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Characterization of Metabolites of Fungicidal Cymoxanil in a Sensitive Strain of *Botrytis cinerea*

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The metabolism of cymoxanil [1-(2-cyano-2-methoxyiminoacetyl)-3-ethyl urea] by a very sensitive strain of *Botrytis cinerea* toward this fungicide was studied by using [2-^{14}C]-cymoxanil. Labeled cymoxanil was added either in a culture of this strain or in its enzymatic extract. The main metabolites, detected in biological samples, were isolated and identified by mass and NMR spectrometry. Their identification allowed us to show that this strain quickly metabolized cymoxanil according to at least three enzymatic pathways: (i) cyclization leading, after hydrolysis, to ethyl parabanic acid, (ii) reduction giving demethoxylated cymoxanil, and (iii) hydrolysis and reduction followed by acetylation leading to *N*-acetylcyanoglycine. In a cell-free extract of the same strain, only the first and the second enzymatic reactions, quoted above, occurred. Biological tests showed that, among all the metabolites, only *N*-acetylcyanoglycine is fungitoxic toward this sensitive strain.

KEYWORDS: Metabolism; fungicide; cymoxanil; mass spectrometry; *Botrytis cinerea*

INTRODUCTION

Cymoxanil, 1-(2-cyano-2-methoxyiminoacetyl)-3-ethyl urea, **Ia** is a fungicidal cyanooxime effective against plant diseases caused by fungi belonging to the Perenosporales. In practice, it is mainly used against downy mildew of vine (*Plasmopara viticola*) and potato blight (*Phytophthora infestans*) *1*. Although this compound is not effective against the gray mold, in laboratory tests, a few strains of *Botrytis cinerea* Pers. Ex Fr. appeared sensitive *2–4*. In previous works *5, 6*, we showed, by monitoring the disappearance of cymoxanil in culture media of different phenotypes of *B. cinerea*, that the sensitivity of strains toward cymoxanil was correlated to its capacity to metabolize it. In a culture of a highly sensitive strain, it was completely and quickly metabolized (half-life time $\leq$ 2 h). This correlation suggests that cymoxanil is a profungicide. That means that it must initially undergo a biotransformation by the fungus to be activated into one (or several) fungitoxic metabolite(s).

The cymoxanil metabolism has been study in several conditions *7, 8*. Cymoxanil is rapidly degraded in neutral to alkaline solutions, and its rate of degradation is strongly dependent on both pH and temperature *8, 9*. Morrica et al. *9* reported that the degradation in solution takes place according to three competing degradation pathways. Two pathways involve an initial cyclization to form either a six-membered ring compound, 1-ethyl-2,4-dioxo-5-methoxyimino-6-imino(1*H*,3*H*)pyrimidine **1**, or a five-membered ring compound, 1-ethyl-5-methoxyamino-5-cyano-2,4-dioxoimidazolidine **2**, which is thereafter rapidly degraded into 1-ethyl-5-methoxyimino-2,4-dioxoimidazolidine (**IIa**) and then 1-ethyl-2,4,5-trioxoimidazolidine (**IIb**). The third degradation pathway involves a direct cleavage of the C-1 amide bond to give 2-cyano-2-methoxyimino acetic acid **3** (**Figures 1** and **2**). All three pathways eventually lead to the formation of oxalic acid. In soil, cymoxanil is degraded by both hydrolytic and microbial actions with half-life time < 2 days *8*. The degradation in soil also follows a series of C5- and C6-cyclization and/or hydrolysis reactions similar to those observed in solution. Thus, during the initial phase of degradation, the metabolites **1**, **3**, and 1-ethyl-5-methoxyamino-2,4-dioxo-5-imidazolidinecarboxamide are detected as major soil degradation products and 2-cyano-2-hydroxyiminoacetic acid **4** and **IIa** as minor products. Cymoxanil metabolism in plants (grapes, tomato fruit, and potatoes) *10–12* and in animals (rats) *13* was also reported. In these living organisms, it is rapidly metabolized, mainly to glycine which is reincorporated into natural components such as sugars. In the case of rats, most of the metabolites formed are eliminated via urinary excretion. In addition to glycine, metabolites synthesized by the pathways previously quoted such as the hydrolysis products, **3** and **4**, and the cyclization products, **1** and **IIb**, are also identified in urine.

In this article, we present our results concerning the biotransformation of cymoxanil by a highly sensitive strain of *B. cinerea*. Previously *6*, we were able to follow the disappearance of

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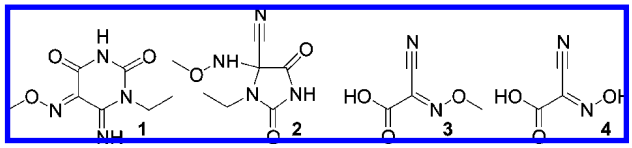

Figure 1. Structures of cymoxanil metabolites according to refs *8, 9, 13*.

cymoxanil but not the appearance and evolution of the main metabolites, in particular in the mycelium. For this reason, we used [2-^{14}C]-cymoxanil, which was added in a culture medium containing the mycelium of a highly sensitive strain and in an enzymatic extract obtained from its mycelium. The labeled metabolites were directly monitored by thin-layer chromatography (TLC). After these chromatographic analyses, the main metabolites were isolated from biological samples and identified by mass and NMR spectrometry analyses.

MATERIALS AND METHODS

Chemicals. Cymoxanil **Ia** was prepared according to a method described in the literature *13* (**Figure 2**). Cymoxanil with ^{15}N into the oxime function and deuterium into the methoxy group were respectively obtained with sodium ^{15}N-nitrite- and d_6-dimethyl sulfate. The product **IIb** *14, 15* was obtained by slowly adding oxalylchloride (0.87 mL, 10 mmol) in a solution of ethyl urea (0.88 g, 10 mmol) in 15 mL of Et_2O. The mixture was refluxed during 2 h. After evaporation, 20 mL of H_2O was added. The reaction mixture was extracted with AcOEt. The solvent was evaporated, and the product **IIb** was obtained as a solid (yield = 80%). *N*-Acetylcyanoglycine **IId** was prepared as follows *16, 17*: ethyl acetamidocyanoacetate (1.7 g, 10 mmol) was added to a solution of KOH (0.56 g, 10 mmol) in 5 mL of H_2O and 10 mL of EtOH and stirred at room temperature for 3 days. Most of the solvent was then removed in vacuo, and 3 mL of H_2O was added to the residue. This aqueous solution was extracted with CH_2Cl_2 to remove the unreacted ester. The aqueous layer was adjusted at pH 0.5 with concentrated HCl to precipitate **IId**. This one was filtered and washed with ice water and then Et_2O to give 0.6 g of **IId** (yield = 42%). ^{1}H NMR 300 MHz (DMSO-d_6) δ (ppm) 1.95 (s, 3, CH_3), 5.6 (d, 1, J = 6 Hz, CHNH), 9.1 (d, 1, J = 6 Hz, CHNH), 13.8 (s, 1, OH); ^{13}C NMR 75.5 MHz (DMSO-d_6) δ (ppm) 22.82 (CH_3), 44.96 (CH), 117.15 (C≡N), 166.47 (C═O), 170.74 (C═O).

[2-^{14}C]-Cymoxanil provided by Du Pont de Nemours had a radiochemical purity of about 95% and a specific activity of 2.79 MBq/mg. Radiolabeled cymoxanil ethanolic solution used in our studies was prepared by dissolving unlabeled cymoxanil (1.75 mg, 8.75 μmol) and [2-^{14}C]-cymoxanil (0.25 mg, 1.25 μmol, 0.67 MBq/mg) in 500 μL of EtOH.

[2-^{14}C]-Cymoxanil Metabolism in a *B. cinerea* Culture. In our study, we used the strain "L" of *B. cinerea* collected near a Bordeaux vineyard that is very sensitive to cymoxanil *5*. It was maintained on a malt agar medium containing yeast extract. After 15 days under light, mycelial pellets of *B. cinerea* were prepared by seeding about 10^6 conidia/mL in a yeast extract liquid medium *18* and incubating at 23 °C on a rotary shaker at 150 rpm for 24 h. Then, 3 mL of this culture was taken to inoculate 150 mL of yeast extract medium in 250-mL Erlenmeyer flasks and incubated again for 24 h as described above to obtain the mycelium used in the cultures. The mycelium was harvested by filtration through a bolting cloth of 100 μm, washed with 50 mL of H_2O, and resuspended in 150 mL of H_2O. The [2-^{14}C]-cymoxanil metabolism studies were carried out on 5 mL of these suspensions dropped in 25-mL Erlenmeyer flasks. After 25 μL of 2 × 10^{-2} M ethanolic solution of cymoxanil was added, the treated cultures were incubated as described above. After 0, 15, 30, 60, 120, 240, and 360 min, each mycelium was separated by filtration from its culture medium. The mycelium was washed with 2.5 mL of H_2O. Both the culture medium and the mycelium were analyzed by TLC deposit. The filtrate was lyophilized and dissolved in 1.25 mL of a mixture of H_2O−EtOH (75:25, v/v), and then an aliquot of 50 μL of this solution was deposited on a plate. The mycelium was extracted with 5 mL of a mixture

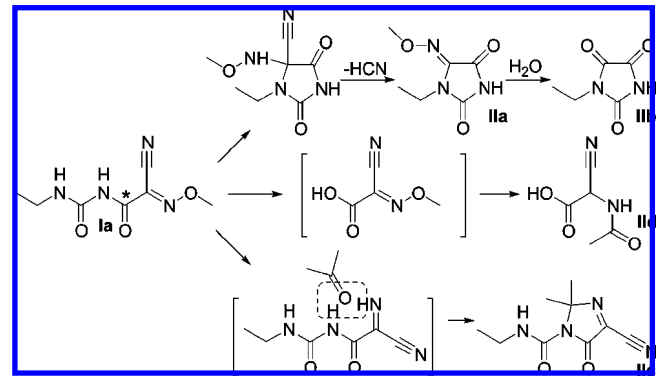

Figure 2. Cymoxanil metabolism in a culture of the strain "L" of *B. cinerea* (* indicates ^{14}C localization in radiolabeled cymoxanil).

acetone−EtOH (50:50, v/v). After 48 h at 4 °C, it was filtered through a Whatman GFA filter and washed with 5 mL of acetone−EtOH (50:50, v/v). The filtrate was evaporated in vacuo. Then, 200 μL of acetone−EtOH (50:50, v/v) was added and 50 μL was deposited on a plate.

[2-^{14}C]-Cymoxanil Metabolism in a Crude Enzyme Extract. Mycelial pellets of *B. cinerea* strain "L" were prepared by seeding 150 mL of yeast extract liquid medium (about 10^6 conidia/mL) in a 250-mL Erlenmeyer flask *18* and incubating at 23 °C on a rotary shaker at 150 rpm for 24 h. The mycelium was harvested by filtration through a bolting cloth of 100 μm and resuspended in 1200 mL of yeast extract liquid medium in a 2000-mL Erlenmeyer flask and incubated again for 24 h as described above. The mycelium was harvested by filtration through a bolting cloth of 100 μm, washed with H_2O, and lyophilized for 24 h. This one was kept cold until use. Cell-free extracts were prepared by grinding the mycelium (500 mg) in a mortar with liquid nitrogen. To this ground mycelium was added 8 mL of cold phosphate buffer (50 mM, pH = 6.65), containing ethylenediaminetetraacetic acid (1 mM) and dithiothreitol (1 mM). This mixture was crushed with Potter-Elvehjem for 2 min, and 8 mL of phosphate buffer was added. After centrifugation at 9000 rpm at 4 °C for 30 min, the supernatant obtained was collected under vacuo through a 0.22-μm membrane (PVDF) in an Erlenmeyer flask and kept in ice, giving a filtrate to be used in all enzyme assays. In a tube of 2 mL were added 500 μL of crude enzyme extract and then 20 μL of 2 × 10^{-2} M ethanolic solution of cymoxanil. The mixture was incubated at 38 °C, and after 0, 10, 20, and 30 min, the enzymatic reactions were stopped by addition of 500 μL of acetone. The samples were centrifuged at 5000 rpm during 10 min. The supernatant (1 mL) was filtered through 0.2-μm membranes (regenerated cellulose) before deposit of an aliquot of 50 μL.

TLC Procedure. The disappearance of [2-^{14}C]-cymoxanil, correlated with the appearance of labeled metabolites in the cultures (medium and mycelium) and in the cell-free extracts of *B. cinerea*, was followed according to time, by TLC. This technique has the advantage to avoid the samples preliminary purification and the problematic extraction of the polar metabolites. Chromatographies were performed both on silica gel (60 F_{254} plates, 0.2-mm thick, 10 × 10 cm, Merck KGaA Germany, cat no. 1.05628) and reverse-phase (RP 18WF_{254s} plates, 0.2-mm thick, 10 × 10 cm, Merck KGaA Germany, cat no. 1.13124). The presence of the most polar products was investigated by ion-pairing TLC. This technique consists in pretreating the reverse-phase plates with quaternary ammonium salts before the deposit of samples to separate the products from the medium during elution and also to slow down their migration. Before samples were laid down, the reverse-phase plates were impregnated by vertical dipping for 5 min in a methanolic solution of tetrabutylammonium bromide at the concentration of 70 mM and then dried at 80 °C for 5 min. Fifty microliters of each sample was deposited onto HPTLC plates which were eluted in a horizontal development chamber. The silica gel plates were eluted with a solution of hexane−AcOEt−AcOH (70:30:1, v/v/v) up to 70 mm and the reverse-phase plates with a solution of phosphate buffer (0.01 M, pH = 6.0)−MeOH (55:45, v/v) up to 50 mm. The plates were scanned for radioactivity using a Berthold (LB 2832) automatic TLC-linear analyzer and then were subjected to autoradiography to locate the radioactive bands.

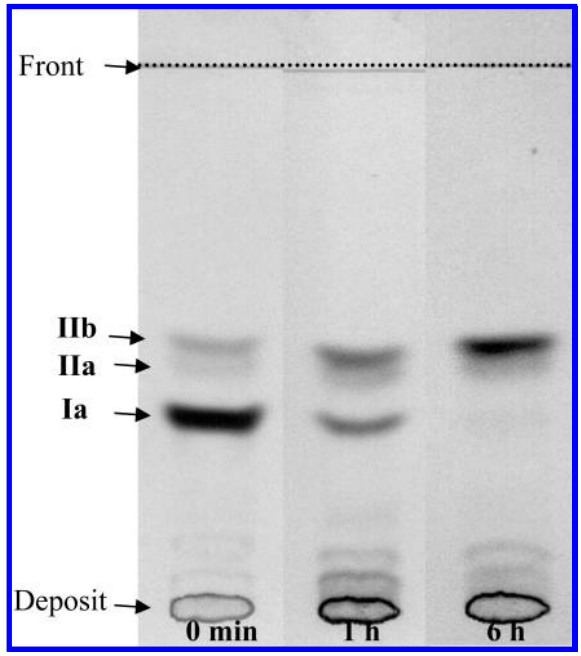

Figure 3. TLC monitoring of [2-^{14}C]-cymoxanil metabolism in the culture medium: Autoradiography of a Si chromatoplate. Migration with hexane−AcOEt−AcOH (70:30:1, v/v/v) up to 70 mm.

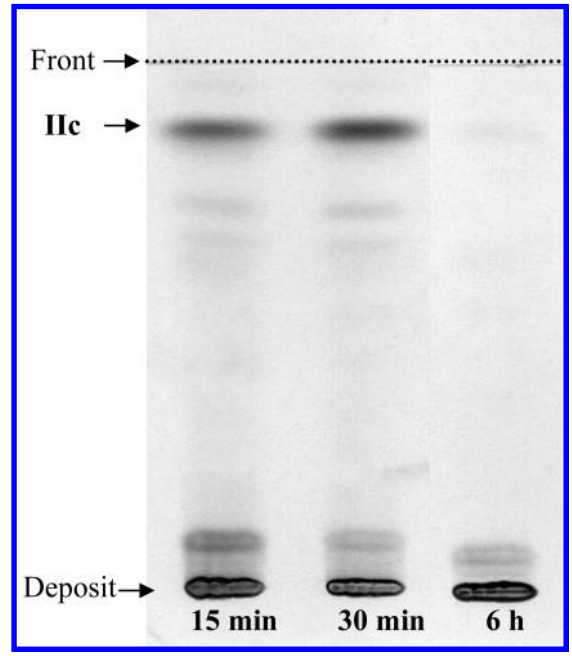

Figure 4. TLC monitoring of [2-^{14}C]-cymoxanil metabolism in the mycelium: Autoradiography of a Si chromatoplate. Migration with hexane−AcOEt−AcOH (70:30:1, v/v/v) up to 70 mm.

LC−MS and NMR Analyses. LC−MS and LC−MS/MS studies were carried out with a Waters (Alliance 2695) HPLC system coupled to a Quattro LC instrument (Micromass) equipped with an electrospray ionization (ESI) or an atmospheric pressure chemical ionization (APCI). Data acquisition and processing were performed by the MassLynx NT 4.0 system. The ESI and APCI source potentials were as follows: capillary voltage, 3.0 kV (positive mode) or 2.75 kV (negative mode); corona voltage 2.5 kV (in APCI mode); extractor voltage, 2 V. The sampling cone voltage was varied usually from 8 to 20 V for mass spectra, and the specific value of the cone voltage for each collision-induced dissociation (CID) experiment was set to optimize the parent ion. The source block and desolvation gas were respectively heated at 120 and 400 °C. Argon as collision gas was set at 3.5×10^{-3} mbar for the CID experiments. Separation was achieved on a reverse-phase column (Uptisphere C_{18} 5 μm, 150 × 2 mm i.d., Interchrom) equipped with a precolumn (Interchrom). Isocratic elutions were performed using a mixture H_2O-CH_3CN with 0.5% acetic acid [for **IIb**: (75:25, v/v); for **IIc**: (50:50, v/v); for **IId**: (95:5, v/v)] (the flow rate was 0.2 mL/min and column temperature was constant at 24 °C). For **IIc**, UV detection was set at 255 nm. ^{1}H NMR and ^{13}C NMR spectra were recorded on Varian VXR 300 (^{1}H: 300 MHz and ^{13}C: 75.5 MHz) and Bruker Avence 600 (^{1}H: 600 MHz and ^{13}C: 150 MHz) spectrometers.

Metabolite IIb. Metabolite **IIb** was studied from the culture medium obtained after 6 h. To this end, the culture medium was concentrated by freeze-drying and then dissolved in H_2O. The aqueous solution was extracted with AcOEt. **IIb**, present in the organic layer, was analyzed by mass spectrometry. The spectral data of the metabolite **IIb** obtained from the culture medium extract were identical to those of the synthetic product.

LC−MS (ESI$^-$): peak at 5.4 min, *m/z* 141 [M − H]$^-$; MS/MS CID 141 (15 eV), *m/z* 113, 42.

Metabolite IIc. Metabolite **IIc** was isolated from the cell-free extract obtained after 20 min. It was purified by solid-phase extraction on a C_{18} cartridge (Varian); the lyophilized extract was dissolved in H_2O-CH_3CN (95:5, v/v) and deposited on a column that was first washed with H_2O and then eluted with the mixture H_2O-CH_3CN (25:75, v/v). It was detected in the fractions on a TLC plate by viewing under UV light. After being freeze-dried, **IIc** was obtained pure as a white solid. This solid being unstable, it was dissolved in CH_3CN until it was studied. LC−MS (APCI$^-$): peak at 4.3 min, *m/z* 208 M$^{\bullet -}$, 193, 136, and 122; MS/MS CID 208 (5 eV), *m/z* 193, 122; MS/MS CID 136 (15 eV), *m/z* 121, 109, 42, 26. ^{1}H NMR 300 MHz (DMSO-d_6) δ (ppm) 1.16 (t, 3, J = 7.2 Hz, $\underline{CH_3}CH_2$), 1.74 (s, 6, 2 CH_3), 3.32 (qd, 2, J = 7.2 Hz, J = 5.7 Hz, $CH_3\underline{CH_2}NH$), 8.0 (t, 1, J = 5.7 Hz, $CH_2\underline{NH}$); ^{13}C NMR 75.5 MHz (DMSO-d_6) δ (ppm) 15.16 ($\underline{CH_3}$), 24.01 ($\underline{CH_3}$), 34.67 (CH_2), 90.36 (C quat.), 111.80 (C≡N), 144.92 (C=N), 149.77 (C=O), 159.28 (C=O).

Metabolite IId. Metabolite **IId** was studied from the mycelium extract obtained after 6 h. **IId** undergoes three successive steps of purification. First, this extract was purified by solid-phase extraction on a C_{18} cartridge (Varian). The extract was dissolved in H_2O and deposited on a column and eluted with H_2O. The presence of **IId** was detected on the TLC plate by staining with iodine vapors. Then, the solution of **IId** was concentrated by freeze-drying before purification by preparative TLC. An aqueous solution was deposited onto the silica gel plate, which was eluted with a mixture of CH_2Cl_2−MeOH (60:40, v/v) up to 80 mm. Then, silica was scraped from 30 to 45 mm and extracted with MeOH. Methanolic solution was evaporated and then H_2O was added. The aqueous solution was acidified with 0.1 N HCl to pH 2 and extracted with AcOEt. **IId** was in the organic layer which was analyzed by mass spectrometry. The spectral data of the metabolite **IId** obtained from the mycelium extract were identical to those of the synthetic product. HRMS (ESI$^-$): *m/z* = 141.0298 [M − H]$^-$ (calcd *m/z* = 141.0300 for $C_5H_6N_2O_3$); LC−MS (ESI$^-$): peak at 3.8 min, *m/z* 283, 141 [M − H]$^-$, 97; MS/MS CID 141 (10 eV), *m/z* 97, 70, 42, 41, 26; MS/MS CID 97 (15 eV), *m/z* 70, 42, 41, 26. MS/MS CID 70 (10 eV), *m/z* 42, 41; MS (ESI$^+$): *m/z* 181, 165, 143 [M + H]$^+$; MS/MS CID 143 (10 eV), *m/z* 125, 115, 73, 69, 43. **IId** obtained from [^{15}N]-cymoxanil: MS (ESI$^-$): *m/z* 142 [M − H]$^-$; MS/MS CID 142 (10 eV), *m/z* 98, 71, 26; MS/MS CID 98 (15 eV), *m/z* 71, 43, 41, 26; MS/MS CID 71(10 eV), *m/z* 43, 41; MS (ESI$^+$): *m/z* 144 [M + H]$^+$; MS/MS CID 144 (10 eV), *m/z* 126, 116, 74, 70, 43. Derivatization of **IId** with diazomethane: MS (ESI$^-$): *m/z* 155 [M − H]$^-$; MS/MS CID 155 (15 eV), *m/z* 123, 95, 79, 64, 53, 26; MS/MS CID 123 (10 eV), *m/z* 79, 64; MS/MS CID 79 (10 eV), *m/z* 64, 52, 26; MS/MS CID 64 (15−25 eV), *m/z* 26; MS (ESI$^+$): *m/z* 195, 179, 157 [M + H]$^+$. Derivatization of **IId** obtained from [^{15}N]-cymoxanil with diazomethane: MS (ESI$^-$): *m/z* 156 [M − H]$^-$; MS/MS CID 156 (20 eV), *m/z* 124, 96, 80, 65, 54, 26.

Bioassays. In vitro effects toward mycelial growth and germ-tube elongation of *B. cinerea* were studied. To study the mycelial growth, a conidia suspension (10^6 conidia mL^{-1}) was mixed with a molten agar

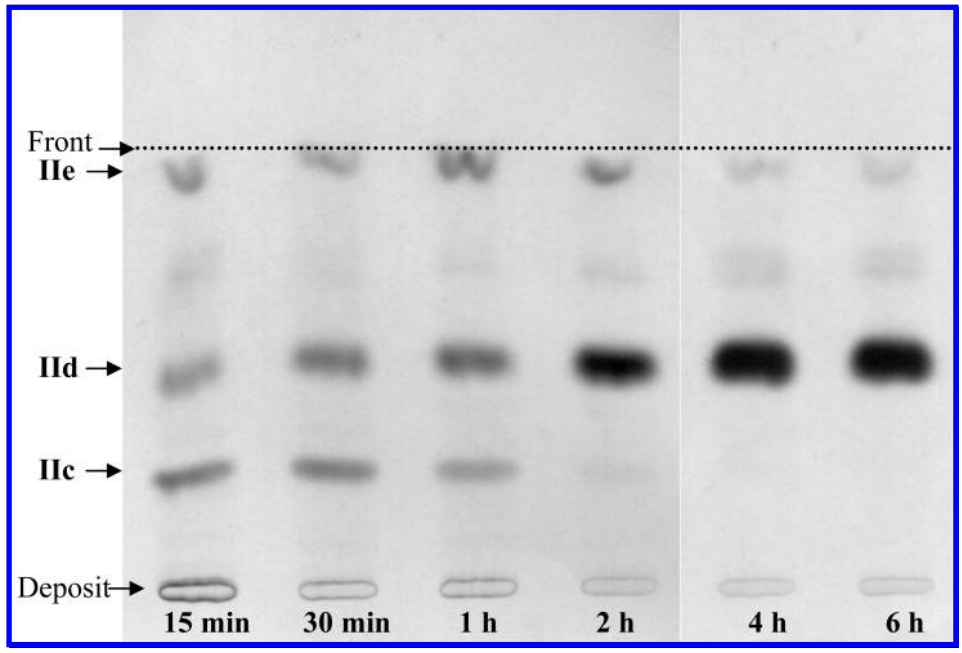

Figure 5. TLC monitoring of [2-^{14}C]-cymoxanil metabolism in the mycelium: Autoradiography of a C_{18} chromatoplate impregnated with 70 mM TBAB. Migration with phosphate buffer−MeOH (55:45, v/v) up to 50 mm.

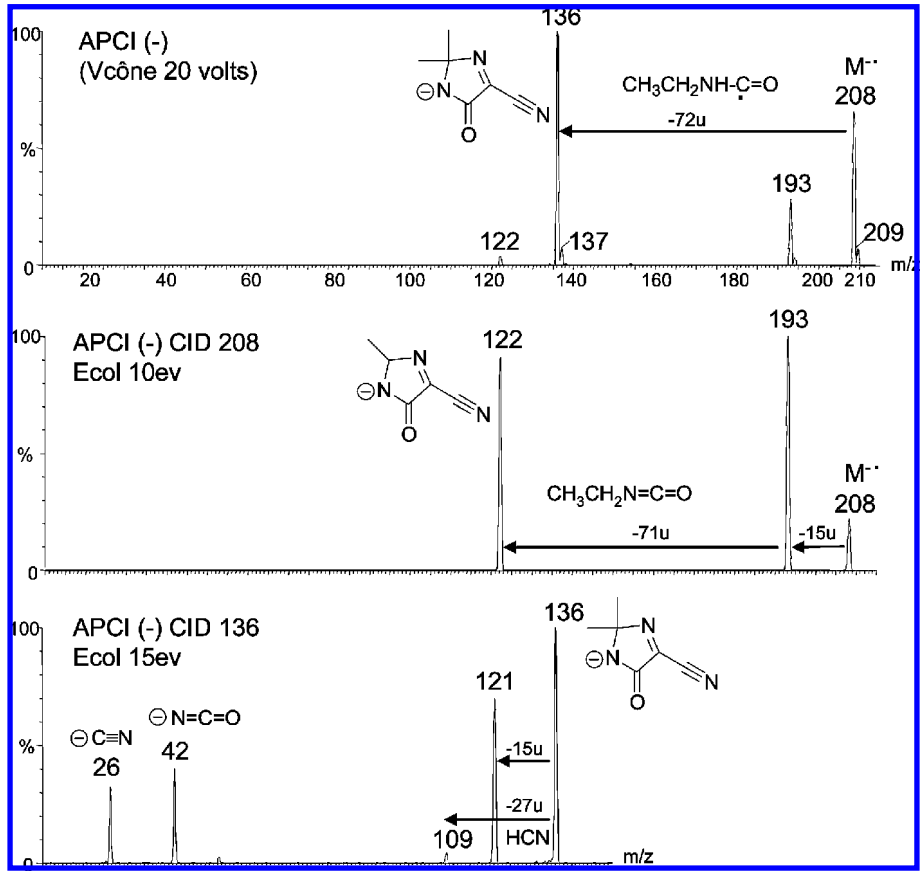

Figure 6. (APCI$^-$)−MS spectrum of **IIc**. APCI$^-$−CID spectrum of ions *m*/*z* 208 and *m*/*z* 136.

medium (20 g of malt, 5 g of yeast extract, and 10 g of agar per liter). Five-millimeter diameter paper filters, on which were deposited the product (0.02 μmol) to be tested, were put on this medium. After being incubated for 48 h at 19 °C in the dark, we checked if the mycelial growth was inhibited or not. To test germ-tube elongation, a conidia suspension (10^6 conidia mL^{-1}) was spread on the surface of an agar medium containing 10 g of glucose, 2 g of K_2HPO_4, 2 g of KH_2PO_4, and 10 g of agar per liter. The medium was amended with the product to be tested dissolved in ethanol (with a range of concentrations from 0.025 to 25 mg L^{-1}). After being incubated for 48 h at 19 °C in the dark, the lengths of germ tubes were estimated under a microscope, using a micrometer. For each compound, the effective concentration reducing germ-tube elongation by half (EC 50) was determined in milligrams per liter.

RESULTS AND DISCUSSION

Labeled Cymoxanil Metabolism in a Culture. Cymoxanil metabolism was studied by monitoring [2-^{14}C]-cymoxanil, added

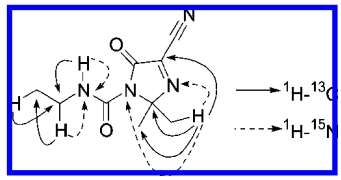

Figure 7. ^{1}H−^{13}C HMBC and ^{1}H−^{15}N HMBC correlations for compound **IIc**.

in a culture of *B. cinerea* strain, highly sensitive to this cyanooxime. The mycelium was removed from the culture medium immediately, 15 min, 30 min, 1 h, 2 h, 4 h, and 6 h after the adjunction of labeled cymoxanil. The main labeled metabolites and cymoxanil were quantified at different times both in the culture medium and in the mycelium after extraction. With time, the radioactivity quickly decreased in the medium and increased in the mycelium. After 15 min, 6% of the radioactivity was found in the mycelium and 40% after 6 h.

The culture media were analyzed by using a silica gel plate that was eluted with hexane−AcOEt−AcOH (70:30:1, v/v/v) (**Figure 3**). This plate showed that cymoxanil (R_f = 0.35) rapidly disappeared in less than 2 h (only 3% of **Ia** still remained after 1 h), whereas two main labeled metabolites, less polar than **Ia**, **IIa** (R_f = 0.43), and **IIb** (R_f = 0.46) appeared. Products **IIa** and **IIb** were hardly separable and quantified together. The quantity of (**IIa**, **IIb**) increased with time reaching 40% of the starting radioactivity after 6 h. The mycelium extracts were initially analyzed with a silica gel plate eluted as previously (**Figure 4**). We detected on this plate a product **IIc**, less polar (R_f = 0.88) than **Ia**. The compound **IIc** increased during 30 min (reaching 6% of the starting radioactivity) then decreased and disappeared after 4 h. No trace of remaining cymoxanil was detected in the mycelium extracts. In these TLC conditions, the major part of the radioactivity was not eluted. To detect in the mycelium extracts the more polar products, a reverse-phase plate impregnated with ion-pairing reagent was necessary (**Figure 5**). On this pretreated plate eluted with phosphate buffer−MeOH (55:45, v/v), we noted, in addition to **IIc**, the presence of two other main labeled metabolites **IId** and **IIe**. The product **IId** increased with time and represented 90% of the radioactivity present in the mycelium and 32% of the starting radioactivity after 6 h. The compound **IIe** increased for 1 h to reach 6% of the starting radioactivity then decreased and represented 2% after 6 h. **IIe** could not be studied because of its instability. It is interesting to note that the silica gel plate was necessary to check the complete disappearance of **Ia** because, on the pretreated reverse-phase plate, **Ia** and **Ic** were observed at the same R_f value. Metabolites **IId** and **IIe** could also be detected on a silica gel plate eluted with a polar mixture CH_2Cl_2−MeOH−AcOH (80:20:1, v/v/v). Their R_f values were, respectively, 0.15 and 0.60. The ion-pairing reverse-phase plate, however, advantageously enabled the monitoring of the three main metabolites at the same time.

In conclusion, after 6 h, the main labeled metabolites of cymoxanil in a culture were (**IIa**, **IIb**) in the medium and **IId** in the mycelium. The study of cymoxanil metabolism by resistant strains allowed us to show that cymoxanil needs to penetrate the mycelium to be metabolized. In these strains, the rate of metabolism is low and 4% cymoxanil was detected in the mycelium. In the highly sensitive strain used in this study, it was not detected because, once penetrated, it was metabolized.

Labeled Cymoxanil Metabolism in a Crude Enzyme Extract. After having followed the metabolism of cymoxanil in a culture, we were interested in its biotransformation in an enzymatic extract. The labeled cymoxanil was added to cell-free extracts prepared from mycelium, and after 0, 10, 20, and 30 min, the enzymatic reactions were stopped by addition of acetone. The different extracts obtained were analyzed on a silica gel plate. We observed that cymoxanil quickly disappeared (its half-life time is less than 10 min). After 20 min, it was completely metabolized into three main metabolites (**IIa**, **IIb**) and **IIc**, which represented, respectively, 25 and 55% of the starting radioactivity. The extracts were also deposited on a reverse-phase plate, but the metabolite **IId** was never detected. This fact means that the enzymatic systems leading to **IId** missed or were inefficient in the crude enzyme extracts.

Identification of Metabolites IIa and IIb. In the monitorings of the labeled cymoxanil, the products **IIa** and **IIb** were detected both in the culture medium and in the cell-free extract. The metabolite **IIb** was analyzed by mass spectrometry after extraction from the culture medium and identified as ethyl parabanic acid (**Figure 2**). The structure of **IIb** was confirmed by comparing MS data of the product present in the beforehand extracted biological samples, with those of the synthesized product. LC−MS/MS spectra of ions *m/z* 141 ($[M - H]^-$) from the two compounds, performed at 5, 10, 15, and 20 eV, were identical, exhibiting mainly two ion products in various proportions, *m/z* 113 ($[M - H]^- - 28$) and *m/z* 42, corresponding to the loss of CO and to the formation of ion $[N{=}C{=}O]^-$, respectively. The metabolite **IIa** could not be detected in the mass spectrometry analyses. **IIa** is supposed to be, according to the literature *9, 11, 13*, a cyclization product of cymoxanil, and **IIb** would result from the hydrolysis of the oxime function (**Figure 2**). Morrica et al. reported that cymoxanil under certain conditions of light and pH was unstable and led to the mixture **IIa**−**IIb** *9*. In our case, **IIa** was not due to a chemical degradation of cymoxanil but to the presence of an enzymatic system. Control experiments indeed showed that **Ia** was stable both in our experimental conditions (pH, temperature) and incubated in an inactivated crude extract.

Identification of Metabolite IIc. The product **IIc** was detected both in the mycelium and in the cell-free extract. It was purified from the cell-free extract by reverse-phase solid-phase extraction. **IIc** was obtained as a pure but unstable white solid. Its structure was assigned by combining MS and NMR studies (**Figures 6** and **7**).

The $(APCI^-)$−MS spectrum of **IIc** showed an ion at *m/z* 208 that was interpreted as a $M^{\bullet -}$ radical anion containing the same even number of nitrogen atoms as that of **Ia**. Fragment ions at *m/z* 193, *m/z* 136, and less so at *m/z* 122 were also present (**Figure 6**). The CID spectrum of ion *m/z* 208 showed two main product ions, indicating successive losses of a CH_3 radical and a 71 u molecule, likely $CH_3CH_2N{=}C{=}O$, leading to *m/z* 122. The CID spectrum of the in-source produced ion *m/z* 136 gave ion products at *m/z* 121, 109, 42, and 26 corresponding, respectively, to losses of $[CH_3]^\bullet$ or HCN and formation of ions $[N{=}C{=}O]^-$ and $[N{\equiv}C]^-$. Ion *m/z* 136, not found in the CID spectrum of ion *m/z* 208, might rather originate from an undetected $[M - H]^-$ ion at *m/z* 207 by elimination of $CH_3CH_2N{=}C{=}O$. Its elemental composition was determined as $C_6H_6N_3O$ by ESI−HRMS. To progress in the structure determination of **IIc**, we compared these MS data with those of the derivative obtained after PtO_2/CH_3OH catalytic hydrogenation. The (ESI^-)−MS spectrum of hydrogenated **IIc** exhibited a $[M - H]^-$ ion at *m/z* 209, indicating the addition of a single molecule of hydrogen. The CID spectrum of ion *m/z* 209 gave ion products at *m/z* 182, 138, 111, and 95, resulting, respectively, from the loss of either HCN or $CH_3CH_2N{=}C{=}O$,

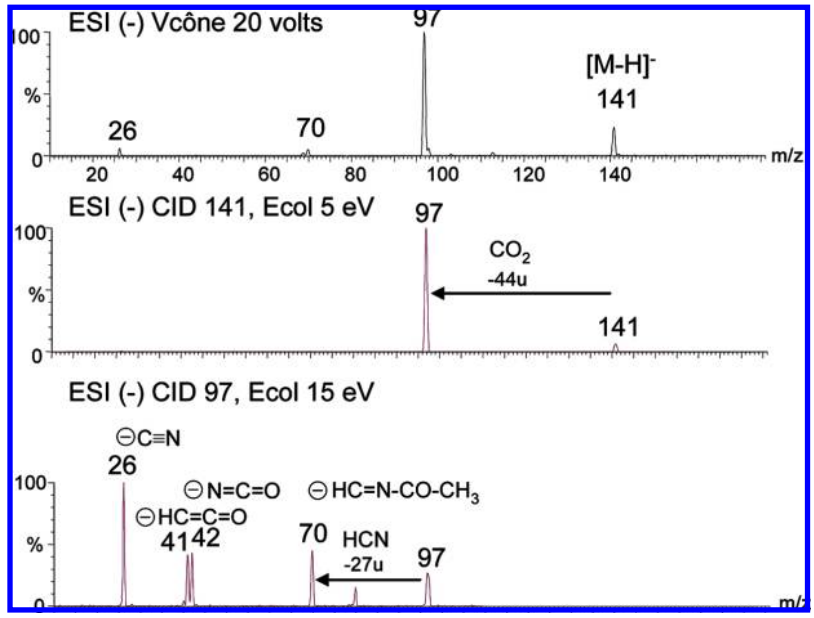

Figure 8. (ESI^-)−MS spectrum of **IId**. (ESI^-)−CID spectrum of ions *m/z* 141 and *m/z* 97.

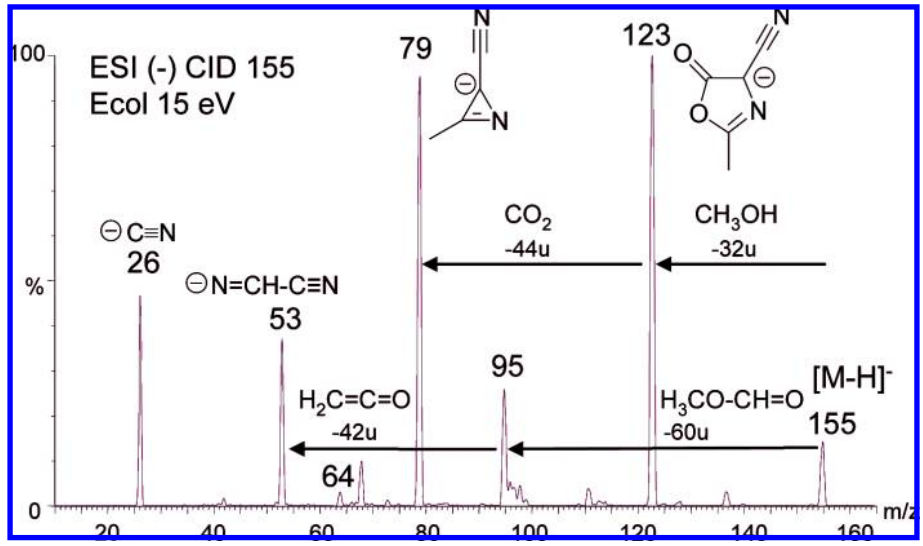

Figure 9. (ESI^-)−CID spectrum of ion *m/z* 155 obtained from the ester derivative of **IId**.

or successively the latter followed by HCN or HN=C=O elimination. These ions and their complementary anions $[N{=}C{=}O]^-$ and $[N{\equiv}C]^-$ also observed claimed for the conservation of most functional groups (nitrile, external or internal amide). This decomposition pathway was confirmed by the CID spectrum of the in-source produced ion *m/z* 138 that exhibited the same ions *m/z* 111, 95, 42, and 26 as the parent deprotonated molecule. The precursor of ion *m/z* 138 in **IIc** (i.e., *m/z* 136) should thus contain the original unsaturation and be cyclic (cf. unsaturation degree).

^1H and ^{13}C NMR spectra were obtained for compound **IIc**. 1D spectra indicated the presence of nine carbon and 12 proton resonances. The ^1H spectrum first confirmed the presence of the ethyl amide moiety, whereas the six remaining protons of the molecule appeared as a singlet at 1.74 ppm. Since no resonance was observed at a chemical shift compatible with the presence of a methoxy group, this indicated that the *o*-methyloxime moiety had been transformed in the reaction. $^1H-^{13}C$ HSQC and HMBC as well as $^{15}N-^1H$ HMBC experiments (**Figure 7**) were thereafter performed to complete the structure elucidation. In the $^{15}N-^1H$ HMBC experiment, three resonances were observed for the nitrogen atoms, one of which (99.4 ppm) was attributed to the ethyl amide moiety and the two remaining resonances (−14.2 and 174.9 ppm) were correlated to the proton singlet at 1.74 ppm. This proton resonance was thereafter easily attributed in the $^1H-^{13}C$ HMBC experiment to a *gem*-dimethyl moiety also correlated to two carbon resonances at 90.36 and 144.92 ppm.

According to the MS and NMR data, the metabolite **IIc** was identified as 1-(2,2-dimethyl-5-oxo-4-cyano-3-imidazolin-1-yl)-*N*-ethyl amide (**Figure 2**). **IIc** probably resulted from the reaction of an undetected cymoxanil metabolite with acetone, which was used either to extract the mycelium or to stop the enzymatic reactions in the cell-free extract. The precursor of **IIc** is likely the demethoxylated imine, which might be obtained by regiospecific reduction of cymoxanil. The cell-free extract was analyzed before addition of acetone by MS spectrometry but the precursor of **IIc** could not be detected. However, it was present and stable in the extract because if acetone was added after analysis, the product **IIc** was again obtained.

Identification of Metabolite IId. The product **IId** was only

found in the mycelium. Being polar and hardly separable from other constituents, it could not be obtained sufficiently pure for NMR analyses. The structure of **IId** was thus investigated only by MS approaches (**Figure 8**). This study was made easier by the introduction of stable isotopes into the starting cymoxanil like ^{15}N into the oxime function or deuterium into the methoxy group. Comparison of spectra of **IId** obtained from labeled and nonlabeled cymoxanil showed in **IId** the presence of ^{15}N but not that of the perdeutered methyl moiety. Moreover, we showed by dissolving **IId** in D_2O that three atoms of hydrogen in **IId** were exchangeable. The ESI^-−HRMS spectrum of **IId** exhibited a $[M - H]^-$ ion signal at *m/z* 141.0298 corresponding to the $C_5H_5N_2O_3$ formula. The low-resolution ESI spectrum of **IId** showed that $[M - H]^-$ was accompanied by the $[2M - H]^-$ ion. In the positive mode, the $[M + H]^+$ ion at *m/z* 143 was observed together with the cationized adducts $[M + Na]^+$ and $[M + K]^+$, confirming the molecular mass 142. The CID spectrum of $[M - H]^-$ ion *m/z* 141 (**Figure 8**) exhibited ion products at *m/z* 97, 70, and 26, indicating successive losses of CO_2 (44 u) and HCN (27 u) and the production of the anion $[N{\equiv}C]^-$. The initial loss of CO_2 from the deprotonated molecule suggested the occurrence of a CO_2H group in **IId**. The CID spectrum of the in-source generated ion *m/z* 97 confirmed that this ion was thereafter responsible for the production of ions *m/z* 70 and 26 during its decomposition. Interestingly also, ion *m/z* 97 was found to produce two other ions at *m/z* 41 and 42, likely issuing directly from *m/z* 70. Comparing to spectra of the metabolite obtained from the ^{15}N-labeled cymoxanil, it appeared that, in the low mass region, only ion *m/z* 42 (i.e., shifted to *m/z* 43) contained the labeled nitrogen atom. It was thus interpreted as a $[N{=}C{=}O]^-$ anion, whereas ion *m/z* 41 might correspond to a C_2HO structure. To complete identification, **IId** was subjected to derivatization by diazomethane. The ESI^-−MS spectrum of the obtained derivative (**Figure 9**) exhibited a $[M - H]^-$ ion at *m/z* 155, confirming the presence of an acid function in **IId**. At low energy, this ion *m/z* 155 could undergo successive losses of CH_3OH and CO_2 to lead to the ion product *m/z* 79. The latter was dissociated into ion products *m/z* 64 and 26, corresponding, respectively, to the loss of $[CH_3]^{\bullet}$ and the formation of the anion $[N{\equiv}C]^-$. The use of $[^{15}N]$-cymoxanil showed that the ion at *m/z* 64 contained also the second nitrogen atom. A second decomposition pathway was observed that showed an alternative transformation of ion *m/z* 155 into ion *m/z* 53 by elimination of 60 and 42 u neutrals that could be interpreted as $H_3CO{-}CHO$ and CH_2CO, respectively. From the elemental composition of the parent ion *m/z* 155 (deduced from the exact mass of **IId**), ions *m/z* 79 and *m/z* 53 were, respectively, determined as $C_4H_3N_2$ and C_2HN_2. The presence of the two nitrogen atoms in these ions indicated a cyanoamino acid substructure for **IId**. On the basis of these results, the metabolite **IId** was identified as *N*-acetylcyanoglycine. The structure of **IId** was confirmed by comparing the mass spectra data of the metabolite isolated from the mycelium extract with those of the synthesized product. LC−MS/MS spectra of ions *m/z* 141 ($[M - H]^-$) and *m/z* 143 ($[M + H]^+$) from both compounds recorded at a variety of collision energies were found to be identical. In conclusion, it can be postulated that cymoxanil was initially hydrolyzed, reduced, and finally acetylated (**Figure 2**). The *N*-acetyl group should originate from the cellular metabolism.

The identification of the main metabolites allowed us to determine the general scheme of the cymoxanil metabolism by a culture of *B. cinerea* strain, highly sensitive to this cyanooxime (**Figure 2**). Thus, cymoxanil was completely metabolized according to at least three enzymatic pathways: a cyclization leading after hydrolysis to ethyl parabanic acid, a reduction giving the demethoxylated cymoxanil, and a hydrolysis and a reduction, followed by an acetylation leading to *N*-acetylcyanoglycine. In a cell-free extract of the same strain, only the two first enzymatic reactions, quoted above, occurred. Among the identified metabolites, **IIb** is ubiquitous in the different cymoxanil metabolism quoted previously *8, 9, 13*, whereas **IIc** and **IId** are obtained by specific pathways to cymoxanil metabolism by a highly sensitive strain of *B. cinerea*.

Fungicidal Activity. The fungicidal activity of the main metabolites obtained, **IIb**, **IIc**, and **IId**, and also one of the cell-free extract before addition of acetone containing **IIa**, **IIb**, and the precursor of **IIc**, was tested in vitro toward *B. cinerea* strains. Only the metabolite **IId** exhibited a fungitoxic activity toward the highly sensitive strain. The inhibition of the germ-tube elongation with this metabolite was more effective (EC 50 = 0.025 mg L^{-1}) than one obtained with cymoxanil (EC 50 = 0.25 mg L^{-1}). On the other hand, the comparison of cymoxanil metabolism by a highly sensitive strain to those obtained by less sensitive ones showed that **IId** was obtained in 32% yield in a highly sensitive strain, 12% in a moderately sensitive strain, and only 2% in a resistant one. These results suggested that the sensitivity of *B. cinerea* strains could be correlated with cymoxanil biotransformation into this metabolite. The formation of *N*-acetylcyanoglycine probably plays an important role in the fungitoxicity of cymoxanil, but its presence is not sufficient to explain the fungicidal activity because it was ineffective on the resistant strains. It is difficult to explain the inefficiency of *N*-acetylcyanoglycine on these resistant strains of *B. cinerea*. One could make the assumption that cyanoglycine would be the final active compound rather than *N*-acetylcyanoglycine. Indeed, cyanoglycine is reported in the literature as a competitive inhibitor of enzymes implied in the biosynthesis of aminoacids. For example, α-cyanoglycine strongly inhibits some pyridoxal phosphate-dependent enzymes such as glutamate decarboxylase, tryptophan synthase, threonine dehydratase, and cystathionine synthase *19–22*. Cyanoglycine as active principle would mean that the acetylated metabolite must be hydrolyzed by an enzymatic system that would be ineffective in the resistant strains. On the other hand, the inefficiency of acetylcyanoglycine on the resistant strains could be also due to a weaker uptake of the metabolite when added in the nutrient medium.

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