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Phenazines and Phenoxazinones from Some Novel *Nocardiaceae**

Nancy N. Gerber

ABSTRACT: Microorganisms forming a novel group of *Nocardiaceae* were seen to produce 1,6-phenazinediol 5,10-dioxide (iodinin) crystals. Careful study of one strain in submerged culture revealed the formation of 1,6-phenazinediol, 1,6-phenazinediol 5-oxide, 2-amino-3H-phenoxazin-3-one, and 2-acetamidophenoxazin-3-one, all substances which are produced along with iodinin by *Microbispora aerata*, *Brevibacterium iodinum*, and *Streptomyces thioluteus*. In addition, four other compounds in yields of 0.1–0.5 mg/l. were detected and identified by comparison with authentic samples. Two, previously known from other microorganisms, were phenazine-1-carboxylic acid and 3,6-dibenzylidene-2,5-dioxopiperazine. A new compound was 1-phenazinol 10-oxide (orange crystals, mp 165–167°; λ_{max}^{EtOH} 468 (ϵ 2120), 387 (3600), and 279 mμ (67,800)) which was also formed from 1-phenazinol by disrupted cells of *B. iodinum* and was synthesized chemically in low yield by the peracetic acid oxidation of 1-phenazinol.

Also new was 2-amino-1-carboxy-3H-phenoxazin-3-one (dark crystals, decomposing at 310–320°; λ_{max}^{EtOH} 425 (ϵ 10,620), 442 (10,380), and 233 mμ (28,220); methyl ester, orange crystals, mp 210–215°) which was also synthesized chemically in low yield by the ferricyanide oxidation of a mixture of *o*-aminophenol and 3-hydroxyanthranilic acid. The position of the carboxy group in the new phenoxazinone (1 not 9) was proved by its conversion in aqueous base to 2-hydroxy-3H-phenoxazin-3-one.

The morphology of a novel group of *Nocardiaceae* (Lechevalier and Lechevalier, 1965) is being investigated in these laboratories. The first organism of this group, called the "Malloch strain," and received from Dr. R. E. Gordon, formed slants with lustrous coppery needles on the mycelium and in the agar. This is the characteristic appearance of iodinin, 1,6-phenazinediol 5,10-dioxide, as we have seen it before on cultures of *Microbispora aerata* and *Brevibacterium iodinum*.[1] When the Malloch strain was grown in submerged culture on a variety of media used for actinomycetes (Becker *et al.*, 1964), it produced the mixture of phenazines and phenoxazinones that usually accompany iodinin (Gerber and Lechevalier, 1964). These mixtures were extracted from whole broth by chloroform and separated by column chromatography on silica gel, thin layer chromatography on silica gel G, and paper chromatography on fully acetylated paper. The separated pure substances were identified by ultraviolet and visible absorption spectra and shown to be identical with authentic samples by their color, fluorescence, and R_F values in at least four different solvent systems, usually three on paper and one on thin layer plates. Identified in this way were iodinin, 1,6-phenazinediol, 1,6-phenazinediol 5-oxide (Gerber and Lechevalier, 1965), 2-amino-3H-phenoxazin-3-one, and 2-acetamidophenoxazin-3-one. In addition to these known materials, when the Malloch strain was grown on a soybean meal–peptone–glucose–salt medium, we isolated four unknown compounds, all in amounts too small for ordinary analyses. Therefore we deduced their structures from spectra, color tests, and chromatographic behavior, then confirmed them by comparison with authentic synthetic products.

The first of these was observed as a separate orange band from column chromatography. Its absorption spectrum was characteristic of a phenazine, maxima at 468, 387, and 279 mμ. The yields of pure material, orange crystals, mp 165–167°, were about 0.5 mg/l. of whole broth. With sodium hydrosulfite this orange phenazine was reduced to 1-phenazinol, identical

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[1] Previously referred to as *Pseudomonas iodina*. As Sneath (1956) observed, it is a Gram-positive diptheroid bacterium and is probably best included with the brevibacteria.

with an authentic sample. It was identical with the orange product formed in high yield when 1-phenazinol was incubated with disrupted cells of *B. iodinum* by the method used with 1,6-phenazinediol (Gerber and Lechevalier, 1965). Finally, it was identical with one of the mono-*N*-oxides formed from 1-phenazinol by peracetic acid. In this oxidation, two different mono-*N*-oxides can be formed as well as the di-*N*-oxide. Only one mono-*N*-oxide of 1-phenazinol (yellow, mp 190° dec) had been reported in the literature. It was a by-product from a different chemical synthesis and the position of the oxide oxygen had not been established with certainty (Pachter and Kloetzel, 1952). Both steric and electronic effects favor oxidation at the nitrogen in position 5 rather than the one in position 10. In the peracetic acid oxidation of 2-methoxyphenazine where no steric effects are involved, Otomasu *et al.* (1964) found that the electron-donating properties of the methoxy group increased the electron density at nitrogen in the 10 position and thus facilitated oxidation at this position. We obtained all three oxides from 1-phenazinol by treatment with peracetic acid. They were easily separated by paper or column chromatography. After an 8-hr reaction, the major product was the yellow mono-5-oxide, mp 191°, 20% yield; after 26 hr the 5,10-dioxide, red, mp 185–186°, 7% yield (lit. mp 182–183°: Yoshioka and Kidani, 1952). At all reaction times between 4 and 22 hr, the previously unknown orange mono-10-oxide, identical with the natural product, was present in small amounts, 0.5% yield. Its structure is shown in Chart I.

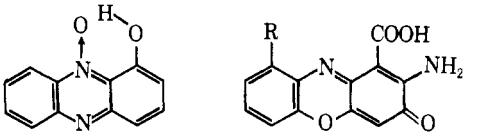

CHART I

The second unknown material was observed as a yellow spot on thin layer plates when the second extract (of whole broth adjusted to pH 8.5) was chromatographed. It was not present in the first extract done at pH 5.5. Another strain, L-13, produced more of the yellow spot and was used for subsequent work; however the yields declined in successive batches. Column chromatography of the second extracts effected only partial purification; fractions were examined by thin layer chromatography since no clear-cut band corresponding to the unknown product was observed. After a second column chromatography of relevant fractions final purification was accomplished on thin layer plates. The pure substance, dark orange needles which sublimed away from 250 to 300° without melting, had absorption spectra characteristic of a 2-amino-3H-phenoxazin-3-one, maxima at 425–441 and 230 mμ in neutral solution. The yields were 0.5 mg or less/l. of whole broth. Its spectra and color tests were identical with those of cinnabarin, 2-amino-1-carboxy-9-hydroxymethyl-3H-phenoxazin-3-one (Chart I, R = CH_2OH), which has been isolated from *Coriolus sanguineus* (Cavill *et al.*, 1953, 1957, 1959; Gripenberg, 1951); however its chromatographic behavior, by direct comparison, was that of a less polar molecule. Therefore, by an oxidative mixed condensation of *o*-aminophenol and 3-hydroxyanthranilic acid using potassium ferricyanide in neutral solution, we prepared two new carboxy-2-amino-3H-phenoxazin-3-ones (none are known in the literature) as well as the expected known "unmixed" products, 2-amino-3H-phenoxazin-3-one and 2-amino-1,9-dicarboxy-3H-phenoxazin-3-one (cinnabarinic acid; Gripenberg *et al.*, 1957; Gripenberg, 1958; Butenandt *et al.*, 1957). Of the two new substances, the less polar and more abundant one (4–7% yield) was identical with the natural product and the methyl esters prepared with diazomethane, mp 210–215°, were also the same. In order to determine the location of the carboxyl group both carboxyaminophenoxazinones were treated with dilute base. Under these conditions in phenoxazin-3-ones a 2-amino group is hydrolyzed and a carboxyl in position 1 but not 9 is lost (Schäfer, 1964). With sodium hydroxide solution the more abundant isomer was transformed to 2-hydroxy-3H-phenoxazin-3-one identical with an authentic sample (Cavill *et al.*, 1961). Thus the new natural phenoxazinone was shown to be 2-amino-1-carboxy-3H-phenoxazin-3-one (Chart I, R = H).

The third unknown substance proved to be phenazine-1-carboxylic acid by comparison with an authentic sample (Kögl and Postowsky, 1930). The yield of pure material, mp 240–245°, from 4 l. of whole broth was unweighable, less than 0.2 mg. It was separated from five other products on thin layer chromatograms of a certain fraction, detected as a dark spot in ultraviolet light and identified after elution by its absorption spectrum and melting point. The fraction used was one of several resulting from two successive preliminary separations on silica gel columns. Previously known from *Pseudomonas aureofaciens* (Kluyver, 1956; Haynes *et al.*, 1956; Toohey *et al.*, 1965), *P. aeruginosa* (Takeda and Nahanishi, 1959), and *Streptomyces misakiensis* (Isono *et al.*, 1958) it has never before been found occurring with iodinin.

The fourth unknown substance was shown to be 3,6-dibenzylidene-2,5-dioxopiperazine by comparison with an authentic sample. Again, the yield of pure material, (mp 300–303°) from 4 l. was unweighable. The dioxopiperazine was detected in strain L-13 by its characteristic bright fluorescence and R_F value on thin layer chromatograms of certain fractions from column chromatography. It was identified by its absorption spectrum and melting point and could also be isolated directly from chloroform extracts by successive thin layer chromatography in two different solvent systems. Brown *et al.* (1965) has summarized its previous isolations from *Streptomyces noursei*.

Discussion

Phenazines and phenoxazinones have now been

found to occur together in four genera of microorganisms. This suggests related biosynthetic paths. Phenoxazinones have been formed from *o*-aminophenols by enzymes present in *Streptomyces antibioticus* (Katz and Weissbach, 1962), *Pycnoporus coccineus* (Nair and Vining, 1964), *Tecoma stans* (Nair and Vaidyanathan, 1964), rat livers (Subba Rao *et al.*, 1964), and poikilothermic animal livers (Morgan and Weimorts, 1964). However, little is known about the immediate precursors of the phenazine ring, although the incorporation of shikimic acid into 2-hydroxyphenazine (Levitch and Reitz, 1966), phenazine-1-carboxylic acid (Levitch and Stadtman, 1964), and pyocyanine (Millican, 1962) has been demonstrated.

The conversion of 1-phenazinol to its 10-oxide by a sonicated cell suspension of *B. iodinum* is notable for its ease and specificity. Paper chromatography clearly showed the absence of 1-phenazinol 5-oxide or the di-*N*-oxide. Thus the oxidation is truly enzymatic and not occurring by some unsuspected chemical process. (For example, Rauser (1959) has found that hydrogen peroxide is formed in ultrasonic fields.) The two methods for the oxidation of 1-phenazinol are complimentary. The enzymatic one gives the 10-oxide; the chemical one produces mainly the 5-oxide. Apparently the enzyme is specific, in phenazines, for the oxidation of nitrogen atoms which have a perihydroxy group. Thus *N*-oxides are formed from 1,6-phenazinediol, 1,6-phenazinediol 5-oxide, 1-phenazinol, and 6-methoxy-1-phenazinol while under identical conditions phenazine and phenazine-1-carboxylic acid are recovered unchanged.

Experimental Section

Melting points were determined using the Kofler micro hot stage. Paper chromatography (descending method), unless otherwise noted, used Schleicher and Schuell No. 2497 (fully acetylated) paper to which a leader strip of Whatman No. 1 paper had been sewed. Thin layer chromatography used silica gel G (E. Merck Ag. Darmstadt; Brinkmann Instruments Inc., Westbury, Long Island, N. Y.) plates which were prepared without a spreader, dried overnight at 50°, stored over calcium chloride, and redried for 10 min at 50° after spotting. Solvent systems employed were: A, toluene–ethanol–water (4:17:1); B, butanol–acetic acid–water (4:1:1); C, ethanol–water (1:1); D, chloroform–acetic acid (9:1); E, methanol–water (1:1); F, methanol–10% hydrochloric acid (1:1); G, chloroform–absolute ethanol (99:1); H, ethyl acetate–acetic acid (9:1); and I, chloroform–acetone (95:5). Mallinckrodt silicic acid 100 mesh (powder) or SilicAR CC-7 100–200 mesh was used for column chromatography. Column lengths were seven to twelve times their diameters. Ultraviolet and infrared spectra were measured on a Cary Model 14 recording spectrophotometer and a Perkin-Elmer Infracord, respectively. All media were prepared with tap water.

Fermentations, Preliminary Separations, and Identification of Several Known Compounds. The Malloch strain was maintained on YD slants (10 g of Difco yeast extract, 10 g of Cerelose, 15 g of agar/l., pH 7.0–7.2), transferred every 4–6 weeks, incubated at 28°, and when well grown, stored at 5°. For production, 7-day-old slants were used to inoculate four to six flasks of YD broth, 100 ml/250-ml flask. After 3 days at 215 rpm (Rotary action shaker, Model V, New Brunswick Scientific Co., New Brunswick, N. J.) at 28° the resulting whole broth was inoculated at 5% into 40 flasks of SBM/J (10 g of soybean meal, 10 g of Wilson's peptone no. 851,[2] 20 g of Cerelose, 5 g of sodium chloride/l., pH 7.5), 100 ml/250-ml flask. After 5 days at 28° and 215 rpm the resulting whole broth was shaken overnight at 28° with one-half its volume of chloroform. The red-orange chloroform extract was concentrated to 100 ml and after standing overnight the bulk of the iodinin was removed by filtration. The yields varied from 10 to 120 mg of iodinin which was identical with an authentic sample; $\lambda_{max}^{CHCl_3}$ 290, 350, and 530 mμ; R_F 0.096 and 0.17 in solvents A and B; spots traveled 2 mm in solvent C overnight; thin layer chromatography in solvent D, R_F 0.9, wet and dry color purple. The filtrate was chromatographed on a 200-g silica column eluting with chloroform and collecting 50-ml fractions. Paper chromatography in solvent A disclosed iodinin in fractions 1–9 and 1,6-phenazinediol 5-oxide in fractions 2–7. Fraction 5 was separated by preparative paper chromatography in solvent A and the 1,6-phenazinediol 5-oxide, obtained by extracting the orange bands with toluene, was shown to be identical with an authentic sample; $\lambda_{max}^{CHCl_3}$ 487 mμ and 283 mμ; R_F 0.40 and 0.35 in solvents A and B; spots traveled 3.1 cm in solvent C during 48 hr; thin layer chromatography in solvent D, R_F 0.9, wet color orange, dry color brown.

From another batch, slower moving components were eluted from the column with solvent G; thin layer chromatography disclosed 2-amino-3H-phenoxazin-3-one in some of them. After a second column chromatography of the relevant fractions and preparative thin layer chromatography, the 2-aminophenoxazinone was obtained by eluting with methanol, identical with an authentic sample; λ_{max}^{MeOH} 235 mμ and 415–432 mμ; $\lambda_{max}^{MeOH-HCl}$ 235 mμ and 462 mμ; R_F 0.47 in solvent A; 0.40 and 0.59 in solvents E and F on Whatman No. 1 paper; thin layer chromatography in solvent D, R_F 0.3, wet color brown, dry color brown with bright fluorescent edges when viewed with ultraviolet light. Among the slower moving compounds, after a second separation on a silica column, thin layer chromatography also disclosed 2-acetamido-3H-phenoxazin-3-one and phenazine-1-carboxylic acid. Using preparative thin layer chromatography eluting with methanol, both were obtained in pure form and shown to be identical with authentic samples;

[2] The Wilson Co., Chicago, Ill.

for the phenoxazinone: λ_{max}^{EtOH} 405 mμ and 235 mμ; R_F 0.38 and 0.56 in solvents A and B; 0.84 in solvent A on Whatman No. 1 paper; thin layer chromatography in solvent D, R_F 0.5, wet color yellow, dry color yellow with bright red-orange fluorescence in ultraviolet light; for the phenazine: mp 240–245°, mixture melting point undepressed; λ_{max} 440, 364, and 250 mμ; R_F 0.76 and 0.58 in solvents A and B; spots traveled 12 cm in solvent C overnight; thin layer chromatography in solvent D, R_F 0.6, wet and dry color pale yellow, dark when viewed by ultraviolet light.

When the Malloch strain was grown in PGB (5 g of Wilson Peptone 851,[2] 70 ml of glycerol, 5 g of Amber BYF 50X[3]/l., pH 7.5), inoculated at 5% from 3-day-old submerged growth in YD (50 ml/250-ml erlenmeyer flask), the original chloroform extract was yellow and the main fraction from column chromatography was 1,6-phenazinediol identical with an authentic sample; λ_{max} 445, 374, and 273 mμ; blue with alcoholic ferric chloride; R_F 0.52 and 0.57 in solvents A and B; spots traveled 4 cm in solvent C overnight; thin layer chromatography in solvent D, R_F 0.85–0.9, wet color dirty yellow, dry color blue.

2-Amino-1-carboxy-3H-phenoxazin-3-one. A. From Nocardia Strains. Among the slower moving components eluted from the column with solvent G (previous section) thin layer chromatography in solvent D disclosed a yellow spot with a deep red fluorescence at R_F 0.55. An examination of 12 strains of this group by thin layer chromatography of their chloroform extracts showed that No. L-13 furnished the highest yield of this material. Furthermore, it was extracted by chloroform at pH 8.5 but not at pH 5.5. Therefore, strain L-13 was grown on SBM/J as described previously for the Malloch strain except that fermentation was continued for 10 days. The whole broth was first extracted at pH 5.5 then 8.5. The second extract was concentrated and chromatographed on a 40-g silica column, eluting with chloroform. In a typical run 76 20-ml fractions were collected; thin layer chromatography in solvent D disclosed the desired product in fractions 21–24. These fractions were combined, rechromatographed on a 10-g column, then the fractions containing the product were combined and separated by thin layer chromatography in solvent D. After elution with solvent D, yields were 1–3 mg/batch or about 0.5 mg/l. This product sublimed away above 250°; no melting could be observed. It gave no color with alcoholic ferric chloride and was reversibly decolorized by sodium hydrosulfite. It was soluble in 2 N sodium hydroxide (violet solution changing to dull red), slowly soluble in 3 N sodium carbonate (gray solution), soluble in saturated sodium bicarbonate with heating (yellow solution), insoluble in 1 N hydrochloric acid, and soluble in concentrated hydrochloric acid (orange solution). It was extracted from chloroform very slightly by 5% aqueous sodium bicarbonate and not at all by 10% hydrochloric acid; λ_{max}^{EtOH} 425–440 mμ and 230 mμ, unchanged when acid was added; R_F 0.26 and 0.34 in solvents A and B and 0.58 on Whatman No. 1 paper in solvent F; thin layer chromatography in solvent D *vs.* authentic cinnabarin, the R_F values were 0.6 and 0.05, respectively.

B. Synthesis. To a mixture of 100 mg each of *o*-aminophenol and 3-hydroxyanthranilic acid in 100 ml of 10% aqueous ammonia was added 10 ml of 10% aqueous potassium ferricyanide. After 4 hr at room temperature the reaction mixture was adjusted to pH 8.5 and extracted three times with chloroform. Thin layer chromatography of the extract in solvent D showed 2 spots: 2-aminophenoxazinone (brown, R_F 0.3) and the desired product (yellow, R_F 0.6); they were separated satisfactorily by column chromatography eluting with chloroform. Pure 2-amino-1-carboxyphenoxazinone (4–7% yield) decomposed at 310–320° without melting; λ_{max}^{Nujol} 3.05, 3.20, 6.05, 6.18, 6.35, 6.45, 10.3, 10.6–10.8, and 12.5 μ. The ultraviolet spectrum was identical with that of the natural product: λ_{max}^{EtOH} 425 (ε 10,620), 442 (10,380), and 233 mμ (28,220); and the two samples had identical mobilities, color, and fluorescence in solvents A and B (R_F 0.22 and 0.32) and on thin layer chromatography in solvent D. When the reaction was carried out in 10% ammonium acetate thin layer chromatography in solvent D of the chloroform extract disclosed an additional spot (red-brown, R_F 0.25). This product could be extracted into aqueous sodium bicarbonate or 10% hydrochloric acid and after purification on a silica column crystallized as its chloroform solution was concentrated (less than 4% yield). It did not melt but decomposed at 330–350°; λ_{max}^{EtOH} 420 mμ; $\lambda_{max}^{EtOH-acid}$ 465 mμ; λ_{max}^{Nujol} 3.05, 3.15, 5.95, 6.3, 6.4, 6.48, 6.8, 7.8, 10.3, 11.75, 12.3, and 13.05–13.3 μ. When treated with aqueous base overnight it did not give 2-hydroxyphenoxazinone (see next section) but rather a slower moving spot (R_F 0.3) on thin layer chromatography in solvent H identical with that from cinnabarinic acid and base. Probably this is the isomeric 2-amino-9-carboxy-3H-phenoxazin-3-one but the structure proof is not complete. Its solubility and carboxyl absorbtion in the infrared indicate a more polar, less internally hydrogen bonded molecule than the 1-carboxy isomer as would be expected.

C. Methyl Ester. Both synthetic and natural samples of 2-amino-1-carboxyphenoxazinone in chloroform were treated with diazomethane in ether. The bright orange methyl ester from the synthetic sample melted at 210–225° after purification on a silica column; λ_{max}^{EtOH} 428 mμ (ε 6050) and 234 mμ (ε 8100); $\lambda_{max}^{EtOH-acid}$ 432 mμ; the two samples had identical mobilities in solvents A and B (orange spots, bright yellow-orange fluorescence) and on Whatman No. 1 paper; in solvent F, R_F 0.40, 0.43, and 0.58.

D. Conversion to 2-Hydroxy-3H-phenoxazin-3-one. A few milligrams of 2-amino-1-carboxyphenoxazinone was allowed to stand overnight in 1 ml of 1 N sodium hydroxide. The solution was adjusted to pH 2–3

[3] A fraction of autolyzed brewers yeast, sold by Amber Laboratories, Inc., Milwaukee, Wis.

with sulfuric acid then taken to dryness in a vacuum oven. Organic products were extracted with pyridine and purified by chromatography on silica gel eluting with ethyl acetate. The main band was 2-hydroxyphenoxazinone identical with an authentic sample: λ_{max}^{EtOAc} 395 mμ; $\lambda_{max}^{EtOH-NH_3}$ 432 mμ (sh) and 412 mμ; R_F 0.54 and 0.53 in solvents A and B; R_F 0.48 on Whatman No. 1 paper in solvent F; thin layer chromatography in solvent H, R_F 0.5, wet color brown, dry color brown with bright fluorescent edges under ultraviolet light.

1-Phenazinol 10-Oxide. A. From Malloch Strain. When chloroform extracts of whole broths were chromatographed in silica columns (first section), a weak orange band could be observed following the dark red iodinin band. Eluted with chloroform it furnished dark orange crystals which after trituration with hexane and recrystallization from ethanol–water weighed 2 mg (yield about 0.5 mg/l.) and melted at 165–167°; λ_{max}^{EtOH} 468 (ε 2120), 387 (3600), 380, 368, 334, 326, and 279 (67,800) mμ; R_F 0.46 and 0.45 in solvents A and B; spot traveled 4.5 cm in solvent C overnight; thin layer chromatography in solvent D, R_F 0.8, wet color orange, dry color brown. A solution of this product in ethanol was treated with dilute sodium hydrosulfite solution until colorless. It was then diluted with water, aerated, and extracted with chloroform. The chloroform solution had maxima at 416, 368, 359, 350, 342, 335 (s), and 246 mμ in agreement with 1-phenazinol, not 1,6-phenazinediol or 6-methoxy-1-phenazinol. The residue gave a black ferric chloride test and after recrystallization from ethanol–water melted at 155–158° undepressed when mixed with authentic 1-phenazinol. Paper chromatography fails to distinguish conclusively between 1-phenazinol, 1,6-phenazinediol, and 6-methoxy-1-phenazinol.

B. By Enzymatic Oxidation of 1-Phenazinol. The experiments were carried out as for 1,6-phenazinediol (Gerber and Lechevalier, 1965) except that 1 mg of 1-phenazinol in 1 ml of dimethyl sulfoxide was added to 100 ml of saline–buffer containing 5 ml of sonicated cell suspension. The reaction mixture was agitated gently overnight at 28°, then acidified to pH 3.5. After 1 hr the orange aqueous solution was filtered from coagulated protein and extracted with chloroform. Conversion was essentially complete under these conditions; the 1-phenazinol 10-oxide melted at 164–165°, undepressed when mixed with material from part A; $\lambda_{max}^{CHCl_3}$ 468, 388, 380, 369, 360, 340, and 282 mμ. Paper chromatographic comparisons of the orange products from parts A and B with 1,6-phenazinediol 5-oxide gave R_F 0.42, 0.41, and 0.37 in solvent A; spots moved 21, 21, and 18 cm in solvent B during 12 hr and 3.6, 3.6, and 0–3.1 cm in solvent C during 48 hr.

C. Synthesis. A solution of 1-phenazinol (80 mg) in 4 ml of glacial acetic acid and 400 μl of 30% hydrogen peroxide was kept at 52° for 24 hr. The mixture was poured into water and extracted three times with chloroform. Chromatography of the concentrated extract on a 50-g silica column eluting with chloroform gave first a pale orange fraction of 1-phenazinol 10-oxide, identical with the products from parts A and B in ultraviolet and visible absorbtion spectra and paper chromatographic behavior, R_F 0.38 in solvent A. The crystalline material obtained was less than 0.5 mg, mp 150–151° not enough for recrystallization. The next fraction was a mixture but by a second column chromatography small amounts of two pure substances could be obtained from the first and last fractions; from the first, 1-phenazinol 5-oxide; yellow; mp 190–191°; $\lambda_{max}^{solvent\ A}$ 448 (ε 2755), 383 (3390), 374 (2680), 363 (2820), and 355 mμ (2680); λ_{max}^{EtOH} 440, 381, 372, 362, 352, and 274 mμ; R_F 0.55 in solvent A; from the last, 1-phenazinol 5,10-dioxide; red; mp 185–186°; $\lambda_{max}^{solvent\ A}$ 502 (ε 5230), 382 (sh) (2965), and 374 mμ (3110); λ_{max}^{EtOH} 498, 382 (sh), 372, and 287 mμ. The reaction was repeated withdrawing 100-μl samples at 0, 4, 8, 11, 22, and 26 hr. Each was treated as above then the entire residue from each chloroform extraction was separated by paper chromatography in solvent A. The resulting well-separated bands (7.4, 12.3, 17, and 21 cm) were cut apart, extracted with solvent A, and assayed spectrophotometrically using the known absorbance of the pure materials in solvent A as standards. At the times given above the micrograms recovered were: 1-phenazinol 960, 390, 104, 0, 0, and 0; 1-phenazinol 5-oxide 0, 210, 356, 220, 150, and 69; 1-phenazinol 10-oxide 0, 10, 10, 9, 6, and 4; 1-phenazinol 5,10-dioxide 0, 0, 32, 55, 140, and 130.

3,6-Dibenzylidene-2,5-dioxopiperazine. Among the slower moving compounds eluted from the silica column of Malloch strain extract (first section) thin layer chromatography disclosed a colorless spot with a bright yellow fluorescence in ultraviolet light, R_F 0.9 in solvent D. It resembled the title dioxopiperazine which has been isolated in quantity from *Streptomyces thioluteus* (N. N. Gerber, unpublished data) and was shown to be identical with an authentic sample of 3,6-dibenzylidene-2,5 dioxopiperazine, R_F 0.078 and 0.26 in solvent A and B; spots traveled 15 cm overnight in solvent A; thin layer chromatography in solvent I, R_F 0.8, colorless when wet, very pale yellow when dry. This product was also shown by thin layer chromatography in strain L-13 extract fractions. For isolation in quantity the first chloroform extract from 4 l. of whole broth was concentrated to 100 ml, thin layer chromatography (24 ml on eight plates) in solvent D gave yellow fluorescent bands at R_F 0.9 which after elution with solvent I were run on two plates in solvent I. Several fluorescent bands were visible ahead of the purple iodinin band. The second or third one proved to be the desired product after elution with solvent I; $\lambda_{max}^{CHCl_3}$ 338 mμ, $\lambda_{max}^{1\%\ NaOH}$ 395 μ and 310 μ (sh), mp 300–303° after trituration with hexane; undepressed when mixed with an authentic sample.

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