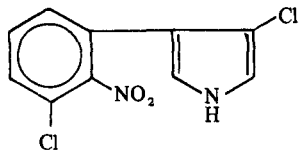
Biological Inactivation of Pyrrolnitrin. Identification and Synthesis of Pyrrolnitrin Metabolites

Patrick J. Murphy* and Terry L. Williams

The Lilly Research Laboratories, Indianapolis, Indiana 46206. *Received August 18, 1971*

The absorption, excretion, and metabolic conversion of [^{14}C]pyrrolnitrin (1) have been examined in the rat using both *in vivo* and *in vitro* techniques. Antibiotic activity was rapidly destroyed *in vivo*. Although 29% of the administered radioactivity was excreted in the urine in 24 hr and 50% in the bile in the same time period there was no pyrrolnitrin-like antifungal activity found in either plasma, urine, or bile. Pyrrolnitrin was readily metabolized *in vitro* using rat liver microsomes supplemented with NADPH. Three of the products have been isolated and identified by comparison to chemically synthesized standards. These compounds are 3-(3-chloro-2-nitrophenyl)succinimide (2), 4-chloro-3-(3-chloro-2-nitrophenyl)-3-pyrrolin-2-one (3), and 4-chloro-3-(3-chloro-2-nitrophenyl)-5-hydroxy-3-pyrrolin-2-one (4). A fourth product has been identified by "trapping" the metabolite as a $HS(CH_2)_2OH$ adduct and identifying this adduct by comparative tlc. This product is 4-chloro-3-(3-chloro-2-nitrophenyl)maleimide (5). The chemical synthesis of each of these compds is described. Each of the compds has been found to be devoid of pyrrolnitrin-like antifungal activity.

Pyrrolnitrin, 3-chloro-4-[2-nitro-3-chlorophenyl]pyrrole (1), is a broad-spectrum antifungal agent first reported by Arima and coworkers.[1] Although this antibiotic is effective



1

as a topical preparation, minimal activity is noted after oral administration.[2] Gastric acidity has been mentioned as a possible cause of this decreased activity, but active metabolic degradation has not been thoroughly investigated. In the course of our studies on the pharmacology of pyrrolnitrin we have examined its metabolism in the rat using both *in vivo* and *in vitro* techniques. We have found that pyrrolnitrin disappears rapidly from plasma, and the parent compd cannot be detected in either the urine or the bile after ip administration. When radioactive pyrrolnitrin was used, a number of metabolites were detected in the plasma, none of which possessed pyrrolnitrin-like antifungal activity. *In vitro* studies have shown that the pyrrole ring of pyrrolnitrin is readily oxidized by enzymes having the properties of mixed-function oxidases. The oxidation products include a potent alkylating agent, 4-chloro-3-(3-chloro-2-nitrophenyl)maleimide (5).

Results and Discussion

Excretion. The results obtained from iv administration of [^{14}C]pyrrolnitrin (Table I) indicate that after 24 hr approximately 30% of the radioactivity is excreted in the urine. When [^{14}C]pyrrolnitrin was administered to rats having cannulated bile ducts, approximately 50% of the radioactivity was excreted in the bile in the first 24 hr (Table II).

Metabolic Conversion of [^{14}C]Pyrrolnitrin *in vivo*. Bile. The radioactive materials excreted in the bile were extd and examined by tlc. Extn of the bile with PhH removed between 5 and 10% of the total radioactivity from the aq phase. The ext was concd and chromatogd by tlc. The tlc plate was analyzed by radioautography. Although a minimum of 6 radioactive materials were present, no radioactive zone corresponded to unchanged pyrrolnitrin.

The absence of pyrrolnitrin in the bile samples was further indicated by microbiological assay. A bile sample (0.8 ml) containing an amount of radioactivity corresponding to 100 μg of pyrrolnitrin was found to be devoid of pyrrolnitrin-like antifungal activity (level of detection >0.3 μg).

Table I. Urinary Excretion of Radioactive Metabolites[a]

Time after injection, hr	Per cent radioactivity excreted
1	1.7
2	8.1
4	7.8
24	29.0

[a] Each of four 200-g male rats was given an iv injection of 2 mg of [^{14}C]pyrrolnitrin (6.0×10^6 dpm/mg) in 0.2 ml of polyethylene glycol.

Table II. Biliary Excretion of Radioactive Metabolites[a]

Time interval, hr	% radioactivity recovered 1[b]	2[c]
0-1	0.4	2.8
1-2	2.2	5.2
2-3	2.6	6.3
3-4	5.7	6.7
4-5	6.1	12.4
5-22	35.4	15.3
Total	52.4	48.7

[a] The bile ducts of two 200-g rats were cannulated. After the cannulae were in place and the bile flow resumed, the rats were given a single dose of [^{14}C]pyrrolnitrin by injection into the stomach. Bile was collected during the indicated periods. [b] 4.0 mg of pyrrolnitrin administered (6×10^6 dpm/mg). [c] 3.2 mg of pyrrolnitrin administered (6×10^6 dpm/mg).

Urine. Tlc of PhH extracts of urine from rats that had been given [^{14}C]pyrrolnitrin by iv injection showed no unchanged pyrrolnitrin. Microbiological assay of a sample of urine (0.4 ml) containing an amount of radioactivity equivalent to 100 μg of pyrrolnitrin showed no detectable pyrrolnitrin-like antifungal activity.

Plasma. Six rats (150 g) were given a dose of [^{14}C]pyrrolnitrin (4 mg, 1.2×10^6 dpm/mg) sc. Blood samples were taken at intervals from 30 min up to 24 hr. The plasma was isolated by centrifugation, and an aliquot was used for liquid scintillation counting. One third of the plasma was used for bioassay, while the remainder was extracted, and the extract was analyzed by glc. Analysis of radioactivity indicated that the extracts contained the equivalent of from 1.0 to 4.4 μg of pyrrolnitrin. However, no pyrrolnitrin could be detected by glc (limit 0.01 μg), nor was there any detectable pyrrolnitrin-like antifungal activity. The results obtained from the plasma as well as from urine and bile indicated that pyrrol-

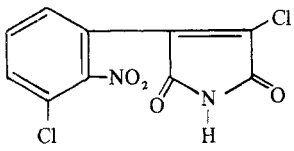
nitrin is rapidly converted to a series of metabolites that are devoid of antifungal activity.

In Vitro **Conversion of [^{14}C]Pyrrolnitrin.** In order to learn more about the metabolic conversion products of pyrrolnitrin *in vitro* incubations were conducted using various liver subcellular components. The mitochondrial and 100,000*g* supernatant fractions had no effect on pyrrolnitrin. The microsomal fraction had a minimal ability to alter the drug when incubated without cofactors, but in the presence of NADPH or (less effectively) NADH there was a significant conversion (up to 15%) of added pyrrolnitrin in 30 min. Tlc indicated that a minimum of 6 products were formed during these incubations. Because a number of these metabolites seemed to correspond chromatographically to the *in vivo* metabolites, further studies were undertaken using large-scale incubation of pyrrolnitrin with isolated rat liver microsomes.

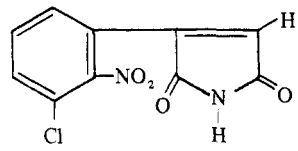
The metabolites of pyrrolnitrin were isolated by column chromatography of the extracts. The distribution of the metabolites is shown in Figure 1. The individual peak fractions were combined, concd, and analyzed.

Fraction 1 was found to contain only unchanged pyrrolnitrin. Fraction 2 contained a component, R_f (5% MeOH-*i*-Pr_2O) 0.38. This material was isolated by prep tlc. The mass spectrum of the purified material was identical with that of 3-(3-chloro-2-nitrophenyl)succinimide (**2**). Fractions 3 and 4 were not analyzed further. Fraction 5 was purified by prep tlc in 10% MeOH-*i*-Pr_2O. Two radioactive bands were observed (**5a**, R_f −0.32; **5b**, R_f −0.44). These bands were eluted separately, and the purified compds were subjected to mass spectrometric analyses. The mass spectrum of **5a** was identical with that of 4-chloro-3-(3-chloro-2-nitrophenyl)-3-pyrrolin-2-one (**3**). The mass spectra of **5b** was identical with that of 4-chloro-3-(3-chloro-2-nitrophenyl)-5-hydroxy-3-pyrrolin-2-one (**4**). Fractions 6 and 7 were chromatographed in a number of solvent systems and were found to contain a mixture of components. None of these metabolites has been identified.

The route of metabolism indicated by the identified compounds suggested that structures such as **5** and **6** might be



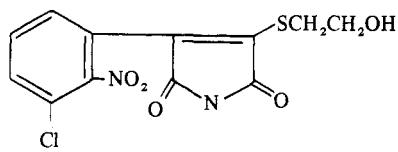
5



6

readily formed by common oxidation reaction. Since both of these compds are substituted maleimides, their generation in a biological system might be expected to lead to rapid binding to proteins and/or reaction with biological SH compds.

A further study of **5** was undertaken in order to assess this possibility. When **5** was allowed to react with $HS(CH_2)_2OH$, a quantitative formation of **7** occurred. With the hope of trapping intermediates such as **5** a series of incubations were performed with pyrrolnitrin in the presence of β-mercaptoethanol with and without NADPH. The ex-



7

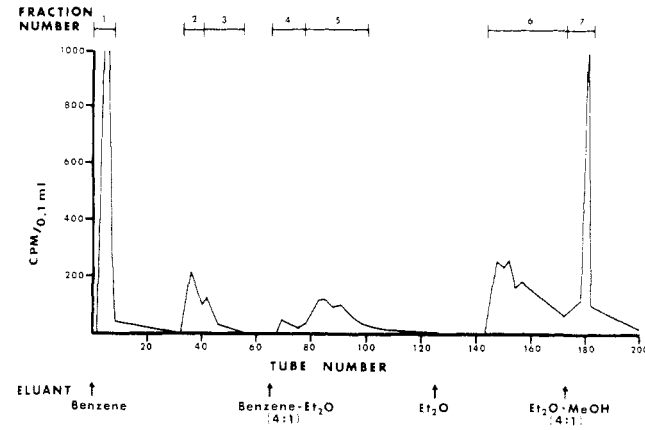


Figure 1. Column chromatography of pyrrolnitrin and pyrrolnitrin metabolites. The concentrated extract was dissolved in benzene and placed atop a 240 × 22 mm column of silicic acid. The eluant was changed as indicated; each fraction was 10 ml. The radioactive fractions were combined as indicated by fraction numbers 1–7.

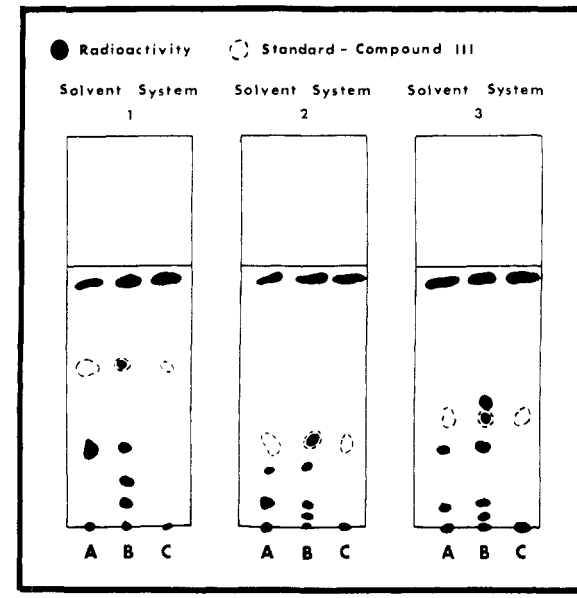


Figure 2. Thin-layer chromatographic analyses of extracts from *in vitro* incubation of [^{14}C]pyrrolnitrin with rat liver microsomes: A, pyrrolnitrin + NADPH; B, pyrrolnitrin + NADPH + $HS(CH_2)_2OH$; C, pyrrolnitrin + $HS(CH_2)_2OH$. Solvent system 1, 15 cm in PhH followed by 10 cm in *i*-Pr_2O–MeOH (9:1); solvent system 2, 15 cm in PhH followed by 10 cm in *i*-Pr_2O–HOAc (95:5); solvent system 3, 15 cm in PhH followed by 10 cm in EtOAc–PhH (1:1). The R_f of pyrrolnitrin in all 3 systems is 0.65.

tracts were then cochromatographed with **7** in 3 solvent systems. The results are shown in Figure 2. In the presence of β-mercaptoethanol and NADPH a number of radioactive zones are formed which are not present in the extracts from incubations containing either of these compds alone. One of the compds formed cochromatographs with **7** in each of the solvent systems tested.

Thus it seems that at least one, if not more, maleimide type compounds are generated during the microsomal oxidation of pyrrolnitrin.

A summary of these metabolic conversions of pyrrolnitrin by rat liver microsomes is given in Figure 3. All of the metabolites identified were oxidized pyrroles. The pyrrole ring seems to be particularly susceptible to oxidation, which may account for the rapid degradation of pyrrolnitrin *in vivo*. Compds **2**, **3**, **4**, **5**, and **6** were found to have no significant antifungal activity. The generation of a substituted maleimide by microsomal oxidation is most interesting in

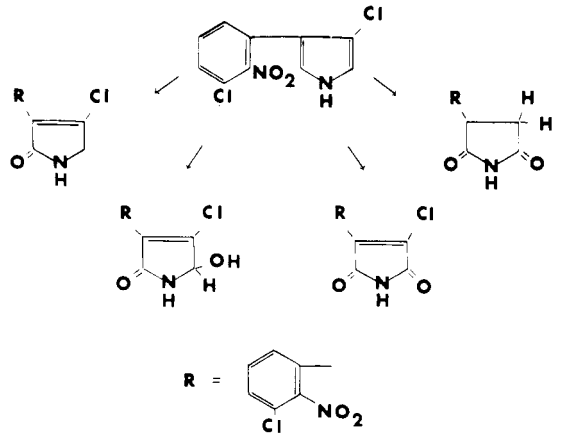


Figure 3. Metabolic conversion of pyrrolnitrin *in vitro* by rat liver microsomes.

light of the known reactivity of maleimides with proteins and SH groups. To our knowledge the conversion of a pyrrole to a maleimide has not been previously reported. Whether significant amounts of the types of maleimides reported herein are formed *in vivo* can only be assessed by further study.

Experimental Section

[^{14}C]Pyrrolnitrin labeled in the 2 position of the pyrrole ring was prepd biosynthetically from [^{14}C]tryptophan by the method of Hamill, *et al.*[3] The isolated material was assayed for purity by tlc and exhibited a single radioactive zone corresponding to authentic pyrrolnitrin.

All enzyme cofactors were obtained from Boehringer-Manheim Corp. Silicic acid (100–200 mesh) was purchased from Bio-Rad Laboratories.

Assays. Plasma samples were assayed for radioactivity by digestion with a mixt of 0.2 ml of $HClO_4$ (70%) and 0.4 ml of 30% H_2O_2 at 70° for 45 min in Teflon-capped vials. The samples were cooled rapidly in ice and dissolved in 20 ml of a soln contg 11 ml of PhMe, 9 ml of methyl Cellosolve, and 6 mg of PPO. All other samples were dissolved directly in 10 ml of dioxane-based scintillation fluid prepd by combining 104 g of naphthalene, 75 ml of Permafluor (Packard Instruments), 425 ml of PhMe, 500 ml of dioxane, and 300 ml of MeOH. Radioactivity was detd using a Packard liquid scintillation counter with external standard.

Microbiological activity was assayed using *Neurospora crassa* (Lilly M45-846) as the test organism and pyrrolnitrin as the standard. Glc was carried out according to the method of Hamill, *et al.*[4]

Urinary Excretion. Four male 200-g Sprague-Dawley rats were given a single injection of 2 mg of [^{14}C]pyrrolnitrin (6×10^{-6} dpm/mg) in 0.2 ml of polyethyleneglycol iv. The urine was collected for the indicated periods, and total radioactivity was detd by counting an aliquot of the sample.

Biliary Excretion. Male Sprague-Dawley rats (200 g) were anesthetized with Et_2O and their bile ducts were cannulated. A single dose of [^{14}C]pyrrolnitrin in 0.2 ml of PEG_{200} was administered by injection into the stomach. Bile was collected over the indicated period, and then the total radioactivity was detd by analyzing an aliquot of the sample.

In Vitro **Incubation.** Rat liver microsomes were prepd by differential centrifugation of 10% liver homogenates prepd in 0.25 *M* sucrose.[5] The 100,000*g* pellet was resuspended in 0.05 *M* phosphate buffer, pH 7.0. NADPH was added at 5×10^{-4} *M*. [^{14}C]Pyrrolnitrin was dissolved in 0.05 ml of 0.1% Tween 80 in acetone prior to addn to the incubation medium (final concn, 0.1 m*M*). Following the addn of the enzyme prepn (1.5 ml) the final vol was adjusted to 2 ml with 0.05 *M* phosphate buffer, pH 7.0. After the indicated time the reactions were stopped with 2 ml of Me_2CO and then were extd with three 1-ml portions of PhH. Under these condns the recovery of unchanged pyrrolnitrin averaged 90%. The recovery of the metabolites varied due to production of nonextractable material in the course of metabolic degradation.

Large-scale incubations were performed using male rats that had been treated for 5 days with phenobarbital (40 mg/kg, ip). Microsomes were prepd in the usual manner and were resuspended in 0.05 *M* phosphate buffer, pH 7.0 (200 ml/10 g of liver). Microsomal suspension (30 ml) was placed in a 125-ml Erlenmeyer flask. An NADPH-generating system consisting of isocitrate dehydrogenase, $NADP^+$, and isocitrate was added to give a final concn of NADPH equal to 2.5×10^{-4} *M*. [^{14}C]Pyrrolnitrin (1 mg in 0.1 ml of 0.1% Tween 80–Me_2CO) was added to each flask. The reaction mixt was incubated 45 min at 37° with vigorous shaking in air. The reaction was stopped with an equal vol of Me_2CO. The protein ppt was collected by centrifugation, and the aq phase was extd with PhH. The exts were concd and used for column chromatog.

Column Chromatography. In one series of incubations a total of 100 mg (0.6×10^6 dpm/mg) of [^{14}C]pyrrolnitrin was utilized. The PhH exts contd 1×10^7 dpm. The concd PhH ext was placed atop a silicic acid column (42 g, 240 × 22 mm) packed in PhH. The column was eluted with increasing concns of Et_2O and MeOH. The peak fractions were pooled and concd.

Chemical Syntheses. Oxidation of Pyrrolnitrin with CrO_3–HOAc. Pyrrolnitrin (2.5 g) was dissolved in 100 ml of glac AcOH. A soln of CrO_3 (2 g) in 80% AcOH (200 ml) was added over a 1.5-hr period with stirring at room temp. After 15 min of addl stirring 10 ml of abs EtOH was added, and the mixt was stirred for 15 min. The dark green soln was concd *in vacuo* at 50°. The residue was triturated with PhH and H_2O, and the aq phase was extd 3 times (PhH). The extracts were washed with H_2O and concd. Upon concn a ppt formed which was isolated and recrystd from EtOH to yield pure 4-chloro-3-(3-chloro-2-nitrophenyl)-5-hydroxy-3-pyrrolin-2-one **(4).** The structure was confirmed by mass spectrometry, ir, nmr, uv, and elemental analyses.

The PhH supernatants were then placed atop a column of silicic acid (240 × 22 mm). The column was eluted with PhH. After elution of unchanged pyrrolnitrin a fraction was eluted which contd 2-chloro-3-(3-chloro-2-nitrophenyl)maleimide **(5).** This material pptd upon concn and was washed (PhH) and analyzed by nmr, mass spectrometry, uv, ir, and elemental analyses.

Oxidation of Pyrrolnitrin with Chloroperbenzoic Acid. Pyrrolnitrin (10 g, 39 mmoles) was dissolved in 60 ml of CH_2Cl_2. Solid Na_2HPO_4 (22.4 g) was added. A CH_2Cl_2 soln of *m*-chloroperbenzoic acid (7.8 g in 60 ml of CH_2Cl_2) was added slowly (75 min) to the stirred slurry at room temp. After this time the CH_2Cl_2 slurry was treated with a 2% aq $Na_2S_2O_3$ until a neg peroxide test was obtd with starch-iodide paper. The soln was then extd with 3 vol of H_2O and dried ($MgSO_4$). The org layer was taken to dryness. The residue was extd with Et_2O leaving a blue polymeric ppt. The Et_2O ext was taken to dryness and then the residue was triturated with PhH and placed atop a column of silicic acid packed in PhH. The column was eluted with PhH followed by increasing concns of EtOAc. The initial benzene eluate contd pyrrolnitrin followed by a yellow compd, which was chromatog distinct from pyrrolnitrin. Mass spectral, nmr, ir, and uv evidence indicated that it was 3-(3-chloro-2-nitrophenyl)maleimide **(6).** The fraction eluted with 5% EtOAc–PhH was concd, and the crude ppt was recrystd from EtOAc–PhH to yield 3-(3-chloro-2-nitrophenyl)succinimide **(2).** The structure was confirmed by nmr, ir, uv, and mass spectrometric data.

The fraction eluted with 10% EtOAc–PhH was concd and a ppt appeared. This was washed (PhH) to give essentially pure 4-chloro-3-(3-chloro-2-nitrophenyl)-3-pyrrolin-2-one **(3).** The structure was assigned on the basis of mass spectral, ir, uv, nmr, and elemental analyses. The assignment of the carbonyl position was supported by chemical reactivity of the 4-Cl substituent. Although a number of other products were observed, none was sufficiently purified for identification.

Acknowledgment. We would like to thank Dr. R. Hamill, Mr. C. B. Carrell, Mr. J. A. Mabe, and Dr. D. R. Brannon for preparing pure [^{14}C]pyrrolnitrin for use in the above studies. We would also like to thank Mr. J. Westhead for the microbiological analyses and Mr. H. Sullivan for assistance in the glc analyses.

References

(1) K. Arima, H. Imanaka, M. Kousaka, A. Fukuda, and G. Tamura, *J. Antibiot., Ser. A,* **18,** 201 (1965).
(2) M. Nishida, T. Matsubara, and N. Watanabe, *ibid., Ser. A,* **18,** 211 (1965).
(3) R. Hamill, R. Elander, J. Mabe, and M. Gorman, *Antimicrob. Ag. Chemother.,* **1967,** 388 (1968).
(4) R. L. Hamill, H. R. Sullivan, and M. Gorman, *Appl. Microbiol.,* **18,** 310 (1969).
(5) G. H. Hogeboom, *Methods Enzymol.,* **1,** 16 (1955).