV spectra ($\lambda_{max}^{abs\ EtOH}$ 239, 287, 328, 373 (sh) and 399 (sh) mμ) were identical.

The picrate salt of cyanomycin was reported to have a melting point of 157.5°C and a UV spectrum ($\lambda_{max}^{abs\ EtOH}$ 238, 286, 324 mμ) that changed somewhat with concentration[1]. It should be mentioned that the picrate of pyocyanine has been reported in the literature to have a melting point of 194~195°C (dec.)[5].

We also prepared the crystalline perchlorate salt of the isolated antibiotic for comparison of its melting point with that reported for the perchlorate of pyocyanine. This derivative, after recrystallization, gave a melting point of 220~221°C. The melting point reported for pyocyanine perchlorate is 221~223°C (dec.)[5].

The picrate of pyocyanine was dissolved in aqueous alkali at pH 9. Extraction with chloroform yielded a blue compound, identical in its UV spectrum (in absolute ethanol) and Rf value (TLC-12% methanol: 88% $CHCl_3$) with the original pyocyanine; this result suggests that the picrate was not the derivative of a degradation product.

Discussion

These results indicate that pyocyanine, isolated from pseudomonads in this and other laboratories[2], is identical with the antibiotic cyanomycin, previously reported from a *Streptomyces* species.

There have been numerous reports of the biosynthesis of phenazines by actinomycetes (for review see LECHAVELIER[6]) but, to our knowledge, pyocyanine itself has not previously been encountered in Actinomycetales.

The points of similarity between cyanomycin and pyocyanine, as presented above, are summarized in Table 1. The elemental analyses reported by FUNAKI *et al.*[1] for cyanomycin picrate do not agree well with either the theoretical values calculated for pyocyanine picrate or with the values calculated for a picrate based on their cyanomycin formula, $C_{15}H_{12}N_2O_2$. Although the melting point for cyanomycin picrate agrees well with that of our pyocyanine picrate, which appears to be non-solvated, there is evidence that pyocyanine picrate picks up water of hydration from the air. This is not sufficient, however, to explain the low N content[1] for cyanomycin picrate, as shown below.

Calculated for: $C_{21}H_{15}O_9N_5$: C 52.39, H 3.14, N 14.55.
Found[1]: C 50.51, H 3.12, N 12.74.

Hence, we feel that our assignment of the formula, $C_{13}H_{10}ON_2 \cdot C_6H_3O_7N_3$, to cyanomycin picrate is as consistent with the data of FUNAKI *et al.*[1] as is their own assignment of $C_{15}H_{12}O_2N_2 \cdot C_6H_3O_7N_3$.

The melting point of the picrate salt of pyocyanine, previously reported as 194~195°C[5], was observed in this laboratory, both on material isolated from fermentation broths and on chemically synthesized pyocyanine, to be 158~159°C. This discrepancy might be attributable to the existence of either various solvated forms of pyocyanine picrate or polymorphic crystal forms. The preparations obtained under controlled conditions, however, *e. g.*, working under N_2 to minimize oxidative demethylation, melted at 158~159°C. Finally, it should be emphasized that a sample of crystalline, homogeneous pyocyanine, on storage in contact with air or light, gave rise to several colored derivatives, one of which was identified as 1-phenazinol. This conversion of pyocyanine to 1-phenazinol under various conditions is well known[7]. Thus, 1-phenazinol identified in fermentation mixtures, especially those containing pyocyanine, may represent an artifact

of the isolation procedure rather than a true fermentation product.

It is also possible that the antibiotic preparation described by MUKHERJEE and NANDI[8] from *P. aeruginosa* is a mixture of phenazines similar to that described in our work.

Acknowledgements

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