New Microbial Phenazines (1)

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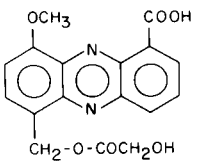
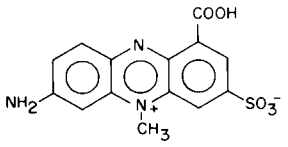
Eleven phenazines were identified as fermentation products of a bacterium. They included the previously unknown 2,9-dihydroxyphenazine-1-carboxylic acid (**4**), 1,8-phenazinediol-10-oxide (**5**) and 8-amino-1-phenazinol (**6**) as well as phenazine-1,6-dicarboxylic acid (**1**), 9-hydroxyphenazine-1-carboxylic acid (**2**) and 1,8-phenazinediol (**3**) not isolated before from natural sources.

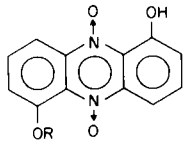
About twenty-one naturally occurring phenazines are known, all from microbial sources (2a-g). Three examples are shown to illustrate the structural diversity. Mono-*N*-oxides and β-hydroxy substituents have also been found.



Griseolutein A



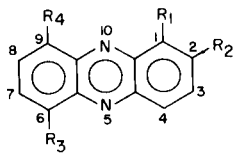
Aeruginosin B

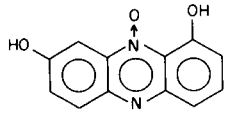


R = H, Iodinin
R = CH_3, Myxin

From an unidentified bacterium (3) growing on a well-shaken, dilute peptone-glycerol medium, we have isolated by solvent extraction and purified by column (CC) and thin-layer chromatography (TLC), as described earlier (4), eleven phenazines. Phenoxazin-3-ones were not observed. Five of the phenazines: iodinin, 1,6-phenazinediol, 1,6-phenazinediol-5-oxide, phenazin-1-carboxylic acid and 2-hydroxyphenazine-1-carboxylic acid were previously known from other microorganisms and were identified by comparison with authentic samples (5). Three other known compounds (**1**, **2** and **3**) had not been isolated before from natural sources and were shown to be identical with authentic synthetic specimens (6). (For **3** the dimethoxy derivatives were compared). Finally, **4**, **5** and **6** were new compounds. Iodinin, 1,6-phenazinediol, phenazine-1-carboxylic acid, **2**, **3**, **4** and **5** could be obtained in 10-50 mg. amounts from 4 liters of whole broth; the others were isolated in 0-5 mg. quantities.

The isolation of **1** makes it easier to visualize the biosynthesis of phenazines from shikimic acid (7). The diacid **1** could not be completely separated from phenazine-1-carboxylic acid by any single chromatographic procedure. Phenazines **2** and **4** could be separated only in the form of derivatives. The methoxy acids were separated easily; by TLC small amounts of the acetyl derivatives or the methoxy-methyl esters could usually be resolved. Acids **1**, **2** and **4** were chloroform-insoluble when pure. Apparently **3** and **4** exist in keto and enol forms since both were easily soluble in bicarbonate and each showed a carbonyl band in the infrared. When treated with diazomethane **3** furnished, in addition to the yellow dimethoxy derivative, a minor purple product.





5

(**1**) $R_1 = R_3 = COOH$, $R_2 = R_4 = H$.
(**2**) $R_1 = COOH$, $R_4 = OH$, $R_2 = R_3 = H$.
(**3**) $R_2 = R_4 = OH$, $R_1 = R_3 = H$
(**4**) $R_1 = COOH$, $R_2 = R_4 = OH$, $R_3 = H$.
(**6**) $R_2 = NH_2$, $R_4 = OH$, $R_1 = R_3 = H$.

Phenazines **4**, **5** and **6** were identified by conversion to **3** or the dimethyl ether of **3**. Thus, the methoxy derivative of **4** was decarboxylated with copper powder in diphenyl ether at 240° for 4 hours; **5** was reduced with zinc dust in warm pyridine containing a trace of dilute hydrochloric acid (8) and **6** was hydrolyzed with 6 *N* hydrochloric acid at 100° for 5 hours to a product similar

TABLE I

Preparation and Properties of Some Microbial Phenazines and Their Derivatives

Compound	Method of Purification or Synthesis	Properties
1	CC of A and C on acid-washed air-dried silica, elute with $CHCl_3$ or EtOAc-$CHCl_3$ 1:20. Best from 3 day fermentation.	M.p. >300°; UV max 250, 370 mμ, dark (a), yellow (b).
dimethyl ester of **1**	**1** + diazomethane	M.p. 229-230 subl. (c) UV max 250, 365 mμ, dark (a), yellow (b), OCH_3 4.12δ (6H)
2	Same as **1**, elute with MeOH	M.p. >270°, blue FeCl test, dark (a), UV max 268, 370, acid-EtOH 271, 365, 373 mμ.
methyl ether methyl ester of **2**	**2** + diazomethane	M.p. 115-120°, yellow (a), red-orange (b), UV max 265, 348, 363 mμ, $C_{15}H_{12}N_2O_3$ (mass spec.)
methyl ether of **2**	methyl ether methyl ester of **2** + 10% KOH in MeOH-H_2O, 1:1, 37° overnight.	M.p. 258-261°, yellow (a), UV max 264, 368, 427 mμ.
acetyl derivative of **2**	**2** + Ac_2O + trace pyridine, room temperature overnight.	M.p. 210-220°, yellow solid
3	TLC of C. Best from 6 day fermentation.	M.p. 230°, yellow brown solid, dark (a), brown FeCl test, UV max 268, 383, 432, acid-EtOH 270, 421 mμ.
dimethyl ether of **3**	Natural **3** + diazomethane, or *o*-nitroanisole and *p*-anisidine (d)	M.p. 150-152°, bright yellow (a), orange (b), 154-155° (e), UV max 267, 375, 415, acid-EtOH 267, 375, 410 mμ.
diacetyl derivative of **3**	Same as **2**	M.p. 178-181° (f), colorless crystals.
dimethyl ether methyl ester of **4**	Same as for **2**	M.p. 148-153°, red orange solid yellow (a), orange (b), UV max, 268, 365-373, 420 mμ. $C_{16}H_{14}N_2O_4$ (mass spec.)
4	Same as **1** elute with EtOAc-$CHCl_3$ 1:4 or 1:1.	M.p. >270°, black $FeCl_3$ test.
methyl ether of **4**	Same as for **2**	M.p. 245-248° dec., yellow (a), UV max 272, 380, 432 mμ.
acetyl derivative of **4**	Same as for **2**	M.p. 215-218°, orange solid.

TABLE 1 (continued)

Preparation and Properties of Some Microbial Phenazines and Their Derivatives

Compound	Method of Purification or Synthesis	Properties
5	TLC of A or C. CC of A or C on regular silica eluting with increasing amounts of EtOAc in $CHCl_3$. Best from 6 day fermentation.	M.p. 235-240° dec., dark red solid, black $FeCl_3$ test, dark (a), with hydrosulfite color change: orange to colorless to yellow. UV max 285, 405, 540, acid-EtOH 283, 403, 470 basic-EtOH 295, 440, 550 mμ. $CHCl_3$ 283, 400, 468.
methyl ether of **6**	From B + CH_2N_2 by TLC also from crude **2** and **4** + CH_2N_2.	M.p. 180°, bright orange solid, resolidifies, then 240-243° dec. Bright orange (a): UV max 275, 376, 480, acid-EtOH 272, 379, 400, 480. Basic-EtOH 277, 372, 485 mμ. With hydrosulfite color change: pink to yellow to pink.

(a) Appearance of TLC spots in UV light. (b) Color of spots in HCl fumes. (c) Mixed m.p. with authentic material was undepressed. (d) I. Yoshioka and H. Otomasu, *Pharm. Bull.* (Japan), 1, 66 (1953). (e) Lit. m.p. 154-155° in d. (f) Lit. m.p. 181° in d.

to **3** which, with diazomethane, gave the dimethoxy derivative of **3**.

The assignment of the carboxyl group in **4** to position 1 rather than 4 or 6 followed from its TLC behavior. Like 2-methoxyphenazine-1-carboxylic acid, the methoxy derivative of **4** does not move from the origin in chloroform-acetic acid (99:1) whereas phenazine, phenazine-1-carboxylic acid, 1-phenazinol, 1,6-phenazinediol, 1,6-dimethoxy or 1,8-dimethoxyphenazine and the methoxy derivative of **2** all have R_F values of 0.5-0.6. Thus, like 2-methoxyphenazine-1-carboxylic acid, the methoxy derivative of **4** must have a methoxy group *ortho* to the carboxyl group which prevents the latter from achieving coplanarity with the ring and hydrogen-bonding with the nitrogen. This internal hydrogen-bonding occurs with the hydroxy and carboxyphenazines cited above so that their TLC mobility is essentially equal to that of phenazine.

The assignment of the *N*-oxide oxygen in **5** to position 10 rather than 5 follows from the peracid oxidation of **3**. Both steric and electronic effects favor oxidation at position 5 (9). The main product from **3** after 5 hours at 50° with excess peracetic acid was distinctly different from **5**. After a 25-hour reaction period the pink di-*N*-oxide of **3** was observed which could also be obtained by direct peracid oxidation of **5**.

The aminophenazine **6** was isolated only in the form of its methoxy derivative. The yellow methoxy *N*-acetyl derivative hydrolyzed on TLC plates over a period of a few days. This loss of acetate had been observed before with n_1-pyrromycinone triacetate (10) and apparently can occur where keto-enol tautomerism of the parent hydroxy or amino group is a factor. The methoxy nmr band of the methoxy derivative of **6** is found at δ 4.13; those of the dimethoxy derivative of **3** at δ 3.97 and 4.13. Since in all other dimethoxyphenazines α-methoxy groups have a higher δ value than β (11), the hydroxy group in **6** is placed as shown.

EXPERIMENTAL (12)

Fermentation and Preliminary Fractionation.

Strain V15295 was maintained on Bennett's agar (13) by incubation at 28° for 2-3 days and then refrigerated for storage. For production, 2-3 day-old slants were used to inoculate seed flasks (50 ml. of YD/250 ml. flask). After 24-28 hours the whole broth from these was inoculated at 5% into peptone-glycerol (3 g. Difco peptone, 5 ml. glycerol/1; 50 ml./250 ml. flask, pH 6.0

after autoclaving). After 3 or 6 days the whole broth was centrifuged at about 5000 rpm for 10 minutes and the sediment, including considerable iodinin, was discarded. The cloudy supernatant liquid was acidified to *p*H 3 and extracted twice with an equal volume of chloroform, each extraction carried out overnight on the shaking machine. The chloroform solution, after concentration, was extracted 4-6 times with 5% sodium bicarbonate and the combined extracts were acidified. Filtration, after overnight cooling, furnished a solid which was sometimes further subdivided into ethyl acetate soluble (A) and insoluble (B) portions. The aqueous acidic filtrate was extracted several times with chloroform and isopropyl acetate (C). Details are shown in Table I.

Phenazine-1,6-dicarboxylic Acid.

In our hands, the published method (14) failed unless the mixture of anthranilic acid, *o*-nitrobenzoic acid and powdered potassium hydroxide was allowed to become somewhat moist and "sticky" before heating. No crystalline dipotassium salt could be observed; instead, the crude acidic product was triturated thoroughly three times with 95% ethanol, centrifuging each time. The dark brown, ethanol-insoluble material gave, after treatment with diazomethane and column chromatography, the desired dimethyl ester, m.p. 228-230°.

Acknowledgment.

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(3) A gram-negative, non-motile rod, received in 1967 from Dr. Enrique Tejera, Caracas, Venezuela.

(4) N. N. Gerber, references in 2c and e.

(5) F. G. Holliman kindly supplied recently synthesized 2-hydroxyphenazine-1-carboxylic acid, R. B. Herbert, F. G. Holliman and J. D. Kynnersley, *Tetrahedron Letters*, 1907 (1968).

(6) We wish to thank K. Yoshida, *Yakugaku Zasshi*, **84**, 703 (1964) for a sample of **2**.

(7) For a review of the biosynthesis of phenazines see M. Podojil and N. N. Gerber, *Biochemistry*, **6**, 2701 (1967).

(8) This method was found to be superior for the reduction of iodinin to 1,6-phenazinediol.

(9) In 2-methoxyphenazine electronic effects favor oxidation at nitrogen 10, H. Otomasu, H. Takahashi and K. Yoshida, *Yakugaku Zasshi*, **84**, 1080 (1965). However, in **3** because of the partial ketolization of the 8-hydroxy group the electronic and steric effects of the 1-hydroxy function should dominate.

(10) By TLC three spots were observed corresponding to hydrolysis of one, two or three acetate groups, J. R. Hegyi and N. N. Gerber, *Tetrahedron Letters*, 1587 (1968).

(11) Y. Morita, *Chem. Pharm. Bull.* (Tokyo), **14**, 433 (1966).

(12) For details of experimental methods see reference 4. Nmr data were obtained on a Varian A-60 spectrophotometer using tetramethylsilane as an internal standard. Submerged fermentations were carried out on a rotary action shaker Model V, New Brunswick Scientific Company, New Brunswick, N. J. at 250 rpm and 28°. All paper and thin layer chromatographic comparisons were with authentic samples run on the same paper or plate. In every case for confirming identity three or more solvent systems were employed.

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