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# Effects of Pesticides on the Bacterial Production of Pyrrolnitrin

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Pyrrolnitrin is a halogenated bacterial metabolite with antifungal and antibacterial activities which served as a lead structure of synthetic fungicides. Several pyrrolnitrin-producing bacteria are considered to be promising biopesticides. However, the application of these microorganisms is not straightforward since many synthetic pesticides usually coexist in agricultural fields and inevitably affect the efficacy of biocontrol agents. In this regard, effects of 25 xenobiotics, including 18 pesticides, were investigated for pyrrolnitrin biosynthesis by *Burkholderia* sp. O33 and *Pseudomonas fluorescens* Pf-5. Strong inhibition of pyrrolnitrin synthesis was observed in 9 chemicals, including 6 pesticides, while glyphosate and validamycin enhance biosynthesis. Fenpiclonil and fludioxonil strongly inhibit the oxidative transformation of aminopyrrolnitrin to pyrrolnitrin. Halogenation reaction to aminopyrrolnitrin was reduced by methimazole, a well-known flavin-dependent monooxygenase inhibitor. Most pesticides gave moderate growth inhibitory effects. The results suggested that synthetic chemicals can modulate the efficacy of pyrrolnitrin producing bacteria, through the inhibition of cell growth or pyrrolnitrin biosynthesis. Pathway specific inhibition by fenpiclonil, fludioxonil, and methimazole will give structural insights of corresponding enzymes.

**KEYWORDS: Pyrrolnitrin; pesticide toxicity; fenpiclonil; fludioxonil; biosynthesis**

## INTRODUCTION

Synthetic pesticides are commonly used in most of the agricultural fields. Their structural diversity ensures the control of a wide array of pathogenic organisms. Because of the strong concerns of toxicological problems, rigorous evaluation of the toxicity to higher animals and plants is commonly performed to newly registered synthetic pesticides. However, the effects on soil- or plant-dwelling microorganisms with beneficial activities were not well understood. Low toxicity to nontarget organisms and continuous regeneration of bioactive metabolites make the biopesticides or organisms promising alternatives to synthetic pesticides. However, coapplication or coexistence of biopesticides and synthetic chemicals are inevitable in general agricultural practices since the biological spectra of these metabolites are generally limited to a narrow range of pathogens. In consideration of these problems, interaction between synthetic pesticides and biopesticides should be evaluated in further detail to improve the efficiency of both pesticides.

Various bacterial secondary metabolites from polyketides, proline, or tryptophan metabolism have antifungal activities, including 2,6-diacetylphloglucinol, pyoluteorin, pyrrolnitrin, and phenazines. Pyrrolnitrin (Prn) is produced from several bacterial species, including *Pseudomonas*, *Burkholderia*, *Serratia*, and *Myxococcus* (*7*, *10*, *23*, *26*). Because of its strong antifungal activities, some Prn-producing bacteria get interest as topical medicine or biopesticides (*23*). In addition, some of the above-mentioned metabolites served as synthetic leads of novel fungicides (e.g., fludioxonil and fenpiclonil from pyrrolnitrin). Prn and pyoluteorin are produced from tryptophan and proline, respectively (*9*, *12*, *26*, *27*, *32*). Various aspects of gene regulations and environmental factors were evaluated to improve the biosynthetic yield or adaptation in the natural environment (*6*−*8*, *28*, *29*).

In this study, we evaluated the effects of several pesticides, commonly applied in agricultural fields on the biosynthesis and growth of Prn-producing bacteria. Some additional xenobiotics, including drugs and environmental contaminants were tested. Their mechanistic aspects of inhibitory effects were also discussed.

## MATERIALS AND METHODS

**Chemicals.** The following reagents were obtained from Aldrich Korea (Seoul, Korea): 7-chloroindole, 7-bromoindole, Prn, antipyrine, aminoantipyrine, methimazole, sodium sulfite, sodium bromide, and triclosan. Pesticide standards were obtained from commercial suppliers (Aldrich Korea; ChemService Inc.; Kyungnong Inc.), and their purities were over 98%. A polychlorinated biphenyl mixture (PCB, Aroclor 1016) was purchased from Accustandard Inc. (New Haven, CT). A list of pesticides and test concentrations is given in **Table 1**. Nutrient broth was from BD Korea (Seoul, Korea). Solvents were of HPLC grade or higher.

**Culture of Bacterium.** *Burkholderia* sp. O33 and *Pseudomonas fluorescens* Pf-5 were obtained from Korean Agricultural Culture Collection (KACC # 12815) and American Type Culture Collection (ATCC # BAA-477), respectively, and maintained in nutrient agar at 27 °C. Both strains were cultured in phosphate-buffered nutrient broth (NBM) medium, supplemented with different pesticides (**Table 1**). NBM medium comprised $Na_2HPO_4$ (1.20 g), $KH_2PO_4$ (0.25 g), and nutrient broth (1.5 g) in deionized water (500 mL) at pH 6.8. Stock solutions of pesticides in

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**Table 1.** List of Pesticides and Other Xenobiotics in This Study and Concentrations

| name | classification | concentration ($\mu$M) | name | classification | concentration ($\mu$M) |
|---|---|---|---|---|---|
| acifluorfen | herbicide | 5 | fenpiclonil[a] | fungicide | 0.5, 1, 5, 10, 25 |
| alachlor | herbicide | 10 | fludioxonil[a] | fungicide | 1, 5, 10, 25, 50 |
| atrazine | herbicide | 6 | glyphosate | herbicide | 10, 20 |
| aminoantipyrine | drug | 5 | imazalil | fungicide | 6 |
| antipyrine | drug | 5 | imidacloprid | insecticide | 10 |
| atrazine | herbicide | 5 | methimazole | drug | 6 |
| biphenyl-2-amine | misc | 5, 30 | nitrofen | herbicide | 10 |
| carbaryl | insecticide | 6 | paraquat | herbicide | 15 |
| celestine blue | misc | 5 | PCB | misc | 5 |
| chlorpyrifos oxon | insecticide | 5 | tebufenpyrad | acaricide | 5 |
| chlorpyrifos | insecticide | 5 | triclosan | bactericide | 10 |
| chloridazon | herbicide | 10 | validamycin | fungicide | 20 |
| cyprodinil | fungicide | 10 | | | |

[a] Concentration dependent kinetic experiments were performed with *Burkholderia* sp. O33 and *Pseudomonas fluorescens* Pf-5.

dimethyl sulfoxide (typically 0.5 mL) were added after sterilization to make specified concentrations. The culture was maintained at 27 and 30 °C, 160 rpm for strain O33 and Pf-5, respectively. Three replicates were prepared for all experiments. To test the effects of fenpiclonil on the biosynthesis of brominated Prn, sodium bromide (500 mg/L) and fenpiclonil (5 mg/L) were supplemented in NBM (1 L) and cultured for 10 days. The control was prepared according to the same procedures without fenpiclonil.

**Extraction of Pyrrolnitrin and Precursors.** For quantitative analysis, an aliquot amount of culture (60 mL) was centrifuged (3500 rpm, 50 min). The supernatant was saturated with NaCl and extracted with ethyl acetate (80 mL × 3). The organic layer was dried over anhydrous sodium sulfate. After removing the organic solvent, we redissolved the residue in ethyl acetate (1.2 mL) and analyzed it with GC-MS. The weight of the cell pellet after centrifugation was measured and compared with that of others.

For the preparation of large quantities of metabolites, the supernatant from batch cultures (2 L) was saturated with sodium chloride and extracted with ethyl acetate (500 mL × 3). The combined organic layer was dried over anhydrous sodium sulfate. After removing the solvent, we redissolved the residue in ethyl acetate (2 mL) and purified it with preparative silica gel TLC (*13*).

**Instrumental Analyses.** Prn and its precursors were analyzed with GC-MS (Shimadzu GCMS QP-2000 and GC-2010), equipped with a DB-5MS column (60 m, 0.25 $\mu$m film thickness, 0.25 mm i.d.; Agilent Technologies, USA). Helium was a carrier gas at a flow rate of 1 mL/min. The column temperature was programmed as follows; 95 °C (10 min) and raised to 295 °C at a rate of 2 °C/min and held for 20 min. The mass spectra of metabolites were obtained in full scan or selected ion monitoring (SIM) mode. Quantitation ions for Prn, aminopyrrolnitrin (AmPrn), and monochloroaminopyrrolnitrin (MAmPrn) were 256, 226, and 192, respectively. Because tryptophan and its halogenated analogues were quantitatively degraded into corresponding indole in GC-MS, these amino acids in supernatants were determined as corresponding indoles in full scan mode. Retention times (*Rts*) of indole, 7-chloroindole, and 7-bromoindole were 27.8, 31.2, and 36.8 min, respectively.

**Molecular Modeling.** Energy minimized structures of Prn, AmPrn, MAmPrn, fenpiclonil, and fludioxonil were calculated with HyperChem ver. 8.0.4 (Hypercube Inc., Gainesville, FL). Electron densitiy isosurface maps were calculated after energy minimization with a PM3 semiempirical forcefield. Molecular volumes of the compounds were calculated with the add-on QSAR properties module of the same program.

## RESULTS

**Effects of Xenobiotics on Pyrrolnitrin Biosynthesis.** Toxic effects of xenobiotics were tested with *Burkholderia* sp. O33, and more detailed studies with selected fungicides have been performed with two Prn-producing strains (*Burkholderia* sp. O33 and *P. fluorescens* Pf-5). From the previous study, Prn biosynthesis by strain O33 reached a maximum at approximately 4−5 days of incubation (*18*). Concentrations of Prn or metabolites were negligible in biomass, and most portions were found in culture supernatants. In general, no halogenated metabolites, other than 7-chloroindole, MAmPrn, AmPrn, and Prn were observed in culture supernatants in both strains (data not shown). The concentrations of Prn were much higher than those of AmPrn or MAmPrn (**Table 2**).

Differential effects on Prn biosynthesis were observed in xenobiotic-treated cultures (**Table 2**). Prn biosynthesis was diminished by many xenobiotics, except for some pesticides (e.g., chloridazon, cyprodinil, glyphosate, and validamycin), where the apparent enhancement of biosynthesis was observed in glyphosate- and validamycin-treated cultures of strain O33 (**Table 2**). Among the tested chemicals, 8 compounds markedly inhibit Prn biosynthesis, including acifluorfen, biphenyl-2-amine, carbaryl, chlorpyrifos oxon, fenpiclonil, methimazole, nitrofen, and triclosan (**Table 2**). Comparative analysis of the metabolite profiles revealed interesting aspects of these chemicals. For example, suppression of Prn and/or metabolites was observed in acifluorfen, biphenyl-2-amine, carbaryl, chlorpyrifos oxon, nitrofen, and triclosan. However, accumulation of Prn precursors with a reduced amount of Prn was observed in fenpiclonil and methimazole-treated cultures of strain O33 (**Figure 1** and **Table 2**). Kinetic analysis of Prn and its metabolites indicates that the AmPrn level rapidly increased in fenpiclonil-treated strains O33 and Pf-5 (**Figure 2B** and **E**, respectively). In addition to fenpiclonil, the accumulation of AmPrn was also observed in fludioxonil-treated Pf-5 (**Figure 2F**). Ratios between the concentrations of Prn and AmPrn in control cultures were approximately 6−15. However, it has changed to 0.02−0.04 by the increasing concentration of fenpiclonil in strains O33 and Pf-5 (**Figure 3**). The concentrations of MAmPrn were at trace levels (average of 0.04 mg/kg). However, an approximately 30-fold increase of its concentration was observed in methimazole-treated cultures of strain O33 (**Table 2**). Similarly, fenpiclonil treatment also increases the level of MAmPrn as well as AmPrn.

**Polychlorination of AmPrn and Test Chemicals.** Instrumental analysis of culture extracts indicates that 7-chloroindole, MAmPrn, AmPrn, and Prn were the only halogenated metabolites from the control and those of most xenobiotic treatments. However, detailed GC-MS analyses of fenpiclonil and biphenyl-2-amine-treated culture extracts suggested that additional metabolites were produced by strain O33 or Pf-5. For example, the mass spectrum of a peak (*Rt*, 64.6 min) in fenpiclonil-treated Pf-5 indicates that the metabolite contains at least three halogen (chlorine) atoms (**Figure 4**). Similar peaks were also found in strain O33 with the same fungicide and Pf-5 with fludioxonil, while no trace was found in the control or other chemicals.

**Table 2.** Concentration of Pyrrolnitrin and Metabolites in 5 Day Cultures of *Burkholderia* sp. O33, Treated with Xenobiotics and Growth Inhibition

| | concentration (mg/L) | | | | | |
|---|---|---|---|---|---|---|
| chemical[a] | Prn[b] | AmPrn | MAmPrn | ClInd | Ind | growth (% of control) |
| control | 7.73 ± 2.52 | 0.37 ± 0.08 | 0.03 ± 0.01 | 0.03 ± 0.01 | 0.03 ± 0.01 | 100 |
| acifluorfen | 1.68 ± 0.51 | 0.05 ± 0.01 | ND[c] | 0.01 ± 0.01 | 0.03 ± 0.01 | 78 ± 12 |
| alachlor | 4.22 ± 1.19 | 0.06 ± 0.01 | ND | 0.03 ± 0.01 | 0.05 ± 0.01 | 86 ± 18 |
| aminoantipyrine | 6.23 ± 1.08 | 0.20 ± 0.05 | ND | 0.03 ± 0.01 | 0.02 ± 0.00 | 100 ± 8 |
| antipyrine | 6.53 ± 1.03 | 0.12 ± 0.04 | ND | 0.04 ± 0.00 | 0.03 ± 0.01 | 105 ± 6 |
| atrazine | 5.33 ± 0.96 | 0.08 ± 0.01 | ND | 0.08 ± 0.01 | 0.04 ± 0.02 | 95 ± 11 |
| biphenyl-2-amine | 2.82 ± 0.83 | 1.72 ± 0.27 | ND | 0.01 ± 0.00 | 0.05 ± 0.01 | 72 ± 19 |
| carbaryl | 3.00 ± 1.18 | 0.36 ± 0.09 | ND | 0.03 ± 0.01 | 0.02 ± 0.00 | 85 ± 15 |
| celestine blue | 4.74 ± 1.18 | 0.20 ± 0.08 | 0.04 ± 0.01 | 0.09 ± 0.02 | 0.03 ± 0.01 | 89 ± 17 |
| chloridazon | 8.09 ± 1.55 | 0.39 ± 0.06 | ND[c] | 0.05 ± 0.01 | 0.03 ± 0.01 | 95 ± 3 |
| chlorpyrifos | 5.61 ± 0.95 | 0.03 ± 0.01 | ND | 0.02 ± 0.00 | 0.02 ± 0.00 | 70 ± 11 |
| chlorpyrifos oxon | 2.11 ± 0.64 | 0.030 ± 0.01 | ND | 0.01 ± 0.00 | 0.03 ± 0.01 | 61 ± 14 |
| cyprodinil | 7.31 ± 1.41 | 0.27 ± 0.08 | 0.05 ± 0.01 | 0.02 ± 0.01 | 0.02 ± 0.01 | 95 ± 7 |
| fenpiclonil | 1.33 ± 1.33 | 4.55 ± 0.59 | 0.19 ± 0.05 | 0.04 ± 0.01 | 0.05 ± 0.02 | 100 ± 4 |
| fludioxonil | 6.23 ± 1.06 | 0.22 ± 0.07 | 0.01 ± 0.00 | 0.02 ± 0.00 | 0.05 ± 0.01 | 89 ± 13 |
| glyphosate | 11.19 ± 1.82 | 0.31 ± 0.03 | ND | 0.02 ± 0.00 | 0.02 ± 0.01 | 100 ± 7 |
| imazalil | 6.80 ± 1.27 | 0.01 ± 0.01 | ND | 0.04 ± 0.01 | 0.02 ± 0.00 | 100 ± 11 |
| imidacloprid | 5.40 ± 0.97 | 0.06 ± 0.01 | ND[c] | 0.01 ± 0.00 | 0.03 ± 0.01 | 96 ± 9 |
| methimazole | 3.45 ± 0.55 | 0.03 ± 0.01 | 0.94 ± 0.16 | 0.01 ± 0.00 | 0.03 ± 0.00 | 85 ± 7 |
| nitrofen | 1.68 ± 1.21 | 0.03 ± 0.01 | ND | 0.01 ± 0.00 | 0.04 ± 0.00 | 89 ± 13 |
| paraquat | 6.42 ± 1.60 | 0.08 ± 0.02 | 0.06 ± 0.01 | 0.04 ± 0.01 | 0.04 ± 0.01 | 76 ± 6 |
| PCB | 4.25 ± 0.94 | 0.12 ± 0.06 | ND | 0.01 ± 0.01 | 0.03 ± 0.01 | 78 ± 12 |
| tebufenpyrad | 6.66 ± 2.00 | 0.11 ± 0.03 | 0.05 ± 0.01 | 0.13 ± 0.02 | 0.04 ± 0.01 | 89 ± 7 |
| triclosan | 3.76 ± 1.40 | 0.06 ± 0.01 | ND | 0.02 ± 0.01 | 0.03 ± 0.00 | 71 ± 5 |
| validamycin | 11.82 ± 1.72 | 0.27 ± 0.04 | 0.03 ± 0.01 | 0.02 ± 0.00 | 0.01 ± 0.00 | 82 ± 11 |

[a] Concentrations of fenpiclonil, fludioxonil, and biphenyl-2-amine were 10, 10, and 5 $\mu$M, respectively. Concentrations of other chemicals were as described in **Table 1**. [b] Abbreviations: Prn, pyrrolnitrin; AmPrn, aminopyrrolnitrin; MamPrn, monochloroaminopyrrolnitrin; ClInd, 7-chloroindole; Ind, indole. [c] ND, not detected.

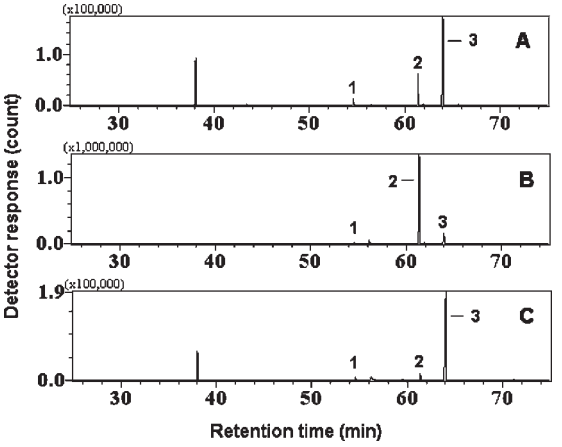


**Figure 1.** Representative GC-MS SIM chromatograms of culture extracts of *Burkholderia* sp. O33, control (**A**), treated with fenpiclonil (**B**), and fludioxonil (**C**). Extracts were prepared after 4 days of incubation. Arabic numerals denote monochloroaminopyrrolnitrin (1), aminopyrrolnitrin (2), and pyrrolnitrin (3).

From the culture extracts of strain O33, treated with biphenyl-2-amine, several peaks with possible indication of chlorine atoms were found. Mass spectra of four peaks (*Rts*, 47.5, 50.8, 55.8, and 60.9 min) gave characteristic halogen (Cl or Br) isotope patterns (**Figure 5**). For example, the relative abundance of *m*/*z* 233, 234, and 235 ions of peaks 4 were 72, 11, and 24, respectively.

**Growth Inhibition by Xenobiotics.** Approximately 7 chemicals have shown weak to moderate growth inhibitory effects on strain O33, including acifluorfen, biphenyl-2-amine, chlorpyrifos, chlorpyrifos oxon, paraquat, PCB, and triclosan, while fenpiclonil and fludioxonil did not inhibit bacterial growth (**Table 2**).

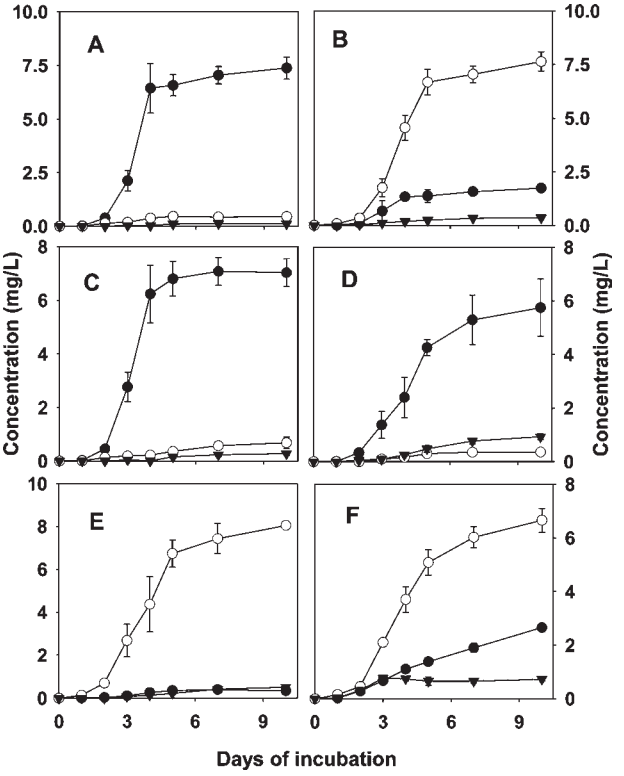


**Figure 2.** Time-dependent accumulation of pyrrolnitrin (●), aminopyrrolnitrin (○), and monochloroaminopyrrolnitrin (▲) in the cultures of *Burkholderia* sp. O33 control, fenpiclonil, and fludioxonil (**A**, **C**, and **E**) and *Pseudomonas fluorescens* Pf-5, control, fenpiclonil (**B**, **D**, and **F**), respectively. Concentrations of pesticides were 10 $\mu$M. Values of three replicates and error bars for standard deviations are shown.

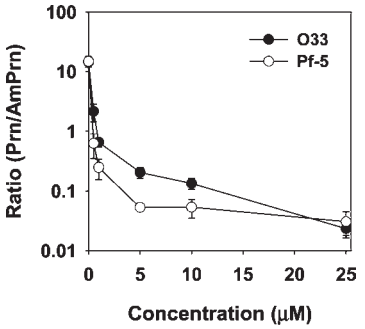


**Figure 3.** Fenpiclonil concentration-dependent changes of the relative ratio of pyrrolnitrin (Prn) and aminopyrrolnitrin (AmPrn) in *Burkholderia* sp. O33 (●) and *Pseudomonas fluorescens* Pf-5 (○) after 5 days of incubation. The *x*-axis is the concentration of fenpiclonil, and the *y*-axis is the ratio between the concentration (mg/L) of Prn to AmPrn.

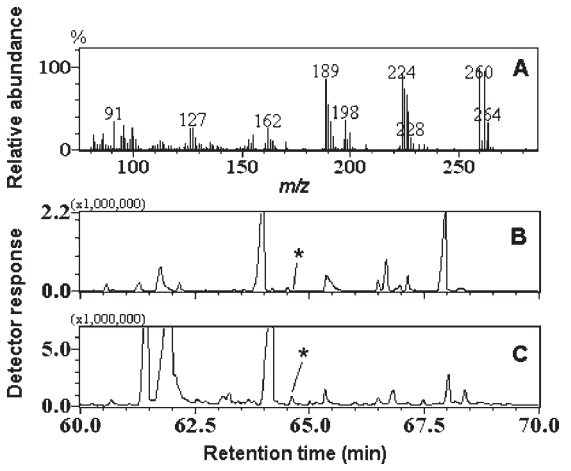


**Figure 4.** Mass spectrum of the trichlorinated derivative of aminopyrrolnitrin (**A**) and GC-MS total ion chromatograms of culture extracts of no-pesticide control and fludioxonil-treated *P. fluorescens* Pf-5 (**B** and **C**). Asterisks in chromatograms denote the trichlorinated derivative of aminopyrrolnitrin.

Phenylpyrazole-type drugs (antipyrine and aminoantipyrine) give no effects.

**Effects of Fenpiclonil on the Biosynthesis of Brominated Pyrrolnitrins.** Both bacterial strains could produce brominated analogues of Prn, AmPrn, and MAmPrn in NaBr-supplemented NBM. In comparison with Prn, the biosynthesis of brominated analogues proceeded very slowly, and multiple products were obtained (Supporting Information, Figure S-1). For example, the concentration of brominated Prn reached a maximum at 7−9 days. Prn and its chlorinated precursors were found as minor constituents in NaBr-supplemented culture, while approximately 17 brominated or mixed halogen (bromine and chlorine in the same molecules) metabolites were found as major products (Supporting Information, Figure S-1). The relative proportion of MAmPrn analogue to AmPrn/Prn was much higher than those of non-NaBr supplemented cultures. Because no synthetic standards were available, the concentrations of these metabolites were evaluated with GC-MS peak area. In general, strain O33 produced a larger amount of pyrrolnitrin analogues than Pf-5. However, a similar response to fenpiclonil treatment was observed in both strains. The ratio between the concentrations of Prn derivatives and AmPrn analogues was much smaller in fenpiclonil-treated cultures than those of the control (**Figure 6**).

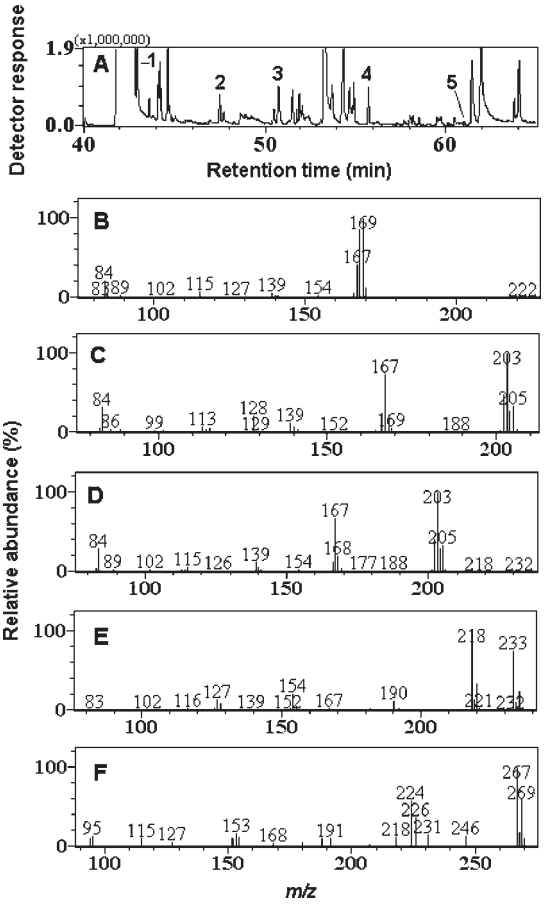


**Figure 5.** GC-MS total ionchromatograms of culture extracts of *Burkholderia* sp. O33, treated with biphenyl-2-amine (30 μM) after 5 days of incubation (**A**) and mass spectra of biphenyl-2-amine and its halogenated derivatives (**B**−**F**). Arabic numerals in insert **A** correspond to the mass spectra of **B**, **C**, **D**, **E**, and **F** for 1, 2, 3, 4, and 5, respectively.

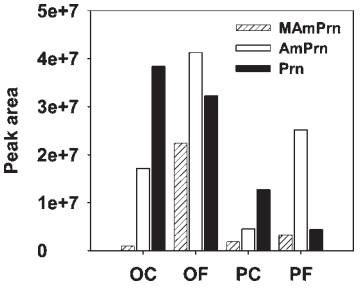


**Figure 6.** Effects of fenpiclonil on the biosynthesis of brominated analogues of pyrrolnitrin by *Burkholderia* sp. O33 and *P. fluorescens* Pf-5. Abbreviations: OC and OF, *Burkholderia* sp. O33, control, and fenpiclonil-treated; PC and PF, *P. fluorescens* Pf-5, control, and fenpiclonil-treated, respectively. The following abbreviations, including monochloroaminopyrrolnitrin (MAmPrn), aminopyrrolnitrin (AmPrn), and pyrrolnitrin (Prn), denote the summed peak areas of each class of metabolites (brominated analogues).

## DISCUSSION

In this study, 25 xenobiotics, including 18 pesticides were selected to determine the effect of pyrrolnitrin biosynthesis and bacterial growth. The pesticides are either commonly used in current agricultural practices or have novel modes of action. Antipyrine, aminoantipyrine, and biphenyl-2-amine were chosen as structural analogues of pyrrolnitrin, being derivatives of biaryl

compounds. Aroclor 1016 (PCB) and triclosan were common environmental contaminants. Celestine blue and methimazole are known inhibitors of Rieske-type dioxygenase and flavin-dependent monooxygenase, respectively (*3*, *33*).

Among several aspects of Prn biosynthesis, the inhibitory activities of fenpiclonil and fludioxonil were of particular interest. These two phenylpyrrole fungicides were developed from pyrrolnitrin as the lead structure. Fenpiclonil inhibits the conversion of AmPrn to Prn from both strains in a concentration-dependent manner, while fludioxonil-mediated inhibition was observed only in strain Pf-5 (**Figures 2** and **3**). Prn is produced from tryptophan through the action of four different proteins (*13*, *20*). Aminopyrrolnitrin oxidase (*PrnD*) catalyzes the oxidation of amine (AmPrn) to the nitro group (Prn). *PrnD* is an unusual Rieske-type oxygenase, oxidizing aromatic amines (*20*, *22*). Rapid and continuous accumulation of AmPrn indicates that fenpiclonil and fludioxonil may specifically inhibit *PrnD*. Concentration of Prn in fludioxonil-treated Pf-5 was gradually increased (**Figure 2F**) while the conversion of AmPrn to Prn was almost completely inhibited by fenpiclonil throughout the experiment (**Figure 2E**). The results may suggest that fludioxonil and AmPrn competitively interact with *PrnD*, while the affinity of fenpiclonil may be much stronger than that of AmPrn. Analysis with purified enzyme will give better insight into the inhibitory effects of these fungicides. Differential sensitivity to fludioxonil of strains O33 and Pf-5 indicates the structural difference of the corresponding enzyme (*PrnD*) between the strains. One novel metabolite was continuously found in fenpiclonil (strain O33 and Pf-5) and fludioxonil (Pf-5)-treated cultures, which was not found in the control or other treatments (**Figure 4**). The mass spectrum was closely related to AmPrn. However, its isotope distribution of molecular ions indicates that approximately three chlorines are in this metabolite. The metabolite was tentatively identified as the trichlorinated derivative of AmPrn.

In comparison with the control without sodium halide amendment, large numbers of polyhalogenated analogues (more than 2 halogens) were obtained in NaBr- or NaBr-fenpiclonil cosupplemented cultures (Supporting Information, Figure S-1). However, the inhibitory effect of fenpiclonil was also evident. The portions of AmPrn analogues were much higher than Prn analogues in fenpiclonil-treated cultures for both strains (**Figure 6**). These findings again suggested that the phenylpyrrole fungicides may be effective inhibitors of *PrnD*.

Comparative analysis of the electrostatic properties indicated that AmPrn and fenpiclonil were very similar to each other, while the charge distribution of Prn is largely different from those of the others (Supporting Information, Figure S-2). In addition to the charge distribution, molecular shapes or related properties (e.g., surface area and volumes) also determine the biological activity of chemicals with specific enzymes. Estimated molecular volume of fenpiclonil (618.8 $A^3$) is close to that of AmPrn (608.4 $A^3$), while Prn and fludioxonil were much larger: 641.4 and 635.5 $A^3$ for Prn and fludioxonil, respectively.

Biphenyl-2-amine also inhibited the oxidation of AmPrn (**Table 2**). Structural similarities between AmPrn and biphenyl-2-amine may cause such effects as those of phenylpyrrole fungicides. Another interesting effect of biphenyl-2-amine was the halogenation/oxidation of the supplemented substrates. Although the transformation rate was much slower than that of the natural substrates (AmPrn and MAmPrn), several halogenations or oxidation products of biphenyl-2-amine were observed (**Figure 5C**−**F**). Mass spectra of two peaks (*Rts*, 47.5 and 50.8 min) gave *m*/*z* 203 (205) as molecular ions. Abundance of isotope peaks suggested that the metabolites are regioisomers of monochlorinated biphenyl-2-amine. Trace peaks at 55.8 and 60.9 min have shown *m*/*z* 233(235) and 267(269) as molecular ions, respectively. It was difficult to identify the structure of these metabolites because of the strong interference of other metabolites. However, the isotope patterns indicate that the metabolites may be mono- and dichlorinated derivatives of biphenyl-2-amine, of which the amine was oxidized to the nitro group. It is noteworthy that halogenase from pyrrolnitrin biosynthesis can introduce chlorine to analogues of natural substrates (*15*). In addition, some pyrrolnitrin-producing bacteria have halogenase, other than tryptophan-7-halogenase (*PrnA*) and monochloroaminopyrrolnitrin halogenase (*PrnC*) (*19*).

The concentration of MAmPrn was usually far less than that of other metabolites (Prn and AmPrn). However, alarge amount of MAmPrn (0.94 mg/L) was accumulated in methimazole-treated O33 (**Table 2**). Amino acid sequences of two halogenases (*PrnA* and *PrnC*) in Prn biosynthesis are weakly similar with flavin-dependent monooxygenases (FMOs) (*5*,*34*). It is noteworthy that methimazole and its analogues inhibit FMO and haloperoxidase (*17*, *24*). Accumulation of MAmPrn by methimazole suggested that some of the above-mentioned enzymes may be inhibited.

It is well known that glyphosate is a competitive inhibitor of 5-enoylpyruvylshikimate-3-phosphate synthase (EPSP synthase), a key enzyme of aromatic amino acid biosynthesis (e.g., phenaylalanine and tryptophan). Because tryptophan is a precursor of Prn, inhibition of EPSP synthase may reduce pyrrolnitrin biosynthesis. However, the concentration of Prn or its precursors was not decreased by glyphosate-treatment (**Table 2**). It has to be mentioned that several types EPSP synthases with different sensitivity to glyphosate are reported (*31*). In addition, glyphosate triggers the imbalance of energy in some bacterial species (*11*). Results of growth rates and Prn biosynthesis, however, reveal that strain O33 is tolerant to glyphosate toxicity.

For the proper application of microorganism as biocontrol reagents, pesticide tolerance of the organism is an important prerequisite. Bacterial growth inhibition by several pesticides, including chloroacetanilides, triazines, organophosphorus insecticides, paraquat, and nitrodiphenyl ethers, has been reported (*2*, *16*, *35*). However, the toxicity of these pesticides is highly dependent on species or strains. Test chemicals gave moderate growth inhibition of strain O33. But suppression of the specific Prn biosynthesis step was not observed, except for the above-mentioned chemicals. Both chlorpyrifos and the oxon metabolite inhibit bacterial growth. However, significant suppression of Prn biosynthesis was observed only in oxon treatment. Instrumental analysis reveals that a much higher level of 3,5,6-trichloropyridin-2-ol is present in oxon-treated culture than in chlorpyrifos (data not shown). Halogenated phenols are toxic to many bacterial species (*25*). Accumulation of the phenolic metabolite may cause growth inhibition by the oxon metabolite. It is noteworthy that strain O33 gave the same response with chlorpyrifos oxon. Carbaryl, an *N*-methylcarbamate insecticide, can be hydrolyzed to the phenolic metabolite (e.g., 1-naphthol). Tebufenpyrad and the related analogue (e.g., Fenpyroximate) are known to inhibit mitochondrial electron transport at the NADH−coenzyme Q reductase of complex I (*4*). It is interesting that the similar site in the Prn-producing pseudomonad is related to the glucose suppression of pyoluteorin biosynthesis (*29*). However, neither cell growth nor Prn biosynthesis was inhibited by tebufenpyrad in strain O33.

Some pseudomonads are sensitive to PCB toxicity (*1*). Aroclor 1016 inhibits cell growth and Prn biosynthesis in strain O33 (**Table 2**). Triclosan is commonly used in hygienic products as

bacteriostats, which inhibit fatty acid biosynthesis (*14*). Growth of strain O33 was moderately inhibited by triclosan (**Table 2**). The reduced amount of Prn in PCB and triclosan treatment may be a consequence of limited biomass rather than inhibition of a specific step of Prn biosynthesis. Slight bacterial growth inhibitory effects were reported with antipyrine and an amino analogue (*30*). However, Prn biosynthesis and bacterial growth were not inhibited in strain O33. In addition, halogenations or other metabolic transformations of the test chemical were not observed, indicating the high substrate specificity of Prn biosynthesis enzymes.

In summary, synthetic pesticides and some environmental contaminants can modulate the efficacy of pyrrolnitrin-producing bacteria, through the inhibition of cell growth or pyrrolnitrin biosynthesis. Phenylpyrrole fungicides, derived from pyrrolnitrin, inhibit oxidative transformation of AmPrn to Prn, while methimazole, an FMO inhibitor, suppresses halogenations to AmPrn. Information from the study will provide better structural insight of the corresponding enzymes.

**Supporting Information Available:** GC-MS TIC and mass spectra of brominated Prn and precursors, and electron density isosurface maps of Prn, AmPrn, fenpiclonil, and fludioxonil. This material is available free of charge via the Internet at http://pubs.acs.org.

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