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Revised Structure for the Phenazine Antibiotic from *Pseudomonas fluorescens* 2-79 (NRRL B-15132)

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A phenazine antibiotic (mp, 243 to 244°C), isolated in a yield of 134 μg/ml from cultures of *Pseudomonas fluorescens* 2-79 (NRRL B-15132), was indistinguishable in all of its measured physicochemical (melting point, UV and infrared spectra, and gas chromatography-mass spectrometry data) and biological properties from synthetic phenazine-1-carboxylic acid. Gurusiddaiah et al. (S. Gurusiddaiah, D. M. Weller, A. Sarkar, and R. J. Cook, Antimicrob. Agents Chemother. 29:488–495, 1986) attributed a dimeric phenazine structure to an antibiotic with demonstrably similar properties obtained from the same bacterial strain. Direct comparison of the physicochemical properties of the authentic antibiotic obtained from D. M. Weller with synthetic phenazine-1-carboxylic acid and with the natural product from the present study established that all three samples were indistinguishable within the experimental error of each method. No evidence to support the existence of a biologically active dimeric species was obtained. Phenazine-1-carboxylic acid has a pK_a of 4.24 ± 0.01 (25°C; I = 0.09), and its carboxylate anion shows no detectable antimicrobial activity compared with the active uncharged carboxylic acid species. These data suggest that phenazine-1-carboxylic acid is probably not an effective biological control agent for phytopathogens in environments with a pH greater than 7.

Weller and Cook (10) reported that the wheat root disease take-all, which is caused by *Gaeumannomyces graminis* var. *tritici*, can be suppressed by inoculating wheat seeds with *Pseudomonas fluorescens* 2-79 (NRRL B-15132). Gurusiddaiah et al. (6) characterized an antibiotic (mp, 242°C) produced by this culture and considered to be significant in this suppressive activity as having an unusual phenazine-1-carboxylic acid dimer structure. However, the physicochemical properties (UV and infrared [IR] spectra and the melting point) of this compound are similar to those of the phenazine-1-carboxylic acid (mp, 238 to 241°C), isolated from *P. aeruginosa* by Chang and Blackwood (4), and also to the properties of a material (mp, 243 to 244°C) isolated from *P. fluorescens* 2-79 in our laboratory. These similarities warranted a critical assessment of the data and a direct comparison with synthetic phenazine-1-carboxylic acid.

MATERIALS AND METHODS

Organisms. *Bacillus cereus*, *Escherichia coli* HB101, and fluorescent pseudomonad strains A37, 2-79, K2-79, and PGPR were routinely maintained on tryptic soy agar (8) or nutrient agar (Difco Laboratories). For long-term storage, bacteria were kept in 40% glycerol at −12°C. The fungi *G. graminis* var. *tritici* 500 and *Rhizoctonia solani* Rh21 were maintained on half-strength PDA medium (potato-dextrose agar powder [Oxoid Ltd.], 19.5 g; Difco agar, 7.5 g; water, 1 liter).

Media. A liquid glycerol-peptone-phosphate medium (glycerol [30 ml], peptone [10 g], K_2HPO_4 [0.5 g], and $MgSO_4 \cdot 7H_2O$ [0.5 g] in 1 liter of distilled water) was used for phenazine-1-carboxylic acid production. One liter of medium per 5-liter Erlenmeyer flask was inoculated with 2 ml of starter culture and grown without agitation at 25°C. Phenazine-1-carboxylic acid yields obtained from cultures incubated 4 weeks were 134 mg for strain 2-79, 61 mg for K2-79, 62 mg for PGPR, and <1 mg for A37. As noted by other researchers (4, 6), the yields were variable.

Antibiotic isolation. An authentic sample of the Gurusiddaiah et al. antibiotic was obtained from D. M. Weller. The natural antibiotic was isolated in our laboratory by the procedure of Chang and Blackwood (4). Synthetic phenazine-1-carboxylic acid was prepared by the method of Clemo and McIlwain (5). The yellow synthetic product was sublimed in vacuo (250 millitorr [ca. 33.33 Pa], 200°C) and crystallized from ethanol.

Antibiosis bioassays. The PDA medium was adjusted by the addition of a suitable pH buffer (20 mM phosphate, 20 mM citrate) instead of water. Bacteria were mixed in 5 ml of molten 2% agar containing 0.1% (wt/vol) peptone and poured over the PDA medium. Portions of the 1.5 mM phenazine-1-carboxylic acid in ethanol were evaporated on sterile paper disks (11-mm diameter) and applied to the agar. Fungi were centrally inoculated onto buffered PDA plates with 3-mm agar plugs obtained from the outer perimeter of actively growing mycelia, and the paper disks treated with phenazine-1-carboxylic acid were applied 2 to 3 days later.

Sources. *P. fluorescens* 2-79 (10) was kindly supplied by D. M. Weller, U.S. Department of Agriculture, Pullman, Wash. Strain K2-79 (originally from D. M. Weller) was supplied by J. F. Kollmorgen, Victorian Crops Research Institute, Horsham, Victoria, Australia. Strain A37 was isolated from South Australian soil by D. M. Weller. Strain PGPR was provided by J. L. Parke, Department of Plant Pathology, University of Wisconsin-Madison, Madison. *B. cereus*, *E. coli*, and *R. solani* were provided by J. R. Harris, M. Ryder, and H. J. McDonald, respectively, all from the Commonwealth Scientific and Research Organisation Division of Soils. The fungus *G. graminis* var. *tritici* 500 was supplied by C. A. Parker, University of Western Australia, Nedlands, Western Australia, Australia.

Physicochemical data. Melting points were determined with a Kofler hot-stage microscope, UV spectra were ob-

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tained with a Perkin-Elmer Lambda 5 recording spectrophotometer, and IR spectra were obtained by using 0.5% antibiotic in KCl disks or 1% solutions in $CHCl_3$ with a Perkin-Elmer 983 spectrophotometer recording from 4,000 to 400 cm^{-1}. GC-MS data were obtained with a Hewlett-Packard 5992b capillary gas liquid chromatograph-quadrupole mass spectrometer. This apparatus used electron impact ionization (70 eV) and was fitted with a bonded-phase (BP1) fused silica column (12 m by 0.2 mm; S.G.E., Melbourne, Australia). To examine methyl esters (prepared with diazomethane or methanol-hydrochloric acid), the column was maintained at 200°C for 1 min and then the temperature was increased by 10°C/min to 220°C; the injection port temperature was 350°C. Acids were analyzed isothermally at 250°C. Nuclear magnetic resonance spectra were recorded with samples dissolved in deuterochloroform (with tetramethylsilane as an internal standard) with a JEOL FX 90Q Fourier transform instrument. Silica gel thin-layer chromatography was carried out with solvent A (dichloromethane) (6) or solvent B (chloroform-methanol, 9:1 [vol/vol]) (4) on fluorescent (254 nm) precoated plastic sheets (Plastikfolien 60 F254; E. Merck AG). Ionization constants were determined spectrophotometrically (1) at a wavelength of 373.3 nm with 0.54 mM phenazine-1-carboxylic acid in 0.09 M sodium formate solutions which were titrated with a solution which was 0.54 mM with respect to phenazine-1-carboxylic acid, 1 M with respect to hydrochloric acid, and 0.09 M with respect to formic acid. pH and $A_{373.3}$ were measured after each of 30 additions in the pH range 5.0 to 2.8. The anion spectrum was unchanged in the pH range 7 to 11 and exhibited an absorption maximum at 366.6 nm with a molar absorption coefficient of 13,000. The ionization constant and molar absorption coefficient of 14,900 at 373.3 nm for the neutral species was obtained by a least-squares analysis of the data as described by Albert and Serjeant (1).

RESULTS

Identity criteria. Results showing the identical characteristics of the synthetic phenazine-1-carboxylic acid, the natural antibiotic isolated from *P. aeruginosa* 2-79 in this laboratory, and the authentic sample of the antibiotic "dimer" of Gurusiddaiah et al. are presented in Table 1 and Fig. 1. Other relevant data are listed below. Various studies have shown the acid's mp to be 242°C (6), 239°C (5), 238 to 241°C (4), 242°C (3), or 243°C (7). The methyl ester has shown mp's of 126 to 128°C (3) and 120 to 123°C (7). The UV spectra of phenazine-1-carboxylic acid in $CHCl_3$ showed absorbance at 251 and 361 nm (4). The IR data of the "dimer" were 3,040, 3,020, 1,740, 1,625, 1,605, 1,565, and 1,525 cm^{-1} and Fig. 4 in reference 6. GC/MS data (6), with the ionization technique not specified and abundance values interpolated from Fig. 1 in reference 6, were as follows (*m/z*, abundance): 448, 0.3; 431, 1.4; 404, 5.6; 360, 7.0; 225, 80.3; 208, 8.5; 207, 44.4; 180, 100; 179, 21.1; and 153, 7.0.

In further comparisons (data not shown), the 2-79 antibi-

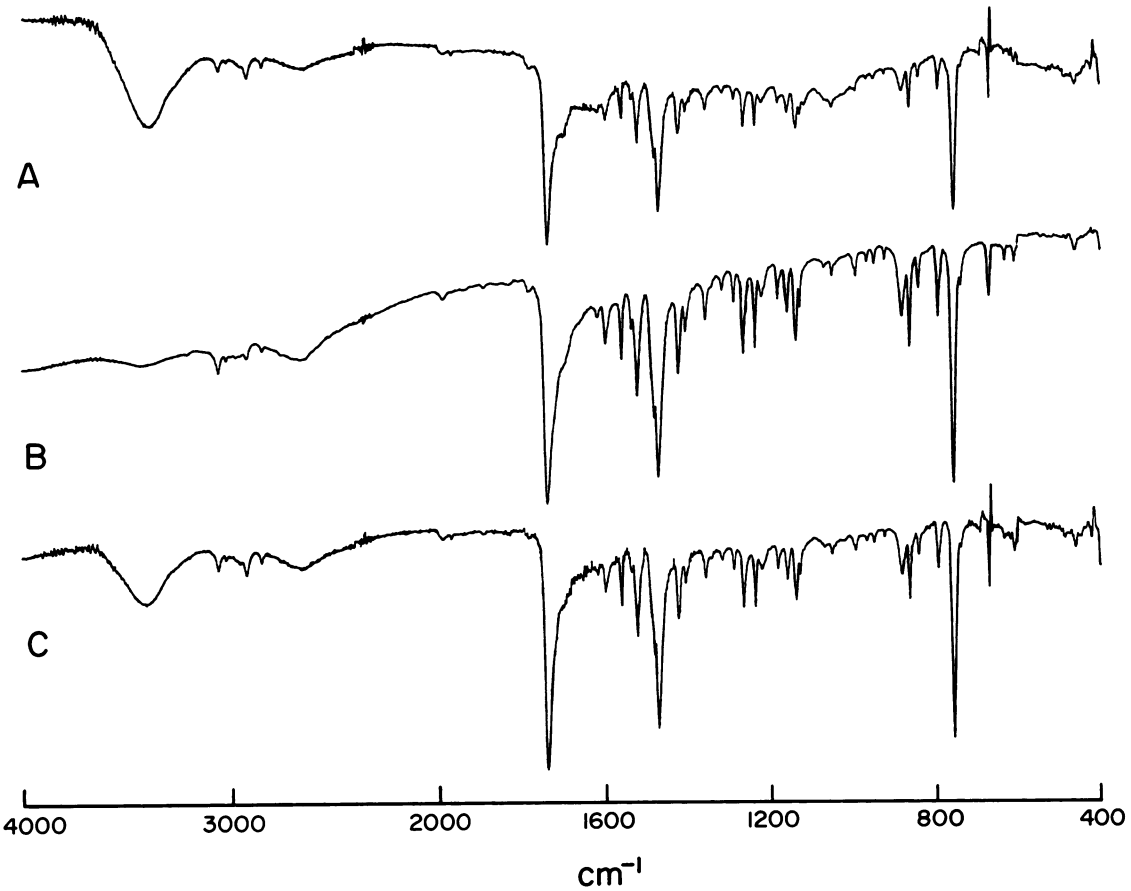


FIG. 1. Comparison of IR spectra at 0.5% in KCl disks of sublimed samples of synthetic (A) and natural (B) phenazine-1-carboxylic acid from *P. fluorescens* 2-79 and of the "dimer" species (C) obtained from Gurusiddaiah et al. (6).

TABLE 1. Comparison of physicochemical properties of free acid and its methyl ester

Physicochemical property	Phenazine-1-carboxylic acid type		
	Synthetic	Natural	"Dimer"
Elemental analysis (% C/H/N/O)	69.6/3.6/12.5/14.3	70.1/3.9/12.6/13.7	69.3/3.6/12.5/14.2
mp (°C)			
Acid	243–244	243–244	244
Mixture mp with Natural	243–244		243–244
Methyl ester	123–124	123–124	123–124
Mixture mp with Natural	123–124		123–124
Thin-layer chromatography			
Acid			
Solvent A	0.16	0.16	0.16
Solvent B	0.59	0.59	0.59
Methyl ester			
Solvent A	0.16	0.16	0.16
Solvent B	0.68	0.68	0.68
UV spectra, nm (ε)[a]			
Acids (CH_2Cl_2)	251 (97,000)	251 (97,000)	251 (97,000)
	348 sh		
	356 sh		353 sh
	364 sh		364 sh
	370 (19,000)	370 (19,000)	370 (19,000)
	408 sh		409 sh
Methyl ester (ethanol)	248 (73,000)	248 (73,000)	248 (73,000)
	364 (13,200)	364 (13,200)	364 (13,200)
GC/MS			
Acid			
Retention time (min)	2.1	2.1	2.1
m/z (abundance)	224 (0.3)	224 (0.3)	224 (0.3)
	207 (0.3)	207 (1.0)	207 (0.5)
	181 (5.2)	181 (5.3)	181 (5.3)
	180 (100)	180 (100)	180 (100)
	179 (16.2)	179 (16.4)	179 (16.1)
	154 (0.5)	154 (0.5)	154 (0.4)
	153 (1.6)	153 (1.8)	153 (1.6)
	152 (1.4)	152 (1.6)	152 (1.4)
	102 (0.8)	102 (0.7)	102 (0.8)
	76 (1.5)	76 (1.8)	76 (1.7)
	75 (1.2)	75 (1.4)	75 (1.2)
Methyl ester			
Retention time (min)	2.9	2.9	2.9
m/z (abundance)	238 (19.7)	238 (21.4)	238 (20.2)
	207 (16.7)	207 (13.7)	207 (12.9)
	181 (7.4)	181 (6.0)	181 (5.3)
	180 (100)	180 (100)	180 (100)
	179 (28.8)	179 (29.7)	179 (29.7)
	153 (2.0)	153 (1.6)	153 (1.6)
	152 (3.8)	152 (3.7)	152 (3.3)

[a] Values in parentheses are molar absorption coefficients (ε). sh, Shoulder.

otic ^1H and ^{13}C nuclear magnetic resonance chemical shifts and intensities in deuterochloroform agreed with the findings of Gurusiddaiah et al. (6). The IR spectra of chloroform solutions of our natural antibiotic, were indistinguishable from those of the Gurusiddaiah et al. authentic sample. Likewise, the UV spectra of all three stock ethanolic solutions were superimposable when the solutions were diluted with either ethanol or ethanol which was 0.2 M with respect to HCl.

Phenazine-1-carboxylic acid toxicity and pH. The paper disk bioassay with the agar medium at pH 6 showed that *R. solani* was inhibited by 25 μg of phenazine-1-carboxylic acid, *G. graminis* by 13 μg, and *B. cereus* by 0.5 μg, but that *E. coli* was not inhibited by 40 μg of the antibiotic.

Figure 2 shows that the inhibition of both *G. graminis* and *B. cereus* steadily decreases as the pH increases. The antimicrobial activity against both fungi and bacteria is related to the concentration of protonated phenazine-1-carboxylic acid. The pK_a was 4.24 ± 0.01 (25°C; I = 0.09); the ionic strength and temperature correction detailed by Albert and Serjeant (1) provided a thermodynamic pK_a of 4.34. An independent estimate obtained by the solubility procedure (1) gave a pK_a of 4.25 ± 0.02 (25°C; I = 0.1). A previously established pK_a value for phenazine-1-carboxylic acid of 7.0 (7), which is well outside the range expected for aromatic carboxylic acids (1), was obtained in 66.7% dimethyl formamide. This value is therefore unsuited for comparison with the current aqueous pK_a determinations or

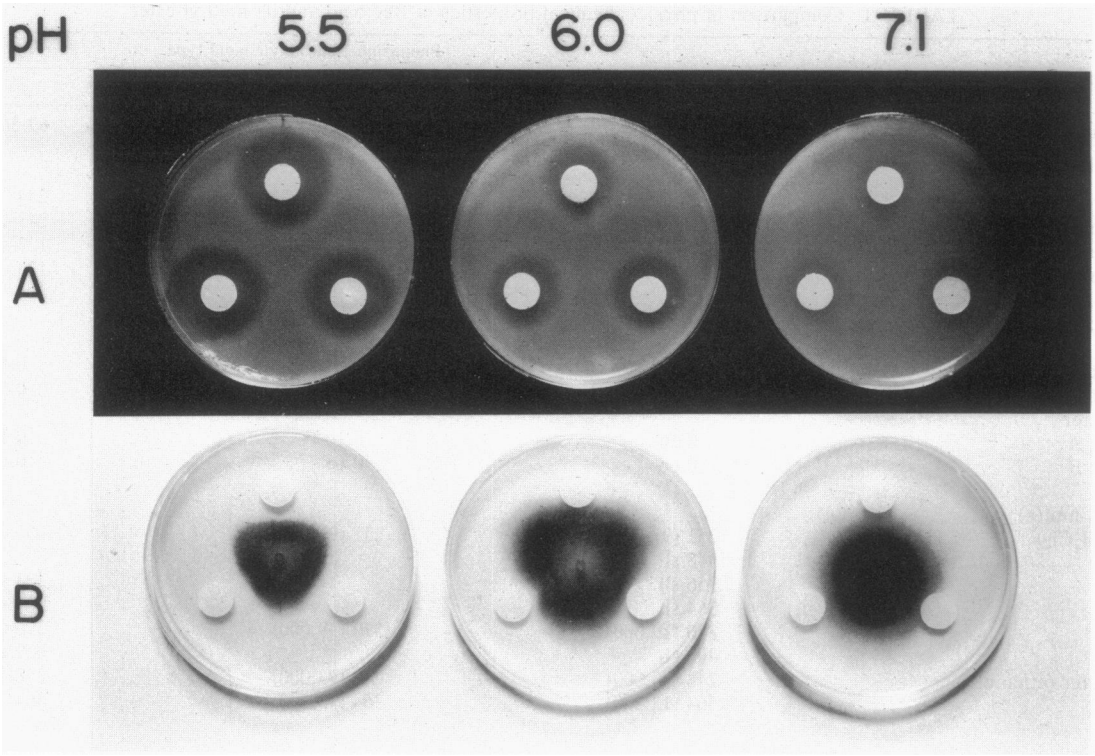


FIG. 2. Bioassay at pH values of 5.5, 6.0, and 7.1 for *B. cereus* with 0.5 μg of antibiotic per disk (A) and *G. graminis* var. *tritici* with 13 μg of antibiotic per disk (B). In each plate, the top disk has the natural antibiotic, the left disk has the synthetic antibiotic, and the right disk has the "dimeric" species obtained from Gurusiddaiah et al. (6).

for the interpretation of the behavior of biological systems which exhibit a pH dependency.

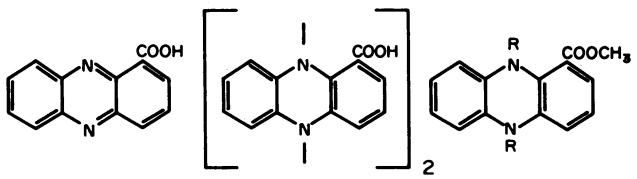
DISCUSSION

Production. The production by *Pseudomonas* spp. of phenazine-1-carboxylic acid (mp, 239 to 242°C), structure I in Fig. 3, is well documented (4). However, a recent study by Gurusiddaiah et al. (6) reported the isolation of an antibiotic (mp, 242°C) from *P. fluorescens* 2-79 for which an unusual, highly symmetrical, nitrogen-cross-linked phenazine-1-carboxylic acid dimer was proposed. The possible three-dimensional structure of this dimer is shown as structure II in Fig. 3 for the purpose of this discussion.

Physicochemical properties. We have independently isolated an antibiotic (mp, 243 to 244°C) from the same *Pseudomonas* strain as that used to produce the sample of the Gurusiddaiah et al. antibiotic provided to us by D. Weller.

The synthetic reference sample of phenazine-1-carboxylic acid prepared by the method of Clemo and McIlwain (5), the natural product isolated here, and the Gurusiddaiah et al. sample were indistinguishable within experimental error. Except for the mass spectrum, the physicochemical properties reported by Gurusiddaiah et al. (6) for this antibiotic are in excellent agreement with our data.

Two problems are apparent in the interpretation of data by Gurusiddaiah et al. (6) for their proposed dimeric structure. The first concerns the long-wave absorption band near 370 nm, which is apparent in the yellow color of the crystals. The bridgehead nitrogen bond geometries for the proposed dimeric structure would impose such severe restrictions upon any orbital overlap with the π electrons of the adjacent aromatic ring systems that they would severely interrupt delocalization, compared with the corresponding phenazine-1-carboxylic acid. A dimeric structure such as structure II in Fig. 3 can therefore reasonably be predicted (cf. Scott [9]) to show no significant absorption in the visible region of the spectrum. A good example of this is the methyl ester of *N,N*-diacetyl dihydrophenazine-1-carboxylic acid (structure III in Fig. 3; $R = CH_3CO$), which exhibits only a single absorption band with a maximum near 260 to 265 nm (2). However, dihydrophenazine-1-carboxylic acids bearing hydrogen atoms on the nitrogen atoms, such as methyl ester III (Fig. 3; $R = H$), do show long-wavelength bands at 350 and 450 nm. Birkofer (2) has suggested that these long-wave bands arise from quinonoid structures; quinonoid species are planar molecules, unlike the nitrogen-cross-linked dimer proposed by Gurusiddaiah et al. (6). The second problem with the dimeric structure proposed by Gurusiddaiah et al. concerns the lithium aluminum hydride reduction of the antibiotic to produce 1-hydroxymethyl phenazine. This



I II III

FIG. 3. Phenazine-1-carboxylic acid structures. (I) Phenazine-1-carboxylic acid. (II) Phenazine-1-carboxylic acid dimeric structure proposed by Gurusiddaiah et al. (6). (III) Dihydrophenazine-1-carboxylic acid methyl ester ($R = H$) or its diacetyl derivative ($R = CH_3CO$).

phenazine is the expected product from the reduction of phenazine-1-carboxylic acid; however, reductive cleavage of structure II in Fig. 3 would produce dihydro-1-hydroxymethyl phenazine.

The primary physical data presented by Gurusiddaiah et al. (6) to support the dimeric structure concern the existence of ions with mass spectrum *m/z* values of 448, 431, 404, and 360. The identity criteria followed here show that these ions were not present (i.e., <0.1% base peak) in the underivatized authentic Gurusiddaiah et al. antibiotic, as shown by electron impact ionization, nor were the corresponding masses observed for the methyl ester derivative, even with the aid of selective ion monitoring (*m/z*, 476). The methyl ester derivative did exhibit the expected strong molecular ion (*m/z*, 238), whereas the free acid showed only a weak molecular ion, with the bulk of the ion current being carried by the base peak fragment ion (*m/z*, 180; M^+-CO_2). Although the high-mass ions of ≥360 *m/z* are consistent with a dimeric structure, the ionization technique cannot show what proportion of the sample produced these ions or whether they are the consequence of the particular ionization procedure or instrument used for the mass spectrum published by Gurusiddaiah et al. (6). Their spectrum in the range of 153 to 225 *m/z* is consistent with a strongly protonated molecular ion (MH^+; *m/z*, 225) and with its subsequent fragmentation pattern with ion assignments as already indicated (6).

Biological properties. The biological properties of the antibiotic were reported (6) to differ from those for phenazine-1-carboxylic acid structure I. However, since no direct comparison with synthetic phenazine-1-carboxylic acid was done, these data must be considered inconclusive. Figure 2 shows that the antibiotic (mp, 243 to 244°C) isolated here, an authentic sample of the *P. fluorescens* 2-79 "dimer" provided by D. Weller, and the synthetic phenazine-1-carboxylic acid (5) all have indistinguishable biological activities toward the indicator strains, as shown by the overlay bioassays, and that their toxicities also exhibit the same pH dependency. It is therefore unlikely that the observed activity is due to some potent but minor constituent of the natural product samples. The observed (Fig. 2) pH dependency of the biological activity against both indicator strains, taken together with the measured phenazine-1-carboxylic acid ionization constant (pK_a = 4.24 ± 0.01), is consistent with the conclusion that, compared with the un-ionized species, the carboxylate anion is biologically inactive toward the indicator strains (Fig. 2). Two conclusions may be drawn from these findings. First, without a knowledge of both the pH of the medium and the pK_a of the antibiotic, the measurement of MICs gives no indication of the actual concentration of the biologically active species that would be required to inhibit an organism. For example, at pH 6.0 the minimum amounts of phenazine-1-carboxylic acid required for detectable inhibition of various organisms were as follows: *R. solani*, 25 μg; *G. graminis*, 13 μg; and *B. cereus*, 0.5 μg. For any pH, the actual concentration of the active neutral species can be readily calculated from the following equation (1): % nonionized = 100/[1 + antilog (pH − pK_a)]; at pH 6.0, the active species represents only 1.7% of the applied loadings, and at pH 7, it diminishes to only 0.17%. Thus, the biological control of susceptible phytopathogens by organisms producing only phenazine-1-carboxylic acid will probably be ineffective in alkaline (pH > 7) environments. However, these data do not exclude the possibility that other antibiotics may play a role in the successful biological control of pathogens by strain 2-79 in high-pH environments.

The simplest interpretation of the data supplied by Gurusiddaiah et al. (6), taken in conjunction with the data presented here, is that the major component of the antibiotic (mp, 242 to 244°C) obtained from *P. fluorescens* 2-79 is phenazine-1-carboxylic acid and that the existence of a biologically active, covalently linked dimer must be considered doubtful. The usefulness of phenazine-1-carboxylic acid (pK_a, 4.24) as a biological control agent for some plant pathogens in alkaline environments will be severely limited by its almost complete ionization to an inactive carboxylate ion.

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LITERATURE CITED

1. **Albert, A., and E. P. Serjeant.** 1971. The determination of ionization constants, 2nd ed. Chapman & Hall, Ltd., London.
2. **Birkofer, L.** 1952. Konstitution von Dihydrophenazin derivaten. Chem. Ber. **85**:1023–1034.
3. **Breitmaier, E., and D. U. Hollstein.** 1976. Carbon-13 nuclear magnetic resonance chemical shifts of substituted phenazines. J. Org. Chem. **41**:2104–2108.
4. **Chang, P. C., and A. C. Blackwood.** 1969. Simultaneous production of three phenazine pigments by *Pseudomonas aeruginosa* Mac 436. Can. J. Microbiol. **15**:439–444.
5. **Clemo, G. R., and H. McIlwain.** 1934. A new phenazine synthesis, the phenhydrazins. J. Chem. Soc. **1934**:1991–1993.
6. **Gurusiddaiah, S., D. M. Weller, A. Sarkar, and R. J. Cook.** 1986. Characterization of an antibiotic produced by a strain of *Pseudomonas fluorescens* inhibitory to *Gaeumannomyces graminis* var. *tritici* and *Pythium* spp. Antimicrob. Agents Chemother. **29**:488–495.
7. **Isono, K., K. Anzai, and S. Suzuki.** 1959. Tubermycin A and B, new antibiotics. J. Antibiot. Ser. A **11**:264–267.
8. **Martin, J. K.** 1975. Comparison of agar media for counts of viable soil bacteria. Soil Biol. Biochem. **7**:401–402.
9. **Scott, A. I.** 1964. Interpretation of the ultraviolet spectra of natural products, p. 89–134. *In* D. H. R. Barton and W. Doering (ed.), Comprehensive organic chemistry. Pergamon Press, Oxford.
10. **Weller, D. M., and R. J. Cook.** 1983. Suppression of take-all of wheat by seed treatments with fluorescent pseudomonads. Phytopathology **73**:463–469.