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Metabolism of Piromidic Acid, a New Antibacterial Agent. II.[1] *In Vitro* Metabolic Pathway of Piromidic Acid in Rats

Yutaka Sekine, Mie Miyamoto, Masahisa Hashimoto, and Kiyoshi Nakamura

Research and Development Division, Dainippon Pharmaceutical Co., Ltd.[2]

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The metabolism of piromidic acid (PA, 5,8-dihydro-8-ethyl-5-oxo-2-pyrrolidino-pyrido[2,3-*d*]pyrimidine-6-carboxylic acid=pyrrolidino-PPA) by rat liver preparations was studied. The first step of PA metabolism was found to be hydroxylation at the 2- or 3-position in the pyrrolidine ring by the mixed-function oxidase system in liver microsomes to form M-II (2-hydroxypyrrolidino-PPA) or M-V (3-hydroxypyrrolidino-PPA). M-V did not undergo successive oxidation with hepatic $105000\times g$ supernatant and microsomal preparations, whereas M-II was further metabolized not only by the hepatic $105000\times g$ supernatant, to form the corresponding γ-aminobutyric acid derivative ($RNHCH_2CH_2CH_2$-$COOH$, M-IV), but also converted by the hepatic microsomes to 2,5-dihydroxypyrrolidine derivative (M-VI), which was in turn converted to form amino derivative (M-III, amino-PPA). This conversion is highly likely to be non-enzymatic degradation. Glucuronic-acid conjugate of PA was found to be produced from PA with hepatic $9000\times g$ supernatant preparations containing uridine-5′-diphosphoglucuronic acid and D-saccharic acid-1,4-lactone. Any evidences implying extrahepatic biotransformation by the blood or kidney preparation were not found of PA and its metabolites except M-II which was found to be converted to M-IV in both incubation media.

Piromidic acid (PA, 5,8-dihydro-8-ethyl-5-oxo-2-pyrrolidinopyrido[2,3-*d*]pyrimidine-6-carboxylic acid) is an antibacterial agent[3] for infections in the gastrointestinal, urinary and biliary tracts.

In the previous paper,[1] it was reported that ten metabolites containing three new types of pyrrolidine-ring metabolites together with unchanged PA were found in the urine of rats and human subjects receiving oral PA. The metabolism of PA was also found to be characterized that pyrrolidine moiety was extensively metabolized. Metabolism of the pyrrolidine moiety have been reported for nicotine by McKennis, *et al.*[4] and Hucker, *et al.*,[5] tremorine by Hammer, *et al.*[6] and prolintane by Yoshihara, *et*

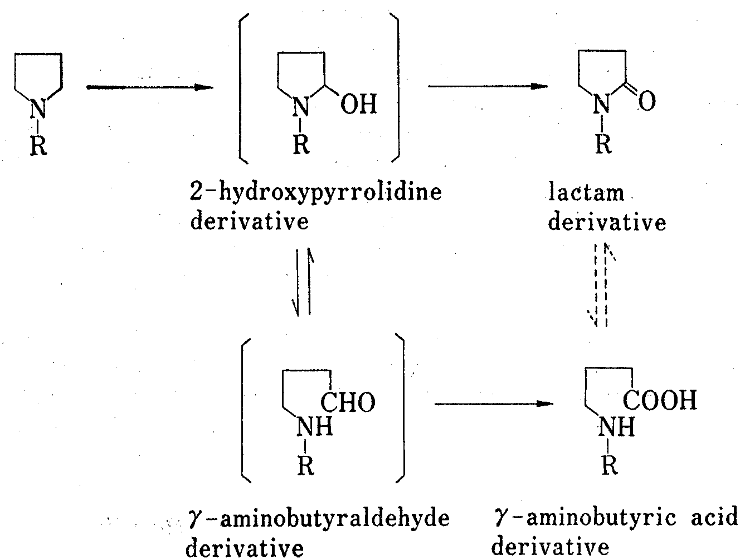

Chart 1

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al.[7] Major metabolites of these drugs are their lactam derivatives and γ-aminobutyric acid derivatives ($RNHCH_2CH_2CH_2COOH$). Concerning the formation of these metabolites, the following scheme has been proposed (Chart 1). Since there found marked differences in pyrrolidine-ring metabolisms of PA and other drugs, the present study is undertaken to elucidate the *in vitro* metabolic pathway of PA together with enzymic systems involved in the biotransformation in rats.

Experimental

Materials——Authentic samples of PA and its derivatives used were the same as those described in the previous paper,[1] unless otherwise indicated. The ^{14}C-labeled PA (^{14}C-PA, ethyl-1-^{14}C) was kindly supplied by Mr. A. Kagemoto in our laboratory and its specific activity was 28 μCi/mg. The radiochemical purity was 98% as assayed by thin-layer chromatography. β-Glucuronidase (Type I) and uridine-5′-diphosphoglucuronic acid (UDPGA) from Sigma Chemical Co. were used.

Liver Preparations and Incubation——Male Wistar rats weighing 190—200 g, starved for 24 hr prior to preparation, were used in this study. Hepatic $9000\times g$ supernatant, microsomal and $105000\times g$ supernatant preparations were obtained according to the procedure reported by Guarino, *et al.*[8] Protein assay was carried out by the method of Lowry, *et al.*[9] In experiments with phenobarbital induced rats, the animals were treated with sodium phenobarbital (16 mg/ml in aqueous solution, 80 mg/kg, intraperitoneally) daily for 4 days, the last dose being administered 24 hr before sacrifice.

A typical incubation mixture (5 ml) consisted of hepatic $9000\times g$ or $105000\times g$ supernatant solution (*ca.* 10 mg of protein), PA (0.3 μmoles), nicotinamide-adenine dinucleotide phosphate (NADP, 2 μmoles), glucose-6-phosphate (G-6-P, 50 μmoles), $MgCl_2$ (25 μmoles) and Tris–HCl buffer containing 1.15% KCl (pH 7.4). The corresponding microsomal incubation mixture contained, in addition, G-6-P dehydrogenase (2 units). Incubations were carried out aerobically at 37° for 10 to 60 min. The reaction was stopped by addition of 2 ml of water saturated with NaCl. Under these conditions, the amounts of unchanged PA *versus* time was found to be linear up to 15 min.

Incubation with Kidney Preparation and Blood——The kidney $9000\times g$ supernatant was obtained by the same way as in hepatic preparations. An incubation mixture (5 ml) consisted of renal $9000\times g$ supernatant (*ca.* 10 mg of protein) or whole blood (1 ml), PA (0.3 μmoles, or a metabolite), NADP (2 μmoles), G-6-P (50 μmoles), $MgCl_2$ (25 μmoles) and Tris–HCl buffer containing 1.15% KCl (pH 7.4). In the incubation mixture, in addition, nicotinamide-adenine dinucleotide (NAD) (5 μmoles) was added as a cofactor of aldehyde dehydrogenase. Incubations were performed at 37° for 30 min.

***In Vitro* PA-glucuronide Formation**——PA-glucuronide formation was examined under the following conditions. An incubation mixture (5 ml) consisted of rat hepatic $9000\times g$ supernatant solution (*ca.* 20 mg of protein), PA (0.3 μmoles), UDPGA (1.5 μmoles), D-saccharic acid-1,4-lactone (5 μmoles) and Tris–HCl buffer containing 1.15% KCl (pH 7.4). Incubations were performed at 37° for 30 min.

Thin-Layer Chromatography (TLC)——Two ml of the reaction mixture, after addition of 8 ml of a buffer solution (pH 3.0, 1/5 M Na_2HPO_4–1/10 M citric acid), was extracted with 20 ml of $CHCl_3$ and the chloroform phase was subjected to TLC examinations. When ^{14}C-PA as a substrate was incubated, 1/2 volumes of EtOH were added to the reaction mixture and the salt precipitated was removed by centrifugation at $6000\times g$ for 10 min. The supernatant was subjected to TLC examinations. TLC was performed according to the same way as that described in the previous paper[1] except that radioactivity on the TLC plate was measured with a radiochromatogram scanner, Nuclear-Chicago Actigraph III.

Determination of PA and Its Metabolites——The determination of PA and its metabolites in the incubation mixture was performed by TLC separation followed by ultraviolet (UV) assay as described in the separate paper.[10]

Results

I. Enzymatic Formation of PA-metabolites

After incubation of ^{14}C-PA with hepatic $9000\times g$ supernatant for 30 min, the reaction mixture was examined by TLC. Six spots were detected on radiochromatograms and termed

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as metabolite I (M-I) to metabolite VI (M-VI) in order of decreasing *Rf*-values in the solvent system, chloroform–formic acid–ethyl formate (10:1:4). After extraction and preparative TLC, the metabolites associated with respective spots were separated. Since M-III and M-VI were found in trace amounts after the incubation, phenobarbital-treated liver preparation was also used in the incubation for characterization and identification of these metabolites. The characteristic properties of metabolites such as absorption maxima of UV spectra, and *Rf*-values and color of fluorescence in UV-lamp on a TLC plate are summarized in Table I. In comparison with authentic samples, they were identified as listed in Table I, except M-VI being identified by TLC as one of urinary metabolites of rats, for which a probable structure has been proposed to be 2-(2,5-dihydroxypyrrolidino)-5,8-dihydro-8-ethyl-5-oxo-pyrido[2,3-*d*]-pyrimidine-6-carboxylic acid.[1] The amounts of metabolites formed after the incubation are shown in Table II. Incubation of PA with hepatic $9000 \times g$ supernatant was found to afford all the metabolites, except glucuronides, detected in the urine of rats and humans receiving PA.

Table I. *Rf*-Values, Color of Fluorescence in UV-lamp and Absorption Maxima of UV Spectra of *in Vitro* Metabolites after Incubations[a] of ^{14}C-PA with Rat Hepatic $9000 \times g$ Supernatant Prepations

Metabolite	TLC[b] Solvent (A)	Systems (B)	Color of fluorescence	UV[c] $\lambda_{max.}$ (nm)	Structure
M-I	0.53	0.83	light blue	274, 331	PA
M-II	0.48	0.78	light blue	265, 330	2-(2-hydroxypyrrolidino)-PPA[d]
M-III	0.35	0.58	violet	253, 326	2-amino-PPA
M-IV	0.27	0.57	dark blue	264, 329	2-(3-hydroxycarbonylpropylamino)-PPA
M-V	0.25	0.41	light blue	272, 333	2-(3-hydroxypyrrolidino)-PPA
M-VI	0.12	0.22	light blue	265, 330	probable 2-(2,5-dihydroxypyrrolidino)-PPA

a) Incubations were carried out as described in Experimental in the text.
b) TLC: solvent system (A) $CHCl_3$–HCOOH–$HCOOC_2H_5$=10:1:4, (B) $CHCl_3$–AcOH=5:1, plate, Eastman Chromagram 6061
c) in a phosphate buffer solution (pH 7.4, 1/15 M Na_2HPO_4–1/15 M KH_2PO_4)
d) PPA: 5,8-dihydro-8-ethyl-5-oxo-pyrido[2,3-*d*]pyrimidine-6-carboxylic acid

Table II. *In Vitro* Metabolites formed after Incubations[a] of PA with Rat Hepatic $9000 \times g$ Supernatant Preparations

Metabolite	Formed (%)[b] 10 min	15 min	20 min
M-II	26.3	35.5	35.8
M-V	3.1	4.3	5.4
M-IV	0.9	2.8	9.9
M-III	0	trace	trace
M-VI	0	trace	trace

a) Incubations were carried out as described in the text.
b) expressed as percent of added PA

II. Intracellular Localization of Enzyme Activity and Requirement for Cofactors in Metabolism of PA

Conversion of PA or its metabolites after *in vitro* incubation with hepatic microsomal or $105000 \times g$ supernatant preparations was examined by TLC and the results are summarized in Table III. Incubations of PA with hepatic microsomal preparations were found to yield M-II and M-V with trace amounts of M-III and M-VI, but not to afford M-IV. However, an addition of $105000 \times g$ supernatant to the incubation mixture of PA afforded M-IV together

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Table III. *In Vitro* Metabolites formed after Incubations[a] of PA or its Metabolites with Rat Hepatic Microsomal or $105000 \times g$ Supernatant Preparations

Substrate	Liver preparation	Metabolite
PA	microsomal (Ms)	M-II, M-V, M-III (trace) M-VI (trace)
PA	$105000 \times g$ sup.[b]	not detected
PA	Ms + $105000 \times g$ sup.	M-II, M-IV, M-V M-III (trace), M-VI (trace)
M-II	Ms	M-III (trace), M-VI (trace)
M-II	$105000 \times g$ sup.	M-IV
M-V	Ms + $105000 \times g$ sup.	not detected
M-III	Ms + $105000 \times g$ sup.	not detected
M-IV	Ms + $105000 \times g$ sup.	not detected
M-VI	none	M-III

a) Incubations were carried out as described in the text.
b) hepatic $105000 \times g$ supernatant

Table IV. *In Vitro* Conversion of M-II to M-IV and M-III after Incubations[a] with Non-treated and Phenobarbital-treated Rat Hepatic $9000 \times g$ Supernatant Preparations for 15 min

Metabolite	Found (%)[b]	
	Non-treated	Phenobarbital-treated
M-IV	13.5	21.10
M-III	trace	1.15
M-VI	trace	0.48

a) Incubations were carried out as described in the text.
b) expressed as percent of added PA

Table V. Requirement for Cofactors in Metabolism of PA by Rat Liver Preparations

Enzyme system[a]		PA recovered(%)
Liver preparation	Cofactors	
$9000 \times g$ sup.[b]	none	94.9
$9000 \times g$ sup.	NADP, G-6-P $MgCl_2$[c]	9.2
$9000 \times g$ sup.	NADPH	8.3
microsomal	NADP, G-6-P $MgCl_2$ G-6-P-dehydrogenase[d]	42.8
microsomal + $105000 \times g$ sup.[e]	NADP, G-6-P $MgCl_2$	6.9
$105000 \times g$ sup.	NADP, G-6-P $MgCl_2$	102.2

a) The reaction mixture (5 ml) consists of hepatic $9000 \times g$ supernatant, microsomal or $105000 \times g$ supernatant preparation (*ca.* 10 mg of protein), PA (0.3 μmoles) and Tris–HCl buffer containing 1.15% KCl (pH 7.4). Incubations were carried out aerobically at 37° for 30 min.
b) hepatic $9000 \times g$ supernatant
c) NADP (2 μmoles), G-6-P (50 μmoles), $MgCl_2$ (25 μmoles)
d) G-6-P-dehydrogenase 2 units
e) hepatic $105000 \times g$ supernatant

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with M-II, M-V, M-III and M-VI. M-V, M-III and M-IV did not undergo successive oxidation with 105000×g supernatant or microsomal preparations, whereas M-II was further metabolized to form the corresponding γ-aminobutyric acid derivative (M-IV) in hepatic 105000×g supernatant and was also converted by hepatic microsomes to M-VI and M-III. The conversion of M-VI to M-III is highly likely to be non-enzymatic degradation since M-III was formed qualitatively in equal amounts either by incubation with or without hepatic preparations. The conversion of M-II to M-III *via* M-VI described above was more evidently demonstrated with hepatic preparations of phenobarbital-treated rats as shown in Table IV.

The metabolism of PA by a hepatic microsomal fraction was dependent on the presence of NADPH or a NADPH-generating system as shown in Table V. Under nitrogen atmosphere PA could not be metabolized by the hepatic microsomal preparation including NADPH-generating system.

III. Enzymatic Formation of PA-glucuronide *in Vitro*

Glucuronic acid conjugation of PA was examined with rat hepatic 9000×g supernatant containing UDPGA and D-saccharic acid-1,4-lactone. PA was converted to its glucuronide as shown in Table VI.

Table VI. Glucuronide Formation of PA after Incubation[a] with Rat Hepatic Preparation

Time (min)	PA (recovered %)[b]	PA-glucuronide (formed %)[b]
0	98.8	0
30	90.0	10.4

a) An incubation mixture (5 ml) consisted of hepatic 9000×g supernatant preparation (*ca.* 20 mg of protein), PA (0.3 μmoles), UDPGA (2 μmoles), D-saccharic acid-1,4-lactone (5 μmoles) and Tris-HCl buffer containing 1.15% KCl (pH 7.4).
b) expressed as percent of added PA

IV. Metabolism of PA by Blood or Kidney Preparation of Rats

Any evidences implying extrahepatic biotransformation of PA and its metabolites except for M-II were not found by the blood or kidney preparation of rats. On the other hand, M-II was found to be converted to M-IV in both incubation media as revealed by TLC.

V. Metabolic Pathway of PA

The first step of PA metabolism was found to be hydroxylation in the pyrrolidine ring to form M-II and M-V by the mixed-function oxidase system in liver microsomes as shown in Tables II and III. M-V did not undergo successive oxidation. On the contrary M-II was further metabolized to form M-IV in the hepatic 105000×g supernatant, kidney preparation or blood. M-II was also converted by hepatic microsomes to M-VI which was in turn metabolized non-enzymatically to form M-III. M-III and M-IV did not exhibit any indications for successive biotransformation by hepatic microsomes or 105000×g supernatant. From these results, the metabolic pathway of PA in rats is summarized as shown in Chart 2.

Discussion

The first step of PA metabolism was found to be hydroxylation at the 2- or 3-position in the pyrrolidine ring. The *in vitro* metabolic rate of 2-hydroxypyrrolidine formation of PA was found to be about ten times faster than that of 3-hydroxypyrrolidine formation (Table II). This indicates that there existed marked differences in enzymatic reactivity between the 2- and 3-positions in the pyrrolidine ring to the mixed-function oxidase system in liver microsomes. 2-Hydroxypyrrolidine derivatives are generally so unstable that their isolation has not been successful. However, 2-hydroxypyrrolidine derivative of PA (M-II), was definitely stable enough to be isolated from urine, bile or the reaction mixture containing hepatic prepara-

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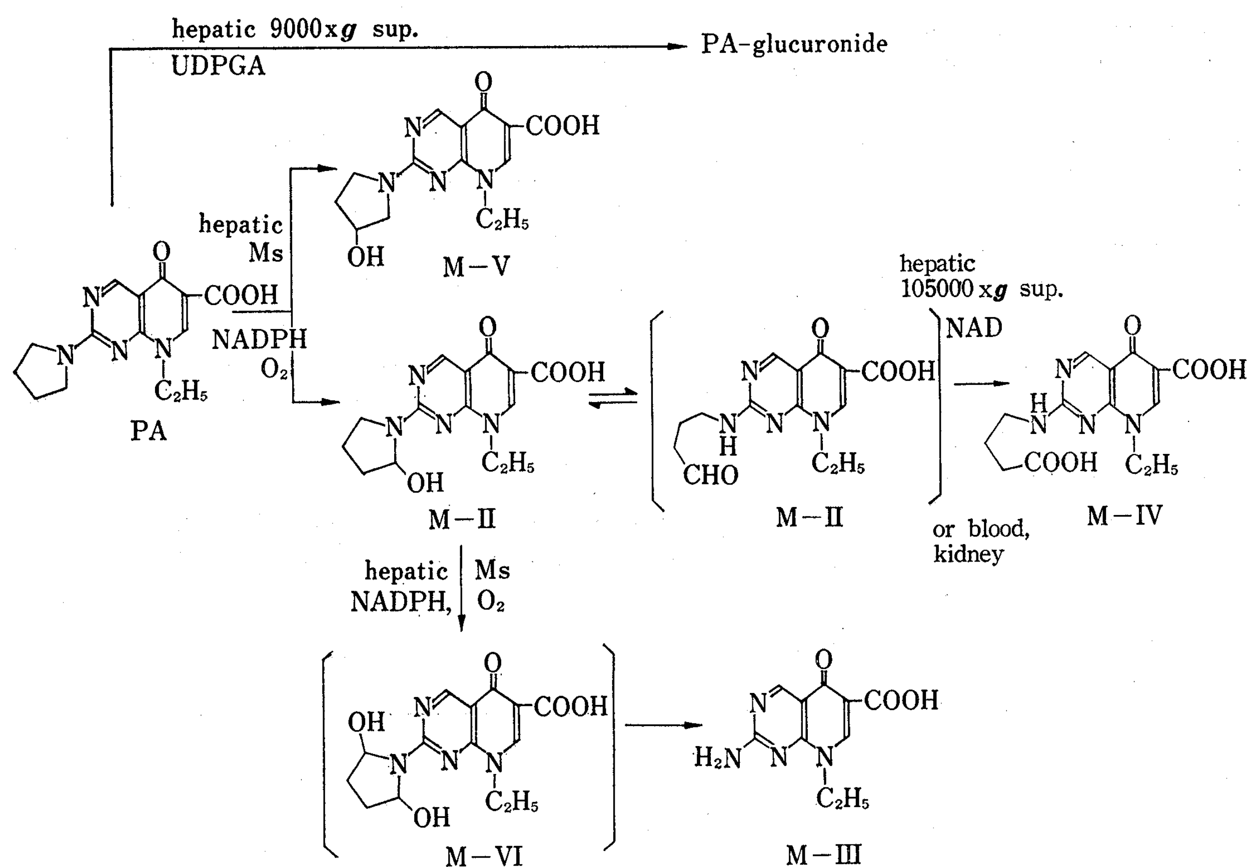

Chart 2. *In Vitro* Metabolic Pathway of PA

tions. In this case, M-II is likely to be stabilized by a hydrogen bonding between a hydroxyl group in the pyrrolidine ring and one of nitrogen atoms in the pyrimidine ring. M-II is further metabolized by hepatic microsomes at the 5-position in the pyrrolidine ring to form 2,5-dihydroxypyrrolidine derivative (M-VI), and M-VI was found to be successively converted toM-III. This conversion is highly likely to be non-enzymatic degradation since M-III was formed qualitatively in equal amounts either by incubation with or without hepatic preparations. It was also found that M-II was converted to γ-aminobutyric acid derivative by the action of aldehyhydededrogenase (Grade II from Sigma Chemical Co.). This indicates that M-II partly existed as an aldehyde form, a ring-opened tautomer of 2-hydroxypyrrolidine derivative, in aqueous solutions. The formation of M-III *via* M-VI and none of M-III formed *via* M-IV are probably ascribed to the stability of M-II in a ring-closed alcohol form even in aqueous solution, since hydroxylation at the 1-position of γ-aminobutyric acid derivatives has never been reported to occur. No evidences implying extrahepatic biotransformation by the blood or kidney preparation were found of PA and its metabolites except M-II which is likely to be converted to M-IV by the action of aldehydedehydrogenase in both incubation media. Any evidences implying the formation of lactam derivative from 2-hydroxypyrrolidine and γ-aminobutyric acid derivatives of PA were not found. In contrast to M-V, successive biotransformation of M-II is likely due to the fact that M-II has a practically unchanged partition ratio as compared with PA, whereas M-V has a much decreased partition ratio.[1] It seems to depend on physicochemical properties of the 2-hydroxypyrrolidine derivative whether it is successively converted to a γ-aminobutyric acid derivative or to a lactam as reported in metabolism of nicotine[4,5] and tremorine.[6]

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