Metabolism of Tryptophan by *Pseudomonas aureofaciens*

III. Production of Substituted Pyrrolnitrins from Tryptophan Analogues

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Exogenous tryptophan is metabolized by *Pseudomonas aureofaciens* to yield pyrrolnitrin [3-chloro-4-(2'-nitro-3'-chlorophenyl)-pyrrole], an antifungal agent. The ability of this culture to metabolize tryptophan analogues in a similar manner was investigated by addition of the appropriate compound to the fermentation. Tryptophan precursors and metabolites or nonphenyl-substituted tryptophans had little effect on pyrrolnitrin biosynthesis, but simple derivatives of indole inhibited the production of pyrrolnitrin. Tryptophans substituted at the 4 position decreased pyrrolnitrin production and were converted into the corresponding substituted indoles. Tryptophans substituted at the 5, 6, and 7 position with fluorine or at the 5 and 7 position with methyl yielded new pyrrolnitrin derivatives. Substitution of larger groups (such as chloro, bromo, trifluoromethyl, and methoxy) at these positions led to the formation of the intermediate, amino pyrrolnitrin [3-chloro-4-(2'-amino-3'-chlorophenyl)-pyrrole], with the appropriate new substituent. The trifluoromethyl group at the 6 position of tryptophan prevented chlorination at the 3 position of pyrrolnitrin.

Biosynthetic studies in our laboratory (2, 4) have shown that D-tryptophan is a direct precursor of the antifungal substance pyrrolnitrin [3-chloro-4-(2'-nitro-3'-chlorophenyl)-pyrrole]. Addition of d-tryptophan to a medium inoculated with *Pseudomonas aureofaciens* ATCC 15926 increased the yield of pyrrolnitrin, and up to 18% of added dL-tryptophan-3-14C was converted into pyrrolnitrin. Since the added tryptophan is such an efficient precursor, it was of interest to determine whether related compounds might substitute in the biosynthetic sequence and lead to new substances related to pyrrolnitrin. Two derivatives, 3-chloro-4-(2'-nitro-3'-chloro-4'-fluorophenyl)-pyrrole and 3-chloro-4-(2'-nitro-3'-methylphenyl)-pyrrole, were isolated from fermentations supplemented with 6-fluorotryptophan and 7-methyltryptophan, respectively (1). These derivatives have antifungal activity similar to that of pyrrolnitrin. This paper reports amplification of the above studies to include the effects of a series of tryptophan analogues, precursors, and related compounds on pyrrolnitrin biosynthesis.

1 Part of this study was reported at the 154th meeting of the American Chemical Society, Chicago, Ill., September 1967.

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MATERIALS AND METHODS

Materials. Those tryptophan analogues not commercially available were prepared by E. C. Kornfeld, Eli Lilly and Co. (E. C. Kornfeld and T. Suarez, 154th Amer. Chem. Soc. Meeting Abstr. Q49, 1967). The indole derivatives and other nontryptophan compounds were obtained commercially.

Silica gel thin-layer chromatography (TLC) plates were used throughout this work (E. Merck, AG, Darmstadt, Germany). Nuclear magnetic resonance (NMR) spectra were determined with a Varian HA-60 spectrometer; mass spectra were determined with a CEC model 21-110B spectrometer. Infrared spectra were determined as chloroform solutions, and ultraviolet spectra were determined as ethanol solutions.

Organism. The strain used in this study, *P. aureofaciens* (Lilly A10338.5, *P. fluorescens* biotype E), was described previously (4).

Fermentation procedure. The slant cultures of A10338.5 were grown on Trypticase Soy Agar Medium (BBL) at 30 C for 24 to 48 hr and stored at 4 C. The composition of the medium and fermentation procedures were the same as described previously (4). The complex medium (CMM) used for pyrrolnitrin production was used throughout these studies. Erlenmeyer flasks (250 ml) containing 60 ml of medium were sterilized by autoclaving at 121 C for 20 min (pH 6.2 to 6.4 after autoclaving). Inoculated flasks were incubated at 30 C on a rotary shaker for 24 to 48
hours to attain the maximal stationary phase of growth before the additions were made, and the incubation was continued for 120 hr after the additions. The precursor compounds were generally added at concentrations of 250, 500, and 1,000 μg/ml.

Microbiological assay method. The quantitative assay method for pyrrolnitrin and pyrrolnitrin analogues was an agar diffusion paper disc assay with Neurospora species (M45-846) as the test organism, and crystalline pyrrolnitrin as the assay standard at 1,000 μg/mg. Whole broth was diluted with 2 volumes of methanol to release the antibiotic from the cells, and the filtrate from the diluted broth was assayed directly.

Extraction of pyrrolnitrin and pyrrolnitrin analogues. The extraction procedure was the same as described previously (4). The broth was diluted with 2 volumes of methanol, 3% Hyflo Super-cel (Johns-Manville, New York, N.Y.) was added, and the suspension was filtered. The filtrate was concentrated in vacuo to remove the methanol, adjusted to pH 10.5, and extracted twice with equal volumes of toluene. A subsequent extraction with chloroform was necessary at times to remove certain pyrrolnitrin analogues. The combined extracts were evaporated in vacuo, and the residue was dissolved in 10 ml of methanol for assay and TLC determination.

Separation of pyrrolnitrin and pyrrolnitrin analogues. The methanolic concentrate was evaporated in vacuo, and the residue was dissolved in chloroform and filtered over carbon columns (Pittsburgh, 12 X 40). The chloroform eluate was evaporated to dryness, and the residue was dissolved in benzene or mixtures of benzene-hexane and chromatographed on silica gel (Woehm activity grade 1). The eluted fractions were monitored by TLC, using benzene as a developer and an H₂SO₄ spray for detection. The appropriate fractions were combined, evaporated, and crystallized from chloroform-hexane. A typical TLC plate, shown diagramatically in Fig. 1, illustrates the approximate movement of each class of compound. The isolation and structure of the dialkylresorcinols have recently been reported (M. Gorman, H. Boaz, and E. Lavagnino, 5th Int. Symp. Chem. Nat. Prod., London, Abstr. G24, p. 426, 1968). Repeated development of the TLC plates (five or six times) in hexane-benzene (7:3) was necessary to effect separation of some of the pyrrolnitrin derivatives from pyrrolnitrin.

RESULTS

Tryptophan precursors and metabolites. The tryptophan precursors and metabolites (serine, kynurenine, anthranilic acid, indoleacetic acid, tryptamine, and indolepyruvic acid) had little or no effect on pyrrolnitrin yields. Addition of shikimic acid resulted in a 20% increase over the control. Phenylalanine had a slight inhibitory effect. Indole and indole derivatives (3-Cl-indole, 2-methyl-5-Cl-indole, 5-Br-indole, 5-F-indole, 5-Cl-indole, and 5-F-gramine) greatly reduced pyrrolnitrin production.

Nonphenyl-substituted tryptophans. The tryptophan derivatives substituted in the alanine portion on the nitrogen-containing ring of the indole (α-methyltryptophan, β-methyltryptophan, 1-methyltryptophan, 2-methyltryptophan, and glycyltryptophan) had little or no effect on pyrrolnitrin yields. Chloroacetyltryptophan is apparently rapidly hydrolyzed to tryptophan, as it brought about a similar increase in the levels of pyrrolnitrin as DL-tryptophan.

4-Substituted tryptophans. The 4-substituted tryptophans generally decreased the pyrrolnitrin yields (Table 1). The halogen-substitution at the 4-position appeared to alter the metabolism of the tryptophan, and zones with the Rₚ values and the characteristics of 3-chloroindoles were observed on TLC plates. The 4-methyltryptophan caused an accumulation of a substituted amino derivative of pyrrolnitrin.

5-Substituted tryptophans. The 5-substituted

![Fig. 1. Thin-layer chromatography of extracts. Silica Gel G; solvent system, benzene or hexane-benzene (7:3); and sulfuric acid spray. The colors of each class were chloroindoles (C), red; pyrrolnitrins (B), greenish-purple; resorcinols, brown; and aminopyrrolnitrins (A), yellowish-brown.](image-url)
The Table 1. Metabolism of 4-substituted tryptophans

| DL-Substituted tryptophan | Effect on yield (µg of pyrrolnitrin/ml) | Metabolites |
|---------------------------|----------------------------------------|-------------|
| Fluoro                    | 9.5                                    | C           |
| Chloro                    | 22                                     | C           |
| Bromo                     | 22.5                                   | C           |
| Methyl                    | 12.5                                   | A           |
| Control                   | 50                                     |             |

a Sixty milliliters of inoculated medium per 250-ml flask incubated at 30 C. After 24 to 48 hr of incubation, 250, 500, or 1,000 µg of precursor per ml was added and incubated for 120 additional hours. The controls averaged 50 µg of pyrrolnitrin per ml.

b New metabolites as identified by thin-layer chromatography: A = aminopyrrolnitrin derivatives; B = pyrrolnitrin derivatives; and C = chloroindole derivatives.

Table 2. Metabolism of 5-substituted tryptophans

| DL-Substituted tryptophan | Effect on yield (µg of pyrrolnitrin/ml) | Metabolites |
|---------------------------|----------------------------------------|-------------|
| Fluoro                    | 38                                     | B           |
| Bromo                     | 31.5                                   | A           |
| Hydroxy                   | 25                                     | A           |
| Benzyloxy                 | 50                                     |             |
| 5,6-Methylenedioxy        | 21                                     | A           |
| Methyl                    | 27.5                                   | B           |
| Chloro                    | 25                                     | A           |
| Methoxy                   | 26.5                                   | A           |
| Control                   | 50                                     | A           |

a See Table 1, footnote a.

b See Table 1, footnote b.

tryptophans generally reduced the amount of biological activity produced. By using the bromo-, chloro-, hydroxy-, and 5,6-methylene-dioxysubstituted tryptophans, a series of compounds identified as aminopyrrolnitrin derivatives were produced. Incubation of fluoro- or methyltryptophan yielded new pyrrolnitrin derivatives (Table 2). The 5-benzyloxytryptophan apparently was not utilized by the organism.

6-Substituted tryptophans. The addition of fluorotryptophan (Table 3) yielded a new pyrrolnitrin derivative, 4'-fluoropyrrolnitrin [3-chloro-4-(2'-nitro-3'-chloro-4'-fluorophenyl)-pyrrole]. Its properties were recently reported by Gorman et al. (1), and its structure was confirmed by X-ray diffraction analyses (3). The biological activity of the extract was greater than that of the control fermentation. This increase was attributed to the fact that the new derivative was more active than the pyrrolnitrin (1,200 and 1,000 µg/mg, respectively). A range of 250 to 500 µg of 6-fluorotryptophan per ml resulted in maximum production of 4'-fluoropyrrolnitrin, whereas higher levels inhibited production (Fig. 2). Incubation of the 6-bromo-, chloro-, methyl-, methoxy-, and trifluoromethyltryptophans yielded large quantities of the corresponding aminopyrrolnitrin derivatives with concomitant lowering of pyrrolnitrin yields.

The bromo-, methyl- and chlorotryptophandervied extracts were subjected to preparative TLC, and a series of amino derivatives with various degrees of chlorination were observed. Mass spectral analysis of three of these compounds indicated that they were the amino derivatives corresponding to the 4'-substituted pyrrolnitrin, isopyrrolnitrin [2,3-dichloro-4-(2'-

Table 3. Metabolism of 6-substituted tryptophans

| DL-Substituted tryptophan | Effect on yield (µg of pyrrolnitrin/ml) | Metabolites |
|---------------------------|----------------------------------------|-------------|
| Fluoro                    | 55                                     | B           |
| Chloro                    | 17.5                                   | A           |
| Bromo                     | 16                                     | A           |
| Methyl                    | 8.5                                     | A           |
| Methoxy                   | 37.5                                   | A           |
| Carboxy                   | 1.5                                     |             |
| Methysulfonyl             | 50                                     |             |
| Trifluoromethyl           | 18                                     | A           |
| Nitro                     | 15.5                                   | A           |
| i-Butyl                   | 50                                     |             |
| Control                   | 50                                     |             |

a See Table 1, footnote a.

b See Table 1, footnote b.
TABLE 4. Metabolism of 7-substituted tryptophans

| dl-Substituted tryptophan | Effect on yield (µg of pyrrolnitrin/ml) | Metaboliteb |
|---------------------------|----------------------------------------|-------------|
| Fluoro-                   | 18.5                                   | B           |
| Chloro-                   | 87.5                                   | A           |
| Methyl-                   | 11                                     | B           |
| Methoxy-                  | 48                                     | A           |
| Aza-                      | 50                                     |             |
| Trifluoromethyl-          | 50                                     |             |
| Bromo-                    | 47.5                                   | A           |
| Control                   | 50                                     |             |

a See Table 1, footnote a.  
b See Table 1, footnote b.

to pyrrolnitrin, [3-chloro-4-(2'-amino-3'-chlorophenyl)-pyrrole], were produced when large amounts of tryptophan were added (4). The two compounds played an important role in the interpretation of the results of experiments with tryptophan derivatives. We have speculated that one of the first steps in the metabolism of 7-tryptophan by the pseudomonom is chlorination of the 3 position. A loss of the alanine moiety at this point would result in the formation of 3-chloroindole. For pyrrolnitrin biosynthesis, the chlorine would facilitate the opening of the indole ring between the nitrogen and the 2-carbon. Subsequent condensation of the α-amino nitrogen with the ring carbon would result in a 3-(2'-aminophenyl)-pyrrole. The enzymatic conversion of aminopyrrolnitrin to pyrrolnitrin has been reported by Hamill et al. (2). Substitution of the 4 position of tryptophan appears to divert metabolism from pyrrolnitrin synthesis primarily to production of a 3-chloroindole derivative.

The nature of the substituent at position 5, 6, and 7 has a considerable effect upon the oxidation of the amino group, possibly due to steric inhibition. Thus, the 5-, 6-, and 7-fluorotryptophans and 5- and 7-methyltryptophans led to new pyrrolnitrin derivatives, whereas the chloro-, bromo-, trifluoromethyl-, and methoxytryptophans were converted to aminopyrrolnitrin derivatives. The results of mass spectral analyses of the chloro- and bromoamino-pyrrolnitrins indicate that in vivo chlorination is stepwise in that at least three distinct chloroderivatives (mono-, di-, and tri-) are detected. Several variations in the chlorination pattern have been reported to occur naturally without precursor addition (2). The trifluoromethyl group prevented the chlorination at the 3 position.

The biological activity of the isolated derivatives indicates that the nitro group is necessary for antifungal activity, but that 3'-chloro substitution is not necessary. Derivatives with increased biological activity can be obtained with the proper selection of substituted tryptophans as precursors.
The inert character of those tryptophan analogues substituted other than in the benzene ring is thought to define the specificity of the first chlorination step.

The metabolism of these tryptophan derivatives by *P. aureofaciens* to produce substituted phenylpyrroles has made available a novel group of antifungal compounds.

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