THE BIOSYNTHESIS OF THE ANTIBIOTIC PYRROLNITRIN BY *PSEUDOMONAS AUREOFACIENS*

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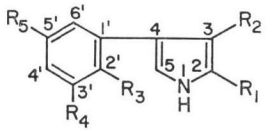
Feeding experiments with tryptophan samples labeled specifically with radioactive and stable isotopes have shown that *Pseudomonas aureofaciens* converts this amino acid into pyrrolnitrin in such a way that the indole nitrogen gives rise to the nitro group, the amino group becomes the pyrrole nitrogen, C-3 of the precursor side chain becomes C-3 of the antibiotic, and H-2 of the indole ring and H-α of the side chain give rise to H-5 and H-2 of pyrrolnitrin, respectively. Only the L-isomer of tryptophan is incorporated with retention of the α-hydrogen and the amino nitrogen. From the D-isomer the labels from these two positions are lost. The obvious conclusion that L-tryptophan is the more immediate precursor is, however, contradicted by the better incorporation of D- than L-tryptophan into the antibiotic. Several potential pathway intermediates were evaluated for incorporation and 4-(*o*-aminophenyl)-pyrrole was found to be a good precursor. The results are discussed in terms of a plausible pathway for pyrrolnitrin biosynthesis.

In 1965, ARIMA and co-workers[1] reported the isolation of a new potent antifungal antibiotic, pyrrolnitrin (**1**) (Scheme 1), from a culture of a pseudomonad, named *Pseudomonas pyrrocinia*[2]. The same compound was obtained independently at the Lilly Research Laboratories[3] from other *Pseudomonas* species[4]. A number of congeners of pyrrolnitrin, *e.g.*, isopyrrolnitrin (**2**)[5], oxypyrrolnitrin (**3**)[6], 2-chloropyrrolnitrin (**4**)[7], deschloropyrrolnitrin (**5**)[8] and aminopyrrolnitrin (**6**), have been isolated from *Pseudomonas* cultures and others were produced by directed biosynthesis, either in the presence of ammonium bromide[9] or from various tryptophan analogs[10]. The structure of pyrrolnitrin, established by ARIMA's group[11], has been confirmed by syntheses[12,13] and by an X-ray crystal structure analysis[14]. The clinical use of this antibiotic is based on its good activity against dermatophytic fungi and its low toxicity[15].

Scheme 1. Structures of pyrrolnitrin and analogs.



	R_1	R_2	R_3	R_4	R_5
1	-H	-Cl	$-NO_2$	-Cl	-H
2	-Cl	-Cl	$-NO_2$	-H	-H
3	-H	-Cl	$-NO_2$	-Cl	-OH
4	-Cl	-Cl	$-NO_2$	-Cl	-H
5	-H	-Cl	$-NO_2$	-H	-H
6	-H	-Cl	$-NH_2$	-Cl	-H
8	-H	-H	$-NO_2$	-H	-H
9	-H	-H	$-NH_2$	-H	-H
10	-CHO	-Cl	$-NO_2$	-Cl	-H
11	-COOH	-Cl	$-NO_2$	-Cl	-H
12	-COOH	-H	$-NH_2$	-Cl	-H

Studies on the biosynthesis of pyrrolnitrin showed that of a number of compounds tested only D- and L-tryptophan were significantly incorporated[3] and that D-[^{14}C]tryptophan was a more efficient

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precursor of the antibiotic than the L-isomer[7]. Addition of tryptophan to the culture medium increased antibiotic production, but only the D-isomer was active in this respect[3]. Several considerations, including the isolation of 3-chloroindole from tryptophan-supplemented cultures[3], led to the proposal of a pathway (Scheme 2) to account for the transformation of tryptophan into the phenylpyrrole system based on an attack on the indole ring system by a chloroperoxidase as the crucial initial step[16]. Except for the last reaction, the demonstrated[7] oxidation of aminopyrrolnitrin to pyrrolnitrin, this pathway was hypothetical and called for further experimental examination.

In the following paper we report studies on the biosynthesis of pyrrolnitrin from tryptophan which shed further light on this unique biochemical transformation. Some of the results have been communicated in preliminary form[17,18].

Experimental Methods

Feeding Experiments

Strain A 10338.5 of *Pseudomonas aureofaciens* was used in these studies and the experiments were conducted as described previously[3,7]. Labeled precursors were added as filter-sterilized aqueous solutions 24 hours after inoculation and the cultures were harvested 5 days later, unless stated otherwise. The isolation of pyrrolnitrin followed the procedure given earlier[3,7]. In experiments requiring only the determination of radioactivity, the toluene extracts were mixed with carrier pyrrolnitrin, which was then reisolated by chromatography on a column of silica gel (Woelm or Macherey and Nagel, 0.05 ~ 0.02 mm particle size, benzene) and crystallized from ether - hexane to constant specific radioactivity. In the ^{15}N experiments the toluene extract of the cultures was subjected to tlc (silica gel G, benzene) followed by preparative glc (6′ × 1/8″ column, 3% SE-30 on Gas Chrom P, 40 ~ 60 mesh, helium 60 ml/minute, column temperature 195°C, thermal conductivity detector) of the eluted pyrrolnitrin. These samples were then analyzed for their ^{15}N content by mass spectrometry in a Hitachi RMU 6A instrument (direct inlet, probe temperature 90°C, ionizing voltage 75 eV), comparing the peak heights of the molecular ion and the peak at *m/z* 229 and their isotope satellites with those of the unlabeled material. Their specific radioactivities were determined by counting one aliquot and quantitating another using the u.v. absorption at 250 nm ($\varepsilon = 7500$). In the other stable isotope experiments, pyrrolnitrin was purified from the toluene extract by chromatography on silica gel and crystallization from ether - hexane.

Radioactive samples were counted in a Beckman LS 100 or LS 250 scintillation counter using PPO and dimethylPOPOP in toluene as scintillator solution and methanol or water/BIO-SOLV BBS-3 (Beckman) as solvents. Counting efficiencies were determined for each sample using internal standards. NMR spectra were recorded on JEOL PFT-100, Varian CFT-20 or Varian EM-360 spectrometers or on a Fourier transform spectrometer operating at 15.0777 MHz (^{13}C) in a Varian H-60 magnet. Mass spectra, except the ones indicated above, were recorded on a DuPont 21-492BR instrument using the direct inlet and 70 eV ionizing voltage.

Labeled Precursors

D,L-[Alanine-3-^{14}C]tryptophan was obtained from Amersham-Searle and D,L-[indole-2-^{14}C-1-^{15}N]-tryptophan was a gift from Professor E. LEETE, University of Minnesota, Minneapolis. D,L-[Alanine-2-^{15}N]tryptophan was material available from previous work[19], as was the D,L-[alanine-2-^{3}H]tryptophan used in the first experiments, while that used in later experiments was prepared more recently by the same procedure[19]. These two samples contained at least 97.6% and 95.8%, respectively, of their tritium in the position indicated, as determined by enzymatic degradation to indoleacetic acid. Double labeled samples were obtained by mixing the appropriate single labeled specimens. The multiple labeled D- and L-tryptophan samples were prepared by treatment of the corresponding D,L-forms with L- or D-amino acid oxidase as described previously[19]. In the case of the D-amino oxidase reaction a large hydrogen isotope effect prevented complete oxidation of the tritiated D-tryptophan. Thus, after

three incubations with the enzyme the material still contained an appreciable amount of tritium (2.3 dpm ^{3}H per dpm ^{14}C), but practically no ^{14}C in the form of D-tryptophan. For the accurate determination of the $^{3}H/^{14}C$ ratios, aliquots of the precursor solutions were cocrystallized with excess non-labeled carrier tryptophan of the appropriate configuration.

D,L-[Alanine-3-^{13}C]tryptophan (60.1 atom% ^{13}C excess) was synthesized from $Ba^{13}CO_3$ by liberation of the $^{13}CO_2$, reduction with $LiAlH_4$ to $H^{13}CHO$, MANNICH condensation to give gramine, followed by condensation with diethyl formaminomalonate and hydrolysis/decarboxylation to tryptophan. The procedures used were those described by WEYGAND and LINDEN[20] for the preparation of the corresponding ^{14}C compound. The preparation of D,L-[indole-2-^{2}H]tryptophan(44 atoms% D) involved conversion of 3-formyl-[2-^{2}H]indole into the amino acid by the hydantoin method[21].

3-Formyl-[2-^{2}H]indole: To a suspension of 220 mg of the aniline SCIFF's base of 3-formyl-2-indolecarboxylic acid[22] in 10 ml D_2O was added NaOD until the precipitate dissolved completely. After 10 minutes, DCl was added to pH 3 and the precipitate was filtered off, washed with D_2O and dried *in vacuo* at 100°C. IR spectroscopy indicated 95% deuterium exchange. This material was refluxed with 3 ml dried anisole under nitrogen for one hour. Ten ml of D_2O were then added and the mixture distilled to remove the anisole and aniline with the water. The 3-formylindole remaining behind was recrystallized from aqueous methanol. Yield 60%, m.p. 190°C.

[3-^{14}C]-3a-Hydroxy-1,2,3,3a,8,8a-hexahydropyrrolo[2,3-b]indole-2-carboxylic acid (**7**): This compound, as the mixture of the two diastereomeric pairs with a *cis* ring junction, was prepared by peroxyacetic acid oxidation of D,L-[alanine-3-^{14}C]tryptophan as described by SAVIGE[23].

4-(*o*-Nitrophenyl)-[5-^{3}H]pyrrole (**8**)[24]: *o*-Nitrophenylpyruvic acid (339 mg, 1.62 mmole), anhydrous sodium acetate (3 g), aminoacetal (385.5 mg, 2.9 mmole), glacial acetic acid (8 ml) and tritiated water (2 ml, containing 25 mCi tritium) were stirred for 12 hours at 115°C. After cooling and dilution with 40 ml water, the mixture was extracted with 3×20 ml ether. The extract was washed with water, 1 N NaOH and water, dried and evaporated. The residue was chromatographed on 15 g silica gel (Macherey and Nagel, 0.05~0.2 mm) with benzene as solvent. The fractions containing only 4-(*o*-nitrophenyl)pyrrole were pooled and evaporated to give 7.1 mg of an oil. ^{1}H-NMR (CCl_4) δ (ppm) (multiplicity, number of hydrogens): 6.3 (m, 1H), 6.8 (m, 2H), 7.5 (m, 4H). IR (KBr): 1620, 1530, 1370 and 748 cm^{-1}. UV (methanol), λ_{max} (nm): 370, 262 (sh), 231 (sh), end absorption. Radioactivity: 1.30×10^{7} dpm tritium.

4-(*o*-Aminophenyl)-[5-^{3}H]pyrrole (**9**)[7]: A mixture of 5.5 mg of the tritiated 4-(*o*-nitrophenyl)-pyrrole, 0.5 ml methanol, 0.25 ml 5 N NaOH and 25 mg sodium dithionite was stirred under reflux for 1 hour, diluted with water and extracted with 3×10 ml CH_2Cl_2. The combined organic phase was extracted with 1 N HCl, the aqueous extract made alkaline with 30% NaOH and extracted with CH_2Cl_2. After drying and evaporating the organic solvent, the residue was purified by tlc on silica gel 60 F_{254} with chloroform as solvent. Elution of the main band gave 0.53 mg 4-(*o*-aminophenyl)pyrrole containing 2.30×10^{5} dpm tritium.

Degradations of Labeled Pyrrolnitrin

The oxidation of pyrrolnitrin with $KMnO_4$ to 2-nitro-3-chlorobenzoic acid was carried out as described by IMANAKA *et al.*[11].

2-Formylpyrrolnitrin (**10**)[25,26]: To a mixture of 1.65 ml dimethylformamide and 0.55 g (0.33 ml) $POCl_3$ were added 289 mg pyrrolnitrin and the reaction mixture was stirred successively for 1 hour at 20°C, 1 hour at 40°C and 2 hours at 60°C. After cooling to room temperature, 6 g ice and 4.5 g sodium acetate·$3H_2O$ were added and the mixture was stirred for 30 minutes at 60°C. After cooling the precipitate was filtered off and taken up in ethyl acetate. The solution was washed with saturated $NaHCO_3$ and water, dried and evaporated, and the residue was recrystallized from benzene to give 240 mg (75%) **10**, m.p. 208~209°C (lit. 208°C[26]). UV(EtOH), λ_{max} (nm)(ε): 291 (18,000), 246 (10,700). IR (KBr): 3210, 1648, 1545, 1534, 1380 cm^{-1}. NMR (d_6-acetone), δ, ppm (multiplicity, number of H) (assignment): 7.31 (s, 1H) (H-5), ~7.7 (m, 4H), 9.72 (s, 1H) (C*H*O). To further confirm the position of the formyl group, the aldehyde was oxidized with Ag_2O as described by TANAKA *et al.*[25] to give the corresponding pyrrolnitrin-carboxylic acid. UV (EtOH): 237 (17,000). IR (KBr): 3380, 1665, 1600,

1533, 1370 cm^{-1}. M.p. 227°C (from ether - hexane) (lit. pyrrolnitrin-2-carboxylic acid, m.p. 215°C[12]; pyrrolnitrin-5-carboxylic acid, m.p. >265°C[27]).

Deuteration of Pyrrolnitrin to Assign ^{1}H-NMR Signals for H-2 and H-5

To 50 ml [OH-D_3]glycerol, which had been deuterated in the exchangeable positions by repeatedly dissolving it in C_2H_5-OD and evaporating the solvent, was added to 100°C 60 mg pyrrolnitrin-2-carboxylic acid. The mixture was heated to 230°C within 3 minutes and after 10 minutes at this temperature was poured on 80 g ice. Extraction with benzene and chromatography on 7 g silica gel with benzene as solvent gave 38 mg (74%) pyrrolnitrin. NMR (d_6-acetone): 6.86 (s, 0.42 H), ~7.6 (m, 4H); no signal at 6.96. VILSMEIER formylation of this material (34 mg) as described above gave 13 mg 2-formylpyrrolnitrin (**10**). NMR (d_6-acetone): 7.31 (s, 0.41 H) (H-5), ~7.7 (m, 4H), 9.72 (s, 1H) (C*H*O).

Results

In order to further evaluate the proposed pathway of pyrrolnitrin formation (Scheme 2), we examined the fate of the two nitrogen atoms of tryptophan during the conversion into **1**. An experiment with D,L-[indole-2-^{14}C,1-^{15}N]tryptophan clearly showed (Table 1, Expt. 1) that the indole nitrogen and the adjacent carbon are utilized to about the same extent, indicating specific incorporation of the indole nitrogen. Mass spectral analysis of the pyrrolnitrin sample from this experiment showed isotopic enrichment both in the molecular ion *m/z* 256 (29.2 atom% ^{15}N-excess, precursor 90 atom% ^{15}N-excess) and in the fragment at *m/z* 229 resulting from the loss of HCN from the pyrrole ring[28]. Hence, the ^{15}N from the indole ring of the precursor has been incorporated into the nitro group of **1**. Another feeding experiment with D,L-[alanine-3-^{14}C,2-^{15}N]tryptophan also gave ^{15}N-labeled pyrrolnitrin, although the incorporation of the ^{15}N was substantially lower than that of ^{14}C (Table 1, Expt. 2). In the

Table 1. Incorporation of multiple labeled tryptophan samples into pyrrolnitrin by *Pseudomonas aureofaciens*.

Expt. No.	Precursor	Pyrrolnitrin formed (μmoles)	% Incorporation of			% Retention of	
			^{14}C	^{3}H	^{15}N	^{3}H*	^{15}N*
1	56.8 μmoles D,L-[indole-2-^{14}C, 1-^{15}N]-tryptophan, 5.17×10^6 dpm ^{14}C and 51.1 μatoms ^{15}N excess	18.75	10.05	—	10.7	—	107
2	49 μmoles D,L-[alanine-3-^{14}C, 2-^{15}N]-tryptophan, 1.13×10^7 dpm ^{14}C and 26.4 μatoms ^{15}N excess	18.35	15.6	—	2.9	—	19
3	19.6 μmoles D,L-[alanine-3-^{14}C, 2-^{3}H]-tryptophan, 4.71×10^6 dpm ^{14}C and 1.70×10^7 dpm ^{3}H	19.9	16.1	4.2	—	26	—
4	48.2 μmoles D-[alanine-3-^{14}C, 2-^{3}H, 2-^{15}N]tryptophan, 1.69×10^6 dpm ^{14}C, 1.03×10^7 dpm ^{3}H and 21.0 μatoms ^{15}N excess	19.8	18.0	0.36	1.76	2	10
5	14.5 μmoles D-[alanine-3-^{14}C, 2-^{3}H]-tryptophan, 1.0×10^6 dpm ^{14}C and 6.35×10^6 dpm ^{3}H	n.d.	15.8	0.52	—	3.3	—
6	14.5 μmoles D,L-[alanine-3-^{14}C, 2-^{3}H]-tryptophan, 1.0×10^6 dpm ^{14}C and 6.40×10^6 dpm ^{3}H	n.d.	13.5	4.07	—	30.2	—
7	14.5 μmoles L-[alanine-3-^{14}C, 2-^{3}H]-tryptophan, 1.0×10^6 dpm ^{14}C and 6.32×10^6 dpm ^{3}H	n.d.	9.6	6.85	—	71.3	—

* % Tritium- and ^{15}N-retention are defined as 100×% incorporation of ^{3}H or ^{15}N/% incorporation of ^{14}C. n.d. = not determined.

mass spectrum of this sample only the molecular ion showed isotopic enrichment (4.2 atom% ^{15}N-excess, precursor 53.8 atom% ^{15}N-excess), but not the fragment at *m/z* 229, demonstrating that the amino nitrogen of the precursor gives rise to the pyrrole nitrogen of **1**.

The low ^{15}N retention, relative to ^{14}C, from the amino group of tryptophan may be due to reversible transamination or deamination/transamination of the substrate prior to its incorporation into **1**. In agreement with this notion, D,L-[alanine-3-^{14}C,2-^{3}H]tryptophan suffers a comparable partial loss of tritium from the α-position of the side chain (Table 1, Expt. 3). Reasoning that D-tryptophan as the better precursor should show less loss of tritium and ^{15}N from the α-position of the side chain, we prepared and fed a sample of D-[alanine-3-^{14}C,2-^{3}H,2-^{15}N]tryptophan (Table 1, Expt. 4). Surprisingly, the D-isomer was incorporated with virtually complete loss of tritium and with an even lower ^{15}N retention than the D,L-form. By extrapolation, the L-isomer must be incorporated with much higher retention of tritium and ^{15}N from the α-position of the side chain than the racemate. A comparison of the tritium retentions upon incorporation of D-, D,L- and L-[alanine-3-^{14}C,2-^{3}H]tryptophan into **1** (Table 1, Expts. 5~7) shows that this is indeed the case. At the same time this series of experiments confirms the earlier observations[3,7] that the D-isomer of tryptophan is incorporated more efficiently than the L-form (15.8 *vs.* 9.6%).

The above data, taken alone, would indicate that L-tryptophan is the immediate precursor of the antibiotic and that the D-isomer must first be converted to the L-form by a process involving loss of tritium and ^{15}N from the α-position of the side chain. This conclusion is, however, contradicted by the better utilization of the carbon skeleton of the D-isomer. Earlier work had shown that slower cellular uptake of L-tryptophan cannot account for its lower incorporation; the L-isomer enters the cells much more rapidly than the D-form. Another possible explanation is that under the standard conditions of the feeding experiments, addition of precursor at 24 hours after inoculation and harvest 5 days later, the L-tryptophan is used extensively for protein synthesis before antibiotic production starts whereas more of the D-isomer, which enters the cells more slowly, is still available during the period of antibiotic synthesis. To test this interpretation, we carried out a time course experiment in which the incorporation of D- and L-tryptophan into **1** was compared at different times of addition and harvest. The results (Fig. 1) clearly show that under all conditions examined the D-isomer was always incorporated more efficiently than the L, rendering the above hypothesis very unlikely.

To obtain further information on the mechanism of the conversion of tryptophan into **1**, the fate of several specific carbon and hydrogen isotopic labels during this transformation was determined. Scheme 2 predicts that C-3 of the tryptophan side chain should become C-3 of pyrrolnitrin, and proposes that halogenation is a late step in the biosynthesis. This requires introducing chlorine into the 3 position of the pyrrole ring. Patterns of electrophilic substitution of pyrroles predict that chlorination usually occurs in the 2 or 5 position, but if the chlorine atom in the 3 position were carried over from an earlier stage of the biosynthesis it would be present before aromatization of the pyrrole ring. For example, the hypothetical hexahydropyrrolo[2,3-b]indole derivative in Scheme 2, following ring opening could undergo a 1,2-aryl shift, as in the biosynthesis of isoflavones, to give ultimately pyrrolnitrin in which the phenyl substituent is attached to the carbon originating from C-3 of the tryptophan side chain. This possibility is supported by the fact that analogs of pyrrolnitrin with varying halogenation patterns are found in *Pseudomonas* (Scheme 1), but none without halogen in the 3 position. To check this possibility we synthesized D,L-alanine-3-^{13}C]tryptophan from Ba^{13}CO$_3$ and fed it to

Fig. 1. Incorporation of D- and L-tryptophan into pyrrolnitrin by *Pseudomonas aureofaciens* upon addition at different times after inoculation.

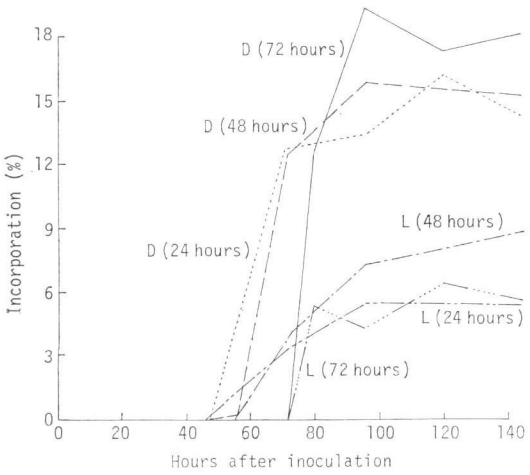
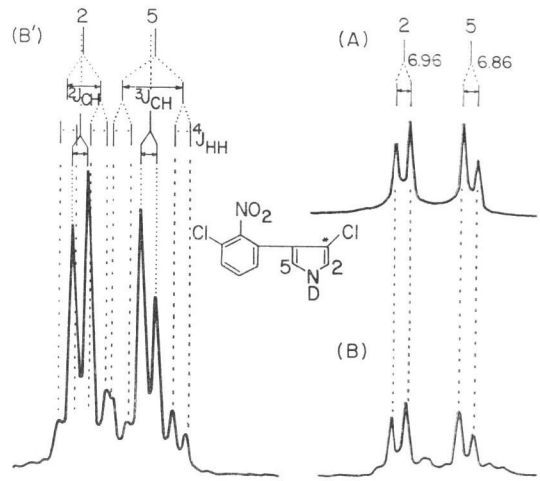
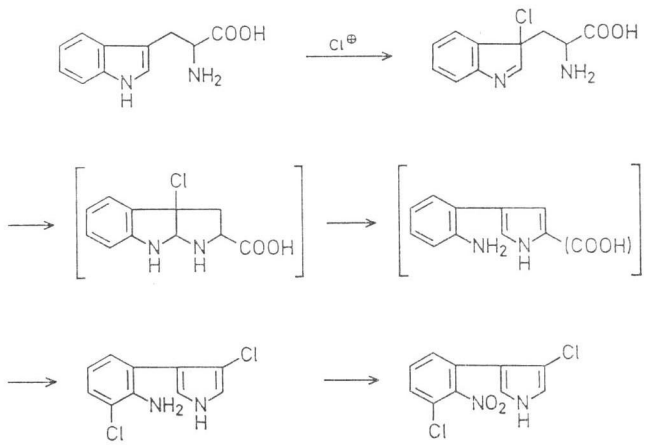


Fig. 2. Proton magnetic resonance spectrum of pyrrolnitrin in deuterio-acetone solution.

Only the H-2 and H-5 signals are shown: (A) normal sample; (B) biosynthetically ^{13}C-enriched sample, 28.5 atom% excess ^{13}C; (B′) expanded portion of (B).



Scheme 2. Hypothetical pathway of pyrrolnitrin biosynthesis according to GORMAN and LIVELY[16].



Pseudomonas cultures (Table 2, Expt. 8). The position of the ^{13}C in the product was determined by ^{13}C- and ^{1}H-NMR spectroscopy.

Assignments of the ^{13}C chemical shifts of the ten carbons of **1**, except for a distinction of the signals for C-2 and C-5 were based on literature data for simple model compounds and on a spectral analysis of 4-phenylpyrrole* and 4-(3′,4′-dichlorophenyl)pyrrole. The critical assignments of the signals for C-3 (111.7 ppm) and C-4 (115.3 ppm) were confirmed by comparing the spectra of **1** and **6**. Reduction of the nitro to the amino group had almost no effect on the signal at 111.7 ppm, whereas the signal at 115.3 ppm was shifted to 118.7 ppm in the spectrum of **6**[18]. In the spectrum of the pyrrolnitrin

* As a matter of convenience we use the pyrrolnitrin numbering system with all the phenylpyrroles encountered in this study.

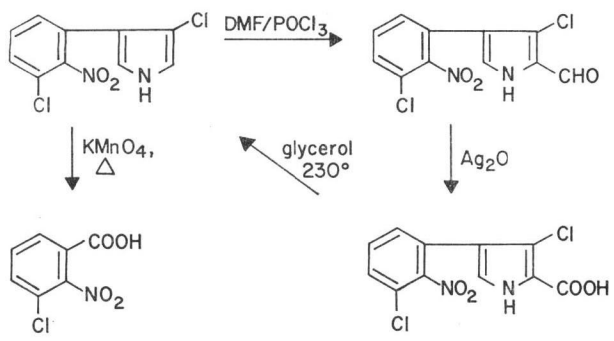
Table 2. Incorporation of labeled potential precursors into pyrrolnitrin.

Expt. No.	Precursor	Pyrrolnitrin formed (μmoles)	Amount of label in **1**	Incorporation (%)	Dilution factor
8	980 μmoles D,L-[alanine-3-^{13}C]tryptophan, 60.1 atom % excess	87.3 (isolated)	28.5 atom % excess ^{13}C	4.2	2.1
9	1,750 μmole D,L-[indole-2-^{2}H]tryptophan, 44 atom % ^{2}H	41.0 (isolated)	19.4 atom % ^{2}H	1.0	2.3
10	10.9 μmoles [3-^{14}C]-3a-Hydroxy-hexahydropyrrolo [2,3-b] indole, 4.85×10^{6} dpm ^{14}C	13.0	$<2.5\times10^{2}$ dpm ^{14}C	<0.01	>20,000
11	11.0 μmoles D,L-[alanine-3-^{14}C]tryptophan, 4.90×10^{6} dpm ^{14}C	14.6	4.27×10^{5} dpm ^{14}C	8.7	15.2
12	5.4 μmoles 4-(*o*-Nitrophenyl)-[5-^{3}H]pyrrole, 2.6×10^{6} dpm ^{3}H	n.d.	9.44×10^{3} dpm ^{3}H	0.36	—
13	3.3 μmoles 4-(*o*-Aminophenyl)-[5-^{3}H]pyrrole, 2.3×10^{5} dpm ^{3}H	n.d.	1.55×10^{4} dpm ^{3}H	6.7	—

sample from experiment 8, only the signal at 111.7 ppm was significantly enhanced above the natural abundance level, indicating that C-3 of the tryptophan side chain labels C-3 and not C-4 of pyrrolnitrin. The same conclusion is reached by proton NMR analysis. In the ^{1}H NMR spectrum of the pyrrolnitrin isolated from the feeding experiment with D,L-[alanine-3-^{13}C]tryptophan, the H-2 and H-5 signals of the pyrrole ring display extra side bands (Fig. 2) which arise from two-bond [^{2}J(C-3-H-2)$=5.3\pm$ 0.3 Hz] and three-bond [^{3}J(C-3-H-5)$=9.0\pm0.3$ Hz] couplings[29]. The assignments of the H-2 and H-5 chemical shifts are based on specific deuteration experiments. D_2O exchange and thermal decarboxylation of pyrrolnitrin-2-carboxylic acid (**11**) gave a sample of pyrrolnitrin in which the signal at 6.96 ppm was absent and the signal at 6.86 ppm was reduced to 42% of the normal intensity (presumably due to exchange during the decarboxylation). VILSMEIER formylation of this material gave 2-formylpyrrolnitrin in which the signal for H-5 was reduced to 41% of its normal intensity. These results establish the assignments for H-2 (6.96 ppm) and H-5 (6.86 ppm). The larger coupling of ^{13}C to H-5 than to H-2 in the labeled sample indicates that the ^{13}C is located at C-3 ($^{2}J_{CH}<{}^{3}J_{CH}$).

The conclusions drawn from this ^{13}C experiment are confirmed by the results of chemical degradations (Scheme 3) of **1** derived from radioactively labeled tryptophan samples. Oxidation of **1** (3140 dpm ^{14}C/μmole) derived from D,L-[alanine-3-^{14}C]tryptophan gave 3-chloro-2-nitrobenzoic acid (6.7 dpm ^{14}C/μmole) containing only 0.21% of the label. Formylation of pyrrolnitrin (^{3}H/^{14}C=2.34) obtained

Scheme 3. Degradations of pyrrolnitrin.

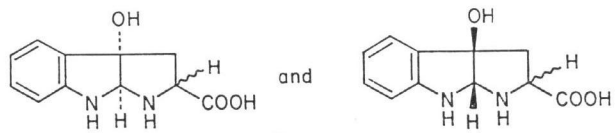


biosynthetically from D,L-[alanine-3-^{14}C,2-^{3}H]tryptophan gave 2-formylpyrrolnitrin (^{3}H/^{14}C=0.23) with loss of most of the tritium, indicating that the tritium from the α position of the L-tryptophan side chain is specifically located at C-2 of **1**.

An alternative to the mechanism suggested in Scheme 2 for the skeletal rearrangement of tryptophan to pyrrolnitrin, is a route proceeding *via* an oxindole intermediate which can undergo transacylation from the ring to the side chain nitrogen. Such a route, in contrast to the one shown in Scheme 2, would involve loss of a hydrogen label at C-2 of the indole ring of tryptophan during the biosynthesis. To probe this possibility we synthesized D,L-[indole-2-^{2}H]tryptophan from 3-formyl-[2-^{2}H]indole, which was prepared by deuterodecarboxylation of the aniline SCHIFF's base of 3-formylindole-2-carboxylic acid. Feeding of this sample (Table 2, Expt. 9) gave **1** which by mass spectral analysis was shown to contain deuterium at 44% of the enrichment of the precursor. Hence, the biosynthesis cannot proceed *via* oxindole intermediates.

Further insight into the biosynthetic pathway was sought by preparing and feeding potential intermediates in labeled form. A key intermediate in the pathway outlined in Scheme 2 is 3a-chloro-1,2,3,3a,8,8a-hexahydropyrrolo[2,3]b-indole. To date this compound has not been synthesized but the corresponding 3a-hydroxy analog (Scheme 4) is accessible by peracid oxidation of tryptophan[28]. Oxidation of D,L-[alanine-3-^{14}C]tryptophan yielded this compound, as a mixture of four stereoisomers with a *cis* ring junction, carrying a ^{14}C-label at C-3. In two parallel experiments we fed this sample and D,L-[alanine-3-^{14}C]tryptophan (Table 2, Expts. 10 and 11). The hexahydropyrrolo[2,3-b]indole derivative was not incorporated whereas under the same conditions tryptophan gave good incorporation. A logical biosynthetic intermediate in light of the available information is 4-(*o*-aminophenyl) pyrrole (**9**)[8]. We synthesized the corresponding nitro analog (**8**) by condensation of *o*-nitrophenylpyruvic acid and aminoacetal in the presence of tritiated water. During the simultaneous decarboxylation tritium is incorporated at C-5, some possibly also at C-2. An aliquot of the tritiated **8** was then reduced to **9**. Both compounds were fed to *Pseudomonas* cultures and the total radioactivity incorporated into pyrrolnitrin was determined (Table 2, Expts. 12 and 13). The good incorporation of **9** suggests that this compound is indeed an intermediate whereas the almost 20 times lower incorporation of **8** points to a preferred, if not obligatory, sequence involving halogenation before amino group oxidation.

Scheme 4. Structures of 3a-hydroxy-1,2,3,3a,8,8a-hexahydropyrrolo[2,3-b]indole isomers fed to *P. aureofaciens*.



7

Discussion

The results of this study confirm unequivocally that tryptophan is a specific and direct precursor of pyrrolnitrin. This is important in view of a report[30] on the incorporation of δ-amino[4-^{14}C]levulinic acid into the pyrrole ring of the antibiotic. The mode of incorporation of tryptophan into **1** established by this work supports in general the scheme of pyrrolnitrin biosynthesis proposed by GORMAN and

LIVELY[16]. That is, as predicted by their hypothesis (Scheme 2), the indole nitrogen of tryptophan gives rise to the nitro group of **1**, the amino nitrogen becomes the pyrrole nitrogen, C-3 of the precursor gives rise to C-3 of **1** and the hydrogen from C-2 of the indole ring is retained during the biosynthesis. Thus, the skeletal rearrangement from tryptophan to **1** involves no 1,2-aryl shift and no oxindole intermediates.

Our results unequivocally establish the operation of a direct pathway from L-tryptophan to pyrrolnitrin with predominant retention of both the amino nitrogen and the α-hydrogen from the tryptophan side chain. Since the tritium from the α-position of the tryptophan side chain, as expected, is located at C-2 of **1**, the removal of the carboxyl group must occur prior to or simultaneously with the formation of a double bond at this carbon. The intermediacy of pyrrole-2-carboxylic acids is incompatible with the experimental results. Just such a compound, **12**, has recently been isolated by SALCHER *et al.*[31] from a pyrrolnitrin-producing *Pseudomonas* culture supplemented with D-tryptophan. The authors postulate this compound as an intermediate in the biosynthesis of pyrrolnitrin, although its conversion into **1** was not reported. However, as pointed out above, **12** cannot be an intermediate on the pathway from L-tryptophan to **1**, although it could be on a separate pathway from D-tryptophan to **1**, not proceeding *via* the L-isomer, if such a pathway should exist. More likely, however, it is a dead-end product or an intermediate on another branch of the pathway.

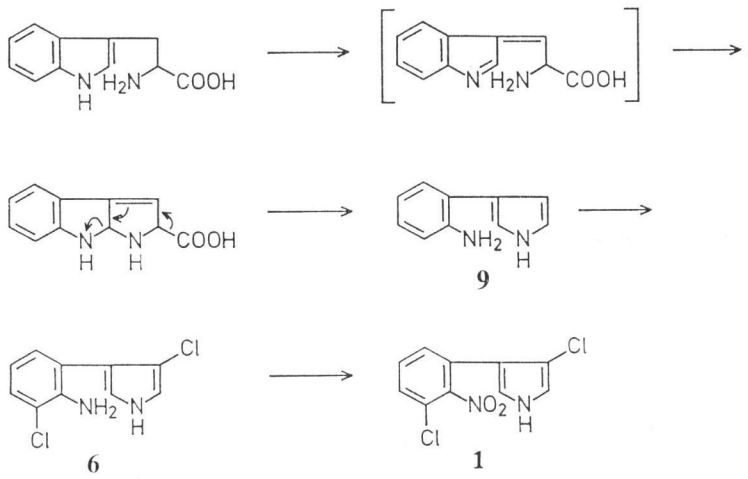
The mode of conversion of D-tryptophan into **1** is still not fully clarified. Clearly, L-tryptophan cannot be converted to **1** *via* the D-isomer, *i.e.*, the latter cannot be a more immediate precursor, despite its better utilization. The complete or almost complete loss of the α-hydrogen and the amino nitrogen from the D-, but not the L-isomer parallels earlier findings on the biosynthesis of ergot alkaloids from tryptophan[19]. However, in that case the conclusion that L-tryptophan is the more immediate precursor was also supported by the better incorporation of the L- compared to the D-isomer. In the present case, the better utilization of the D-isomer is at odds with such a conclusion.

Two explanations can be considered. (1) L-Tryptophan is the immediate precursor of **1** and the D-isomer is incorporated following its conversion into the L-form with loss of the original α-hydrogen and amino nitrogen, *i.e.*, *via* indolepyruvate. This requires an explanation for the better utilization of the D- compared to the L-isomer. L-Tryptophan, but not the D-isomer, induces the kynurenine pathway in *P. aureofaciens*, by which it is rapidly metabolized to anthranilate, which inhibits pyrrolnitrin formation (O. SALCHNER, F. LINGENS, personal communication). Together with strict compartmentation of endogenous and exogenous tryptophan pools this could explain the differential incorporation of the two tryptophan enantiomers, because added D-tryptophan would enter the cells more slowly and thus would only gradually feed the endogenous L-tryptophan pool. Presently this interpretation is entirely speculative. (2) L- and D-tryptophan are converted to **1** separately, the latter with complete loss of the α-hydrogen and the amino nitrogen. While it is not difficult to picture various ways in which the α-hydrogen may be lost in the conversion of the D- and retained in the conversion of the L-isomer to **1**, one cannot rationalize the nearly complete loss of ^{15}N from D-[alanine-2-^{15}N]tryptophan, with its retention in the conversion of the L-isomer, unless one makes the unlikely assumption that the reversible interconversion of D-tryptophan and indolepyruvate is much more rapid than that of L-tryptophan and the keto acid. Although the second possibility cannot be ruled out, the first one is likely.

The later stages of the pyrrolnitrin biosynthetic pathway seem reasonably clear from the available data and are shown in Scheme 5. Whether the sequence tryptophan→**9**→**6**→**1** is an obligatory or only a preferred one, remains to be decided. The latter is quite likely in view of the broad substrate specificity seen in this pathway[32]. Based on their isolation of 7-chloroindoleacetic acid and 3-chloroanthranilic acid from pyrrolnitrin-producing cultures[32], SALCHER *et al.*[31] postulate 7-chlorotryptophan as an intermediate in the biosynthesis of **1**. Since conversion of this compound into **1** has already been demonstrated[32] it seems quite possible that introduction of chlorine into the benzene ring can occur both before and after the skeletal rearrangement.

The exact nature of the compound undergoing the rearrangement to the phenylpyrrole skeleton and the type of reaction leading to its formation are still unclear. Although the non-incorporation of **7** does not rule out the intermediacy of the postulated chloro analog, it must be noted that in the model reactions examined compounds of this type undergo initial ring cleavage between C-8a and N-1,

Scheme 5. Proposed pathway of pyrrolnitrin biosynthesis.



not between C-8a and N-8a[34]. A possible answer to this problem is provided by the recent isolation of an interesting new enzyme from *Pseudomonas* species, indolyl-3-alkane α-hydroxylase[35,36]. Studies on the mechanism of action of this enzyme have shown[37,38] that the initial reaction catalyzed is a 1,4-dehydrogenation of 3-substituted indoles to give 3-alkylidene indolenines, which then undergo secondary reactions, like tautomerization to α,β-unsaturated compounds or addition of water to side-chain α-hydroxylated indoles. As illustrated in Scheme 5, a 3-alkylidene indolenine derived from tryptophan, held on the enzyme in a conformation favoring cyclization to the aminal, would be an attractive intermediate in pyrrolnitrin biosynthesis. The resulting aminal should be very prone to undergo fragmentation with loss of CO_2 and cleavage of the C-8a/N-1 bond. It would be quite conceivable that the entire process from tryptophan to **9** takes place on one enzyme.

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