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Degradation of Acylanilide Pesticides by *Aspergillus niger*

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Aspergillus niger converts the herbicide 3′-chloro-2-methyl-*p*-valerotoluidide (solan) to 3′-chloro-4′-methylacetanilide and the fungicide 2,5-dimethylfuran-3-carboxanilide to acetanilide. The metabolites were formed by hydrolysis with an aryl acylamidase, followed by subsequent acetylation resulting in the corresponding acetanilides. Their structures were elucidated by mass spectrometric analysis and confirmed by comparison with synthetic compounds.

INTRODUCTION

Biochemical activities of soil microorganisms play a major role in the removal of pesticides from soil. Fungi of the order *Mucorales* are known to hydroxylate herbicidal and fungicidal acylanilides and related compounds. The enzyme system responsible for this reaction is a nonspecific, since hydroxyl groups are introduced either on aromatic rings (1), methyl groups attached to the furan ring system (2), or on aliphatic side chains (3, 4).

Among soil fungi, *Phycomycetes* as well as *Ascomycetes* and fungi imperfecti (5–7) are known to be very active in acylanilide decomposition. Therefore it seems reasonable to expect transformations of acylanilides with fungi of the family *Aspergillaceae*, which are commonly found in soil (8). In this investigation the transformation of two representative acylanilides, the herbicide solan and the fungicide 2,5-dimethylfuran-3-carboxanilide, by *Aspergillus niger* was studied.

MATERIAL AND METHODS

Chemicals and Instrumentation

An analytical grade sample of the herbicide 3′-chloro-2-methyl-*p*-valerotoluidide (solan) was a gift from Niagara Chemical Division, Middleport, New York. The fungicide 2,5-dimethylfuran-3-carboxanilide (FA)[1] was obtained from commercial formulations as described previously (2). Silica gel with fluorescent indicator (150 G LS 254, Schleicher und Schüll, Dassel, Germany) was used for thin-layer chromatography. Ultraviolet absorption spectra were recorded on a Beckman DB spectrophotometer, and melting points were determined with a Kofler Hot Stage (Reichert, Austria). Mass spectra were recorded on a Shimadzu LKB 9000-S-instrument. A controlled temperature probe was used for introduction of the samples directly into the ion source.

Culture Methods

Aspergillus niger Van Tieghem was grown in a synthetic glucose medium (9) on a gyratory shaker providing aerobic conditions (temperature 30°C; pH range during growth, 6.8–7.5). Sterile controls containing the pesticides tested were run. Solutions of solan (60 mg/1 ml of acetone) and FA (10.25 mg/1 ml of acetone) were added to the culture medium to final concentra-

[1] FA = 2,5-dimethylfuran-3-carboxanilide.

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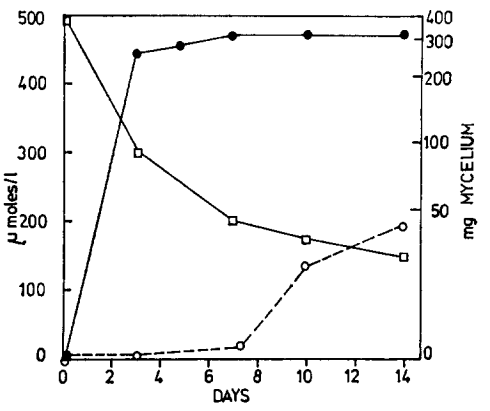

FIG. 1. *Degradation of solan by* Aspergillus niger *in Wegener medium*: (●—●), *growth (dry wt of mycelium)*; (□—□), *solan;* (○—○), *3'-chloro-4'-methylacetanilide.*

tions of 500 μmol/liter (solan) and 100 μmol/liter (FA), respectively.

Analytical Procedures.

Twice a week 10-ml portions of the culture media were extracted three times with 10 ml of ethyl acetate and estimated for pesticide degradation as described previously (2).

Isolation of Pesticide Metabolites

The mycelial mass was separated from the medium by filtration, and the medium

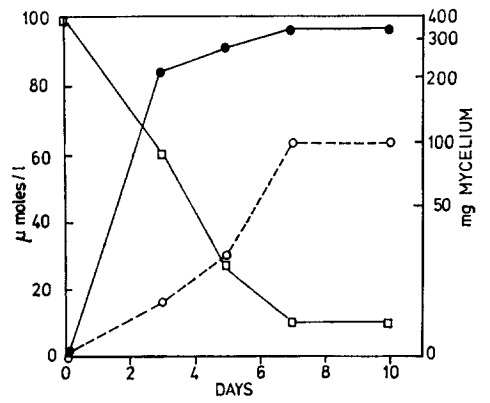

FIG. 2. *Degradation of FA by* Aspergillus niger *in Wegener medium:* (●—●), *growth (dry wt of mycelium);* (□—□), *FA;* (○—○), *acetanilide.*

(500 ml) was extracted with ethyl acetate (1000 ml). The extracts were dried (Na_2SO_4), their volumes were reduced to about 2 ml by rotary evaporation, and the residues were streaked onto preparative silica gel plates (0.5 mm in thickness). After developing with chloroform–benzene (9:1) and benzene–acetic acid (9:1), the bands corresponding to the metabolites were removed from the plates by scraping, and the compounds were eluted with chloroform. The metabolites were recrystallized from chloroform.

RESULTS AND DISCUSSION

General

In the late active growth phase of *A. niger* in a synthetic glucose broth containing either 500 μmol/liter of the herbicide solan or 100 μmol/liter of the fungicide FA, a main metabolite accumulated in both culture media (Figs. 1 and 2). Extracts of the mycelium contained only small amounts of the original compounds (less than 5%) and no metabolites. The metabolites were not further degraded, and their fungitoxicity was not investigated. Within 14 and 10 days, respectively, a 1-liter culture of *A. niger* formed 195 μmol of metabolite from 500 μmol of solan, leaving a residue of 150 μmol of the starting material, and 64 μmol of metabolite from 100 μmole of FA, leaving 10 μmol from the parent compound.

In addition to these metabolites very small amounts of solan were transformed to a yet unidentified product. Because of the very poor yields of this metabolite, no further attempts were made for structure elucidation. With the fungicide FA an additional 8 μmol/liter of 2-methyl-5-hydroxymethylfuran-3-carboxanilide were formed. The structure of this metabolite was confirmed by comparison of its physical properties to be identical to that of the major degradation product of FA in cultures of *Rhizopus japonicus* ATCC 24794 and related *Mucoraceae* (2).

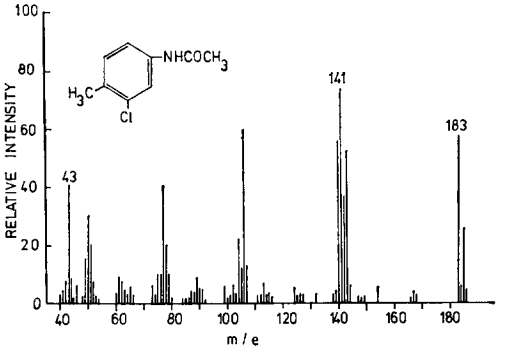

FIG. 3. *The 70-eV mass spectrum of 3′-chloro-4′-methylacetanilide (sample temperature 80°C).*

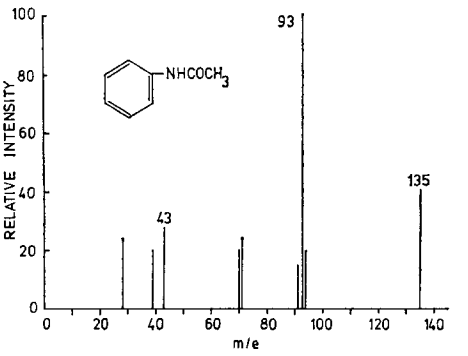

FIG. 4. *The 75-eV mass spectrum of acetanilide (sample temperature 60°C).*

Identification of Metabolites

The mass spectra obtained from the two metabolites indicated that in both pesticides the acyl moieties of the molecules had been changed. The metabolite derived from solan gave a mass spectrum showing a molecular ion at m/e 183, and ions at m/e 141 (base peak) and m/e 43, which indicated cleavage between the amide nitrogen to give an anilino and an acetyl ion, respectively (Fig. 3). The metabolite which was isolated from culture solution of *A. niger* after incubation with FA, exhibited a mass spectrum with a molecular ion at m/e 135, and ions at m/e 93 (base peak) and m/e 43 which also revealed the anilino and acetyl ion (Fig. 4). These results strongly suggest that the anilino moiety of the starting materials remained unchanged. However, the 2-methylvaleric acid moiety of solan and the 2,5-dimethyl-3-furancarboxylic acid moiety of FA were lost during microbial degradation and replaced by acetic acid. These suggestions were confirmed by comparison of the mass spectra of both metabolites, their melting points, ultraviolet absorption spectra, and thin-layer chromatographical data with authentic materials (Table 1). The metabolites were identified as 3′-chloro-4′-methylacetanilide (solan) and acetanilide (FA), respectively.

Formation of acetanilide metabolites from acylanilides may proceed via two mechanisms: Hydrolysis of the parent acylanilide by an aryl acylamidase would result in the formation of the corresponding aniline, followed by subsequent acetylation. A stepwise degradation of the alkyl side chain via β- or ω-oxidation would also

TABLE 1

Physical Data for the Acylanilide Pesticides and Their Metabolites

Compound	mp (°C)	uv λmax (nm) for methanol	tlc R_f-values (×100)[a]		
			A	B	C
3′-Chloro-2-methyl-*p*-valerotoluidide	85–6	246	23	59	96
3′-Chloro-4′-methylacetanilide	95–6	246	10	27	51
2,5-Dimethylfuran-3-carboxanilide	92–4	262	30	45	94
Acetanilide	114.5	240	4	16	44

[a] Solvent systems: A = chloroform–benzene, 9:1; B = benzene–acetic acid, 9:1; C = ethyl acetate–benzene, 6:4.

leave acetanilide as the end product. This mechanism would include an opening of the ring of furancarboxylic acid in the case of FA. Preliminary tests with cell-free extracts of *A. niger* grown in the presence of solan revealed that *A. niger* has "amidase activity," since free aniline could be detected in the incubation mixtures. The same cell-free extract also hydrolyzed L-alanine-4-nitroanilide, a compound which is frequently used for amidase assay. Further attempts are being made to prepare a partially purified enzyme in order to test substrate specificity and to compare this enzyme with other microbial aryl acylamidases.

In growing cultures of *A. niger* no free anilines could be detected. Rapid acetylation of anilines is advantageous for the organism, since the free aniline has a significant higher toxicity against microorganisms than acylanilides (6). Acetylation of anilines seems to be a common reaction among many soil bacteria (10) and has already been described for the fungi *Fusarium oxysporum* (11) and *Talaromyces wortmannii* (12) from degradation studies of the herbicides 3′,4′-dichloropropionanilide (propanil) and 3-(*p*-bromophenyl)-1-methoxy-1-methylurea (metobromuron), respectively.

Hydrolysis of phenylamide-type herbicides and fungicides by microbial aryl acylamidases is well described in the literature. Cultures of *Bacillus sphaericus* which has been isolated from soil, as well as a purified aryl acylamidase derived from this organism, hydrolyze a number of methoxy-substituted phenylurea herbicides, acylanilide herbicides and fungicides, and phenylcarbamate herbicides (13, 14). Partially purified enzyme preparations from a strain of *Pseudomonas striata* (15) were active in degrading phenylcarbamate and acylanilide herbicides. Crude cell-free extracts and a partly purified enzyme from *Fusarium solani* catalyzed the hydrolysis of the herbicide propanil and related compounds (16). In addition acylamidases were induced in the soil fungus *Fusarium oxysporum* by propanil (7). A partially purified acylamidase from cells of *Pencillium* grown in the presence of the 3′,4′-dichloro-2-methylvaleranilide (karsil) could hydrolyze this compound releasing 3,4-dichloroaniline as main metabolite (5). It is an interesting fact that in all these organisms the anilines formed during hydrolysis have accumulated in the culture media. These metabolites apparently may be toxic to soil microorganisms (17). This effect is overcome in many soil microorganisms (10–12) by acetylation of the anilines.

ACKNOWLEDGMENTS

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