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Note

High-performance liquid chromatographic analysis of phenylpyrroles produced by *Pseudomonas cepacia*

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Pyrrolnitrin (III, Fig. 1), 3-chloro-4-(2-nitro-3-chlorophenyl)pyrrole, is a potent antifungal compound first isolated from *Pseudomonas pyrrocinia*[1]. It was subsequently found that a number of Pseudomonads form pyrrolnitrin as a product of tryptophan metabolism[2]. Other less active phenylpyrroles were also produced by this metabolic pathway; these include aminopyrrolnitrin (II)[3], 2-chloropyrrolnitrin (V)[3], isopyrrolnitrin [2,3-dichloro-4-(2-nitrophenyl)pyrrole][4], and oxypyrrolnitrin [3-chloro-4-(2-nitro-3-chloro-6-hydroxyphenyl)pyrrole][5]. Gas chromatographic[6] and thin-layer chromatographic (TLC)[2] techniques have been used to analyze for these phenylpyrroles from Pseudomonads.

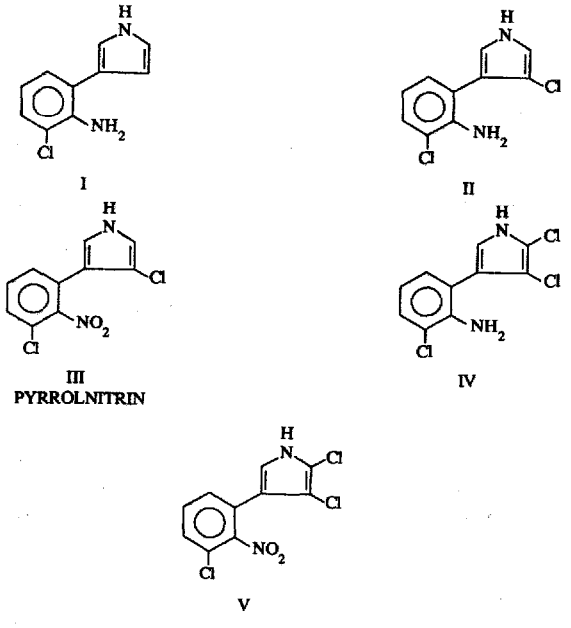

Fig. 1. Structures of phenylpyrroles.

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A strain of *Pseudomonas cepacia*, isolated from apple leaves, inhibited the growth of *Botrytis cinerea* and *Penicillium expansum* on apples and pears, and was found to produce pyrrolnitrin[7]. We have isolated other phenylpyrroles from *P. cepacia*, including compounds II and V. In addition, we found 3-(2-amino-3-chlorophenyl)pyrrole (I), previously isolated from a Pseudomonad grown in the presence of 7-chlorotryptophan[8], and 2,3-dichloro-4-(2-amino-3-chlorophenyl)pyrrole (IV)[9], not reported previously. A high-performance liquid chromatographic (HPLC) method was developed for the separation and quantitative analysis of these five phenylpyrroles in *P. cepacia* fermentations.

EXPERIMENTAL[a]

The medium used in the fermentation of *P. cepacia* consisted of the following: 8 g/l Difco Nutrient Broth, 5 g/l Difco Yeast Extract, and 10 g/l glucose.

Chromatography was performed on an IBM 9533 liquid chromatograph with a Rheodyne 7125 injector, a Waters 450 variable-wavelength detector, and a Hewlett-Packard 3390A reporting integrator. All solvents used were of HPLC grade and were obtained from various manufacturers.

An IBM (Wallingford, CT, U.S.A.) 5-μm C_{18} column (250 mm × 4.6 mm I.D.) and a 50 mm × 4.6 mm I.D. guard column were used for HPLC; analyses were conducted isocratically with an ultrasonically degassed solution composed of acetonitrile–methanol–water (1:1:1.1) at a flow-rate of 1.0 ml/min, and peaks were detected by UV monitoring at 254 nm.

Whatman (Hillsboro, OR, U.S.A.) $KC_{18}F$ plates were used for TLC and were developed in acetonitrile–methanol–water (1:1:1). Phenylpyrrole spots were visualized with diazotized sulfanilic acid (DSA) spray.

Isolation and preparation of standards

Compounds II, III, IV, and V were isolated from *P. cepacia* as previously described[9]. Compound I was isolated using the same procedure with the following changes: *P. cepacia* was grown in the fermentation broth described above. The reversed-phase HPLC fraction containing II was rechromatographed on a Rainin Dynamax (Emeryville, CA, U.S.A.) 8-μm silica column (250 mm × 21.4 mm I.D.) with a 50 mm × 21.4 mm I.D. guard column and an eluent of hexane–chloroform (3:1) at a flow-rate of 20 ml/min and detection at 254 mm. The separation of I (retention time 5.6 min) from II (retention time 6.3 min) was thus achieved. After recrystallization from chloroform–hexane (1:5), compound I was identified from its NMR and mass spectra.

Analysis of standards

Accurately weighed 10-mg samples of each of the five phenylpyrroles were dissolved in 10 ml of methanol. Aliquots of this stock solution were taken such that seven standard solutions were prepared containing 5, 25, 50, 100, 250, 500, and 1000

[a] Reference to a company and/or product named by the Department is only for purposes of information and does not imply approval or recommendation of the product to the exclusion of others which may also be suitable.

μg/ml of each phenylpyrrole. HPLC analyses of 20 μl were made in triplicate from each standard solution. A standard curve correlating the amount of phenylpyrrole in a 20-μl injection to its area was prepared for each phenylpyrrole.

Analysis of fermentation broth

A Fernbach flask (2800 ml) containing 500 ml of sterile fermentation broth was inoculated with *P. cepacia* and agitated on a rotary shaker at 200 rpm at 33°C. Duplicate 10-ml aliquots were removed at 12, 24, 48, and 168 h. Aliquots were centrifuged at 8000 *g*, the broth was discarded, and the cells were extracted by ultrasonification with 10 ml acetone. The suspension was recentrifuged at 5500 *g*, the supernatant evaporated under vacuum, and the residue dissolved in 200 μl methanol. Duplicate 20-μl injections were analyzed by HPLC. The amount of each phenylpyrrole per 20 μl was calculated from its standard curve using a linear regression program. This value is equivalent to the mg/l phenylpyrrole in fermentation broth.

Analysis of a second 10-ml acetone extract of the cells revealed that 98% of the phenylpyrroles are contained in the first extract. To determine if phenylpyrroles are transferred by *P. cepacia* into the broth, an aliquot taken at 48 h was centrifuged and the supernatant freeze-dried. The solids were extracted with chloroform, the solution was filtered, and the solvent removed under reduced pressure. The residue was taken up in methanol for HPLC analysis. Results indicate that the broth contains only 1% of the phenylpyrroles produced by the cells during fermentation.

RESULTS AND DISCUSSION

A chromatogram of the phenylpyrrole standards (Fig. 2) shows baseline separation well suited for quantitative analysis; the capacity factors are listed in Table

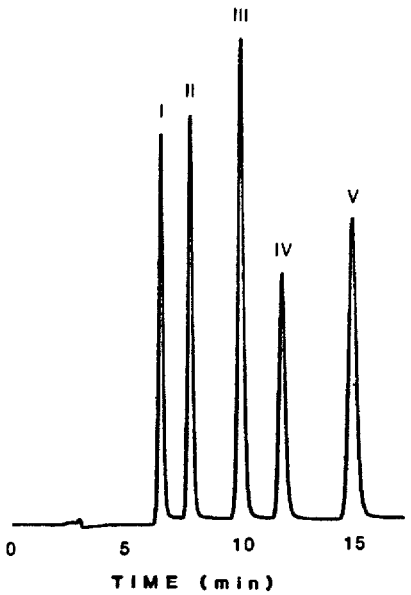

Fig. 2. Chromatogram of standard phenylpyrroles (20 μg each). For chromatographic conditions see text.

TABLE I

HPLC AND TLC CHARACTERISTICS OF *P. CEPACIA* PHENYLPYRROLES

Phenylpyrrole	*HPLC capacity factor (k')*	*TLC R_F value*	*Color reaction with DSA*
I	1.16	0.40	Maroon
II	1.64	0.38	Maroon
III	2.42	0.31	Maroon
IV	3.02	0.28	Orange
V	4.11	0.22	Orange

TABLE II

CONCENTRATIONS OF PHENYLPYRROLES IN *P. CEPACIA* FERMENTATION BROTH AT VARIOUS ELAPSED FERMENTATION TIMES

Phenylpyrrole	*Concentration (mg/l)*			
	12 h	*24 h*	*48 h*	*168 h*
I	0.8	0.2	0.1	0.1
II	0.1	10.4	5.8	4.7
III	0.2	6.8	13.1	13.3
IV	—[a]	2.3	4.1	2.8
V	—[a]	0.4	1.5	1.5

[a] Not detected.

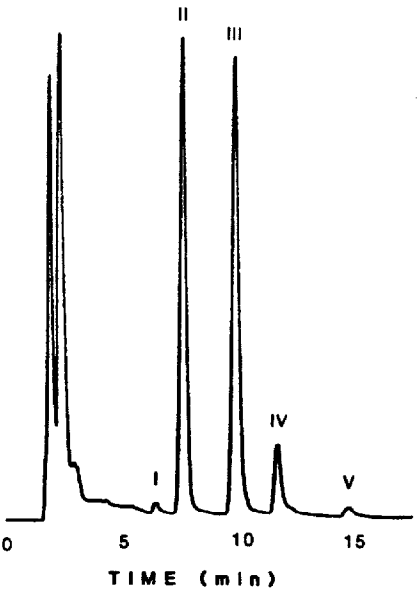

Fig. 3. Chromatogram of phenylpyrroles from *P. cepacia*. I = 0.2 μg; II = 10.4 μg; III = 6.8 μg; IV = 2.3 μg; V = 0.4 μg. For chromatographic conditions see text.

I. For the external standardization, each phenylpyrrole gave a linear response curve in the range tested (0.1–20 μg injected) as shown by correlation coefficients of 0.9994–0.9999.

The concentrations of the five phenylpyrroles at various time intervals during a 168-h fermentation of *P. cepacia* are given in Table II. A chromatogram of a sample taken at 24 h is shown in Fig. 3; note the absence of any interfering peaks. The presence of each phenylpyrrole was verified by reversed-phase TLC as described above; R_F values and the color reactions are given in Table I.

The proposed biochemical pathway for the formation of these phenylpyrroles is corroborated by their changes in concentration during fermentation. Tryptophan is believed to undergo chlorination, rearrangement, and decarboxylation to form compound I[10], which is further chlorinated to II[8], followed by oxidation to III[3]. It is likely that compound IV is similarly formed by chlorination of I or II followed by oxidation to V. The results in Table II show that compound I is present in the highest concentration early in the fermentation. As the fermentation continues compounds II and IV accumulate, followed by a decline in their concentrations and an accompanying rise in the concentrations of III and V, respectively.

With an uncomplicated sample preparation scheme and an HPLC analysis time of only 18 min, this quantitative procedure proves to be an excellent method for monitoring phenylpyrrole production in *P. cepacia* during fermentation.

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