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Inhibition of Methionine Biosynthesis in *Botrytis cinerea* by the Anilinopyrimidine Fungicide Pyrimethanil

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Abstract: When mycelium of *Botrytis cinerea* was treated with low concentrations of the anilinopyrimidine fungicide pyrimethanil the total amount of free amino acids increased. Qualitative variations were also induced: alanine, glutamine, lysine, glycine, histidine, asparagine, arginine, threonine and moreover, α-aminobutyrate and β-alanine were accumulated; cyst(e)ine, valine, leucine and citrulline were reduced. When mycelium of *B. cinerea* was incubated with $Na_2[^{35}S]O_4$, pyrimethanil at 1·5 μM induced a decrease of [^{35}S]methionine and simultaneously an increase of [^{35}S]cystathionine. These data indicate that the anilinopyrimidine fungicide pyrimethanil inhibits the biosynthesis of methionine and suggest that the primary target could be the cystathionine β-lyase.

Key words: pyrimethanil, anilinopyrimidine, *Botrytis cinerea*, methionine biosynthesis

1 INTRODUCTION

Pyrimethanil, along with CGA 219417 (cyprodinil) and mepanipyrim, belongs to a novel chemical class, the anilinopyrimidines (Fig. 1). They are broad-spectrum fungicides highly effective against all strains of *Botrytis* and show no cross-resistance to any current commercially available botryticides.[1–3] This suggests that anilinopyrimidine fungicides have a new mode of action.

Tested *in vitro* on *Botrytis* spp., pyrimethanil does not show any effect on respiration, lipid peroxidation, osmotic stability or on biosynthesis of protein, RNA, DNA, chitin or ergosterol.[1]

In *Botrytis fabae* Sardina and *B. cinerea* Pers. ex Fr., pyrimethanil has been reported to inhibit the secretion of cell-wall-degrading enzymes required for infection at concentrations lower than those needed to inhibit growth.[4,5] This has also been reported for mepanipyrim.[6,7] This mode of action has been suggested as the primary one for pyrimethanil.[5]

Nevertheless, it has been shown that pyrimethanil and the other anilinopyrimidine fungicides are effective on *B. cinerea* growth at low concentrations in culture media lacking amino acids. Addition of several different

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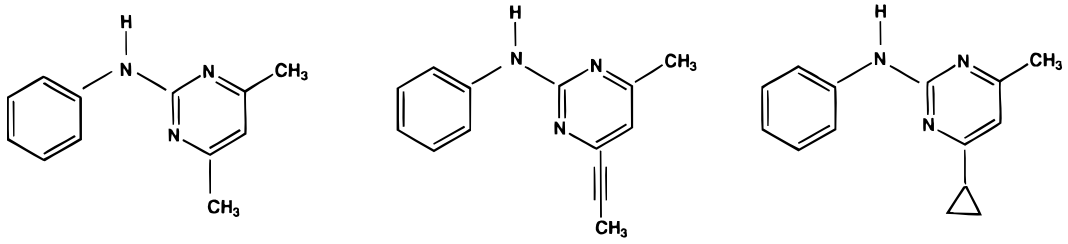

1 **2** **3**

Fig. 1. Chemical structures of the anilinopyrimidine fungicides: (**1**) pyrimethanil; (**2**) mepanipyrim; (**3**) CGA 219417

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amino acids to the culture media reduced the growth-inhibitory activity of pyrimethanil. Among these, methionine did not reverse the activity of pyrimethanil completely but exhibited the highest reversal activity, suggesting a direct effect of pyrimethanil on the methionine biosynthesis pathway in *B. cinerea*.[8,9]

In this paper the methionine biosynthesis inhibition by pyrimethanil in liquid cultures of *B. cinerea* is described. The results are discussed in relation to the metabolism of sulfur amino acids generally proposed for filamentous fungi.

2 MATERIALS AND METHODS

2.1 Chemicals and fungi

Technical grade pyrimethanil was a gift from AgrEvo (UK). This compound was dissolved in absolute ethanol and diluted to give a final solvent concentration of 5 ml litre^{-1}. Carrier-free sodium [^{35}S]sulfate (38·85–59·2 TBq mmol^{-1}) was purchased from Dupont NEN. *N*-Ethylmaleimide and lithium hydroxide monohydrate were from Merck. 5-Sulfosalicylic acid was from Serva. All other chemicals were from Sigma.

All the studies were carried out with the strain 'L' of *B. cinerea* isolated near Bordeaux.[9] This strain was maintained on potato-sucrose agar at 18°C. Spores of 15-day cultures were used as inoculum.

2.2 Liquid culture methods

Liquid cultures were prepared by inoculating 7×10^5 spores ml^{-1} in the medium previously described;[10] yeast extract was omitted in some experiments. Cultures were shaken (orbital shaker; 150 rev min^{-1}) for 24 h at 23°C. To obtain more mycelium, the cultures were diluted 50 times with fresh medium and incubated one more day. Finally, mycelial pellets were harvested, resuspended in fresh medium and treated with pyrimethanil diluted in ethanol as described in Section 2.1. Each fungal sample was lyophilized and weighed.

For $Na_2[^{35}S]O_4$ incorporation, only young mycelium was used in the medium without yeast extract and also without any sulfate source; the sulfates were replaced by equivalent amounts of chlorides for ammonium and magnesium sources.

2.3 Analysis of mycelial amino acid pools

Each sample (60 mg of lyophilized mycelium) was ground in liquid nitrogen and homogenized in ice-cold aqueous 5-sulfosalicylic acid (30 g litre^{-1}; 3 ml), a protein-precipitating agent particularly suitable for extracting free amino acids in our experimental conditions.[11] After 6000*g* centrifugation at 4°C for 10 min, the supernatant was harvested. The pH was adjusted to 2 by adding a few drops of a solution of concentrated lithium hydroxide. The supernatant can be kept a long time at −80°C.

The free amino acids were separated by ion exchange chromatography (Biotronik LC 5001 analyzer, Maintel, Germany; lithium citrate buffers and ninhydrin post-column derivatization), identified using a mixture of amino acids (Benson standard P-ANB, Reno, NV) and quantified using P. E. Nelson 2100 software (Perkin-Elmer).

2.4 Determination of oxidized and reduced glutathione

The reduced (GSH) and oxidized (GSSG) glutathione contents of mycelium and medium were determined by a fluorometric method according to Barak and Edgington.[12] The determinations were made in duplicate on 50-mg samples of lyophilized mycelium and on 0·2-ml aliquots of medium.

2.5 Sodium [^{35}S]sulfate incorporation

Young cultures were prepared by inoculating spores in 10 ml of medium without yeast extract and without any sulfate source. After an incubation time of 5 h, the cultures were treated with pyrimethanil diluted in ethanol as described in Section 2.1 and 370 KBq of $Na_2[^{35}S]O_4$ was added. After 16 h, the cultures were filtered and washed with 10 ml of medium. The mycelium was freeze-dried and weighed. The radio-labelled compounds were extracted according to Antoniewski and Robichon-Szulmajster.[13] After filtration the extracts were lyophilized and dissolved in volumes of water in proportion to the mycelial weights (0·1 ml mg^{-1}). The separation was carried out by thin-layer chromatography (TLC) on cellulose plates (0·1 mm, MN 300, Macherey-Nagel). The plates were run in butanol + acetic acid + water (12 + 3 + 5 by volume). The positions of radioactive bands were determined by autoradiography and the plates were scanned for radioactivity using a Berthold (LB 2832) automatic TLC-linear analyzer. The bands were tentatively identified by co-chromatography with authentic standards and by using different staining procedures for sulfur-containing compounds.[14]

3 RESULTS

3.1 Effect of pyrimethanil on mycelial growth

This study was carried out in triplicate on liquid cultures of *B. cinerea*. The reversal activity of yeast extract on pyrimethanil inhibition was confirmed (Table 1).[8,9] Without yeast extract, the pyrimethanil EC_{50} value (Effective concentration reducing the growth by half) was between 1·5 and 5 μM. With yeast extract, the EC_{50}

TABLE 1
Effect of Pyrimethanil on Liquid Cultures of *Botrytis cinerea* after an Incubation Time of 24 h (Two Experiments)

	Mycelial growth rate (percentage of control)	
Pyrimethanil (μM)	*Yeast Extract*	*No yeast extract*
0·5	100	83
1·5	58	52
5	58	39
15	52	31
50	53	3

value was above 50 μM, but the inhibitory activity of pyrimethanil at 1·5 μM was unaffected. Only at the high pyrimethanil concentrations did the antagonism become apparent. For this reason, all subsequent experiments with pyrimethanil were performed in the medium without yeast extract.

3.2 Effect of pyrimethanil on amino acid pools

This study was carried out twice. As shown in Table 2, pyrimethanil produced an increase in the total amount of free amino acids. In the mycelium of *B. cinerea*, alanine, γ-aminobutyric acid (GABA) and glutamine were the most abundant free amino acids. Among them, only alanine and glutamine were accumulated in the presence of pyrimethanil. Other amino acids, such as lysine, glycine, histidine, asparagine, arginine and threonine, were also accumulated in an equivalent ratio (2 to 3 times), but the greatest accumulations (4 to 10 times) were observed for α-aminobutyric acid and β-alanine, which were, in the control, among the less-abundant

TABLE 2
Composition of Amino Acid Pools in *Botrytis cinerea* after 24-h Treatment with Pyrimethanil

	Experiment 1			*Experiment 2*		
	(*nmol* mg^{-1} *dw*)			(*nmol* mg^{-1} *dw*)		
Amino acid[a]	*PO*[b]	*P1·5*[b]	*Ratio P1·5/PO*	*PO*[b]	*P5*[b]	*Ratio P5/PO*
Ala	42·44	70·32	1·7	31·15	88·10	2·8
GABA	21·31	24·21	1·1	21·90	21·97	1·0
Gln	20·86	31·89	1·5	15·00	34·31	2·3
Cys	10·72	9·03	0·8	6·33	5·35	0·8
Arg	9·70	15·47	1·6	7·58	14·72	1·9
Glu	5·86	5·12	0·9	5·41	6·95	1·3
Leu	5·44	4·45	0·8	2·96	2·47	0·8
Val	3·99	2·72	0·7	2·39	2·10	0·9
Lys	3·79	7·96	2·1	2·46	9·18	3·7
Gly	3·45	6·51	1·9	2·82	8·49	3·0
Asp	2·80	3·83	1·4	3·85	3·99	1·0
Ser	2·65	4·00	1·5	2·73	5·12	1·9
Orn	2·62	4·49	1·7	2·15	3·09	1·4
Pro	2·00	2·23	1·1	1·87	2·68	1·4
Thr	1·75	2·74	1·6	1·94	3·86	2·0
Phe	1·63	1·79	1·1	0·46	0·91	2·0
His	1·32	2·41	1·8	0·79	2·34	3·0
Asn	1·07	2·58	2·4	1·94	3·34	1·7
Cysth	0·57	0·55	1·0	0·50	0·64	1·3
Ileu	0·65	0·85	1·3	0·69	0·96	1·4
Aabut	0·20	0·84	4·2	0·20	2·39	11·9
Cit	1·72	0·70	0·4	nd[c]	nd	—
Tyr	0·20	0·30	1·5	0·24	0·30	1·2
Aaad	0·19	0·11	0·6	0·08	0·15	1·9
BAla	nd	nd	—	nd	0·36	—
Total	146·93	205·10		115·44	223·77	

[a] GABA, γ-aminobutyric acid; Cysth, cystathionine; Aabut, α-aminobutyric acid; Aaad, α-aminoadipic acid; BAla, β-alanine.
[b] PO, control; P1.5, pyrimethanil 1·5 μM; P5, pyrimethanil 5 μM.
[c] nd, non-detectable.

TABLE 3
Effect of Pyrimethanil on the Level of Glutathione produced by Liquid Cultures of *Botrytis cinerea* in the Medium

	GSH (*nmol mg*$^{-1}$ *dw*)			*GSSG* (*nmol mg*$^{-1}$ *dw*)		
Treatments	*6 h*	*16 h*	*24 h*	*6 h*	*16 h*	*24 h*
Control	2·4	1·5	1·2	2·5	1·7	1·5
Pyrimethanil (1·5 μM)	2·4	1·9	1·7	2·8	2·3	2·0
Pyrimethanil (15 μM)	2·5	2·1	1·8	2·8	2·5	2·2

amino acids. Among the other free amino acids, pyrimethanil caused lesser increases, no changes, or slight reductions; this last effect was observed for cyst(e)ine, valine, leucine and citrulline. Methionine, because of its low abundance, was hardly measurable.

3.3 Effect of pyrimethanil on glutathione

This study was carried out twice and the results of the two experiments were close together. As shown in Table 3, we only found a slight increase of GSH and GSSG in the culture medium after a treatment with pyrimethanil. In the lyophilized mycelium we were unable to find any difference between control and treated samples.

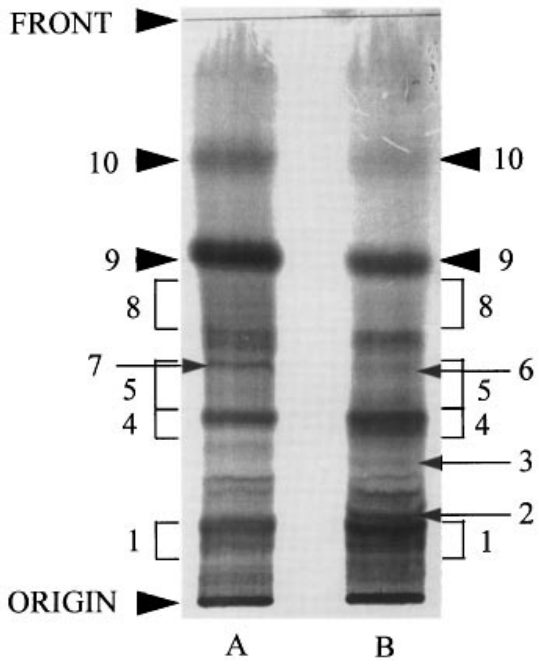

Fig. 2. Autoradiogram of TLC separation of sulfur-containing compounds synthesized from $Na_2[^{35}S]O_4$ in *Botrytis cinerea* mycelium in the absence and presence of pyrimethanil. A: control; B: pyrimethanil 1·5 μM. Migration areas: 1, GSSG + cystine; 2, cystathionine; 3, taurine; 4, homocystine + hypotaurine + methionine sulfoxide; 5, GSH + cysteine (6) + a SAM degradation product (7); 8, homocysteine; 9, methionine; 10, a SAM degradation product: probably methylthioadenosine. The identities of compounds in other bands are unknown.

3.4 Effect of pyrimethanil on sodium [^{35}S]sulfate incorporation

Separation of the sulfur amino acids and related compounds resulted in a typical separation pattern as illustrated by the autoradiogram presented in Fig. 2, and the R_f values are given in Table 4. Methionine was the metabolite which was the more easily separated without ambiguity (**9**, Fig. 2). Cystathionine was also distinguished just above cystine + GSSG (**2**, Fig. 2). Other compounds were more difficult to identify because of oxidation (homocysteine, cysteine, GSH), degradation (*S*-adenosyl-L-methionine, SAM, because of boiling upon extraction), low abundance (taurine) or because of

TABLE 4
Effect of Pyrimethanil on the Incorporation of Sodium [^{35}S]Sulfate in Liquid Cultures of *Botrytis cinerea*

		Radioactivity (%)	
Sulfur-containing metabolite	R_f *value*[a]	*Control*[b]	*Pyrimethanil*[b] (*1·5 μM*)
Methylthioadenosine	0·75	4·8	2·0
Methionine	0·58	21·5	6·0
Homocysteine	0·50	6·0	2·8
SAM degradation product	0·41		
+ Cysteine	0·40	8·1	5·8
+ GSH	0·36		
Methionine sulfoxide	0·31		
+ Hypotaurine	0·31	5·2	6·7
+ Homocystine	0·28		
Cystathionine	0·14	3·2	5·4
Cystine	0·13	11·8	24·4
+ GSSG	0·12		
Other compounds		39·4	46·9

[a] See Fig. 2.
[b] The total radioactivity put down on the plate was 2·32 KBq for the control and 2·27 KBq for the pyrimethanil treatment.

poor separation (methionine sulfoxide/hypotaurine/homocystine and cystine/GSSG). However, it appears that in the treated lane (B, Fig. 2) the radioactivity decreased in methionine and two degradation products of SAM (**7**, **10**; Fig. 2) and increased in the bands corresponding to hypotaurine, taurine, cystathionine and cystine/GSSG.

The quantitative results are shown in Table 4. In the mycelium of *B. cinerea* treated by pyrimethanil at 1·5 μM, methionine level was decreased approximately threefold in comparison to the control, whilst cystathionine and cystine/GSSG increased approximately twofold. Homocysteine and methylthioadenosine levels were also significantly decreased. These results confirm those presented previously.[15] In the present experiment, because of a better TLC separation, we were able to demonstrate more convincingly an accumulation of cystathionine.

4 DISCUSSION

When treated by pyrimethanil, the total amount of free amino acids extracted from the mycelium of *B. cinerea* increased. A similar effect, which could be due to a change in protein turnover, has been reported in plants treated by herbicides known to be amino acid biosynthesis inhibitors.[16] However the amino acid pools pattern obtained in *B. cinerea* treated by pyrimethanil was different from any of those described in plants by Singh and Shaner.[16] This suggests that anilinopyrimidine fungicides have a mode of action different from those of the tested herbicides, which inhibit the biosynthesis of branched-chain amino acids (e.g. imazaquin), aromatic amino acids (e.g. glyphosate), histidine (e.g. amitrole) or glutamine (e.g. phosphinothricin).

Only the reversal activity obtained with methionine and homocyst(e)ine[8,9] together with the non-reversal effect of cystathionine,[9] seem to be directly linked with the action of pyrimethanil on *B. cinerea* metabolism. This was confirmed in our ^{35}S-labelling experiments, in which we observed the depletion of methionine and the accumulation of cystathionine. All these data suggest that pyrimethanil and, most likely, the other anilinopyrimidine fungicides may inhibit cystathionine β-lyase as proposed by Masner *et al.* for CGA 219417.[8]

According to the literature,[17–19] the methionine biosynthesis pathway and the relevant enzymes in filamentous fungi can be represented as in Fig. 3. This pathway has been established in *Neurospora crassa* Shear & Dodge and in *Aspergillus nidulans* (Eidam) Winter. In these fungi, the main route for homocysteine biosynthesis has been ascertained to be the cystathionine pathway[19] *via* *O*-acetyl-L-serine (OAS) sulfhydrylase, cystathionine γ-synthase and cystathionine β-lyase (enzymes 1, 2 and 3, Fig. 3); the *O*-acetyl-L-homoserine (OAH) sulfhydrylase (enzyme 4, Fig. 3) has been proved to be an alternative homocysteine synthase. Inhibition

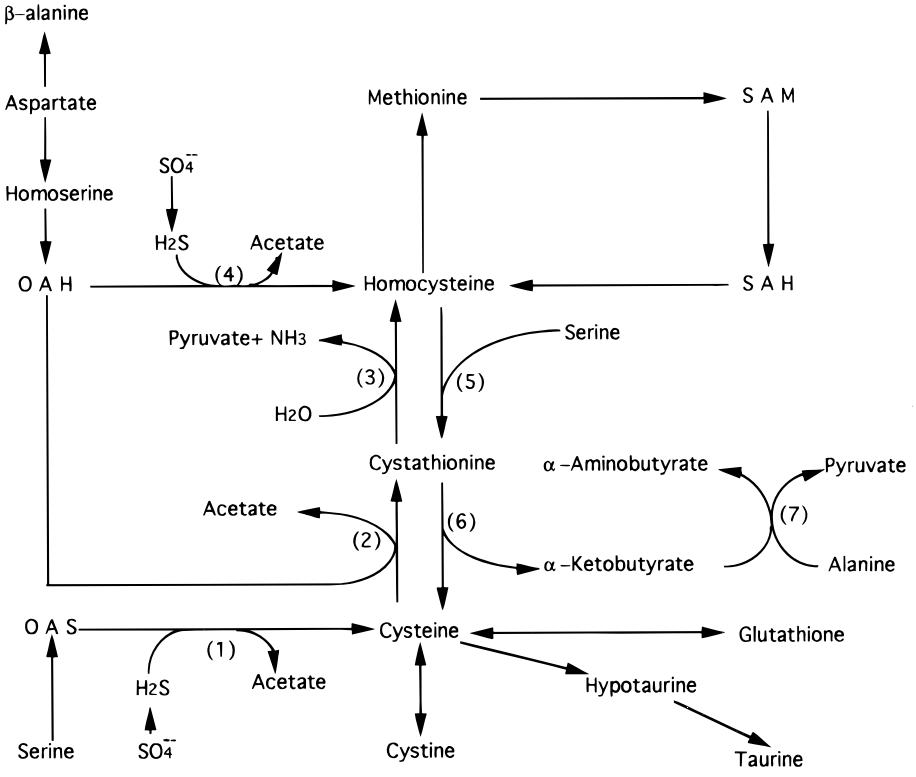

Fig. 3. Methionine biosynthesis pathway modified from Paszewski *et al.*[17] OAS: *O*-acetyl-L-serine; OAH: *O*-acetyl-L-homoserine; SAM: *S*-adenosyl-L-methionine; SAH: *S*-adenosyl-L-homocysteine. Enzymes: (1), OAS sulfhydrylase; (2), cystathionine γ-synthase; (3), cystathionine β-lyase; (4), OAH sulfhydrylase; (5), cystathionine β-synthase; (6), cystathionine γ-lyase; (7), transaminase.

of cystathionine β-lyase (enzyme 3, Fig. 3) in *B. cinerea* by pyrimethanil could support the observation that methionine biosynthesis is not completely stopped because the alternative route generates homocysteine directly from OAH. This inhibition could also explain that, in a sulfate starvation diet, like in our ^{35}S-labelling experiments, *B. cinerea* treated by pyrimethanil gives a larger effect on the accumulation of cystathionine when compared to our experiments on amino acid pools which were done in non-starved conditions. The sulfate starvation strongly decreases the OAH sulfhydrylase activity and furthers the cystathionine pathway, which consequently tends to increase the accumulation of cystathionine. Nevertheless, in our amino acid pools experiments, the accumulation of α-aminobutyrate and β-alanine (Table 2) was still detected as a consequence of pyrimethanil treatment. These data indicate that the accumulation of cystathionine resulting from the pyrimethanil effect cannot be due to an inhibition of cystathionine γ-lyase (enzyme 6, Fig. 3) as reported for a mutant strain of *Saccharomyces cerevisiae* Meyer ex Hansen deficient in cystathionine γ-lyase.[20] The absence of accumulation of cystathionine in our experiments on amino acid pools could also be explained by the lack of sensitivity of the method used. Furthermore, such a method assesses all of the pool of cystathionine and not only the increase of this pool after the treatment by pyrimethanil. The same comment could also be made about the determination of the pools of GSH and GSSG.

Our results can be compared to those reported by Giovanelli *et al.*[21] on the inactivation of cystathionine β-lyase by rhizobitoxine (isolated from *Rhizobium japonicum* Buch) in corn seedlings. In ^{35}S-labelling experiments they obtained an accumulation of cystathionine as a result of rhizobitoxine treatment, but hardly any decrease in methionine level. The amount of cysteine and GSH also increased, probably because direct sulfhydrylation (enzyme 4, Fig. 3) outweighed transsulfuration *via* cystathionine. Similarities also exist between anilinopyrimidine fungicides and the experimental anilide fungicide SC-0858, a botryticide whose action is reversed by methionine, homocysteine and cystathionine.[22] Cystathionine γ-synthase has been suggested as a possible target. However in ^{35}S-labelling experiments conducted in *N. crassa*, no changes in the labelling of methionine or cystathionine were detected.

In our mycelial growth study, the unachieved reversal effect of yeast extract could be explained by another mode of action of pyrimethanil different from cystathionine β-lyase inhibition. This second mode of action could be inhibition of the secretion of cell-wall degradation enzymes.[4,5] But another explanation could be also put forward. Several isoforms of cystathionine β-lyase have been reported in higher plants.[23] If there are also several isoforms of this enzyme in *B. cinerea*, it might be possible that pyrimethanil inhibits only one of these.

In conclusion, the present study on the mode of action of pyrimethanil suggests that cystathionine β-lyase could be a new target for fungicides. To obtain more details about the accumulated metabolites observed in our separation by TLC, we are working on an improved separation using an HPLC method. To confirm the proposed mode of action of anilinopyrimidines, further studies will be conducted on the effect of pyrimethanil on cystathionine β-lyase isolated from *B. cinerea*.

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