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1,6-Phenazinediol-5-oxide from Microorganisms*

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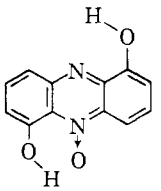
ABSTRACT: The isolation and identification of 1,6-phenazinediol-5-oxide from *Microbispora aerata*, *Pseudomonas iodina*, and *Streptomyces thioluteus* is reported. The title compound was also synthesized by the partial reduction of 1,6-phenazinediol-5,10-dioxide (iodinin) with sodium hydrosulfite. The antimicrobial activity of 1,6-phenazinediol-5-oxide is intermediate between that of iodinin and phenazinediol. Experiments with cell suspensions indicate that 1,6-phenazinediol-5-oxide is an intermediate both in the biosynthesis of iodinin from 1,6-phenazinediol and in the microbial reduction of iodinin.

Recently we reported the isolation of iodinin (1,6-phenazinediol-5,10-dioxide), 1,6-phenazinediol, 2-aminophenoxazin-3-one, and 2-acetamidophenoxazin-3-one from *Microbispora*[1] *aerata* and *Pseudomonas iodina* (Gerber and Lechevalier, 1964). In this paper we noted the presence of an unidentified orange spot on certain paper chromatograms. The orange material could be obtained from either organism. Since in its color, ultraviolet spectrum, solubility, and chromatographic behavior the orange material was invariably intermediate between iodinin and 1,6-phenazinediol we suspected it had the intermediate, hitherto unreported structure, 1,6-phenazinediol-5-oxide.



The structure of the orange material was proved by analysis and by reduction to 1,6-phenazinediol with sodium hydrosulfite. Furthermore, when iodinin was incompletely reduced with sodium hydrosulfite there was obtained a mixture of iodinin, 1,6-phenazinediol-5-oxide, and phenazinediol which could be resolved (as was the naturally occurring mixture) by paper chromatography, countercurrent distribution, or partition chromatography on silica gel. 1,6-Phenazinediol-5-oxide could not be prepared free of iodinin by recrystallization or adsorption chromatography. The natural and synthetic products were identical in every respect. In a typical fermentation (Gerber and Lechevalier, 1964) from *Ps. iodina* 26, 12 and 3.8 mg/liter, respectively, of 1,6-phenazinediol-5-oxide and phenazinediol were obtained; from *M. aerata* the yields were much lower.

These three phenazines were also detected as minor components in chloroform extracts of *Streptomyces thioluteus* 12310[2] which had been grown on Pablum medium. A strain of this organism has been reported to produce aureothin (Hirata, 1961), 1,6-phenazinediol, aureothricin, and thiolutin (Akabori and Nakamura, 1959). The phenazines were separated from other metabolic products and partially from each other by column chromatography on silica gel, eluting with chloroform. Paper chromatography resolved the three phenazines completely and they were found to be identical with authentic specimens by paper chromatographic and spectral comparisons. In two fermentations the yields were in the range of 0.3 to 1 mg/liter for all three phenazines.

Antimicrobial assays of 1,6-phenazinediol-5-oxide were carried out as previously reported (Gerber and Lechevalier, 1964). The results are given in Table I. In most cases the activity was intermediate between that of iodinin and the phenazinediol. The static activity of 1,6-phenazinediol-5-oxide against *Hansenula anomala*, *Saccharomyces cerevisiae*, and *Nocardia coeliaca*

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[1] *Microbispora* = *Waksmania*. The former name has recently been found to have priority.

[2] Strain designations are those of the Institute of Microbiology, Rutgers Uuiversity.

TABLE I: Antimicrobial Activity of 1,6-Phenazinediol, 1,6-Phenazinediol-5-oxide, and Iodinin.

Organism	Phenazinediol	1,6-Phenazinediol-5-oxide	Iodinin
	(mcg/ml for total inhibition)		
Bacteria			
Sarcina lutea 14	5	5–6	0.08
Corynebacterium fimi 22	5	5–6	0.08
Escherichia coli 54	>75	>9.0	>2.0
Proteus vulgaris 73	>75	>9.0	>2.0
Mycobacteria			
Mycobacterium smegmatis 607	25	5.0	1.5
M. rhodochrous 271	37.5	8.0	2.0
Actinomycetes			
Nocardia coeliaca 3520	15	5[a]	0.4
Micropolyspora brevicatena 1086 W/F	37.5	>9	>2.0
Microellobosporia cinerea 3855	12	5	0.5
Actinoplanes sp. W13	10	5	0.1
Fungi			
Saccharomyces cerevisiae 216	50	5[a]	0.4
Hansenula anomala	25	5[a]	0.5
Trichophyton mentagrophytes 171	20	4	0.4
Ceratostomella ulmi 185	15	3	0.5

[a] Static activity.

was shown to be the result of the reduction of 1,6-phenazinediol-5-oxide to the less active phenazinediol by these organisms.

Since the three phenazines were found to be produced by all three organisms, namely, *M. aerata*, *S. thioluteus*, and *Ps. iodina*, it seemed likely that they were related biosynthetically. Phenazinediol or 1,6-phenazinediol-5-oxide in dimethyl sulfoxide solution was added dropwise to a well-stirred suspension of fresh, washed, disrupted *Ps. iodina* cells in phosphate buffer, *p*H 7. Both phenazines were converted almost completely to iodinin after overnight stirring at room temperature. Less active (older) cellular preparations transformed phenazinediol to a mixture of all three phenazines. The product(s) was isolated by chloroform extraction and analyzed by ultraviolet spectroscopy and paper chromatography.

Iodinin in dimethyl sulfoxide was added dropwise with agitation to broth containing cells of one of the following organisms grown in submerged culture: *Candida albicans* 204, *S. cerevisiae* 216, *H. anomala*, or *N. coeliaca* 3520. If the cell-iodinin mixtures were shaken overnight at 28°, only the phenazinediol could be isolated. If they were extracted immediately with chloroform both the phenazinediol and 1,6-phenazinediol-5-oxide were obtained in roughly equivalent yields. Residual substrate was negligible. Another actinomycete, *Micropolyspora brevicatena* 1086 W/F, yielded only the 1,6-phenazinediol-5-oxide when extracted immediately, but a mixture of the phenazinediol and 1,6-phenazinediol-5-oxide when shaken overnight at 37°. Residual iodinin was on the order of 10%. Yields were low, varying from 15 to 30%.

Discussion

Although this is the first report of a phenazine mono-*N*-oxide from natural sources, such structures have frequently been obtained as synthetic products. Mono- and dimethoxy- as well as mono- and diethoxyphenazine mono-*N*-oxides have been prepared both by the Wohl-Aue condensation of nitrophenol ethers with aminophenol ethers in the presence of potassium hydroxide and by the oxidation of the corresponding phenazines with peracids (Yosioka, 1952, 1953, 1954; Yosioka and Otomasu, 1953; Chernetskii and Kiprianov, 1952). Refluxing with acetic anhydride readily reduces the *N*-oxides to the alkoxyphenazines. The only dihydroxyphenazine mono-*N*-oxides known previously, 1,7-dihydroxyphenazine-5-oxide and 1,9-dihydroxyphenazine-5-oxide (Yosioka, 1954), are orange and red, respectively; they melt above 250° with decomposition.

In reporting the isolation of 1,6-phenazinediol and iodinin from *Brevibacterium crystalloiodinum*, Irie *et al.* (1960) suggested that 1,6-phenazinediol was a biosynthetic precursor of iodinin; however until now no evidence for this was available nor was it suspected that 1,6-phenazinediol-5-oxide was an intermediate in this conversion. Our results appear to rule out the suggestion (Witkop and Kissman, 1953) that iodinin is formed via a 5,10-transannular peroxide.

Although the reduction of phenazine di-*N*-oxide by a variety of microorganisms has been reported (Bak and Yosioka, 1963), again, the presence of the monooxide as an intermediate was not demonstrated.

Experimental

All melting points were determined using the Kofler micro hot stage. Analyses were by George Robertson, Florham Park, N.J. Paper chromatography was carried out using the descending method with Schleicher and Schuell No. 2497 (fully acetylated) paper to which a leader strip of Whatman No. 1 paper had been sewed. The solvent systems employed were: A, toluene-ethanol-water (4:17:1); B, butanol–acetic acid–water (4:1:1); C, ethanol-water (1:1).

1,6-Phenazinediol-5-oxide. (A) FROM *Ps. iodina.* Growth of *Ps. iodina* 26 from a 24-hour-old yeast-dextrose slant was used to inoculate two 250-ml Erlenmeyer flasks each containing 50 ml of yeast-dextrose medium (Difco yeast extract, 10 g; Cerelose, 10 g; tap water, 1 liter). After 24 hours' shaking (rotary action shaker, Model V, New Brunswick Scientific Co., Inc., New Brunswick, N.J.) at 215 rpm at 28°, the growth was used to inoculate 20 additional flasks of the same medium which were then incubated similarly for 68 hours. Each flask was then shaken 18 hours with 50 ml of chloroform; the chloroform layers were combined, reduced to 100 ml and, after standing overnight, filtered to yield 165 mg (8.25 mg/flask or 165 mg/liter) of iodinin. The filtrate was extracted four times with 25-ml portions of 4% aqueous sodium hydroxide and centrifuged to break emulsions, and the dark alkaline solution was washed with chloroform and acidified to yield 45 mg of dull-red solid material. Paper chromatography of this product in solvent A resolved it into bands of iodinin, 1,6-phenazinediol-5-oxide, and phenazinediol which were cut apart and eluted with toluene. The yields of each, calculated from the optical density of the eluate and the known $E_{1\text{ cm}}^{1\%}$ of pure materials, were 0.21, 0.55, and 0.18 mg/flask, or 4.2, 12.0, and 3.8 mg/liter, respectively (average of two determinations). Control experiments showed that recovery from the paper was not quantitative; therefore these values represent minimum yields. Small amounts of the pure 1,6-phenazinediol-5-oxide prepared paper chromatographically had λ_{max}^{EtOH} 282, 395, 483 mμ, $\lambda_{max}^{alkaline\ EtOH}$ 299, 575 mμ, identical with the synthetic product described below. Furthermore, its mobility on paper chromatograms was identical with the chemically synthesized material: R_F 0.43 and 0.53 in solvents A and B; 4.4 cm in solvent C after 18 hours.

Dilute aqueous sodium hydrosulfite was added to a dioxane solution of 1,6-phenazinediol-5-oxide until the original orange color had changed to light yellow. The mixture was diluted with water and extracted with chloroform; the chloroform solution was washed with water, dried, and taken to dryness. This gave phenazinediol identical with authentic material in its ultraviolet and visible absorption spectra and in its R_F values in the three solvent systems.

(B) FROM *M. aerata* P-132. This organism was grown as for the production of iodinin (Gerber and Lechevalier, 1964). The chloroform filtrate after the removal of iodinin was treated as in part (A). Pure 1,6-phenazinediol-5-oxide was prepared by paper chromatography in solvent A and found to have ultraviolet and visible absorption spectra and R_F values in the three solvent systems identical with authentic material. The yield was about 50 μg/liter.

(C) FROM *Streptomyces thioluteus* 12310. This organism was maintained on yeast-dextrose slants, transferred every 7–10 days, and not refrigerated for storage. The first generation was grown on yeast-dextrose medium (75 ml per 250 ml flask) for 5 days at 215 rpm and 28°. The resulting growth was used as a 5% inoculum for 40 flasks containing 100 ml of sterile 6% aqueous Pablum per 250-ml flask which were then incubated similarly for 6 days. Each flask was then shaken overnight with 50 ml of chloroform; the chloroform layers were combined, reduced to 40 ml, and applied to a 60-g column of Mallinckrodt silicic acid, 100 mesh. The column dimensions were 2.8 × 17 cm; the silicic acid was placed in the column as a slurry in chloroform. When the column was washed with chloroform the first colored products to be eluted were mixtures of phenazinediol, iodinin, and 1,6-phenazinediol-5-oxide which could be completely resolved by paper chromatography. All three phenazines prepared in pure form by paper chromatography in solvent A had ultraviolet and visible absorption spectra and R_F values in the three solvent systems identical with authentic materials. The yields were in the range of 1 mg/liter for iodinin and 0.3 mg/liter for the others.

(D) SYNTHETIC. Iodinin (100 mg) was completely dissolved in 200 ml of dioxane, with heating. The deep-red solution was cooled to room temperature, then 20 ml of water was added followed by a solution of sodium hydrosulfite (1 g in 10 ml) added dropwise with stirring until the color of the solution was light orange. Although this reaction was carried out five times, it could not always be reliably repeated owing to unpredictable precipitation of either sodium hydrosulfite or iodinin from the reaction mixture; however, partial reduction usually occurred. The reaction mixture was diluted to 2 liters with water and filtered, and the filtrate was extracted with chloroform. Both the solid obtained by filtration and the residue from the chloroform extraction could be used for further purification. Purification was carried out by countercurrent distribution in a toluene–95% ethanol–water (5:4:0.7) system with 100 ml of each layer per tube. The crude mixture (100 mg) was dissolved in the upper layer of tube 1 and twelve transfers were carried out. Paper chromatographic analysis showed that tubes 6 and 7 and the lower layer of tube 8 contained mainly 1,6-phenazinediol-5-oxide, no iodinin, and some phenazinediol, so the contents were combined and reduced to 10 ml. After the mixture had cooled overnight, centrifugation furnished 25 mg of a gummy solid product. Recrystallization from toluene returned 10 mg of dark crystals, mp 244–5° (decomp.) which were homogeneous by

paper chromatography. The analytical sample was prepared from dioxane-water, 7 mg of blood-red triangular plates, mp 248–50° (decomp).

Anal. Calcd for $C_{12}H_8N_2O_3$: C, 63.15; H, 3.53; N, 12.28. Found: C, 63.41; H, 3.72; N, 12.31.

Later batches were purified by partition chromatography on Mallinckrodt silicic acid, 100 mesh, which had been activated overnight at 190°. A two-phase solvent system made up of high-boiling ligroin–toluene–dimethyl sulfoxide–water (5:4:10:1) was used. To 20 g of the activated silicic acid was added dropwise 15 ml of the lower layer of this two-phase system. The mixture was ground thoroughly in a glass mortar with a glass pestle throughout the addition. When the moistened silicic acid appeared homogeneous an excess of the upper layer of the foregoing two-phase system was added and the resulting slurry was poured into the chromatographic column. Two more identically treated portions of silicic acid were added to the column. Then to the fourth portion, lower layer previously saturated with partially reduced iodinin was added. This was necessary because the crude mixture to be separated was not sufficiently soluble in the upper layer (or mobile phase) to be applied to the column in this more usual manner. Elution of the column with the mobile phase (the upper layer of the above two-phase system) yielded first a pink solution of iodinin followed by an orange solution of 1,6-phenazinediol-5-oxide. This orange fraction (about 300 ml) was washed twice with water, dried over sodium sulfate, then reduced to 3 ml. After the solution had stood overnight, 15 mg of pure product was obtained by centrifugation. $\lambda_{max}^{dimethyl\ sulfoxide}$ 283, 393, 487 mμ, $E_{1\ cm}^{1\%}$ 4900, 75, 187; λ_{max}^{Nujol} 3.0, 6.2, 6.4, 6.55, 7.7, 8.0, 8.4, 8.9, 9.3, 9.5, 10.0, 10.3, 11.3, 11.6, 12.4, 12.65, 12.9, 13.65 μ.

Biosynthetic Experiments. (A) FORMATION OF IODININ. The following procedures were carried out at 5° with prechilled sterile solutions and apparatus. Three day old *Ps. iodina* cultures (six flasks) were grown as in the previous section and were chilled and filtered through 24-cm Reeve Angel No. 802 folded filter paper. This removes most of the iodinin crystals; the cells largely pass through. After 0.5 hour the clogged filter paper was replaced with a fresh one. When about 125 ml of filtered broth was obtained it was then centrifuged for 5 minutes at 6000 rpm. The cells were washed with a mixture of saline and 0.05 M phosphate buffer, *p*H 7 (1:1), equal to the original volume of broth, centrifuged again, then suspended in one-half the original volume of saline-buffer mixture and disrupted by sonic treatment at 10 kc for 30 minutes.

Phenazinediol (1 mg in 1 ml of dimethyl sulfoxide) or 1,6-phenazinediol-5-oxide (1 mg in 2 ml of dimethyl sulfoxide) was added dropwise to a magnetically stirred mixture of sterile saline–buffer (200 ml) and sonicated cell suspension (4 ml) at room temperature. After 4 hours of stirring, the warm reaction mixture was allowed to stand overnight. Paper chromatography of a chloroform extract of each reaction mixture showed a trace of recovered starting material and a little 1,6-phenazinediol-5-oxide from phenazinediol, but in both cases the main product was iodinin. The yields of iodinin were 0.29 mg from phenazinediol, 0.35 mg from 1,6-phenazinediol-5-oxide, while control flasks to which no phenazine had been added gave only 0.04 mg. Yields are based on pure materials recovered from paper chromatograms.

(B) MICROBIAL REDUCTION OF IODININ. Growth from slant cultures of the yeasts *C. albicans* 204, *S. cerevisiae* 216, and *H. anomala*, and the actinomycetes *N. coeliaca* 3520 and *M. brevicatena* 1086 W/F, was suspended in sterile water and used to inoculate flasks of yeast-dextrose medium. After 24-hour (yeasts) or 48-hour (actinomycetes) growth on a rotary shaker at 215 rpm, the submerged growth was subcultured at 5% into the same medium. Except for *M. brevicatena*, the cells were used after 21–24 hours' shaking without further treatment. The first four organisms were grown at 28°; *M. brevicatena* was grown at 37° and used after 48 hours' growth.

Iodinin in dimethyl sulfoxide solution was added dropwise with agitation to the broth containing the cells in such a manner that the iodinin concentration did not exceed either the inhibitory concentration for the microorganism (Gerber and Lechevalier, 1964) or 1.5 mcg/ml, whichever was less (iodinin precipitates out of solution at concentrations greater than 2 mcg/ml). The reductions were followed by extraction of small aliquots of the cell-iodinin mixture with chloroform. When the reduction to the more soluble and less antibiotically active phenazinediol and 1,6-phenazinediol-5-oxide had proceeded to completion, more iodinin was added. Reduction rates were relatively rapid, permitting addition of iodinin every 1–7 minutes except with *M. brevicatena*, to which iodinin was added every 25 minutes. It was found that up to 20% by volume of dimethyl sulfoxide could be added without toxicity to the cells. When the cell-iodinin mixture was to be shaken overnight before chloroform extraction, sterile precautions were taken, and iodinin was added only once.

The whole broth-phenazine mixture was shaken overnight with chloroform, the extracts concentrated to dryness and dissolved in toluene, and a known amount was paper chromatographed in solvent A. The bands were cut apart, eluted with toluene, and identified spectrophotometrically as well as by paper chromatography in systems A, B, and C versus authentic materials.

Yields based on spectrophotometric data were as follows for 1,6-phenazinediol-5-oxide (A) and the phenazinediol (B) when the reaction mixture was extracted immediately [concentration of substrate, iodinin, (S)]:

S. cerevisiae: S, 10.6 mcg/ml; A, 0.8 mcg/ml; B, 0.8 mcg/ml

H. anomala: S, 10.6 mcg/ml; A, 1.1 mcg/ml; B, 1.7 mcg/ml

C. albicans: S, 10.6 mcg/ml; A, 0.8 mcg/ml; B, 1.0 mcg/ml

N. coeliaca: S, 19.8 mcg/ml; A, 0.5 mcg/ml; B, 3.0 mcg/ml

M. brevicatena: S, 15.2 mcg/ml; A, 4.0 mcg/ml; B, 0.0 mcg/ml

Under these conditions residual substrate was negligible for the first four organisms, and on the order of 1.5 mcg/ml for *M. brevicatena.*

Acknowledgments

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CORRECTIONS

In the paper by A. Saifer, F. Westley, and J. Steigman in Volume 3, No. 11, November 1964, on p. 1626, in the second, third, and fourth lines, respectively, under equation (11), $(\bar{\nu}_I - \bar{\nu}_{1I}$ *should read* $(\bar{\nu}_I - \bar{\nu}_{1I})$; $(\nu_I - \nu_{1I}/\alpha_I$ *should read* $(\nu_I - \nu_{1I})/\alpha_I$; and $(\nu_I - \nu_{1I}/\alpha_I$ *should read* $(\nu_I - \nu_{1I})/\alpha_I$ (add a closing parenthesis in each instance). On p. 1626, Table II, last column, third line from foot, 700 *should read* 7700. On p. 1627, column 1, line 17, crystalling BSA *should read* crystalline BSA. On p. 1627, Table IV, the heading of the seventh column *should read:* $\alpha = f_{\pm} \cdot \alpha_o{}^a$.

On pp. 1627–28, equations (16), (18), (19), and (20) *should be changed* as follows: (16) Add 1/(B) before fraction and brackets to read thus:

$$\Sigma_i n_i K_{i\mathrm{B}} = \frac{1}{(\mathrm{B})} \cdot \left[\frac{\Sigma_i n_i K_{i\mathrm{A}}}{\Sigma_i n_i K_{i\mathrm{A}}{}^{\mathrm{app}}} - 1 \right]$$

(18), (19), and (20), respectively, add brackets to read:

$$\Sigma_i n_i K_{i\mathrm{Ac}} = \frac{1}{(\mathrm{Ac})_1} \cdot \left[\frac{\Sigma_i n_i K_{i\mathrm{I}}}{\Sigma_i n_i K_{i1_1}{}^{\mathrm{app}}} - 1 \right] \qquad (18)$$

$$\Sigma_i n_i K_{i\mathrm{Ac}} = \frac{1}{(\mathrm{Ac})_2} \cdot \left[\frac{\Sigma_i n_i K_{i\mathrm{I}}}{\Sigma_i n_i K_{i\mathrm{I}_2}{}^{\mathrm{app}}} - 1 \right] \qquad (19)$$

$$\Sigma_i n_i K_{i\mathrm{Ac}} = \frac{1}{(\mathrm{Ac})} \cdot \left[\frac{\Sigma_i n_i K_{i\mathrm{I}}}{\Sigma_i n_i K_{i\mathrm{I}}{}^{\mathrm{app}}} - 1 \right] \qquad (20)$$

On p. 1628, equation (21), the third term, $n_1 K^\circ{}_{i\mathrm{Ac}}$ *should read* $n_1 K^\circ{}_{1\mathrm{Ac}}$.

In the table of contents of Vol. 3, No. 11, November 1964, the title of the article listed beside p. 1783 (Hizukuri and Larner) should end with the words ". . . Independent Form in Liver."