Phenazines and Phenoxazinones from *Waksmania aerata* sp. *nov.* and *Pseudomonas iodina**

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The isolation and identification of 1,6-phenazinediol-5,10-dioxide, I, 1,6-phenazinediol, II, 2-amino-3H-phenoxazin-3-one, III, and 2-acetamido-3H-phenoxazin-3-one, IV, from *Waksmania aerata* sp. *nov.* and *Pseudomonas iodina* are reported. Catalytic hydrogenation of compound I produced II; compound III was synthesized by the oxidation of *o*-aminophenol and subsequently acetylated to yield compound IV. The antimicrobial activity of compounds I–IV is given. Eight isolates of *Waksmania* from soil are classified into two species, one of which, *W. aerata* sp. *nov.*, is described.

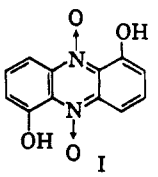
Members of the actinomycete genus *Waksmania*, described by Lechevalier and Lechevalier (1957), are characterized by the formation of longitudinal pairs of spores on the aerial hyphae. Nonomura and Ohara (1957), who described a morphologically identical isolate under the name *Microbispora*, later reported that several new species of this genus formed unidentified crystals on certain solid media (Nonomura and Ohara, 1960). Eight strains producing such crystals have been isolated from French soils by H. A. Lechevalier.

Three of these strains are considered a new species, for which we propose the name *Waksmania aerata* (=covered with bronze) (see Table I). Physiologically they differ from the previously described *Waksmaniae* in diastatic activity, nitrate reduction, and in temperature growth range. Morphologically, *W. aerata* is similar to *W. rosea* (Lechevalier and Lechevalier, 1957) except for spore size. The other five strains are synonymous with previously described Japanese strains.

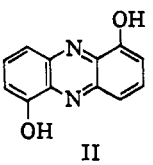
W. aerata W-17[1] produced characteristic crystals on Pablum extract agar (1–2 mg/95 mm Petri dish culture) incubated at 37° for 1 month. Yields up to 180 mg/liter were obtained from shake-flask cultures on Pablum medium at 37° for 2 weeks. The contents of each flask was extracted with chloroform and the deep red organic solution was concentrated to produce fine, glistening, bronze needles.

In dimethyl sulfoxide solution the pure product gave a positive ferric chloride test and was irreversibly decolorized by aqueous sodium hydrosulfite. The ultraviolet spectrum showed maxima at 291, 353, and 534 mμ, and the infrared spectrum displayed a weak O—H band at 3.8 μ indicating that all OH groups were strongly hydrogen-bonded or chelated. The foregoing properties and the molecular formula of $C_{12}H_8O_4N_2$ indicated that it was iodinin, or 1,6-phenazinediol-5,10-dioxide, compound I.

First isolated by Clemo and McIlwain (1938) from *Chromobacterium iodinum* (later named *Pseudomonas iodina* [Sneath, 1956]), compound I has been synthesized (Serebryanyi *et al.*, 1950; Yoshioka and Kidani, 1952) and found to be produced by *Brevibacterium crystalloiodinum* (Irie *et al.*, 1960). The ultraviolet and infrared spectra of our compound I were identical with those published (Sneath, 1956; Irie, *et al.*, 1960;



I

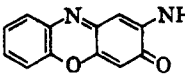


II

Clemo and Daglish, 1950), however neither in capillary tubes nor on the hot stage was a definite melting point at 230° or 236° observed as reported (Clemo and McIlwain, 1938; Irie *et al.*, 1960). Rather, a gradual decomposition above 200° occurred. As a final proof of structure, compound I was reduced with hydrogen and platinum to 1,6-phenazinediol (II), whose melting point and ultraviolet and infrared spectra were identical with published data (Irie *et al.*, 1960).

For comparison, nine *Waksmania* strains were shaken in Pablum medium for 14 days at 37°. Only the three strains of *W. aerata* (W17, P132, and 37-16) produced significant amounts of compound I under these conditions; they yielded 72, 246, and 123 mg/liter, respectively. *W. rosea* 3748 produced no compound I.

Chromatography on silica-gel thin-layer plates of the mother liquors from the production of compound I in an acetic acid–chloroform system revealed two spots, one brown and one yellow, with R_F values of about 0.3 and 0.6, respectively. (Iodinin is violet and in this system has an R_F of 0.8.) Concentrated solutions of both materials were obtained by preparative thin-layer chromatography and the yellow one was isolated as needles melting at 295–297° from chromatography of similar mother liquors on a silica-gel column. The ultraviolet spectra of the brown material in water, dilute acid, dilute base, and cyclohexane suggested that it was identical with questiomycin A or 2-aminophenoxazin-3-one (III), which has been isolated from an unidentified *Streptomyces* (Anzai *et al.*, 1960). The identity was proved by chromatographic comparisons with an authentic sample synthesized by the oxidation of *o*-aminophenol (Osman and Bassiouni, 1960). The yellow product gave negative ferric chloride and silver nitrate tests and its



III, R = —H
IV, R = —$COCH_3$

ultraviolet maxima at 240 and 405 mμ were unchanged in acidic or basic solution. These properties suggested that it was identical with 2-acetamidophenoxazin-3-one (IV), which has never before been isolated from natural sources. The identity was proved by

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[1] The strain designations are those of the culture collection maintained in this laboratory.

TABLE I
MORPHOLOGICAL AND PHYSIOLOGICAL PROPERTIES OF *Waksmaniae*

Organism and Strain Numbers	Spores	Aerial Growth on Oatmeal or Pablum Agar	Temperature Range	Starch Hydrolysis	Gelatin Liquefaction	Peptonization of Litmus Milk	Reduction of Nitrate	Production of Iodinin Crystals
Waksmania rosea 3748	1.6–1.8 μ subglobose	Pale pink	20–45°	−	+	+	−	−
Waksmania aerata sp. *nov.* W17; P132; 37-16	1.2–1.5 μ subglobose	Off-white to faint pink	28–55°	+	+	+	+	++
*Microbispora** *amethystogenes* W55; 37-3; 37-5; 37-6 and 37-68	1.2–1.5 μ subglobose	Off-white to faint pink	20–45°	−	+	+	+	+
Microbispora[a] *parva*	[b]	White to pale pink	[b]	−	±	− or ±	−	±
Microbispora[a] *diastatica*	[b]	Pink	[b]	+	+	±	−	−

[a] *Microbispora* and *Waksmania* are synonyms. [b] Not reported.

mixed melting point and chromatographic comparisons with authentic material prepared by the acetylation of synthetic compound III. The yields of the phenoxazinones from *W. aerata* P-132 were in the range 0.3–1 mg/liter for compound III and 0.6–1.5 mg/liter for compound IV.

In order to compare the antimicrobial activity of iodinin from *Waksmania* with authentic material, compound I was also prepared from two strains of *Pseudomonas iodina*. The bacteria were grown in yeast-dextrose broth shaken at 28° for 2 weeks; compound I was isolated as before. The yields were 216 mg/liter from strain 26 and 37.4 mg/liter from strain E. Thin-layer chromatography of the mother liquors revealed the brown and yellow spots of the phenoxazinones. They were isolated in larger amounts by preparative thin-layer chromatography and their identity was proved by ultraviolet spectra and paper chromatographic comparisons with authentic materials.

The characteristic ultraviolet absorption at 272, 372, and 440-445 mμ indicated that 1,6-phenazinediol (II) was also present in extracts of broths in which the strains of *Ps. iodina* were grown. This phenazine has been reported to be produced by *Streptomyces thioluteus* (Akabori and Nakamura, 1959), and by *Brevibacterium crystalloiodinum* (Irie *et al.*, 1960). It was conveniently prepared from *Ps. iodina* (strain 26) grown in nutrient broth shaken at 28° for 48 hours. After preliminary purification, preparative thin-layer chromatography separated a brown band which was resolved into three components, I, II, and an unidentified orange spot, by chromatography on acetylated paper. Using this procedure, traces of compound II could be detected in extracts of *W. aerata* P-132 grown 4 days in Pablum extract. The identity of compound II from both sources was proved by spectrophotometric and paper chromatographic comparisons with authentic material.

Antimicrobial assays comparing compounds I, II, III, and IV showed them all to be active against certain gram-positive bacteria, fungi, and actinomycetes. They were inactive against the gram-negative bacteria tested. Iodinin was by far the most active of the four. Neither the antifungal activity of iodinin nor the antifungal and antibacterial (other than antimycobacterial) activity of 2-acetamidophenoxazin-3-one has been previously reported. There are no published data to indicate that any of the four compounds has ever been tested for activity against actinomycetes. Otherwise, the inhibitory concentrations reported in Table II are in line with those reported by others (Akabori and Nakamura, 1959; Anzai *et al.*, 1960; DiPaolo and Kolipinski, 1960; Frisk, 1946; Funasaki, 1958; Hirata, 1954; Iland, 1948; Kadoya, 1952; Kawakami *et al.*, 1955; Kitagawa, 1956; McIlwain, 1941, 1943a,b; Noda, 1956; Okamoto, 1948; Okamoto *et al.*, 1954; Wiedling, 1945; Yoshioka *et al.*, 1957; Yoshioka and Tanaka, 1958; Yoshioka and Uehara, 1958; Yoshioka and Zaizen, 1958; Yuasa, 1953).

Comparison of iodinin from *Ps. iodina* with that from *W. aerata* showed the two compounds to have identical antibiotic activity.

The poor water-solubility of iodinin and 2-acetamidophenoxazin-3-one limited assay levels to 2 μg/ml or less for the former compound and 12.5 μg/ml or less for the latter.

DISCUSSION

Phenazines and phenoxazinones are not common products of microorganisms. Until recently, phenazines had been found produced by bacteria only (Waksman and Lechevalier 1962; Miller 1961; Swan and Felton 1957). The production of compounds I-IV by members of two such dissimilar genera, *Waksmania*, an actinomycete, and *Pseudomonas*, a true bacterium, adds further weight to the conclusion that the actinomycetes are more closely related to the bacteria than to the fungi (Lechevalier, 1964). It also suggests the possibility that the four compounds may be produced via related biosynthetic pathways. This possibility is under investigation.

EXPERIMENTAL

All melting points were determined using the Kofler micro hot stage except where otherwise noted. Analyses were made by W. Manser, Zurich, Switzerland, and George Robertson, Florham Park, N. J. Paper chromatography was carried out using the descending method with either Whatman No. 1 paper which had been previously washed by descending chromatography with 2.8% aqueous ammonia, or unwashed Schleicher and Schuell No. 2497 (fully acetylated) paper. Thin-layer chromatography plates were prepared from Silica Gel G distributed by Brinkmann Instruments, Inc., Great Neck, N. Y. Thirty g of powder and 100 ml of water were mixed 1 hour on a rotary shaker. The mixture was spread out evenly on five clean glass plates (4 × 8 in.) with a bent rod.

TABLE II
ANTIMICROBIAL ACTIVITY OF THE PHENAZINES AND PHENOXAZINES

	Minimum Inhibitory Concentration (μg/ml agar)			
	Iodinin	1,6-Phenazinediol	2-Aminophenoxazin-3-one	2-Acetamidophenoxazin-3-one
BACTERIA				
Gram-positive				
Bacillus subtilis 7	0.5	75	—	—
Corynebacterium fimi 22	0.075	5	15	8
Micrococcus lysodeikticus 19	0.08	5	20	7
Sarcina lutea 14	0.08	5	10	7
Staphylococcus aureus 15	>2.0	>50	30	>12.5
Gram-negative				
Pseudomonas iodina E	>2.0	>75	—	—
Escherichia coli 54	>2.0	>75	>75	>12.5
Klebsiella pneumoniae 17	>2.0	>75	—	—
Proteus vulgaris 72	>2.0	>75	>75	>12.5
Pseudomonas aeruginosa 77	>2.0	>75	—	—
ACTINOMYCETES				
Nocardia asteroides 3409	1.0	20–25	5	5
N. coelica 3520	0.4	15	25	>12.5
Micromonospora sp. W50	0.2	10	15	>12.5
Thermoactinomyces sp. W18	0.2	10	—	—
Waksmania rosea 3748	>2.0	20	20	>12.5
Micropolyspora brevicatena 1086 W/F	>2.0	37.5	20	>12.5
Streptomyces antibioticus 3720	0.5	>75	—	—
S. chrysomallus 3657	0.5	>50	30[a]	>12.5
S. griseus 3475	0.4	>75	—	—
S. lavendulae 3440-ly	2.0	37.5	15	>12.5
S. thioluteus 12301	>1.5	>50	—	—
Microellobosporia cinerea 3855	0.5	12	15	10
Streptosporangium roseum W48	0.4	10	10	5
Actinoplanes sp. W13	0.1	10	5	5
Mycobacterium smegmatis 607	1.5	25	5	7
M. phlei W23	1.5	15	—	—
M. avium ATB	>2.0	50	—	—
M. rhodochrous ATCC 271	2.0	37.5	25	10
FUNGI AND YEASTS				
Candida albicans 204	0.4[b]	50 (37.5[b])	50	>12.5
C. tropicalis 205	0.9	>75	>50	>12.5
Saccharomyces cerevisiae 216	0.4	50	25	>12.5
Hansenula anomala	0.5	25	25	>12.5
Aspergillus niger 13	1.0	>75	>75[a]	>12.5
Penicillium notatum 40	0.5	50	25	>12.5
Mucor rouxii 80	0.8	50	—	—
Trichophyton mentagrophytes 171	0.4	20	5	3
Ceratostomella ulmi 185	0.5	15	—	—

[a] Destroys cpd (decolorizes). [b] Static activity.

The plates were dried overnight in an oven at 50° and stored in an air-tight plastic box over anhydrous calcium chloride. After the sample was applied to the starting line the plates were redried at 50° for 15 minutes. The solvent systems employed were: A, acetic acid–chloroform, 1:10; B, toluene-ethanol-water, 4:17:1; C, ethanol–water, 1:1; D, butanol–acetic acid–water, 4:1:1; E, butanol–acetic acid–water, 4:1:5 (upper layer); F, toluene with Whatman No. 1 paper which had been dipped in acetone–dimethyl sulfoxide (3:1) and dried 15 minutes under the hood.

1,6-Phenazinediol-5,10-dioxide (Iodinin), I.—Pablum extract agar (Lechevalier and Lechevalier, 1957) slants of *W. aerata* W-17 or P-132 were washed down and each was used to inoculate two 250-ml Erlenmeyer flasks containing 100 ml of sterile 6% aqueous Pablum. The flasks were shaken at 215 rpm at 37° for 2 weeks; then 100 ml of chloroform was added to each flask and shaking was continued 1 day at 28°. The deep red chloroform solution was separated and centrifuged, if necessary, to break emulsions. The yield of compound I in the chloroform solution was calculated from the optical density at 530 mμ and the previously determined $E^{1\%}_{1\,cm}$ of compound I at 530 mμ, which is 260. If the first extract contained more than 120 μg/ml. a second extraction was carried out. Yields varied from 7 to 25 mg/flask (average 15 mg/flask). The chloroform solution was concentrated to 0.1 volume; cooling overnight furnished glistening bronze needles of compound I, which decomposed with no definite mp. The pure product was soluble in dimethyl sulfoxide, dimethylformamide, benzene, dioxane, and pyridine, slightly soluble in chloroform and isopropyl acetate, and insoluble in hexane and alcohols. Application of the mother liquor to a column of activated silica gel and elution with acetone-chloroform gave a small additional amount of product. The analytical sample was prepared from chloroform, concentrating the solution with filtered air.

Anal. Calcd. for $C_{12}H_8N_2O_4$: C, 59.02; H, 3.30; N, 11.47. Found: C, 58.95, 59.25; H, 3.46, 3.16; N, 11.53, 11.65.

In a similar fashion, *Ps. iodina* from yeast-dextrose

slants was shaken in 50 ml of yeast-dextrose medium in 250-ml Erlenmeyer flasks at 215 rpm at 28° for 2 weeks. Compound I was isolated as before; it was identical with that from *W. aerata* in ultraviolet spectra, melting, and chromatographic behavior. The R_F values of compound I from either source (2–5 μg spots) on acetylated paper in solvents B and D were 0.15 and 0.21; compound I moved 0.4 cm in solvent C after 18 hours.

1,6-Phenazinediol, II.—A. Synthesis.—A mixture of compound I (215 mg) and commercial platinum oxide (50 mg) in absolute ethanol (100 ml) was hydrogenated at atmospheric pressure for 24 hours. After removal of the catalyst, the ethanol solution was taken to dryness in the rotary evaporator and the residue was extracted several times with hot benzene (total volume 100 ml). Reduction of the benzene solution to 20 ml furnished 90 mg of brown crystals. Further concentration to 5 ml yielded 43 mg. The total yield was 133 mg (69%), mp 265° dec in a sealed capillary, $\lambda_{max}^{dimethyl\ sulfide}$ 277, 372, 445 mμ ($E_{1\,cm}^{1\%}$ 5300, 240, 165). The melting point and ultraviolet and infrared spectra agreed with the literature values (Akabori and Nakamura, 1959; Irie *et al.*, 1960).

B. From Microorganisms.—*W. aerata* P-132 and *Ps. iodina* 26 (20 flasks each) were grown as in the previous section for 4 and 2 days, respectively. The contents of the flasks were extracted with chloroform; the chloroform solutions were concentrated to 200 ml, allowed to stand, and filtered to remove compound I. The filtrates were taken to dryness in a rotary evaporator and the residues were refluxed with 50 ml of hexane each. The hexane solutions (after filtering) were concentrated and applied as a line at the starting position of several silica thin-layer plates (2 ml per plate) which were then developed in solvent A. After drying overnight under the hood, the brown bands, R_F about 0.8, were scraped from the plates and packed into two small columns. The column from *W. aerata* was washed with hexane to remove oils and both columns were eluted with ethanol. Chromatography of the brown ethanol solutions on acetylated paper revealed compound II from both organisms as a yellow spot. The R_F values of both were identical with synthetic compound II: 0.61, 0.65, and 0.82 in solvents B, D, and F; 14 cm in solvent C after 18 hours. Preparative-paper-chromatographic separation on acetylated paper (previously washed with ethanol) furnished compound II from each organism as a yellow band which was eluted with ethanol for ultraviolet measurement. The spectra were both identical with that of synthetic compound II.

2-Aminophenoxazin-3-one, III.—A. Synthesis.—The oxidation of *o*-aminophenol with *p*-benzoquinone (Osman and Bassiouni, 1960) furnished compound III as a dark brown product, mp 254–256°, which was used without further purification; $\lambda_{max}^{H_2O}$ 240, 435 mμ ($E_{1\,cm}^{1\%}$ 1240, 1100); $\lambda_{max}^{dil\ HCl}$ 237, 468 mμ (1340, 800); $\lambda_{max}^{dil\ NaOH}$ 335 mμ (580); $\lambda_{max}^{cyclohexane}$ 240, 405, 425 mμ. The melting point and ultraviolet and infrared spectra agreed with the literature values (Osman and Bassiouni, 1960; Cavill *et al.*, 1961; Nagasawa *et al.*, 1959; Anzai *et al.*, 1960).

B. From Microorganisms.—The filtrates remaining after removal of the second crop of compound I from the concentrated chloroform extracts of both *W. aerata* P132 and *Ps. iodina* 26 were concentrated and applied to five silica thin-layer plates (approximately the amount of extract from one flask was applied to one plate) which were then run in solvent A. After drying overnight under the hood, the brown bands, R_F about 0.3, were scraped from the plates, packed into two small columns, and eluted with ethanol. The two eluates were applied to one silica plate each, chromatographed again, and eluted as before. The residues from these purified solutions both had $\lambda_{max}^{H_2O}$ 435; $\lambda_{max}^{dil\ HCl}$ 465; $\lambda_{max}^{dil\ NaOH}$ 330 mμ. The R_F values were both identical with those of synthetic compound III on silica plates in solvent A and on paper in solvent E (0.78), 70% acetone (0.59), 50% methanol (0.22), and methanol–10% HCl 1:1 (0.55). Compound III could not be detected in extracts of *Ps. iodina* E under these conditions.

2-Acetamidophenoxazine-3-one, IV.—A. Synthesis.—A mixture of compound III (1.0 g) and acetic anhydride (5 ml) was heated 1.5 hours on the steam bath then poured into water. The solution was neutralized with bicarbonate and extracted with chloroform, insoluble material being removed by filtering. The chloroform solution was washed once with water then applied to an 80-g column of Davidson activated silica gel. Elution with chloroform removed a brown impurity; acetic acid–chloroform (1:10) eluted the product. This orange solution was freed from acetic acid by shaking with aqueous base, washed with water, and concentrated to 20 ml to produce 160 mg of fine orange needles, mp 295–297°, λ_{max}^{EtOH} 240, 405 mμ ($E_{1\,cm}^{1\%}$ 1400, 1100) unchanged by the addition of acid or ammonia. The melting point and ultraviolet and infrared spectra agreed with the literature values (Cavill *et al.*, 1961; Osman and Bassiouni, 1960; Nagasawa *et al.*, 1959; Brockmann and Muxfeldt, 1958).

B. From Microorganisms.—The same procedure was used as for compound III (see preceding section) except that *Ps. iodina E* was used (compound IV could not be detected in extracts of strain 26 under these conditions) and the yellow band, R_F about 0.6, was chosen. The residues from the purified solution both had λ_{max}^{EtOH} 405 mμ unchanged by the addition of acid or ammonia. The R_F values were both identical with those of synthetic compound IV on silica plates in solvent A, on paper in solvents F (0.9) and E (0.76), and on acetylated paper in solvents D (0.64) and B (0.52). A small amount of solid compound IV was obtained from column chromatography of *W. aerata* W-17 chloroform extract mother liquors on silica gel eluted with solvent A. The yellow and orange fractions 5–10 were washed with aqueous base and the residue from the concentrated chloroform solution washed with hexane. The orange solid thus obtained after recrystallization from ethanol-water and microsublimation on the hot stage melted at 295–297°, undepressed when mixed with synthetic compound IV.

Comparison of Waksmania Strains.—Each strain was grown as described earlier for the production of compound I. The strains which produced no compound I under these conditions were spread on Pablum-extract agar plates and incubated at 37° for 1 month. At this point clusters of compound I crystals would be seen clearly under the microscope. For each strain the entire plate was mixed in the Waring Blendor with chloroform. Chromatography on acetylated paper of the concentrated chloroform solutions revealed pink spots of compound I whose R_F values were identical with that of authentic compound I in solvents B, C, and D. Larger samples, purified by paper chromatography in solvent C, furnished pink bands which, after elution from the paper with toluene, gave the characteristic ultraviolet spectrum of compound I.

Antimicrobial Assays.—Assays were carried out using the agar streak-dilution method (Waksman and

Reilly, 1945) on nutrient agar plus 0.5% NaCl and 1.0% glucose. Solutions of the compounds to be assayed were made in dimethyl sulfoxide as were all subsequent dilutions. Concentrations were verified by spectrophotometric measurements.

Assay Organisms.—Yeasts and bacteria, except for *Corynebacterium fimi*, were used when 24 hours old; *C. fimi*, mycobacteria except *M. avium*, and filamentous fungi except *Ceratostomella ulmi* and *Trichophyton mentagrophytes*, when 48 hours old; *C. ulmi* and *T. mentagrophytes* when 5 days old; and *M. avium* and all actinomycetes when 1 week old.

Inocula of the test organisms were prepared by suspending the growth from a slant culture in sterile water and inoculating with a sterile cotton swab or, in the case of the thermophilic actinomycetes and *M. avium*, with a standard 3-cm wire loop. Thermophilic actinomycetes, mycobacteria, and bacteria were incubated at 37°. All other organisms were grown at 28°.

Final readings were made at 48 hours for bacteria, mycobacteria (except *M. avium*), yeasts, and filamentous fungi, except *C. ulmi* and *T. mentagrophytes* which were read at 96 hours. Actinomycete assays were read after 1 week. The inhibitory concentrations given in Table II are based on at least three assays.

Characterization of Waksmania Strains.—All strains were maintained on Pablum agar (Lechevalier and Lechevalier, 1957) and incubated at 37° for 1 week to 10 days. Determinations of diastatic activity and the capacity to liquefy gelatine and reduce nitrate to nitrite were carried out by the methods recommended by the Society of American Bacteriologists (1957). Growth in litmus milk (Difco Manual, 1953) and degree of peptonization was judged subjectively. Presence or absence of iodinin crystals was confirmed microscopically.

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