In vitro Metabolism of Pyribenzoxim

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The *in vitro* metabolism of a new herbicide pyribenzoxim, {benzophenone *O*-[2,6-bis[(4,6-dimethoxy-2-pyrimidinyl)oxy]benzoyl]oxime} was studied using rice, barnyardgrass and rat liver microsomes. No metabolism of pyribenzoxim was observed with rice and barnyardgrass microsomes although the cytochrome P450 was active, which was evidenced by the metabolism of cinnamic acid. With rat liver microsomes, four metabolites (M1, M2, M3, and M4) were produced while parent compound decreased. M1 and M2 were from the hydrolysis reactions and NADPH-dependent metabolites were M3 and M4 (major metabolite) which were hydroxylated by cytochrome P450. They were identified as bispyribac-sodium (M1), benzophenone oxime (M2), {benzophenone *O*-[2,6-bis[(5-hydroxy-4,6-dimethoxy-2-pyrimidinyl)oxy]-benzoyl]oxime} (M3), and {benzophenone *O*-[2 [(5-hydroxy-4,6-dimethoxy-2-pyrimidinyl) 6-(4,6dimethoxy-2-pyrimidinyl)oxy]benzoyl]oxime} (M4) through LC/MS/MS analyses. Based on the results obtained metabolic map of pyribenzoxim is proposed.

Key words: *metabolism, pyribenzoxim, cytochrome P450, LC/MS/MS.*

Pesticides undergo extensive metabolic transformation in living organisms through various metabolic reactions. Microsomal mixed function oxidase is the primary enzyme for phase one reactions which convert pesticides into more soluble products. Although these products are generally less toxic than the parent compound, more toxic metabolites may also result. Such *in vitro* studies with microsomal preparations provides specific details of the chemical identity of metabolites and intermediates, the pattern of their formation, and the metabolic pathways of pesticides.

Pyribenzoxim was developed as a new herbicide by the LG Chemical Ltd. Korea. This pyrimidinyloxybenzoate was known to inhibit ALS, the enzyme involved in the biosynthesis of the branched chain amino acids in plants.[1] The oral acute LD_{50} (rat) was 5,000 mg/kg and dermal LD_{50} was 2,000 mg/kg, indicative of low mammalian toxicity. There was also no significant maternal or embryonic toxicity, and the bioavailability was negligible in rats by the elimination of radioactivity with feces (~80%) and urine (9%) after 72 hours of treatment.[2]

Bae *et al.* reported that I_{50} value of pyribenzoxim were 14 and 16 mM for rice and barnyardgrass, respectively, indicating inhibition of ALS did not differ significantly between them.[3] However, a high herbicidal selectivity was observed to control barnyard grass without damages to rice plants. This selectivity was explained by the difference of uptake and translocation or by that of metabolism rate between rice and barnyargrass.[4,5]

In the present study, metabolism of pyribenzoxim by rice, banyardgrass, and rat liver microsomes was carried out *in vitro* to investigate the comparative metabolism in plants and animals. The identification of metabolites were also performed using LC/MS/MS and authentic compounds available.

Materials and Methods

Reagents. Pyribenzoxim and bispyribac-sodium were kindly donated from LG Chemical Ltd. (Taejeon, Korea). $NADP^+$, $_D$-glucose-6-phosphate, and $_D$-glucose-6-phosphate dehydrogenase were purchased from Sigma Chemical Co. (St Louis, MO, USA). All other chemicals were the highest grade commercially available.

Instruments. HPLC analysis was conducted with HP1100 (Hewlett Packard, USA) equipped with a variable wavelength detector. The eluent consisted of acetonitrile in water containing 0.1% trifluoroacetic acid (TFA) at a flow rate of 1.0 ml/min. The gradient was programmed linearly as follows; 40% acetonitrile for 3 min, 40-70% acetonitrile for 7 min, 70% for 10 min, and 70-40% for 3 min. The effluent was detected at 244 nm.

Mass spectra were recorded on LC/MS/MS (QUATTRO LC, Micromass co, USA) with electrospray ionization (ESI^+) mode.

Plant culture and induction of microsomal enzyme. Barnyardgrass seeds were germinated and grown on cheese cloth in Ca_2SO_4 solution (1 mM) for 6 days in the dark at 25°C.

1,8-Naphthalic anhydride (0.5%, w/w) was applied

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Abbreviations: ALS, acetolactate synthase.

directly to rice seeds for enzyme activity induction and the seeds were germinated and grown as described above. For the same purpose, 5-day-old rice seedlings were grown in ethanolic solution (5.0%, v/v) of Ca_2SO_4 (1 mM) for 1 day before harvest.

Preparation of microsomes. Rice and barnyardgrass microsomes were prepared using etiolated shoots of 6-day-old seedlings. They were excised and ground in cold buffer containing Na-phosphate (0.1 M, pH 7.5), glycerol (20%, w/v), ascorbic acid (20 mM), mercaptoethanol (14 mM), and EDTA (1 mM). The homogenate was filtered through cheese cloth and centrifuged at 3,000 × g for 20 min to remove nuclei or cell debris fraction. The supernatant was then centrifuged at 10,000 × g for 30 min to separate mitochondria fraction as pellets. It was centrifuged again at 105,000 × g for 2 h to obtain microsomes as pellets and soluble fraction. The microsomal pellet was resuspended in Na-phosphate buffer (0.1 M) containing glycerol (30%, w/v) and mercaptoethanol (1.4 mM). Nuclei or mitochondria pellet were resuspended with the same buffer. All subcellular fractions were stored at -70°C until analysis.[6]

Preparation of rat liver microsomes was based on the method of Lee *et al.*[7] using Specific pathogen-free Sprague-Dawley rat. The liver was homogenized with 4 volumes of ice-cold potassium chloride solution (1.15%, pH 7.4). The liver homogenates were centrifuged at 9,000 × g for 10 min at 4°C, and the resulting post mitochondrial supernatants were centrifuged again at 105,000 × g for 60 min at 4°C. The microsomal pellets were resuspended in 50 mM potassium phosphate buffer (pH 7.4) containing EDTA (0.1 mM). Aliquots of liver microsomes were stored at -70°C until use.

Microsomal protein was determined by the method of Bradford[8] with crystalline bovine serum albumin as a standard.

Preparation of benzophenone oxime. To a mixture of benzophenone (20.0 g), hydroxylamine hydrochloride (11.9 g) in 240 ml of 80% ethanol, sodium hydroxide (97.5 g) was added in portions. Then the mixture was refluxed for 5 min and pored into a diluted hydrochloric acid. The precipitated oxime was filtered, washed with water twice, and dried to give a white solid (10.6 g, 96.3%) [mp ; 53~55°C: CIMS ; *m/z* (%), 198 ($[M+H]^+$, 100), 239 ($[M+CH_3CN]^+$, 40)].

Reaction of pyribenzoxim with rice and barnyardgrass microsomes and subcellular fractions. Reaction mixture consisted of Na-phosphate(0.1 M, pH 7.5), NADPH-regenerating system (0.8 mM $NADP^+$, 10 mM glucose 6-phosphate, and 1 unit glucose 6-phosphate dehydrogenase), 1 mg microsomal protein, 30 μM pyribenzoxim in 1 ml total volume. Reactions were initiated by addition of glucose 6-phosphate dehydrogenase and incubated for 120 min at 37°C. Nuclei, mitochondria and soluble fraction were reacted with pyribenzoxim in the same manner.

Rat liver microsomal reactions of pyribenzoxim. Rat liver microsomes (0.3 mg) were preincubated in 1.0 ml potassium phosphate buffer (50 mM , pH 7.4) in the presence of NADPH-regenerating system containing $NADP^+$ (0.8 mM), glucose 6-phosphate (10 mM) and 1 unit glucose 6-phosphate dehydrogenase for 5 min at 37°C in shaking water bath. The control incubations were conducted with heat-denatured microsomal preparations (80°C for 10 min) with NaF addition (120 mM) or in the absence of NADPH-regenerating system. The reaction was initiated by the addition of 30 μM pyribenzoxim and preceeded for 30 min at 37°C.

Microsomal reaction of cinnamic acid. Reaction mixtures as described above were prepared with rice, barnyardgrass or rat microsomes and 30 μM cinnamic acid. The reactions were carried out and analyzed in the same manner.

Extraction and analysis of metabolites. Reactions of plant microsomes were terminated at 5, 10, 30, 60, and 120 min after treatment by adding 75 μl cold stop solution, a mixture of 4 N HCl and methanol (2 : 1). The reaction mixture was extracted twice with 1ml ethyl acetate. The organic phase was dried under nitrogen gas and redissolved in acetonitrile (250 μl). An aliquot (20 μl) was analyzed through HPLC.

For the rat liver microsomes reactions, 50 μl phosphoric acid (43%) was added at 4, 8, 15, 30 min after treatment to stop the reaction. After mixing with 2 ml of methylene chloride and centrifugation, the organic layer was dried under a stream of nitrogen gas and redissolved in acetonitrile (200 μl), and an aliquot was analyzed as above through HPLC. For the metabolites identification aliquots were analyzed through LC/MS/MS using acetic acid instead of trifluoroacetic acid for the elution solvents of HPLC.

Results and Discussion

Plant culture and enzyme preparations. The culture and enzyme preparations of rice and barnyardgrass were based on the method of Pyon *et al.*[9] in which culture of plants were carried out in dark condition to prevent greening of tissues because chlorophyll and other pigments interfere with the analysis and the inducible herbicide hydroxylase activity in the microsomal fraction drops precipitously during greening.[10]

The major problem in study of plant cytochrome P450 monoxygenases is their low concentration and activity. In plants their contents range from 0.007 to 0.02 nmol/mg microsomal protein as compared with those of rat liver (0.9 to 1.2 nmol/mg microsomal protein).[11] In addition, they are susceptible to degradation by the vacuolar proteases released during tissue homogenization.[12] Therefore, induction of enzyme activity were performed through coating seeds with 1,8-Naphtalic anhydride or growing in ethanolic solution.[10] Furthermore, reactions of cinnamic acid with microsomes were accomplished to confirm the activity of cytochrome P450 in rice and barnyardgrass because the reaction which catalyses hydroxylation of *trans*-cinnamic acid into *p*-

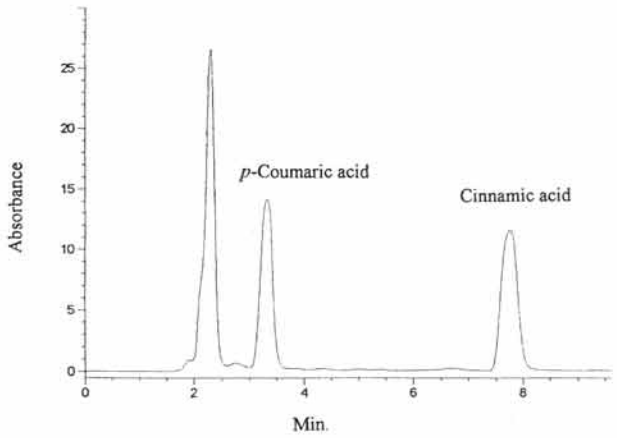

Fig. 1. HPLC chromatogram of cinnamic acid metabolism by barnyardgrass microsomes.

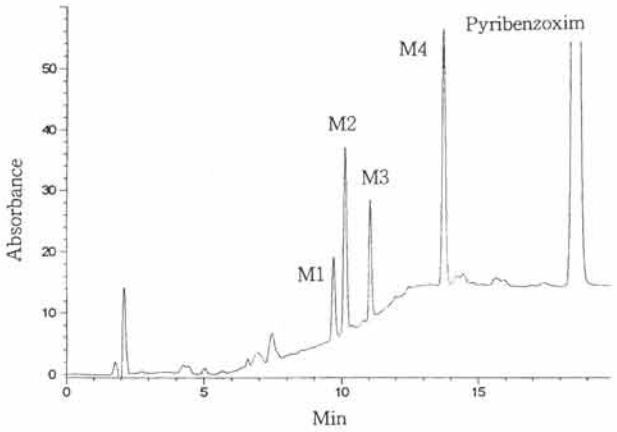

Fig. 2. HPLC chromatogram of rat liver microsomal reaction mixture with pyribenzoxim.

coumaric acid is more feasible than the reaction between pesticide and microsomes, for example, with corn microsomes, cinnamic acid was metabolized much faster than nicosulfuron by 64-fold.[13]

Preparation of benzophenone oxime. An oxime was prepared as a reference compound because the bond cleavage in pyribenzoxim was expected. No difficulties were encountered in the reaction to give good yield.

Microsomal metabolism of pyribenzoxim by rice and barnyardgrass. Cinnamic acid was metabolized by both the rice and barnyardgrass microsomal fractions, indicating cytochrome P450 is active enough (Fig 1). However, pyribenzoxim was not metabolized by microsomes of rice and barnyardgrass. The same results were obtained in the reaction of pyribenzoxim with other subcellular fractions suggesting the selectivity of pyribenzoxim could not arise from cytochrome P450 metabolism. These results may be caused by the low level of cytochrome P450-dependent enzymes[11] in plant microsomes and/or by the low substrate specificity although they could metabolize cinnamic acid.

Metabolism of pyribenzoxim by rat liver microsomes. In the rat liver microsomes reaction with pyribenzoxim, four

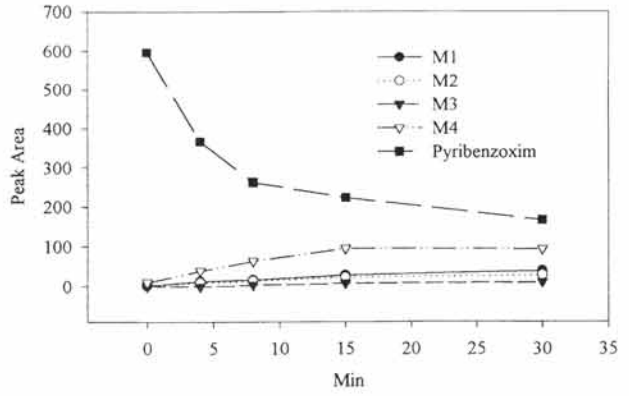

Fig. 3. Formation of metabolites in rat liver microsomal reaction with pyribenzoxim.

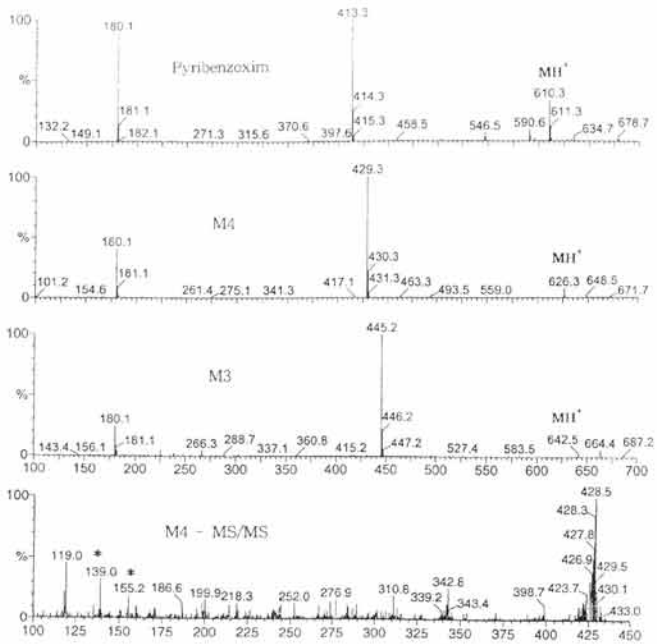

Fig. 4. LC/MS and LC/MS/MS spectra of pyribenzoxim and metabolites.

metabolite peaks (M1, M2, M3, and M4) were observed (Fig. 2) As the reaction preceeded pyribenzoxim decreased and the metabolites increased (Fig. 3). M1 and M2 were again observed in the incubation without NADPH-regenerating system, however, they did not occur in the reaction with heat-denatured rat liver microsomes or with the addition of NaF, an esterase inhibitor.[14] These results suggested that M1 and M2 are from non-oxidative reactions, possibly from enzymatic hydrolysis of pyribenzoxim by microsomal esterases.

Identification of metabolites through LC/MS/MS. From the LC/MS analysis with authentic compounds, M1 ($[M+H]^+$; *m/z* 431) and M2 ($[M+H]^+$; *m/z* 198) were identified as bispyribac-sodium and benzophenone oxime, respectively. The other two metabolites, M3 and M4, have protonated molecular ion peaks $[M+H]^+$ at *m/z* 642 and 626, respectively, suggesting two or one oxygen atom were

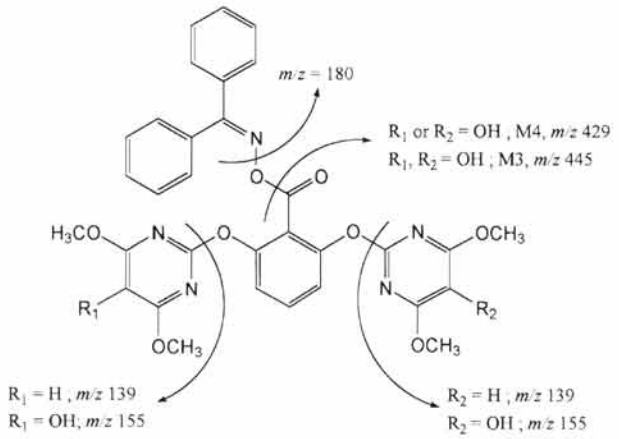

Fig. 5. Fragmentation pattern of pyribenzoxim and metabolites (M3 and M4).

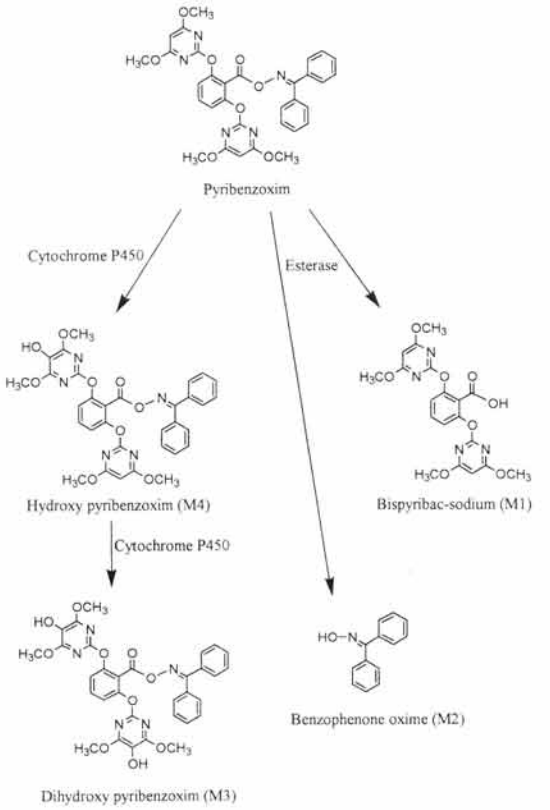

Fig. 6. Proposed metabolic pathway of pyribenzoxim by rat liver microsome.

inserted in the molecule of pyribenzoxim ($[M+H]^+$; *m/z* 610) by cytochrome P450 (Fig. 4). Furthermore, the base ions at *m/z* 445 and 429 for M3 and M4, respectively, compared with *m/z* 413 for pyribenzoxim, suggested the possible hydroxylation of pyrimidine rings because the ion at *m/z* 180 (Figs. 3 and 4) was formed commonly from the three compounds.[15] Therefore, those base ions of M3 and M4 were again fragmented using MS/MS technique to determine the position of hydroxyl groups.

MS/MS spectra of M3 and M4 showed interesting results, in which M3 gave significant ions at *m/z* 155 which is a hydroxylated dimethoxypyrimidine ring fragment. From M4, ions at *m/z* 155 and 139 (M4 MS/MS in Fig. 4) were observed, the latter ion being a dimethoxypyrimidine ring fragment also observed in MS/MS spectrum of pyribenzoxim. These fragmentation patterns (Figs. 4 and 5) suggested M4 is a hydroxy compound containing both a dimethoxypyrimidine ring fragment (*m/z* 139) and a hydroxylated dimethoxypyrimidine ring fragment (*m/z* 155), while two dimethoxypyrimidine rings of M3 were hydroxylated. Therefore, M3 and M4 were identified as {benzophenone *O*-[2,6-bis[(5-hydroxy-4,6-dimethoxy-2-pyrimidinyl)oxy]-benzoyl]oxime} and {benzophenone *O*-[2[(5-hydroxy-4,6-dimethoxy-2-pyrimidinyl) 6-(4,6dimethoxy-2-pyrimidinyl)oxy]benzoyl]oxime}, respectively. On the basis of these results, *in vitro* metabolic map of pyribenzoxim by rat liver microsomes is proposed in Fig. 6.

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