Pigments of *Pseudomonas* Species. Part IV.[1] *in vitro* and *in vivo* Conversion of 5-Methylphenazinium-1-carboxylate into Aeruginosin A

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5-Methylphenazinium-1-carboxylate has been synthesised. It has been converted by aqueous ammonia and cultures of *Ps. aeruginosa* into aeruginosin A.

On the basis of known reactions of the 5-methylphenazinium ion (1), *viz.* its light-induced hydroxylation to give pyocyanin (2) [2] and its reaction with ammonia to give the 2-amino-10-methylphenazinium ion (8),[3] it was proposed [4] that the betaine, 5-methylphenazinium-1-carboxylate (3) was a common intermediate in the biosynthesis of pyocyanin (2) and aeruginosin A (7).[5] We have already reported evidence that this betaine is the immediate biological precursor of pyocyanin [6] and we now describe its *in vitro* and *in vivo* conversion into aeruginosin A.

Although phenazine-1-carboxylic acid (9) (ref. 7) could not be quaternized directly, its methyl ester (10) was successfully treated with methyl 2,4-dinitrobenzenesulphonate [8] under fairly critical conditions to give (5). Anion exchange followed by hydrolysis then gave the betaine (3) in the form of its hydrochloride (4). Microanalyses were inconsistent, but its structure was confirmed both by spectral evidence and its ready conversion into phenazine-1-carboxylic acid (9) with alkali.

The reaction of the phenazinium salt (4) with ammonia rapidly gave aeruginosin A (7) and phenazine-1-carboxylic acid (9), the yield of aeruginosin A depending markedly on the ammonia concentration (Table 1);

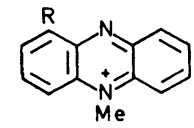
TABLE 1

Reaction of 1-carboxy-5-methylphenazinium chloride (4) with aqueous ammonia

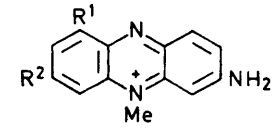
Ammonia concentration (N)	Yield of aeruginosin A (%)
15·1	64
7·6	27·5
3·8	14
1·9	9
0·9	7·5
0·09	4
0·009	0

ammonium salts were ineffective in this reaction. This conversion confirms that methylation of (10) was at the 5- rather than the 10-nitrogen atom.

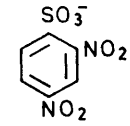
That a single amination product was being formed was confirmed by dequaternization of the reaction mixtures when the products could be compared with samples of 7-aminophenazine-1-carboxylic acid (11) (ref. 9) and 3-aminophenazine-1-carboxylic acid (12): [10] only 7-aminophenazine-1-carboxylic acid was apparent



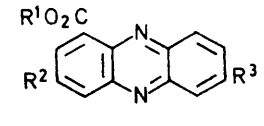
(1) R = H
(2) R = O⁻
(3) R = CO_2^-
(4) R = CO_2H; Cl^-
(5) R = CO_2Me



(7) $R^1 = CO_2^-$, R^2 = H
(8) $R^1 = R^2$ = H

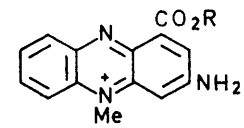


(6) R = CO_2Me; methosulphate



(9) $R^1 = R^2 = R^3$ = H
(10) R^1 = Me, $R^2 = R^3$ = H
(11) $R^1 = R^2$ = H, $R^3 = NH_2$
(12) $R^1 = R^3$ = H, $R^2 = NH_2$

in each reaction mixture. Thus (13) is not a product of amination. This contrasts with the results of reaction of ammonia with the ester (6) where substitution occurs on the methoxycarbonyl-bearing ring to give (14).[11] This difference must presumably be



(13) R = H
(14) R = Me

attributed to a $+I$ effect of the carboxylate anion present in (4) under the reaction conditions as against a $-I - M$ effect of the methoxycarbonyl group in (6).

When 1-carboxy-5-methylphenazinium chloride (4) was added to liquid cultures of *Ps. aeruginosa*, conveniently on the 2nd or 3rd day of growth when no red pigmentation was apparent, aeruginosin A was produced (Table 2). It can be seen that significant amination of (3) occurs at a total ammonia (free + salts) concentration lower than the free ammonia concentration required

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[3] F. Kehrmann and E. Havas, *Ber.*, 1913, **46**, 341.
[4] F. G. Holliman, *S. African Ind. Chem.*, 1961, **15**, 233.
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[6] M. E. Flood, R. B. Herbert, and F. G. Holliman, *Chem. Comm.*, 1970, 1514.
[7] S. R. Challand, R. B. Herbert, and F. G. Holliman, *Chem. Comm.*, 1970, 1423.
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[9] F. G. Holliman, B. A. Jeffery, and D. J. H. Brock, *Tetrahedron*, 1963, **19**, 1841.
[10] D. J. H. Brock and F. G. Holliman, *Tetrahedron*, 1963, **19**, 1903.
[11] V. S. Mokrushin, Z. V. Pashkevich, and E. N. Rysakova, *Khim-Farm. Zhur.*, 1969, **3**, 32 (*Chem. Abs.*, 1970, **72**, 31,750).

for amination of (3) *in vitro*. Thus the amination *in vivo* is clearly enzyme-mediated. Further an exo-enzyme would appear to be operating as a similar intensity of red pigmentation was apparent whether

TABLE 2

Effect of addition of 1-carboxy-5-methylphenazinium chloride (4) to *Ps. aeruginosa*

Days of growth	pH	Ammonia concentration (N) †	Yield of aeruginosin A based on weight of (4) added: With addition	Without addition
2	9·02	0·007	13·5%	0
3	9·17	0·007	22	0
Blank	9·62	0·008	3·7	—

† Free ammonia and ammonium salts.

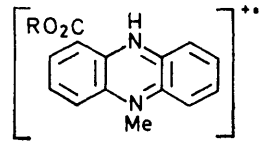
whole cultures or cell-free supernatants were used. These results then show that 5-methylphenazinium-1-carboxylate (3) is a biological precursor for aeruginosin A. We were, however, unable to isolate the betaine (3) from aeruginosin A-producing, and various other strains of *Ps. aeruginosa*.

EXPERIMENTAL

Paper chromatography was performed on Whatman no. 1 paper for qualitative experiments and on 3MM paper for isolation work, with butanol–hydrochloric acid [4 : 1; saturated with water (solvent A)], butanol–pyridine [4 : 1; saturated with water (solvent B)] and butanol–acetic acid [4 : 1; saturated with water (solvent C)] as solvents.

1-*Methoxycarbonyl-5-methylphenazinium 2,4-Dinitrobenzenesulphonate* (5).—Methyl 2,4-dinitrobenzenesulphonate [8] (5·25 g.) was dissolved in dry toluene (200 ml.). The solution was filtered (glass wool) free from insoluble material and then mixed with methyl phenazine-1-carboxylate (3·0 g.). The mixture was stirred at 60° with the exclusion of moisture and light. After 30 hr. the precipitate which had formed was collected and recrystallized from methanol–ether to give 2·87 g. of product, m.p. 183·5—184·5° (Found: C, 50·2; H, 3·2; N, 10·6; S, 6·4. $C_{21}H_{16}N_4O_9S$ requires C, 50·4; H, 3·2; N, 11·2; S, 6·4%), $\lambda_{max.}$ (log ε) in 3N-aqueous hydrochloric acid: 264, 314, and 387 nm. (4·81, 3·83, and 4·25).

1-*Carboxy-5-methylphenazinium chloride* (4).—1-Methoxycarbonyl-5-methylphenazinium 2,4-dinitrobenzenesulphonate (500 mg.) was dissolved in water (200 ml.). The solution was passed through an ion-exchange column (Dowex 1, chloride form). Concentrated hydrochloric acid (10 ml.) was added to the eluate and the solution was heated at 100° for 3 hr. The volume of the solution was reduced to *ca.* 10 ml. and the residual mixture was chilled. The dark green crystals (170 mg.) which separated were collected and recrystallized from 5N-hydrochloric acid. Microanalyses were inconsistent, probably due to partial losses of hydrogen chloride; the material isolated from neutral solution was not tractable. The *phenazinium chloride* was a single spot on paper chromatography with the expected R_F values (solvents A, B); $\lambda_{max.}$ (3N-HCl), 263, 308, and 388 nm. It decomposed thermally in the source of the mass spectrometer. The following major ions consistent with its structure were obtained: *m/e* 240 [$C_{14}H_{12}N_2O_2$, structure (15)], 222 ($C_{14}H_{10}N_2O$), 194 ($C_{13}H_{10}N_2$), and 180 ($C_{12}H_8N_2$). 1-*Methoxycarbonyl-5-methylphenazinium chloride* [$\lambda_{max.}$ (3N-HCl), 260, 318 (sh), and 387 nm.] similarly underwent thermal decomposition in the mass spectrometer and the following major ions were found: *m/e* 254 [$C_{15}H_{14}N_2O_2$, structure (16)], 238 ($C_{14}H_{10}N_2O_2$), 207



(15) R = H
(16) R = Me

($C_{13}H_7N_2O$), 194 ($C_{13}H_{10}N_2$), and 180 ($C_{12}H_8N_2$). On treatment with alkali (2N; 100°, 2 hr.) 1-carboxy-5-methylphenazinium chloride gave a product identical with phenazine-1-carboxylic acid [i.r. spectra (KCl disc)].

Reactions of 1-*Carboxy-5-methylphenazinium Chloride* (4). —*Synthesis of aeruginosin A* (7). 1-Carboxy-5-methylphenazinium chloride (200 mg.) was added to aqueous ammonia (600 ml.; 3·8N). The solution which was formed turned deep red rapidly. It was set aside for 1 hr. with occasional agitation and was then taken to low volume under reduced pressure and acidified. The aqueous solution was thoroughly extracted with chloroform, and its pH was adjusted to 7·0. The volume of the solution was reduced to *ca.* 10 ml. The mixture was chilled to give crystals which were recrystallized from water (Found: C, 58·15; H, 4·85; N, 14·25. Calc. for $C_{14}H_{11}N_3O_2,2H_2O$: C, 58·1; H, 5·2; N, 14·5%). The product was identical with aeruginosin A [paper chromatography (solvent A), u.v.-visible and i.r. spectra (KCl disc)].

Dequaternization (1N-aqueous sodium hydroxide, 100°, 30 min.) of the aeruginosin A, gave a product identical with 7-aminophenazine-1-carboxylic acid [paper chromatography (solvents A, B, C) and i.r. spectra (KCl disc)]; mass spectrum: *m/e* 239·069229 (M^+; calc. for $C_{13}H_9N_3O_2$: 239·069472, Δ 1·2 p.p.m.).

From the chloroform extracts (above) on evaporation a solid was obtained which, after chromatography (Kieselgel G; 0·5-mm. thick plates) and recrystallization from methanol gave yellow crystals identical with phenazine-1-carboxylic acid (i.r. spectra), *m/e* 224·059298 (M^+; calc. for $C_{13}H_8N_2O_2$: 224·058573, Δ 3·5 p.p.m.).

Reaction with various concentrations of aqueous ammonia. Solutions of 1-carboxy-5-methylphenazinium chloride (4 mg. each) in aqueous ammonia of the following normalities were set aside: 15·1, 7·6, 3·8, 1·9, 0·9, 0·09, and 0·009 N. The four solutions of highest normality were evaporated to dryness after 40 min. and the other three after 10 hr. The aeruginosin A produced in each case was isolated on 3MM paper (solvent A). Water was used to elute the aeruginosin A from the paper. Yields were measured spectrophotometrically (0·02N-hydrochloric acid solutions; 538 nm.) using as standards weighed samples of aeruginosin A which had been submitted to the above work up procedure. Percentage yields in Table 1 are calculated on a molecular weight of 274·5 for the phenazinium chloride.

In each experiment the aeruginosin A obtained was dequaternized (as above) and the product compared with 7-aminophenazine-1-carboxylic acid and 3-aminophenazine-1-carboxylic acid by paper chromatography (solvent A). A spot corresponding to 7-aminophenazine-1-carboxylic acid was apparent in each case but not one corresponding to 3-aminophenazine-1-carboxylic acid.

Reaction with various ammonium salts. 1-Carboxy-

[12] E. J. Conway, 'Microdiffusion Analysis and Volumetric Error,' Crosby Lockwood, London, 1950, 3rd edn., 98.

5-methylphenazinium chloride was dissolved in solutions of $(NH_4)_2HPO_4$, $(NH_4)_3PO_4$, and NH_4OAc each 1- and 4N- with respect to ammonia concentration; the pH was in the range 6·2—8. The solutions were kept in the dark. Even after a month there was no observable formation of aeruginosin A.

Biological Formation of Aeruginosin A.—An aeruginosin-producing culture of *Ps. aeruginosa* was grown in liquid culture as described previously.[5] After 2 or 3 days growth and before red pigmentation was apparent the culture fluids were spun free from bacteria. Samples (5 ml.) were each added to an aqueous solution (5 ml.) of 1-carboxy-5-methylphenazinium chloride (1 mg.). The solutions were set aside for 24 hr. and then concentrated. The aeruginosin A was isolated and the yield was determined as described above for the *in vitro* reactions.

A *blank* experiment was also carried out by substituting 0·008N-ammonia in a sample of the culture medium for the cell-free culture fluid above.

Ammonia concentration was measured by Conway's method.[12]

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