Metabolism of *S*-Benzyl *O*, *O*-Diisopropyl Phosphorothioate (Kitazin P®) by Mycelial Cells of *Pyricularia oryzae*[†]

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The metabolism of an organophosphorus fungicide, Kitazin P® (*S*-benzyl *O*, *O*-diisopropyl phosphorothioate) by mycelia of *Pyricularia oryzae*, the rice blast fungus, was studied by using ³²P- and ³⁵S-doubled-labeled, ³⁵S-labeled and non-labeled compounds.

The mycelia incorporated the fungicide from the medium, metabolized most of it to water-soluble metabolites and released them into the medium. The main metabolite from Kitazin P was identified as *O*, *O*-diisopropyl hydrogen phosphorothioate by ion-exchange chromatographic separation of double-labeled metabolites. *S*-Benzyl *O*-isopropyl hydrogen phosphorothioate, diisopropyl hydrogen phosphate and/or isopropyl dihydrogen phosphate were suspected to be minor metabolites. From the toluene-soluble fraction several metabolites were separated by thin-layer chromatography. The most eminent metabolite among them was found to be the *m*-hydroxy derivative of Kitazin P by thin-layer chromatographic identification of the labeled metabolite and gas-liquid chromatographic identification of the metabolite and its methylated and acetylated products with respective authentic samples. The *o*- and *p*-hydroxy derivatives were not found in the metabolites.

No significant difference in the rate and extent of metabolism was found between susceptible and resistant clones against the fungicide, when its concentration was below the growth-inhibitory concentration to the both clones.

The metabolic fate of *O*-ethyl *S*, *S*-diphenyl phosphorodithioate (Hinosan®) in a culture of *Pyricularia oryzae* has been reported in a previous paper.¹⁾ Another organophosphorus fungicide, *S*-benzyl *O*, *O*-diisopropyl phosphorothioate (Kitazin P®) was studied in the present work.

The initial metabolic pathway of Hinosan is mainly the removal of the phenylthio radical. In the present work, whether the first step of the metabolism of Kitazin P is removal of the benzylthio, benzyl or isopropyl radical is one of the subjects to be solved.

From the toluene soluble fraction of the metabolites of Hinosan, the *p*-hydroxy deriva-

tive was identified. Therefore, another subject of the present work is detection of hydroxy derivatives from the metabolites.

Difference in the metabolism of Hinosan was not found between susceptible and resistant clones against organophosphorus fungicides. Difference in the metabolism of Kitazin P between the two clones was also studied in this work.

MATERIALS AND METHOD

Labeled compounds. ³²P, ³⁵S-Double-labeled Kitazin P was kindly supplied by Kumiai Chemical Industry Co. Ltd. It was purified by silica column chromatography with hexane plus chloroform (10:3) as an eluent, followed by thin-layer chromatography in benzene plus methyl ethyl ketone (5:1). The purity of the resulting Kitazin P was ascertained by thin

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layer chromatography to be 99.3% on ^{35}S radioactivity and 99.5% on ^{32}P radioactivity basis, and had about 4,500 cpm/ μg of ^{35}S radioactivity and 350 cpm/ μg of ^{32}P radioactivity at the beginning of the experiment. ^{35}S and ^{32}P of the doubled labeled compound and its metabolites were separately assayed by three channel windows on a Tricarb Liquid Scintillation Spectrometer (model 3003).

^{35}S -Labeled Kitazin P was also used for the identification of the toluene-soluble metabolites. It was synthesized by condensation of benzyl chloride and potassium *O*, *O*-diisopropyl phosphorothioate derived from elemental ^{35}S , diisopropyl hydrogen phosphite and potassium carbonate. The resulting fungicide having radioactivity of about 16,000 cpm/ μg was used for the experiment.

Authentic samples.

Kitazin P and its hydrolysates. Kitazin P, ammonium *O*, *O*-diisopropyl phosphorothioate, and *O*, *O*-diisopropyl hydrogen phosphate were kindly supplied by Kumiai Chemical Industry Co. Ltd.

Acetoxy and hydroxy derivatives of Kitazin P. Three isomers of acetoxy derivatives of Kitazin P, i.e. *o*-, *m*- and *p*-isomers, were prepared and supplied through the courtesy of Kumiai Chemical Industry Co. Ltd. The acetoxy derivatives were able to be hydrolyzed to form the corresponding hydroxy derivatives under a mild condition, for example, under reflux with pyridine plus water (1:5) for several hours. The *m*-hydroxy derivative was purified on TLC in

benzene plus methyl ethyl ketone (3:1) as a developing solvent, and ascertained by its IR spectrum (Fig. 1). It was also identified, after methylation with diazomethane, on GLC with an authentic sample of the *m*-methoxy derivative of Kitazin P.

Methoxy derivatives of Kitazin P. Three, i.e. *o*-, *m*- and *p*-isomers of the methoxy derivatives were synthesized from the corresponding methoxybenzyl chlorides. They are purified on TLC in benzene plus methyl ethyl ketone as a developing solvent. IR $\text{KBr}_{\text{max}} \text{cm}^{-1}$ of *o*-isomer: 2970, 1602, 1497, 1300, 1258 ($-\text{C}-\text{O}-\text{C}$ and/or $\text{P}=\text{O}$), 1105, 1030, 980 ($\text{P}-\text{O}-\text{C}$), 759 ($-\text{o}-\text{C}_6\text{H}_4-$). IR $\text{KBr}_{\text{max}} \text{cm}^{-1}$ of *m*-isomer: 2970, 1602, 1492, 1301, 1264, 1103, 1045, 980, 772 ($-\text{m}-\text{C}_6\text{H}_4-$). IR $\text{KBr}_{\text{max}} \text{cm}^{-1}$ of *p*-isomer: 2975, 1612, 1513, 1308, 1254, 1106, 1034, 980, 833 ($-\text{p}-\text{C}_6\text{H}_4-$).

Test organisms. An isolate of *P. oryzae*, Hoku 373, was used as a test fungus. A clone selected under the pressure of Kitazin® (*S*-benzyl *O*, *O*-diethyl phosphorothioate) was used throughout this experiment as a resistant clone. It is resistant to Kitazin P and other organophosphorus fungicides as well as Kitazin.² The minimum inhibitory concentration of Kitazin P for the mycelial growth of the resistant clone is 0.4 mM (115 $\mu\text{g}/\text{ml}$), while that for the growth of the mother clone is less than 0.05 mM (14.4 $\mu\text{g}/\text{ml}$). Mycelia used in this experiment were cultured and prepared as in a previous work.¹⁾

Incubation of test organism with fungicide. The

FIG. 1. IR Spectrum of *m*-Hydroxy Derivative of Kitazin P (in KBr Tablet).

amount of mycelia used was 1.5 g fresh weight per 10 ml solution of the fungicide in M/30 phosphate buffer containing 1% glucose. Test concentrations of the fungicide were 3 μ g/ml and 50 μ g/ml. The lower concentration does not affect the growth of both the susceptible and the resistant clones, while the higher concentration inhibits the growth of the susceptible clone but it does not inhibit the growth of the resistant clone.

Incubation was made at 27°C with a shaker for given hours.

Isolation and identification of metabolites. As shown in the experiment of Hinosan,¹⁾ incubated mixtures were separated into five fractions, i.e. the toluene soluble and the water soluble fractions of the external solution, the toluene soluble, the water soluble and the insoluble fractions of mycelial cells.

Separation of the water soluble metabolites on Dowex 1-x8 was conducted by the method of Plapp and Casida, and radioactive metabolites were identified by comparing with authentic samples by co-chromatography.¹⁾

The toluene soluble fractions were separated on TLC in benzene plus methyl ethyl ketone (3:1 or 5:1) or hexane plus acetone (3:2). Identification of radioactive metabolites on TLC was also made by co-chromatography with authentic samples.¹⁾ Identification of some metabolites was also conducted by GLC equipped with a flame thermionic detector and columns of NE 60 (2% on Gaschrome Q, 3 mm ϕ $\times$ 1.5 m) and PEGA (2% on Chromosorb W, 3 mm ϕ $\times$ 1.5 m), respectively. Methylation by diazomethane and acetylation by acetic anhydride plus pyridine were conducted before gas chromatographic separation in some cases.

RESULTS

Time course of degradation

The metabolic pattern during the incubation of radioactive Kitazin P with mycelia of *P. oryzae* is shown in Fig. 2. The fungicide applied was taken up by the fungus and was subsequently released into the external medium in a water soluble form. Since a control test without mycelial cells showed no change in distribution of radioactivity, this result indicates the biological degradation of the toluene soluble fungicide to water soluble metabolites.

FIG. 2. Distribution of Radioactivity during Incubation of Labeled Kitazin P with *P. oryzae*.

upper: initial concentration is 3 μ g/ml, lower: initial concentration is 50 μ g/ml.

▨ 32 P in mycelial cells, □ 32 P in medium.

Though Fig. 2 is indicated by the activity of 32 P, the result with 35 S is nearly the same as Fig. 2, suggesting P atom and S atom in the fungicide molecule behave together with one another.

Differences in the rate and mode of the metabolism between the susceptible and resistant clones were not found obviously at the lower fungicide concentration (3 μ g/ml), while the susceptible clone metabolized the fungicide to water soluble metabolites more rapidly at the higher concentration (50 μ g/ml). However, it must be noticed that the higher test concentration inhibits the growth of susceptible clone but it does not inhibit the growth of the resistant clone.

Another resistant clone against Kitazin P, which was selected under a pressure of Hinosan and used for the previous work,¹⁾ was also tested, and nearly the same result was obtained

with the above-mentioned resistant clone selected under a pressure of Kitazin.

Water soluble metabolites

Ion exchange separation of water soluble metabolites, for example, in the external solution after 48 hr incubation of the fungicide (3 µg/ml) with mycelial cells of the susceptible clone, is shown in Fig. 3. The main peak of the radioactivity of the metabolites (I) in the external solution was found at the second gradient of elution throughout the experiment. The ratio of labeled phosphorus and sulfur atoms in its peak on the chromatogram was found to be 1.00 ± 0.05 in all cases of the experiment. The peak coincided with that of an authentic sample of *O*, *O*-diisopropyl phosphorothioate which was co-chromatographed and detected by colorimetry of phosphate.

Two small peaks were found in the first gradient and in the second gradient subsequent to the peak of the main metabolite, respectively (Fig. 3). The former peak had labeled phosphorus atom but no sulfur atom, and was supposed to be inorganic phosphate (V), mono-isopropyl phosphate (IV) and/or diisopropyl phosphate (III) from the result of co-chromatography with inorganic phosphate and di-

isopropyl phosphate. The latter one had labeled phosphorus and sulfur atom in the ratio of 1:1, but could not be identified because of the lack of authentic samples. A later work,³⁾ which was conducted for the identification of the metabolites of Kitazin P on rice plants with the aid of gas-liquid chromatography, revealed that *S*-benzyl *O*-isopropyl phosphorothioate (II) is eluted at the same fractions as a latter small peak in the present work.

From mycelial cells, another small peak of metabolites having phosphorus and sulfur atoms was found, which passed through an ion exchange column at neutral pH before elution with 0.01N hydrochloric acid. The metabolite, therefore, was not exchanged by the anion exchange resin at neutral pH. A similar metabolite has been found in the previous work with Hinosan,¹⁾ but its structure still remains unknown, though the production of neutral conjugates of the fungicide metabolites with fungus components would be suspected.

Any significant difference was not found in the chromatographic pattern of the water soluble metabolites between the susceptible and resistant clones when the experiment was conducted at the lower initial concentration of the fungicide (3 µg/ml).

FIG. 3. Ion Exchange Separation of Water Soluble Metabolites in External Solution on Dowex 1.

Gradient I: 0.01N HCl to 0.1N HCl, II: 0.1N HCl+methanol (1:3) to 1N HCl+methanol (1:3), III: 1N HCl+methanol (1:3) to 6N HCl+methanol (1:3).

○—○ ³²P activity, ○····○ ³⁵S activity.

Toluene soluble metabolites

Several metabolites of the toluene soluble fraction were found on thin-layer chromatogram by radioautography (Fig. 4). Nearly the same pattern of radioautogram was obtained with either the susceptible clone or the resistant one, and with either mycelial cells or the external solution though the major part of the toluene soluble metabolites was located in mycelial cells.

Of these metabolites, production of two metabolites, coded R and S respectively, were more remarkable than the other metabolites (Fig. 4). Both of them have labeled sulfur and phosphorus atoms in the ratio of 1:1

FIG. 4. Radioautogram of TLC of Toluene Soluble Metabolites.
(developing solvent system: hexane+acetone 3:2)

FIG. 5. Vicissitudes of Kitazin P and its Toluene Soluble Metabolites in Culture of *Pyricularia oryzae*.

Change in the total amount of Kitazin P and metabolites R and S in both mycelial cells and external medium are shown in Fig. 5. The results suggest that R and S may be intermediate metabolites of Kitazine P.

Metabolites R and S were collected from thin-layer plates, extracted with ether, methylated with diazomethane, and identified by comparing with authentic samples of methoxy derivatives of Kitazin P by gas-liquid chromatography. The chromatograms, separated on a PEGA column at 200°C (Fig. 6) and a XE 60 column at 180°C, show that the methylated product of metabolite S is *m*-methoxy derivative and that of R contains a small amount of contaminated *m*-derivative but no *o*- and *p*-methoxy derivatives.

The acetylated products of R and S were also chromatographed on a PEGA column at 200°C and a XE 60 column at 180°C. The gas-liquid chromatograms indicate that acety-

FIG. 6. Gas-liquid Chromatographic Separation of *o*-, *m*- and *p*-Isomers of Methoxy Derivatives of Kitazin P (right) and Methylated Metabolite S (left) on PEGA at 200°C. (flow rate of N₂: 40 ml/min)

lated S is the *m*-acetoxy derivative of Kitazin P and acetylated R is not the *p*- or *o*-acetoxy derivative.

Finally, metabolite S was identified as *S*-*m*-hydroxybenzyl *O*,*O*-diisopropyl phosphorothioate (VI) by gas-liquid chromatography with a XE 60 column at 180°C (Fig. 7). Metabolite S labeled with ³⁵S was also identified by thin-layer chromatography with an authentic sample.

The whole toluene soluble fraction of the metabolic products of Kitazin P was gas-

FIG. 7. GLC of *m*-Hydroxy Derivative of Kitazin P (right) and Metabolite S (left).

(XE 60, 180°C, flow rate of N₂: 50 ml/min)

FIG. 8. GLC of Authentic Samples of the Isomers of *o*-, *m*- and *p*-Acetoxy Derivatives of Kitazin P (right) and Acetylated Products of Toluene Soluble Metabolites (left).

(XE 60, 180°C, flow rate of N₂: 50 ml/min)

chromatographed after methylation or acetylation to examine the existence of the *o*- or *p*-hydroxy derivative of Kitazin P in the metabolites, but they were not recognized in the chromatograms (Fig. 8).

DISCUSSION

From the metabolites and their vicissitudes found in this experiment, it may be concluded that the main metabolic pathway of Kitazin P in mycelial cells of *P. oryzae* is removal of the benzyl radical, and the resultant metabolite, *O*, *O*-diisopropyl phosphorothioate, is almost stable in 48 hr (Fig. 3). A part of the fungicide is hydroxylated at the *m*-position of the benzyl radical, and the resultant metabolite, *S*-*m*-hydroxybenzyl *O*, *O*-diisopropyl phosphorothioate, is further metabolized to water soluble metabolites (Fig. 9). Other hydroxylation products, *i.e.* the *o*- and *p*-isomers of *S*-hydroxybenzyl *O*, *O*-diisopropyl phosphorothioate were not recognized in this experiment.

Hydroxylation on the benzene ring has been reported to be caused at *p*-position in the case of the Hinosan metabolism by *P. oryzae*.¹¹ The result of the present experiment is different from that obtained with Hinosan.

Recently, fungal production of *m*-hydroxyphenylacetic acid was reported.^{4,51} It was produced in a large amount when appropriate species of fungi were cultured in a medium containing phenylacetic acid as a precursor.⁶¹ The position of hydroxylation of aromatic carboxylic acids by *Rhizoctonia solani* was reported to depend on the chemical structure of the precursor acids, that is, *m*-hydroxylation occurred when phenylacetic acid was used as a precursor and *p*-hydroxylation occurred in the case of benzoic acid.⁷¹

The results obtained in the present experiment on hydroxylation of organophosphorus fungicides have a similarity to the results on hydroxylation of aromatic carboxylic acid, *i.e.* the benzyl structure adjacent to an acidic functional group was hydroxylated on *m*-position and the phenyl structure adjacent to an acidic group was hydroxylated on *p*-position in both the cases with aromatic acids and organophosphorus compounds.

Detoxification of drugs in the cells of organisms has been one of the important mechanisms of resistance against the drugs. In such

FIG. 9. Metabolic Pathway of Kitazin P by Mycelia of *P. oryzae*.

a case, the metabolism related to the detoxification is more vigorous in resistant organisms than in susceptible ones. The metabolism of Kitazin P to less toxic metabolites in the mycelial cells of a resistant clone was almost at the same level as that in a susceptible one, or rather slower at a higher concentration of the fungicide. However, at the higher concentration, the two test clones were under different physiological conditions, because the test concentration inhibits only the growth of the susceptible clone but not that of the resistant clone. The present results, at least, does not support a possibility of the appearance of resistance by increased detoxification of the fungicide.

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