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A Study on Degradation of Butachlor by a Soil Fungus, *Chaetomium globosum*

[Part I] Identification of major metabolites by GLC-MS

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土壤絲狀菌 *Chaetomium globosum*에 依한 Butachlor의 分解에關한 硏究

(第一報)

주요대사산물의 GLC-MS에 의한 확인

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SUMMARY

In an effort to elucidate degradation mechanisms of an acetanilide herbicide, Butachlor, by soil microorganisms, a common soil fungus, *Chaetomium globosum* which is known to be powerful was selected and incubated in a Butachlor-contained medium. The results obtained from the resulting metabolites are as follows:

(1) Dechlorination from Butachlor occurred very easily, remaining almost constant after 180 hrs. of incubation.
(2) More than 10 metabolites were isolated and characterized, of which the metabolites, m/e 205, 177, 223, 182, and 206 were the main products.
(3) In this paper, the structures and pathways of formation of metabolites, m/e 206, 182, 223, 225, and 189 were tentatively proposed.

INTRODUCTION

Butachlor is the common name approved by the WSSA for 2-chloro-2′, 6′-diethyl-N-(butoxymethyl) acetanilide, also known as N-(butoxymethyl)-2-chloro-2′, 6′-diethylacetanilide. This compound was introduced, in 1969, by the Monsanto Company under the code number "CP 53619", trade mark "Machete". Butachlor is a preemergence herbicide for the control of annual grasses and certain broad-leaved weeds in rice, both seeded and transplanted. It has also shown selec tivity in wheat, barley, sugar beet, cotton, peanuts and several brassica crops. In this country it has now been in wide use for the main crops. In this connection, the investigation on its decomposition products is considered to be very significant in terms of environmental safety.

Microbial degradation provides the major means of detoxification of many classes of herbicides [3,8,9,12,13,14].

Degradation of an analogous herbicide, Alachlor (2-chloro-2′, 6′-diethyl-N-(methoxymethyl) acetanilide) by a common soil fungus, *Chaetomium globosum* produced chloride and four organic metabolites: 2-chloro-2′,6′-diethyl acetanilide, 2,6-diethyl-N-(methoxymethyl) aniline, 2,6-diethylaniline, and 1-chloroacetyl-2,3-dihydro-7-ethylindole [16]. Environmental factors influencing the detoxication and subsequent degradation of Butachlor were studied and three organic soluble and five aqueous soluble metabolites were separated. Two of the organic soluble compounds were tentatively identified as the N-dealkylated and the alpha hydroxy derivatives[1]. Three commercial α-chloro acetanilide herbicides, Alachlor, Butachlor, and Propachlor, were surface-applied in aqueous solution on two soil types in the field to determine their soil half-lives. In the study, dissipation from sterilized soil was 50 times slower than dissipation from viable soil, indicating that microbial decomposition played a major role in herbicide degradation[2]. Nevertheless, no attempt has been made of the elucidation of metabolites of Butachlor by soil microorganisms.

The soil fungus, *Chaetomium globosum*, was used for this study since Taylor[15] had shown that this organism rapidly metabolized ring-labeled ^{14}C-Alachlor without producing $^{14}CO_2$ and Tiedje et al. [16] also adopted it as a powerful microorganism in his research. In the present paper, the major interest concerns the identification and characterization of Butachlor metabolites produced by the soil fungus, *Chaetomium globosum*, and possible pathways of degradation are proposed. For convenience, part of the research is presented this time.

MATERIALS AND METHODS

Purification of Butachlor

Butachlor (technical grade, 85%) was obtained from the Korag Company Limited, Korea. The crude product was passed through a Florisil column with methanol as an eluent to remove impurities. Methanol was evaporated on a rotary evaporator. The resulting colored, oily sample was further purified by modifying the method of Tiedje et al. [16]. Butachlor, 5 gr, was dissolved in 150 ml of methanol and 90 ml of distilled water was added slowly to it until the mixture remained cloudy on swirling by a magnetic stirrer. The aqueous methanolic solution was then extracted with three 200-ml portions of n-hexane. The n-hexane extract was dried over anhydrous magnesium sulfate and evaporated in vacuo to dryness. The yellow-brown oily material was collected. The purity was checked by tlc, glc, and ms. The following three compounds were detected as trace impurities in ms analysis.

(1) 2-chloro-2′, 6′-diethylacetanilide, m/e 225
(2) 2, 2-dichloro-2′, 6′-diethylacetanilide, m/e 259
(3) 2-chloro-2′, 4′, 6′-triethyl-N-(butoxymethyl) acetanilide, m/e 339

Synthesis of 2-chloro-2′,6′-diethyl acetanilide

As one of the possible metabolites of Butachlor, 2-chloro-2′, 6′-diethyl acetanilide was prepared from Butachlor, not from Alachlor, by adopting the method of Hargrove et al.[6] Butachlor, 1.5 gr, was incubated in a mixture of 400 ml of 5N aqueous HCl and 100 ml of acetone at 46°. After 4 days, acetone was allowed to evaporate, and the water insoluble compound precipitated. As the resulting product contained much of black, tarry material, it was recrystallized as follows. The product was refluxed in benzene with some active carbon and filtered by suction. The filtrate was evaporated to dryness and the resulting residue was washed with n-hexane. White needle-like crystals were obtained. The purity was confirmed by tlc, glc, and ms.

Synthesis of 2,6-diethyl phenol

2,6-Diethylphenol was synthesized from 2,6-diethylaniline on the basis of the method of phenol synthesis from aniline[17].

Preparation of potato dextrose broth

250 gr of sliced potatoes dipped in one liter of

distilled water were heated at 60° for an hour and filtered through gauze. 20 gr of glucose was added to the filtrate and the pH was adjusted to 5.1 with 1N HCl. The medium was autoclaved at 120° for 30 min and used for incubation.

Incubation of C. globosum with Butachlor for chlorine ion determination

The medium flask inoculated with *C. globosum* was incubated on a rotary shaker for 5 days at 28°. After incubation, the mycelial pellets were filtered through glass-wool and washed with sterilized distilled water to remove materials interfering with the determination of the inorganic chlorine ion. The filter-washed inoculum was resuspended in 300 ml of 0.02M KH_2PO_4 buffer (pH 5.0) and Butachlor, 0.12 gr, dissolved in 1 ml of acetone was added for further incubation.

Determination of chlorine ion released from Butachlor

Reagent I: 0.3 gr of $Hg(CNS)_2$ was dissolved in 100 ml of 95% ethanol.

Reagent II: 6 gr of ferric ammonium sulfate was dissolved in 100 ml of 6N HNO_3. In order to check the release of the inorganic chlorine ion from Butachlor during incubation, the following procedure was adopted [7]. 50 ml of the incubation mixture was taken from the flask and filtered through glass-wool. 10 ml of it was used for the determination of chlorine ion, and the rest 40 ml used for the metabolite study. 10 ml of the sample was taken in a test tube and 1 ml of reagent I and 2 ml of reagent II were added to it. The mixture was then shaken vigorously and after 10 min, the absorbance was measured at 460 nm using Optica, Milano, Spectrophotometer. The incubation mixture containing the intact Butachlor and the inoculum was used as the reference for the spectrophotometry.

Incubation of C. globosum with Butachlor for metabolites

C. globosum was incubated for 5 days in 300 ml of potato dextrose broth at 28°. At the end of 5 day incubation, 0.12 gr of Butachlor was added to the medium and further incubated for 8 days.

Incubation of Butachlor in 0.02M phosphate buffer

In order to check formation of any decomposition products, 0.02 gr of Butachlor was added to 50 ml of 0.02 M phosphate buffer and incubated for 8 days at 28°.

Incubation of C. globosum in 0.02 M phosphate buffer

As the control, the inoculum cultured for 5 days was filter-washed with distilled water aseptically, added to 50 ml of the phosphate buffer, and incubated for 10 days.

Incubation of F. oxysporum with Butachlor

For metabolites, *F. oxysporum* was also incubated for 5 days in 300 ml of the medium at 28°. All the other procedures were the same as those of *C. globosum*.

Extraction of Butachlor metabolites

The incubation mixture was extracted twice with chloroform on a separatory funnel and the extract was evaporated to dryness on a rotary evaporator. The resulting residue was redissolved in a small volume of acetone for the analyses.

The soil fungus, C. globosum

It was offered by Laboratoire de Microbiologie des Sols, INRA, Dijon-CEDEX, France.

Mass spectra

They were obtained using a CH 5 Massenspektrometer(VARIAN MAT GmbH) and a gas chromatograph(Perkin-Elmer 881). Spectra were determined using the direct probe and/or glc inlet system at an ionizing potential of 70 electron volts.

Gas-liquid chromatography

The analyses were performed with an F & M Scientific 5750 Research Chromatograph, Hewlett-Packard and a TRACOR (TM) 550 Gas Chromatograph, equipped with a flame ionization detector. The columns used were the following:

(A) 6 ft×1/8 in, 3% OV-225 on 60-80 mesh Chromosorb W;

(B) 3 ft×1/4 in, 10% SE-30 on 60-80 mesh Chromosorb W.

Operating parameters were as follows: nitrogen carrier flow, 23 ml/min; injection temp, 250°;

detector temp, 230°. All analyses were made by temperature-programming from 80° to 200° at a rate of 2°/min for column A and from 70° to 240° at a rate of 7.5°/min for column B.

Thin-layer chromatography

It was accomplished on precoated analytical plates of silica gel HF-254 with fluorescent indicator (Art. 5554, DC-Alufolien Kieselgel 60 F 254, 25 Folien 20×20 cm Schichtdicke 0.2 mm, E. Merck, Darmstadt) using solvent systems consisting of (A) benzene-methanol (85:15 v/v), (B) xylene-chloroform-acetone (40:25:35 v/v), and (C) chloroform-acetone (19:1 v/v). The separated substances were detected under a UV lamp (254 nm wavelength).

Microelemental analyses

They were performed with an F-M Carbon Hydrogen Nitrogen Analyzer Model 180, Coleman using acetanilide as the standard.

Derivatization

Trimethylsilyl (Me_3Si) derivatives were made by placing 1-5 mg of the compound(s) to be derivatized in a silli-vial fitted with a teflon and silicone septum. Acetonitrile (0.5 ml) (silylation grade, Pierce Chemical Co.) and 0.5 ml of Regisil(bis(trimethylsilyl) trifluoroacetamide plus 1% trimethyl-chlorosilane, Regio Chemical Co.) were added. The sealed vial was sonicated 5 min and heated to 100° for 15 min. The derivatized products were concentrated approximately tenfold under a stream of N_2 at room temperature.

RESULTS AND DISCUSSION

Determination of chlorine ion

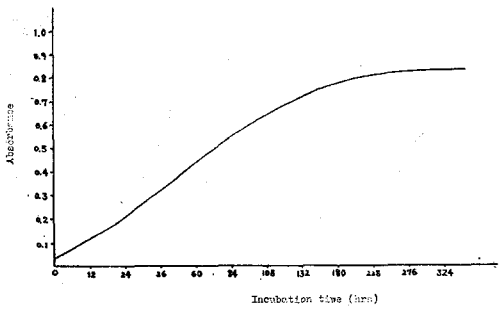

Fig. 1. Dechlorination from Butachlor by *C. globosum* as a function of time

Determination results of the chlorine ion released from Butachlor during incubation are shown in Table I and Figure 1.

Table I. Determination of chlorine ion released from Butachlor during incubation with *C. globosum*

Incubation time(hrs)	Absorbance(at 460 nm)
0	0.053
12	0.159
24	0.207
36	0.294
60	0.508
84	0.565
108	0.575
132	0.637
180	0.791
228	0.792
276	0.794
324	0.797

These figures are plotted against incubation time in Figure 1.

As shown in Table I and Figure 1, it is indicated that chlorine ion is readily released from Butachlor by the soil fungus, *C. globosum*, and, after 180 hrs (7.5 days) of incubation, the amount of chlorine ion did not increase. From these data, it is seen that Butachlor is likely to undergo degradations involving dechlorinaton in the presence of this soil microorganism.

Blank test

No significant metabolites were detected from the control cultures.

Degradation of Butachlor by F. oxysporum

Up to 15 days of incubation, Butachlor remained almost intact in the incubation with this microorganism.

Glc-mass analyses

The chloroform extract of Butachlor incubated with *C. globosum* was subjected to the glc-mass analyses. The gas-liquid chromatogram of the metabolites of 8 day incubation is shown in Figure 2.

Table II shows the masses of the metabolites corresponding to the peaks in the gas-liquid chromatogram.

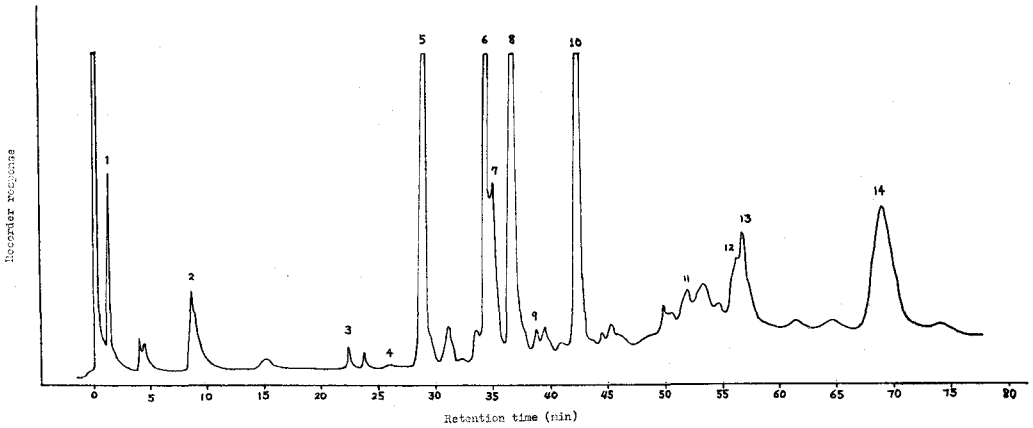

Fig. 2. Gas-liquid chromatogram of Butachlor metabolites produced by *C. globosum* at 8 day incubation, Column A, temp-programmed from 80° to 200° at 2°/min

Table II. Masses of the metabolites corresponding to the peaks in the gas-liquid chromatogram

Peak number	Molecular ion (m/e)
1	115
2	75
3	189
4	147
5	205
6	177
7	165
8	223
9	233
10	182
11	225
12	205
13	259
14	206

Table III. Butachlor metabolites isolated from tlc plates

Metabolites (molecular ions, m/e)	Rf's	
	Solvent A	Solvent B
177	0.85	
165	0.54	
	0.51	
205	0.69	
206	0.56	0.48
222	0.48	
182	0.28	0.45
226		0.02
396	0.22	

Tlc analyses

In order to isolate the metabolites for further identification, the chloroform extract of 8 day incubation was applied on preparative tlc plates as bands using solvent systems A and C.

Table III shows the metabolites isolated from the plates in relatively large amounts.

Of these metabolites, m/e 177, 165, 205, 206, 222, and 396 could be separated with the solvent system A, while m/e 182 and 226 remained at the origin with this solvent. Therefore, the mixture of these two metabolites could be separated by adopting the solvent system C. In the mass analyses with direct probe, the metabolites, m/e 222, 226 and 396 which could not be detected in glc were found. It is thought that these metabolites could not be detected with glc because of their great polarity or large molecular weight.

Identification of the metabolite, m/e 206

The proposed structure and mass spectrum for this metabolite are presented in Figure 3.

In the spectrum, the molecular ion at m/e 206 is strong, and the prominent peaks at m/e 191 (M-15), 164 (M-42) and 136 (M-70) correspond to the losses of CH_3, $COCH_2$, and $COCH_2$, CH_2CH_2, respectively. To further confirm the structure, microelemental analysis was performed. The result is presented in Table IV.

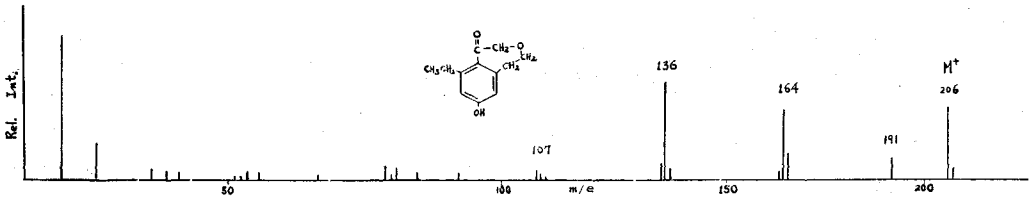

Fig. 3. Mass spectrum of the metabolite, m/e 206

Table IV. Microelemental analysis of the metabolite, m/e 206

element \ amount	calculated (%)	found (%)
C	69.9	70.0
H	6.8	7.8
N	0	0.5

As seen in Table IV, the calculated and determined values are almost identical to support the structure. The trace amount of nitrogen which could not be detected theoretically is thought to result from contamination during instrumentation. The possible pathway of formation of m/e 206 from Butachlor is proposed in Figure 4.

As seen in the scheme, although II was not observed [16], its formation as an intermediate would be quite conceivable, based on the fact that dechlorination occurred very rapidly during incubation. A similar dechlorination has been reported for 2-chloro-N-isopropylacetanilide (Propachlor) metabolism in several plants [11] and by a soil fungus, *F. oxysporum*[10]. The conversions II→III and II→VI will proceed by losses of H_2 and H_2O, respectively. The identity of III will be reported later in the subsequent papers. The conversion III→IV→V will be very important in that no re-

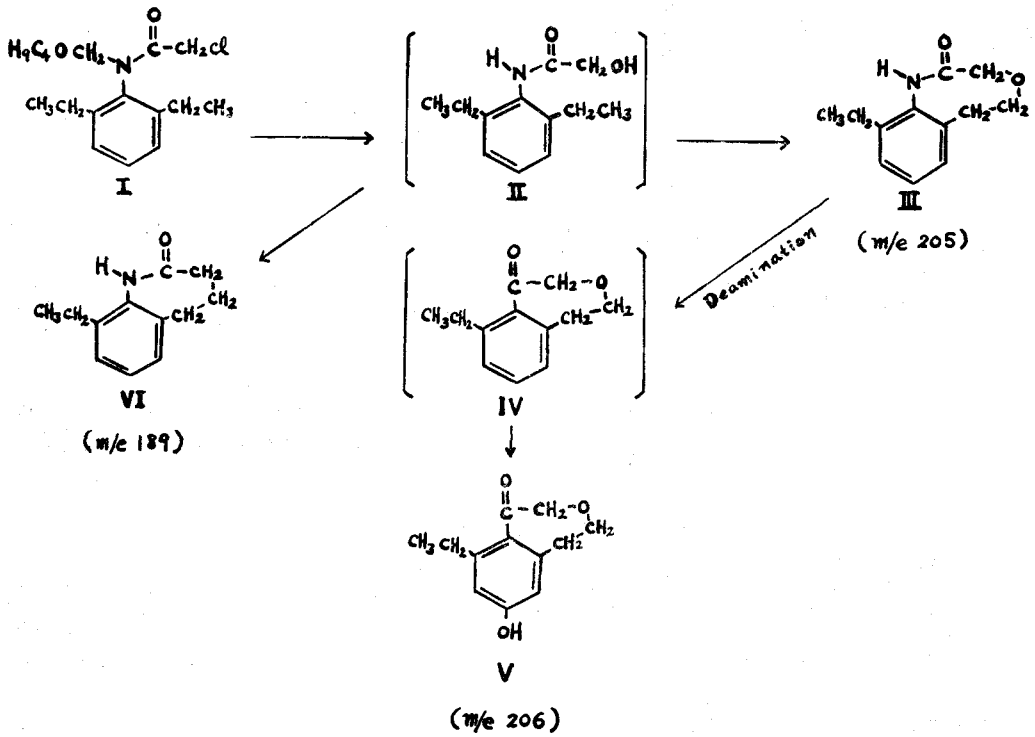

Fig. 4. The possible pathway of formation of the metabolites, m/e 206 and 189.

search has been done on this proposed mechanism. IV will be formed from III by an unknown deamination mechanism linking the carbonyl moiety directly with the benzene ring. Finally, V will be readily formed from IV by hydroxylation.

The proposed structure is thought to be quite reasonable based on the data of microelemental analysis.

Identification of the metabolite, m/e 182

The proposed structure and mass spectrum for this metabolite are presented in Figure 5.

In the spectrum, the weak molecular ion at m/e 182 and the prominent ions at m/e 154(M-CO), 139 (M-CO, CH_3), 126(M-CO, CO), and 111(M-CO, CO, CH_3) are suggestive of the proposed structure. In order to confirm the structure, the

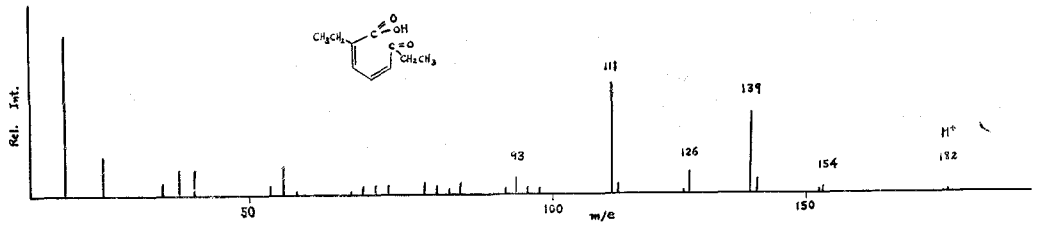

Fig. 5. Mass spectrum of the metabolite, m/e 182

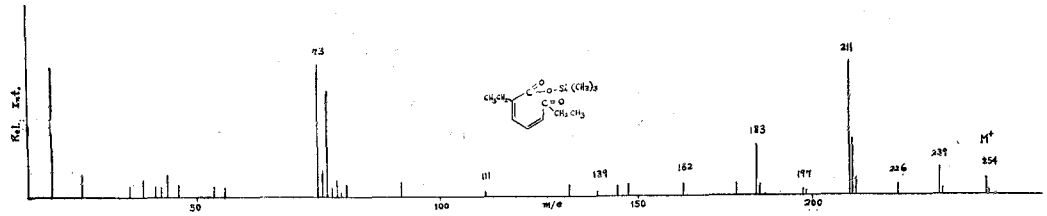

Fig. 6. Mass spectrum of the TMS-derivative of the metabolite, m/e 182

metabolite was derivatized. The mass spectrum of the trimethylsilyl (Me_3Si) derivative is shown in Figure 6.

The molecular ion at m/e 254 indicates the TMS-derivatization of the metabolite. The prominent peaks at m/e 211 and 183 correspond to the peaks at m/e 139 and 111 in the non-derivatized spectrum. The strong peak at m/e 73 indicates the fragment $Si(CH_3)_3$ which is absent in the non-derivatized spectrum.

The possible pathway of formation of this metabolite is proposed in Figure 7.

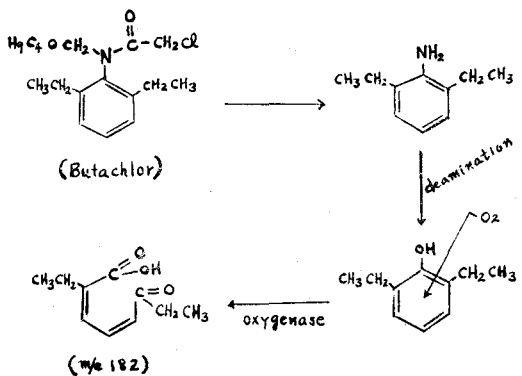

Fig. 7. The possible pathway of formation of the metabolite, m/e 182.

In an effort to verify the fact that the metabolite, m/e 182 is formed from 2,6-diethylaniline via 2,6-diethylphenol, the two compounds were incubated with the microorganism in the same way, respectively. The incubation mixtures were extracted with chloroform to be subjected to tlc and glc analyses. As the tlc analysis showed the expected results, the extract was analyzed by glc. The gas-liquid chromatograms of the metabolites of both compounds are shown in Figure 8.

As seen in the chromatograms, the metabolite, m/e 182 was formed from both compounds(retention time: 13.7 min).

While rather a small amount was formed from

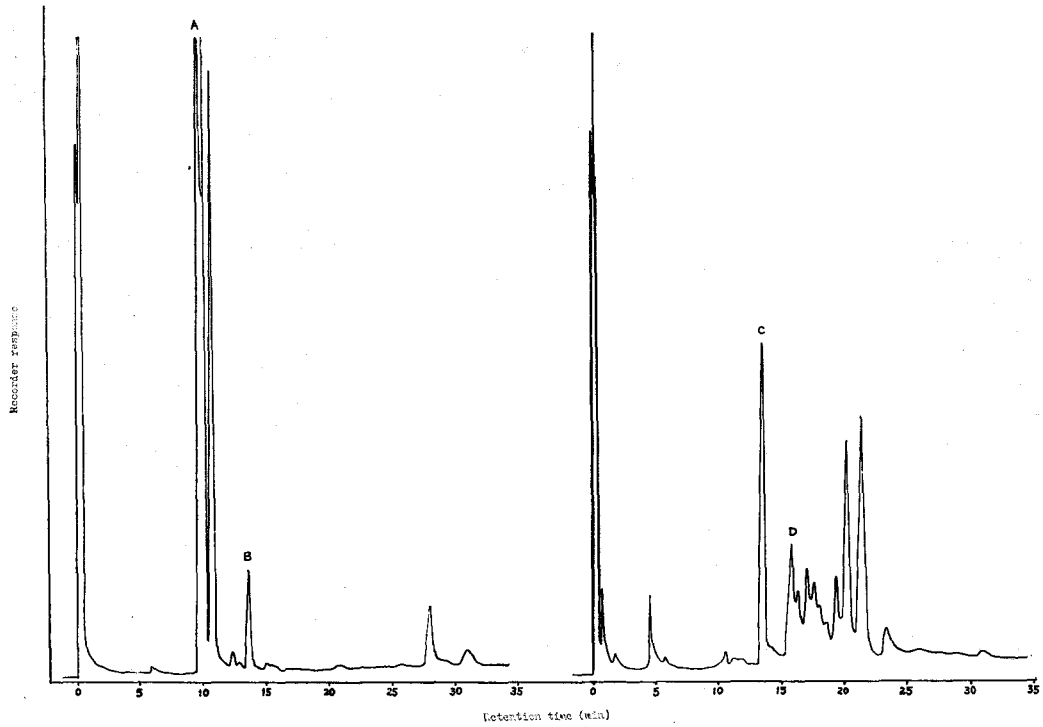

Fig. 8. Gas-liquid chromatograms of the metabolites of 2, 6-diethylaniline (left) and 2, 6-diethylphenol (right)
A: 2, 6-diethylaniline (undecomposed) B: the metabolite, m/e 182
C: the metabolite, m/e 182 D: 2, 6-diethylphenol (undecomposed)
Column B, temp-programmed from 70° to 240° at 7.5°/min

2, 6-diethylaniline, a greater amount formed from 2, 6-diethylphenol, indicating that the latter is the intermediate in the mechanism.

In this scheme, the 2, 6-diethylphenol resulting from 2, 6-diethylaniline will undergo ring fission by the action of an oxygenase to form the proposed formula, even though no reports are available on this mechanism. Oxygenases are a group of enzymes that catalyze the incorporation of molecular oxygen into various organic compounds[5]. They are enzymes catalyzing the addition of molecular oxygen across a double bond between two carbon atoms.

While doing so, they add both atoms of the oxygen molecule to the substrate [4]. Tiedje et al. [16] did not report farther than 2, 6-diethylaniline as the metabolite of Alachlor in his degradation scheme. However, in this investigation with Butachlor, the metabolite, m/e 182 was found as one of the main metabolites, while 2, 6-diethylaniline could not be detected at all.

Identification of the metabolite, m/e 223

As the mass spectrum of this metabolite is completely identical with that of 1-chloroacetyl-2, 3-dihydro-7-ethylindole identified by Tiedje et al. in the degradation of Alachlor[16], it was not presented in this paper. Briefly, the molecular ion at m/e 223 with the M+2 peak in accord with the isotopic abundance ratio indicates the presence of one chlorine. The odd number of the molecular weight suggests the presence of one nitrogen. And, the prominent peaks at m/e 208, 174, and 146 correspond to M-CH_3, M-CH_2Cl, and M-$COCH_2Cl$, respectively. This result indicates that in the degradations of Alachlor or Butachlor by this microorganism, the losses of methoxymethyl or methoxybutyl moiety occur quite readily in like manner to yield this metabolite,

Identification of the metabolite, m/e 225

In the tlc analysis, this metabolite turned out to be completely identical with the authentic 2-chloro-2′,6′-diethylacetanilide which was also isolated and identified in the Alachlor degradation[16], the Rf being 0.51 in the solvent system B.

Although it was originally contained as an impurity, a much more quantity was formed as a metabolite. According to the mass spectrum which is not shown here, the molecular ion at m/e 225 and the M+2 peak indicate the presence of one chlorine. The prominent fragment peak at m/e 176 corresponds to the loss of CH_2Cl(M-49) from the molecular ion. The peak at m/e 148 indicates the subsequent loss of CO(M-CH_2Cl, CO). Further identification and characterization are not attempted.

Identification of the metabolite, m/e 189

The proposed structure and mass spectrum are presented in Figure 9.

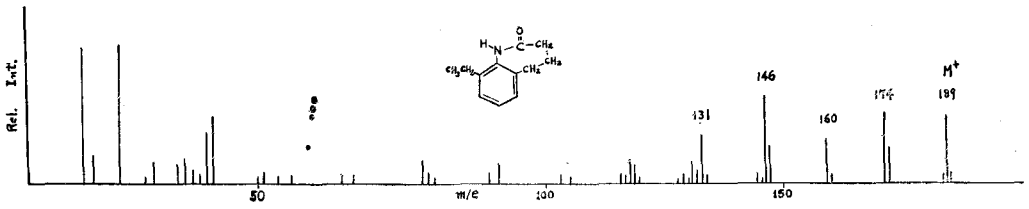

Fig. 9. Mass spectrum of the metabolite, m/e 189

In the spectrum, the molecular ion at m/e 189 is strong and the prominent peaks at m/e 174 (M-CH_3), 146(M-CH_3, CO), and 131(M-CH_3, CO NH) are in good agreement with the expected fragmentation pattern. The possible pathway of formation is shown in Figure 4, where VI represents this metabolite which seems to be a minor product.

요 약

토양미생물에 의한 Acetanilide계 제초제 Butachlor의 분해기구를 구명하기 위하여 분해력이 강력하다고 알려진 *Chaetomium globosum*을 선택하여 Butachlor와 배양한 후 생성된 분해 산물에 관하여 얻은 결과는 다음과 같다.

(1) Butachlor로부터 탈염소화 작용은 아주 용이하게 일어나며 배양후 180시간 부터는 일정하게 유지되었다.

(2) 십여종의 분해 산물을 분리 동정하였고 그 중 질량이 205, 177, 223, 182 및 206인 것이 주된 산물이었다.

(3) 본논문에서는 질량 206, 182, 223, 225 및 189인 분해산물의 구조와 형성 경로를 제안하였다

Acknowledgment

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