Microbial Transformation of [^{14}C]Methabenzthiazuron by the Soil Fungus *Hypocrea* Cf. *pilulifera* Webster St. Con: Isolation, Identification, and Characterization of Some Metabolites from the Chloroform Extract

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Of a series of microorganisms isolated from the soil, *Hypocrea pilulifera*, a fungus of the Ascomycetes class, is the only one able to metabolize the herbicide methabenzthiazuron. In cultures of this fungus the distribution of radioactivity between the individual extract fractions was found to be the following: chloroform extract fraction 73.6–79.4%, water extract fraction 6.2–11.8%, and mycelium extract fraction 3.7–12.1%. As the incubation period is lengthened, the water-soluble metabolites increase while the chloroform-soluble ones decrease. The degradation rate of [^{14}C]methabenzthiazuron is 16% of the [^{14}C]methabenzthiazuron recovered in 7 weeks. Six transformation products of [^{14}C]methabenzthiazuron were isolated with the aid of thin-layer and column chromatography and characterized with the aid of spectroscopic and chromatographic methods.

Methabenzthiazuron, *N*,*N*′-dimethyl-*N*-(2-benzothiazolyl)urea (**1**), the active ingredient in Tribunil, is a wide-spectrum herbicide for use in cereal growing (Hack, 1969). Jarczyk (1972) investigated the migration of methabenzthiazuron in the soil. Cheng et al. (1975) studied the distribution and transformation of methabenzthiazuron in the plant–soil system. Hugé (1970) investigated the influence of methabenzthiazuron on the microorganisms in the soil. The metabolism of methabenzthiazuron in plants was the subject of work by Collet and Pont (1974) who, above all, were able to identify *N*-methylhydroxy-*N*′-methyl-(2-benzothiazolyl)urea (**2**). Also, Wallnöfer et al. (1976) succeeded in identifying *N*-(6-hydroxy-2-benzothiazolyl)-*N*,*N*′-dimethylurea (**6**) and also a demethylated product (**4**) in a Phycomycetes fungus. On the other hand, Mittelstaedt et al. (1977) isolated a major metabolite of methabenzthiazuron after degradation in the soil. The chromatographic properties of this compound were identical with *N*-methyl-*N*-(2-benzothiazolyl)urea (**3**). Sakriss et al. (1976) also found this product as a photochemical degradation product during UV irradiation of Tribunil. The present study has the task of gaining better knowledge about the transformation of methabenzthiazuron by soil microorganisms and also of identifying and characterizing the transformation products.

MATERIAL AND METHODS

Chemicals. [^{14}C]Methabenzthiazuron (ring-labeled) with a specific activity of 42 μCi/mg was kindly made available by Bayer AG, Wuppertal. The substance was radiochemically and thin-layer chromatographically pure (99%). Dimethyl sulfoxide was used to prepare the standard solution, which was kept at −20 °C. Purification of the unlabeled methabenzthiazuron (mp 120–122 °C) was achieved in a dry silica gel column (using benzene as eluent), followed by recrystallization from cyclohexane. The reference substances for the metabolites **2**, **3**, **4**, and **5** were made available by Bayer AG, Wuppertal. Dr. H. G. Aurich of the Faculty of Chemistry of the Technische Universität Marburg provided the compounds **8**–**11**. Compound **12** was prepared from phenyl isocyanate and *N*-methylethanolamine by boiling in petroleum ether. All the solvents used were of analytical grade quality (Merck, Darmstadt); the chloroform alone was distilled twice. Gas chromatography, thin-layer chromatography, or mass spectroscopy methods were used to test all the solvents for their purity before use, and they were found to be free of impurities.

Application of the Methabenzthiazuron. For all the culture preparations 59 mg of unlabeled methabenzthiazuron per liter of medium was used, the specific activity being adjusted according to requirements. The methabenzthiazuron was applied sterilely in acetone solution by way of a membrane filter (Sartorius SM 11605, 0.6 μm). In every case the acetone was evaporated from the culture vessel before addition of the sterile medium. [^{14}C]Methabenzthiazuron was applied from the dimethyl sulfoxide standard (47 μL = 1.04 × 10^6 dpm) in an appropriate amount with a pasteurized Hamilton syringe.

Fungus Culture. The soil fungus *Hypocrea pilulifera*, a member of the Ascomycetes class, was isolated from a sample of soil via a number of concentrating steps. A field cultivated with winter wheat (sL–L) and treated with Tribunil (4 kg/ha) served as the source of the soil sample; sampling was carried out in August. Concentration of the fungus was performed in a modified Hegemann (1966) medium having the following composition per liter of distilled water: 200 mg of EDTA-Na, 580 mg of $MgSO_4 \cdot 7H_2O$, 67 mg of $CaCl_2 \cdot 2H_2O$, 0.2 mg of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 2.0 mg of $FeSO_4 \cdot 7H_2O$, 1.0 g of Na_2SO_4, 3.4 g of KH_2PO_4, 4.44 g of $Na_2HPO_4 \cdot 12H_2O$, 1 mL of trace element solution (Drews, 1968). The pH was adjusted to 7.0 with NaOH. Fifty-nine milligrams/liter of unlabeled methabenzthiazuron served as the source of carbon. The preparations being concentrated were incubated in Erlenmeyer flasks in the dark at 27–30 °C on a rotary shaker. The fungus was kept on malt extract agar at 10 °C.

Inoculum and Incubation of the [^{14}C]Methabenzthiazuron Preparations and of the Methabenzthiazuron Production Batches. In order to study the degradation rates of the [^{14}C]methabenzthiazuron and of the metabolites formed, *Hypocrea pilulifera* fungus cultures were incubated for respective periods of 7 (experiment A), 5 (experiment B), and 4 (experiment C) weeks. The above-described mineral medium with 0.1% added beef extract (Merck, Darmstadt) (experiment A) and with malt extract bouillon (Merck, Darmstadt) adjusted to pH 6.5 with 1 N NaOH (experiments B and C) were used as the culture media. A 3-day culture grown in the appropriate culture medium using the agar slant mycelium served as the inoculum. All the cultures contained the above-described amount of unlabeled metha-

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0021-8561/78/1426-0628$01.00/0

benzthiazuron. In addition, the 30-mL preparations contained 1.04×10^6 dpm [^{14}C]methabenzthiazuron and the 1 L main preparation (malt extract bouillon) contained 9.64×10^6 dpm [^{14}C]methabenzthiazuron. Incubation of the 30-mL preparations was carried out on the rotary shaker in Erlenmeyer flasks with a Kapsenberg closure in the dark at 27–30 °C. The 1-L preparation (with [^{14}C]-methabenzthiazuron) as well as the 5-L production batches (inactive methabenzthiazuron) were incubated in jar-type reagent bottles with purified air (cotton wool, activated charcoal), moistened air (wash-bottle with water), and sterile air (Sartorius SM 11304 0.8-μm membrane filter, cotton wool) at 20–22 °C in daylight and night illumination. Equivalent controls without inoculum were run in every case.

Extraction. Following incubation, the fungal cultures were vacuum-filtered through a Büchner funnel with a circular filter (Schleicher-Schüll No. 589) to separate the mycelium from the aqueous medium. This mycelium-free medium was extracted three times with half the quantity of chloroform in a separating funnel to yield a chloroform and a water extract.

The mycelium was extracted with a 1:1 mixture of chloroform and methanol either in a homogenizer (Braun, Melsungen) or overnight on the shaker in an Erlenmeyer flask. After filtering and washing several times, the mycelium extract fraction was obtained.

Analytical Methods. Both the chloroform extract and the mycelium extract fractions were dried overnight over sodium sulfate. After filtration through fluted filters the extracts were evaporated to dryness in the rotary evaporator and redissolved in 10 mL of chloroform. Liquid scintillation measurements were used to determine the amount of radioactivity. Freeze-drying of the aqueous extract fraction gave a brownish powder which was extracted with acetone and methanol until no residual radioactivity remained. The radioactivity was measured before and after the freeze-drying using the liquid scintillation counter.

Purification and separation of the chloroform extract fraction in the case of the 30-mL preparation was carried out with the aid of thin-layer chromatography on ready-prepared Kieselgel 60 F_{254} silica gel thin-layer chromatography plates (Merck, Darmstadt), which were washed three times with methanol and then activated for 35 min at 110 °C before using. Combined treatment of the chloroform extract fractions of the [^{14}C]methabenthiazuron and of the inactive methabenzthiazuron production batches was performed on a silica gel column using a benzene–ethyl acetate mixture of increasing polarity as the eluent. The radioactive fractions obtained were chromatographed on Kieselgel 60 F_{254} (Merck, Darmstadt) PSC ready-for-use plates with various developers. Isolation and purification of the metabolites proceeded on thin-layer chromatography ready-prepared plates with different developers. The following solvent mixtures were employed: 2:1 benzene–ethyl acetate, 6:4 petroleum ether–acetone, 9:1 chloroform–methanol, 9:1 benzene–methanol, and 1:1 benzene–acetone. Eluting from the silica gel in each case was accomplished with methanol accompanied by ultrasonic treatment.

Type II Berthold-Friesecke thin-layer scanners, one of which was equipped with an integrator, were used for measuring the radioactivity on the silica gel plates. The radioactivity of the solutions was determined with a liquid scintillation counter (BF Betascint 5000). Measurements of the chloroform extract fractions were corrected by means of a quenching curve (Koch-Light quenching set) using the external standard channel ratio, while experimental values on the aqueous solutions were corrected with the aid of a [^{14}C]toluene standard. The samples were measured either in a toluene scintillator solution (Packard Permablend III) or in Bray's scintillator solution (Bray, 1960).

A mass spectrometer (LKB 9000 S, 70 eV, direct inlet) and a CFT 20 Varian NMR instrument were used to carry out the spectroscopic investigations. The mass spectra were plotted via an interface (WDV-Company, Munich) using an IBM 1130 digital computer and a Benson 111 plotter.

Confirmation of the proposed structures for compounds **2–5** resulting from the spectroscopic data was obtained by mass spectroscopic and thin-layer chromatographic comparisons of the metabolites with the corresponding reference substances.

Table I. Distribution of Radioactivity in Different Extract Fractions

	Experiment[a]		
	A, %	B, %	C, %
Chloroform extract fraction	73.6	76.0	79.4
Water extract fraction	11.8	7.7	6.2
Mycelium extract fraction	3.7	12.1	7.4
Recovery rate	89.1	95.8	94.5

[a] Percent of the applied radioactivity.

RESULTS AND DISCUSSION

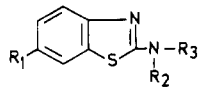
^{14}C Balance Sheet and Degradation Rate of Methabenzthiazuron. After the corresponding, above-described working-up of the [^{14}C]methabenzthiazuron culture preparations it was found that all extracts of experiments A, B, and C were radioactive. Table I shows that the chloroform fractions contain the bulk of the activity. Almost the entire unchanged, recovered [^{14}C]methabenzthiazuron, on the order of 80% of the overall fraction, is contained in these fractions. About 20% of the radioactivity is found in the metabolites which will be discussed in greater detail in this article. No transformation of methabenzthiazuron was found in any of the control preparations without *Hypocrea pilulifera*, and in every case almost 100% of the radioactivity was present in the chloroform extract fraction as unchanged methabenzthiazuron.

A small residue of the [^{14}C]methabenzthiazuron including further transformation products of varying polarity was present in the mycelium fractions. As expected, the water extract fractions contained no [^{14}C]methabenzthiazuron but only metabolites of polar nature. We shall report about the metabolites in these two fractions elsewhere in due course.

Table I reveals that with an increasing duration of incubation the concentration of the water-soluble metabolites rises while that of the chloroform-soluble ones falls. Collet and Pont (1974) made the same observation with plants and Tillmans (1976) with fungi of the order Mucorales. The radioactivity in the mycelium is dependent on the amount of mycelial mass produced, but the various experiments showed that the degradation rate of the methabenzthiazuron does not correlate directly with the amount of mycelium. It is logical to make the assumption that one of the degradation products exercises an inhibitory effect. Such a phenomenon would also explain the relatively slow breakdown of methabenzthiazuron by *Hypocrea pilulifera*.

Isolation of Metabolites from the Chloroform Extract Fraction. The chloroform extract fractions from experiments A and B obtained by means of the above-

Table II. Physical Data of Methabenzthiazuron and Its Degradation Products



	Compound			R_f[a]			
	R_1	R_2	R_3	X	Y	Z	Mp, °C
1	H	CH_3	$CONHCH_3$	0.22	0.68	n.d.	119-120
2	H	CH_3	$CONHCH_2OH$	0.1	0.56	0.54	177
3	H	CH_3	$CONH_2$	0.13	0.52	n.d.	177
4	H	H	$CONHCH_3$	0.1	0.59	0.51	327
5	H	CH_3	H	0.21	0.55	0.28	138
6	OH	CH_3	$CONHCH_3$	n.d.[b]	0.4	0.28	204-205
7	OH	CH_3	CONHO-X	n.d.	n.d.	0.37	n.d.

[a] X = benzene-ethyl acetate 2:1, Y = chloroform-methanol 9:1, Z = petroleum ether-acetone 6:4. [b] n.d. = not detected.

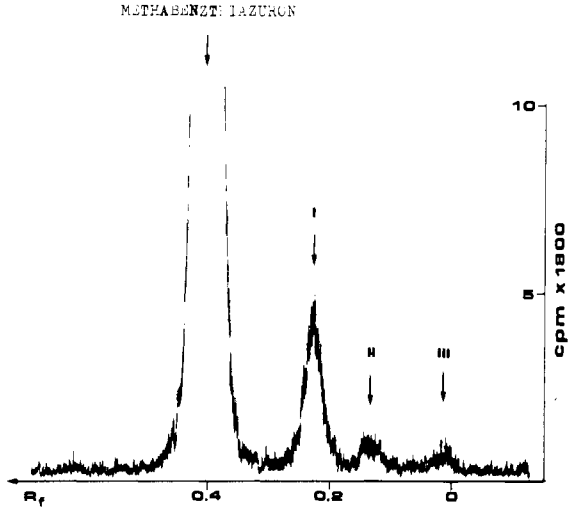


Figure 1. Thin-layer separation on silica gel of chloroform extraction phases of media incubated with [^{14}C]methabenzthiazuron for 7 weeks (solvent mixture: benzene–ethyl acetate 2:1, 2×; measurement range 30 cps, time constant 3 s, scanning speed 120 mm/h).

described methods were applied on thin-layer silica gel plates and developed twice using the solvent mixture benzene–ethyl acetate 2:1. In Figure 1 the scan shows a principal zone for methabenzthiazuron and additional radioactive zones I, II, and III. After scraping off and exhaustive extraction with methanol, the radioactivity of the methabenzthiazuron zone amounted to 84%, that of zone I to 4.8%, and that of the remaining radioactive zones to 11.2% of the chloroform fraction. Renewed thin-layer chromatography of I and II using another developer system (chloroform–methanol 9:1) enabled mass spectra of compounds **3–5** to be prepared following an appropriate treatment. Zone III and additional zones could not be investigated further because of the small quantity of the substances in these experiments.

On account of the larger amount of substance involved, the chloroform extract fractions from experiment C were separated on a silica gel column. Further purification of the individual column fractions was performed on ready-prepared silica gel plates using a variety of suitable developer systems. In this way a number of additional metabolites (**2**, **6**, and **7**) were isolated and purified. Table II lists the R_f values of the compounds **1–7**.

Characterization of Some of the [^{14}C]Methabenzthiazuron Metabolites from the Chloroform Extract Fraction. Predictably, the isolated metabolites from the chloroform extract fraction are predominantly structures which have undergone a change on the urea side chain. The principal metabolite, **3**, has a molecular weight of 207 and is the result of an N-demethylation. Mass spectrometric and thin-layer chromatographic comparisons with the authentic substance indicate the structure of *N*-methyl-*N*-(2-benzothiazolyl)urea for metabolite **3**. In the same way compound **4**, with a molecular weight of 207, was identified as *N*-methyl-*N'*-(2-benzothiazolyl)urea. It is thus possible to state that *N*-demethylation is a first and important step in the degradation mechanism of methabenzthiazuron as other urea herbicides (Börner, 1969; Geissbühler et al., 1963; Geissbühler, 1969; Geissbühler and Voss, 1971; Ross and Tweedy, 1973; Tanaka et al., 1972; Tweedy et al., 1970).

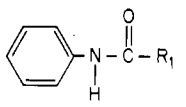
Metabolite **2** with a molecular weight of 237 has a mass which is 16 units higher than methabenzthiazuron. The typical fragmentations of the mass spectrum suggests the introduction of a hydroxy group in the side chain. Following a thin-layer chromatographic comparison with the authentic compound this substance can be identified as *N*-methylhydroxy-*N'*-methyl-(2-benzothiazolyl)urea. Hydroxylation of the side chain was also found by Frear et al. (1972) and by Tanaka et al. (1972) in their work on the breakdown of substituted urea herbicides.

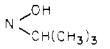
This *N*-methylhydroxy compound can also be converted chemically into *N*-methyl-*N*-(2-benzothiazolyl)urea when adsorbed on silica gel (Göttfert, 1977).

A comparison of the mass spectra of compounds **1** and **5** clearly shows the absence of the methyl isocyanate group in **5**. After a comparison of the data with those of Ogura et al. (1970) and a corresponding comparison by means of thin-layer chromatography this metabolite was identified as methylamino-2-benzothiazole. The mass of 237 suggests the introduction of an oxygen atom in the case of compound **6**. An unchanged side chain fragmentation relative to methabenzthiazuron points to a substitution on the benzothiazole ring. This assumption was confirmed by the ^{1}H NMR spectrum prepared from the isolated metabolite. The signals of the N-methylated urea group appear in the expected position. A multiplet for the aromatic protons is found at δ 7.24. This value corresponds to those established by Wallnöfer et al. (1976) for these metabolites. Based on these comparative spectroscopic data compound **6** can be identified as *N*-(6-hydroxy-2-benzothiazolyl)-*N*,*N'*-dimethylurea. Among others, Belasco and Reiser (1969) also found such a ring hydroxylation for Siduron in animal metabolism and Lee et al. (1973) found it for Monuron in plant metabolism.

Compound **7** is also a ring-hydroxylated derivative of methabenzthiazuron with a mass of 281. The urea grouping with an *N*-methyl group is present. In order to elucidate the N' side group, various possible substances were investigated spectroscopically. However, compounds

Table III. Reference Substances for the Structure Determination of Compound 7



Compound	R_1
8	
9	
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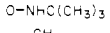
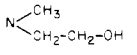
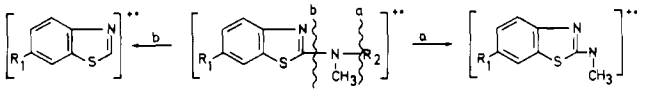
11	
12	



Compound	$[M-N(CH_3)R_2]^+$
1	135
3	135
5	135
6	151
7	151

Compound	$[M]^+$
1	221
3	207
5	164
6	237
7	281

Compound	$[M-R_2]^+$
1	164
3	164
5	163
6	180
7	180

Figure 2. Important fragment ions of compounds **1**, **3**, **5**, **6**, and **7**.

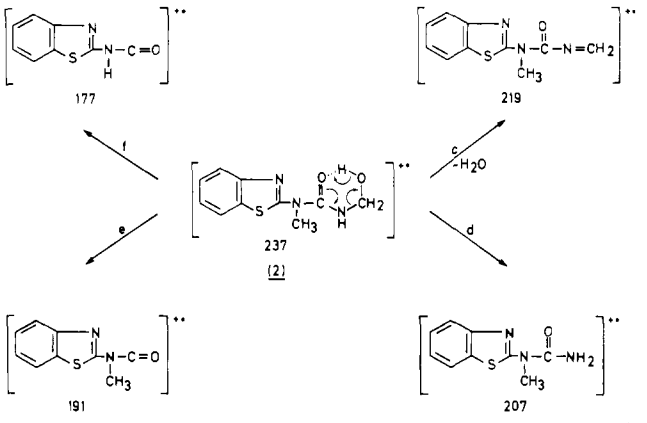


Figure 3. Mass spectral fragmentation pattern of *N*-methyl-hydroxy-*N'*-methyl-(2-benzothiazolyl)urea.

8–**11** and also **12** (Table III) display an entirely different fragmentation behavior than metabolite **7**. An –NHNO–X group remains as the only rational proposed structure which would explain the fragment ions 238 and 222 (see Figure 5). The structure of X, giving rise to observed fragments of mass 17 and 29 in the mass spectrum, cannot be established unambiguously with the available data.

Mass Spectroscopy of Methabenzthiazuron and Its Metabolites. The principal fragmentations of the methabenzthiazuron derivatives (**1**–**7**) are concentrated on the simple C—N cleavages (fragmentation pathway a and b, see Figure 2). These cleavages generally lead to the fragment ions $[M - R_3]^+$ and $[M - N(CH_3)R_3]^+$ recorded as base peaks and whose intensities can be regarded as almost constant within this substance class. The other fragmentation pathways are individual cleavages whose formation is controlled primarily by the structural features of the side chain (R_3). For example, compound **2** with the functional grouping R_3 = $-CONHCH_2OH$ behaves as follows (see Figure 3): Elimination of water from the terminal $-NHCH_2OH$ group is recorded at mass 219. Formation of this fragment ion is promoted by the occurrence of an α,β-unsaturated system (fragmentation pathway c). The McLafferty rearrangement (fragmentation pathway d), which is dependent primarily on the structural feasibility of a six-membered ring transition state being formed, leads to the fragment ion of mass 207. This γ-H shift competes successfully with the α cleavage (fragmentation pathway e), which leads to a fragment ion at mass 191. In this compound, cleavage of the N(2)–CH_3 group leads to a characteristic fragment peak recorded at mass 177 (fragmentation pathway f).

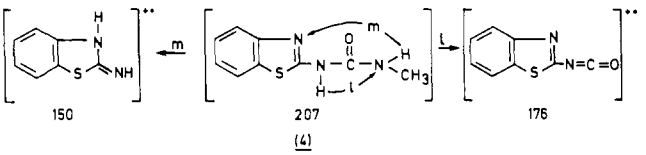


Figure 4. Fragmentation mechanisms of *N*-methyl-*N'*-(2-benzothiazolyl)urea.

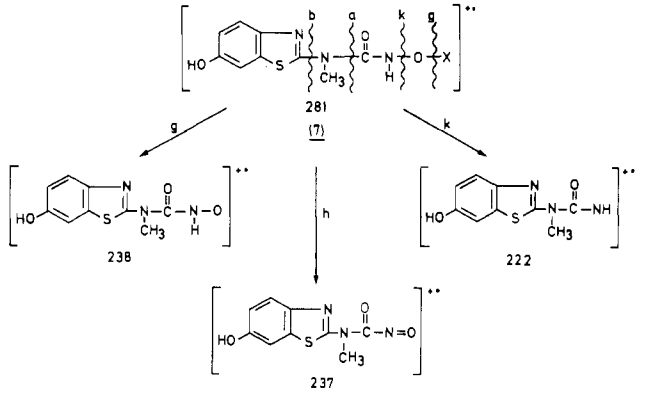


Figure 5. Postulated fragmentation pattern of compound **7**.

In its fragmentation mechanism compound **4** differs from the remaining methabenzthiazuron derivatives by the possibility of a twice-repeated H migration (fragmentation pathways l and m), so that the fragment ions are obtained at mass 176 and 150 (Figure 4). In the lower range the methabenzthiazuron derivatives fragment in the same way as has already been described by Ogura (1970) for 2-methylaminobenzothiazole.

The ring-hydroxylated methabenzthiazuron metabolites **6** and **7** behave similarly to compounds **1**–**3** and **5** (see Figure 2) in their mass spectroscopic behavior. The principal fragmentations via a and b resulting in the fragment peaks at mass 180 and 151, respectively, are very intense. Furthermore, in its mass spectrum, compound **7** (Figure 5) shows the three fragment ions 222, 237, and 238 formed by fragmentation pathways g, h, and k.

CONCLUSIONS

From the investigations forming this study one is able to postulate that, in the soil as well, methabenzthiazuron (**1**) is relatively rapidly metabolized to nonhydrophilic compounds. Further microbial breakdown converts these initial products to hydrophilic metabolites. The unambiguous identification of compounds **3** and **4** formed by N-demethylation of methabenzthiazuron (**1**) is a sure indication that this microbial step may be regarded as a potential first stage during detoxification of this herbicide. Identification of compound **2**, which is formed by hydroxylation of the NCH_3 group, suggests that this reaction is very probably a precursory step of the N-demethylation. Microbial degradation of methabenzthiazuron (**1**) can also take place by direct cleavage of the methyl isocyanate group. The compound **5** resulting from this step confirms the general degradation mechanism of the phenylurea herbicides. From the ring-hydroxylated products **6** and

7 it is seen that also the aromatic ring of methabenzthiazuron (1) can be attacked oxidatively. This occurrence is generally regarded as the first step leading to ring opening and demonstrates the feasibility of the further degradation of this substance class.

ACKNOWLEDGMENT

Special appreciation is expressed to J. Ferguson, Munich, and P. Wallnöfer, Bayer. Landesanstalt für Bodenkultur München, for the advice regarding the microbial problems. We also wish to thank D. Kotzias and E. Schindlbeck, both from the Institut für Ökologische Chemie der Gesellschaft für Strahlen- und Umweltforschung München, for recording the spectra.

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Received for review October 28, 1977. Accepted January 9, 1978. This work is a part of a dissertation, J. Goettfert, Technische Universität München.

Hydroxyterphenyllin: A Novel Fungal Metabolite with Plant Growth Inhibiting Properties

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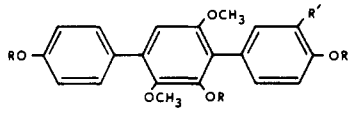
Two metabolites, demonstrating plant growth regulating activity, were isolated from *Aspergillus candidus* found growing on unbleached flour. One of the metabolites was identified as terphenyllin (2′,5′-dimethoxy-4,3′,4″-trihydroxy-*p*-terphenyl); the other was a new metabolite, hydroxyterphenyllin (2′,5′-dimethoxy-3,4,3′,4″-tetrahydroxy-*p*-terphenyl). The former significantly inhibited wheat coleoptile growth at 10^{-3} M; the latter inhibited growth at 10^{-3}, 10^{-4}, and 10^{-5} M. Hydroxyterphenyllin tetraacetate was ineffective against wheat coleoptiles.

During the screening of fungi for metabolites exhibiting plant growth regulating activity, we isolated two biologically active compounds from *Aspergillus candidus* isolated from molded unbleached flour. Both inhibited the growth of wheat coleoptiles. One of these compounds was subsequently identified as terphenyllin (2′,5′-dimethoxy-4,3′,4″-trihydroxy-*p*-terphenyl) (I), a metabolite cytotoxic to HeLa cells (Takahashi et al., 1976) and originally isolated by Marchelli and Vining (1975). The other metabolite, which was more inhibitory to wheat coleoptiles, is a new metabolite, hydroxyterphenyllin (2′,3′-dimethoxy-3,4,3′,4″-tetrahydroxy-*p*-terphenyl) (II). We now wish to report the isolation and identification of hydroxyterphenyllin and the plant growth regulating properties of terphenyllin, hydroxyterphenyllin, and hydroxyterphenyllin tetraacetate (III).



I . R, R′ = H
II . R = H, R′ = OH
III . R = $CH_3C(=O)$, R′ = $CH_3C(=O)O$–

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MATERIALS AND METHODS

Production and Isolation of Active Metabolites. *Aspergillus candidus* (ATCC accession no. 36008) was isolated from a small sample of moldy unbleached flour by culturing on potato dextrose agar (PDA) slants at 26 °C, for 7 days. Cultures were then maintained at 5 °C until transferred to Fernbach flasks (2.8 L), each containing 100

0021-8561/78/1426-0632$01.00/0