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Degradation of the Herbicide Alachlor by Soil Microorganisms

II. Synthesis and Phytotoxicity of Major Degradation Products

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제초제 Alachlor의 토양미생물에 의한 분해

II. 주요 분해산물의 합성과 식물독성

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Abstract

Two of the degradation products of alachlor in soil. product 1, 2, 6-diethyl-N-(methoxymethyl) acetanilide and product 2, 2-hydroxy-2′, 6′-diethyl-(methoxymethyl) acetanilide were synthesized from alachlor reacting with 3 N-hydrochloric acid in the presence of zinc powder at room temperature and a saturated sodium bicarbonate solution at 90°C for 78 hr, respectively. At the concentrations of both 5×10^{-4}M and 1×10^{-3}M, product 2 exhibited almost the same phytotoxicity to rice seedlings, in particular, as alachlor, whereas product 1 lost its phytotoxic effectiveness. It seems that substitution of chlorine atom by hydroxyl group did not affect the phytotoxicity of alachlor, whereas substitution by hydrogen atom did.

Introduction

Alachlor, 2-chloro-2′, 6′-diethyl-N-(methoxymethyl) acetanilide, is a selective preemergence herbicide which has been in wide use for controlling certain annual grasses and broadleaf weeds in corn, potatoes, and some leguminous crops. α-Chloroacetamide herbicides are known to inhibit seed germination and development of germinating seedlings, (1,2,3,4,5) protein synthesis, (6,7,8,9,10) and gibberellic acid (GA_3)-induced hydrolytic enzyme production. (8,11,12)

Hamill and Penner(13) found that more alachlor was absorbed by the susceptible barley than by the tolerant corn. Meanwhile, other investigators correlated resistance with the ability to metabolize the herbicide fast enough to keep the level below that required for growth inhibition.(8) Tolerance of common beans to alachlor is known to depend on cultivars, herbicide placement, temperature, soil moisture, and other soil factors.(14,15).

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Deal and Hess⁽⁵⁾ studied the effects of varying concentrations and duration of alachlor and metolachlor [2-chloro-N-(2-ethyl-6-methylphenyl)-N-(2-methoxy-1-methylethyl) acetamide] treatment on root growth, cell division, and cells enlargement in peas and oats. Chang *et al.*⁽¹⁶⁾ found that R-25788 (N, N-diallyl-2, 2-dichloroacetamide) significantly reduced the phytotoxicity of ten of twenty-two herbicides tested on corn. The antidote R-25788 was also reported to protect corn seedlings from alachlor injury.⁽¹⁷,¹⁸⁾ Spotanski and Burnside⁽¹⁹⁾ reported that alachlor applied preplant incorporated caused sorghum injury. Consequently, the use of crop protectants or antidotes is necessary to minimize the injury in corn or sorghum. According to Beestman and Deming,⁽²⁰⁾ alachlor is readily degraded in soil and the soil microorganisms are involved in the degradation. In the previous investigation on the degradation of alachlor in flooded paddy soils by Lee,⁽²¹⁾ two degradation products were noteworthy. Since the half-life of alachlor in soils is rather short, it is necessary to investigate on the phytotoxicity of the major degradation products from the standpoint of evaluating the effectiveness of the herbicide. Accordingly, in the present investigation, the two major products were synthesized and their phytotoxicity was examined.

Materials and Methods

Synthesis of product 1, 2, 6-diethyl-N-(methoxymethyl) acetanilide

200 mg of alachlor was dissolved with a small amount of acetone in a large screw-capped culture tube (25×150 mm) and an excess of 3 N-hydrochloric acid and zinc powder was added little by little. Hydrogen was evolved vigorously during the reaction. The reaction was allowed to occur at room temperature for 1 hr with agitation on a Vortex mixer. At the completion of the reaction, some distilled water was added and the product was extracted with chloroform. The chloroform extract was then dried over anhydrous sodium sulfate and concentrated to obtain a colorless oily material. For subsequent identification, the product was purified by TLC.

Synthesis of product 2, 2-hydroxy-2′, 6′-diethyl-N-(methoxymethyl) acetanilide

200 mg of alachlor was dissolved with a small amount of acetone in a large screw-capped culture tube (25×150 mm) and an excess of a saturated sodium bicarbonate solution was added. The mixture was reacted with constant shaking at 90°C on a water-bath for 78 hr. At the end of the reaction, some distilled water was added and the product was extracted with chloroform. The chloroform extract was dried over anhydrous sodium sulfate and concentrated to obtain an oily brown product. For purification, preparative TLC plates were used.

Mass spectrometry

Mass spectra of the synthesized compounds were obtained with a Finnigan 3, 200 Gas Chromatograph-Mass spectrometer. The electron impact ionization potential was 70 eV.

Gas-liquid chromatography

An instrument of Shimadzu, Gas chromatograph GC-4C (PTF) equipped with a flame ionization detector was used. The column was a pyrex glass of 6 mm (OD)×8 ft packed with 5% Silicone GE SE-30 on 60-80 mesh Shimalite W(AWDMCS). Operating conditions were as follows: helium carrier flow, 50 ml/min.; air, 0.8 kg/cm^2; hydrogen, 0.7 kg/cm^2; injection port temp., 200°C; detector temp., 230°C; column temp, 150°C.

Infarared spectrometry

The Perkin-Elmer 298 Model was used for the IR spectra. The viscous oily compounds synthesized were applied directly on the cell to obtain neat liquid films.

Thin-layer chromatogrohpy

For purification of the compounds synthesized, Kieselgel GF 254 (Typ 60) (Art. 7730, Merck) was coated on 20×20 cm glass plates and used after activation at 110°C for 3 hr. The precoated analytical plates of silica gel HF-254 with a fluorescent indicator were used for comparison of the Rf's of the compounds. The developing solvents were (A) benzene-methanol (85 : 15, v/v) and (B) xylene-chloroform-acetone (40 : 25 : 35, v/v/v). The spots were detected under a UV lamp with a wavelength of 254 nm.

Preparation of the test solutions

26.9 mg of alachlor was dissolved in 1 m*l* of ethanol and 0.5 g of Triton X-100 as a solubilizer was added to this solution. To this mixture, distilled water was then added up to 100 m*l* of volume to give a 1×10^{-3}M alachlor solution. For a 1×10^{-3}M solution of the degradation product 1, 2, 6-diethyl-N-(methoxymethyl) acetanilide, 23.5 mg of the product synthesized was dissolved in 1 m*l* of ethanol and 0.5 g of Triton X-100 was added. For a 1×10^{-3} M solution of the degradation product 2, 2-hydroxy-2′, 6′-diethyl-N-(methoxymethyl) acetanilide, 25.1 mg of the product was used. The next procedures were the same as the above. The 5×10^{-4}M solutions of each compound were prepared from the respective 1×10^{-3}M solutions by diluting two-fold with distilled water. Therefore, the solutions of 1×10^{-3}M and 5×10^{-4}M of each compund contain 1% ethanol plus 0.5% Triton X-100 and 0.5% ethanol plus 0.25% Triton X-100 as solubilizers, respectively, in addition to distilled water. The control contains none other than 1% ethanol plus 0.5% Triton X-100. Distilled water was also compared with other treatments.

Phytotoxicity test

Two sheets of Whatman filter paper were laid in each petri dish and 5 m*l* of each test solution was added. The seeds were surface sterilized by hypochlorite before germination and 10 germinating seeds of each species were placed on the soaked filter paper. To keep the filter paper moist, certain amounts of the test solutions were added regularly. After certain periods of incubation time at 20°C, the pictures of the seedlings were taken and the growth of shoots and roots was measured, if possible. The species tested include rice (*Akibare* cultivar), radish (*Jin-Ju Dae-Pyung* cultivar), and mungbean.

Results and Discussion

Synthesis of 2, 6-diethyl-N-(methoxymethyl) acetanilide

An outline of the synthetic method of this compound is shown in Fig. 1. In this synthesis, when a mixture of concentrated hydrochloric acid and glacial acetic acid (1 : 1, v/v) was used as the reducing agent in the presence of zinc powder, two products were obtained. One was 2′, 6′-diethyl-N-methoxymethyl-N-(1-hydroxy-ethyl) aniline which resulted from 2, 6-diethyl-N-(methoxymethyl) acetanilide by reduction of the carbonyl group. The other was 2, 6-diethyl-acetanilide which was produced from 2, 6-diethyl-N-(methoxy-methyl) acetanilide by removal of the methoxymethyl group. Their identities were confirmed by MS and TLC.

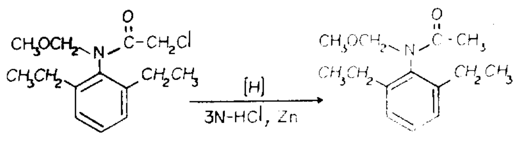

Fig. 1. Scheme for the synthesis of 2, 6-diethyl-N-(methoxymethyl) acetanilide from alachlor

In the meantime, by means of 3 N-HCl without using glacial acetic acid in the presence of zinc powder, the synthesis completed perfectly within 1 hr at room temperature. After purification on preparative TLC plates, the product was subjected to spectrometric identification.

The mass spectrum of this product is presented in Fig. 2. In this spectrum, the molecular ion occurs at m/z 235. The fragment peaks at m/z 220, 204, 192 and 190 correspond to M-CH_3, M-CH_3O, M-

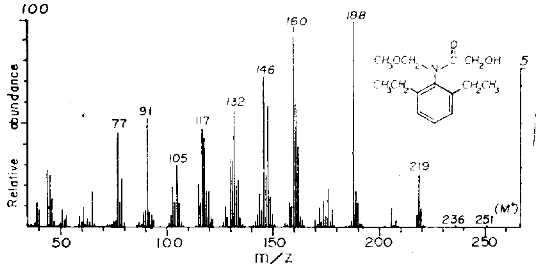

Fig. 2. Mass spectrum of 2, 6-diethyl-N-(methoxymethyl)acetanilide

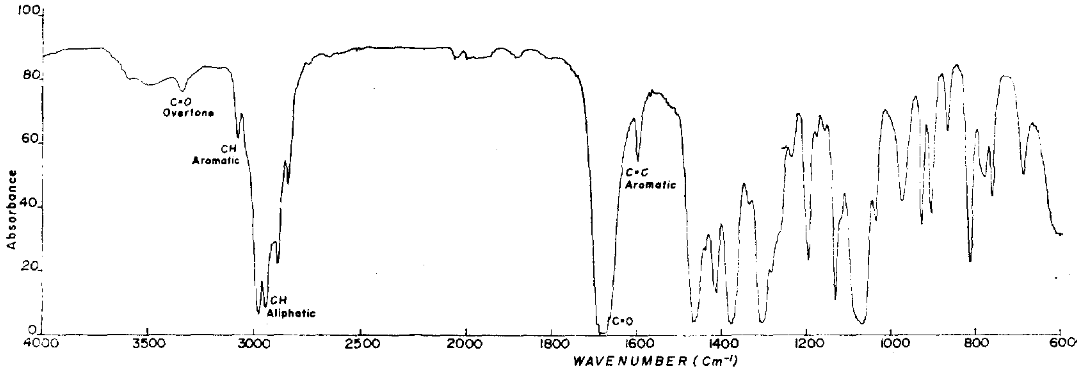

Fig. 3. IR spectrum of 2,6-diethyl-N-(methoxymethyl) acetanilide

$COCH_3$, and $M-CH_3OCH_2$, respectively. The prominent fragment peaks at m/z 178, 162, and 147 correspond to $M-CH_2=C=O$, CH_3, $M-COCH_3$, CH_3, CH_3, or $M-CH_3OCH_2$, $CH_2=CH_2$, and $M-COCH_3$, CH_3OCH_2, respectively. As can be seen, all the fragmentation pattern is in good agreement with what is expected of the chemical structure.

The IR spectrum of this product is presented in Fig. 3. In this spectrum, the characteristic $C=O$ stretching frequency in amides appears at a lower position of 1,680 cm^{-1} than that of a ketone due to the resonance effect. A weak absorption at 3,340 cm^{-1} is thought to be the overtone of the $C=O$ stretching vibration. The aromatic C-H stretching vibration occurs at 3,080 cm^{-1} and the aliphatic C-H stretching vibrations occur at 2,980, 2,950, 2,890, and 2,840 cm^{-1}.

Synthesis of 2-hydroxy-2′,6′-diethyl-N-(methoxymethyl) acetanilide

The synthetic method of this compound from alachlor is outlined in Fig. 4. In this reaction, the chlorine atom present in alachlor is thought to be substituted by the hydroxyl ion which is produced by hydrolysis of sodium bicarbonate. When alachlor dissolved in a small amount of ethanol was allowed to react with a saturated sodium bicarbonate solution at 50°C for 4 and 13 hr, respectively, the synthesis was not completed, leaving the unchanged alachlor behind, as verified on TLC.

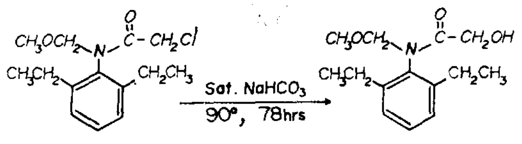

Fig. 4. Scheme for the synthesis of 2-hydroxy-2′,6′-diethyl-N-(methoxymethyl) acetanilide from alachlor

In the long run, it was found that for the complete nucleophilic substitution of the chlorine atom, sufficient time as long as up to 78 hr was needed. The release of chloride ion was checked with a silver nitrate solution. The crude product was purified on preparative TLC plates for further identification. The mass spectrum of this product is shown in Fig. 5. In this spectrum, the molecular ion occurs at m/z 251. The fragment peaks at m/z 236 and 220 correspond to $M-CH_3$ and $M-OCH_3$, respectively. The fragment peaks at m/z 219 and 206 will be due to $M-CH_3OH$ and $M-CH_3OCH_2$. The base peak occurs at m/z 188 which corresponds to either $M-CH_3$, OH, CH_3O or $M-CH_3OCH_2$, H_2O. The most prominent peak at m/z 160 next to the base peak is thought to correspond to either $M-CH_3$, OH, CH_3O, CH_2N or $M-CH_3OH$, CH_2OH, CO. The fragment peak at m/z 146 will correspond to $M-CH_3OCH_2$, CH_2OH, CH_2CH_3. The fragmentation pattern is con-

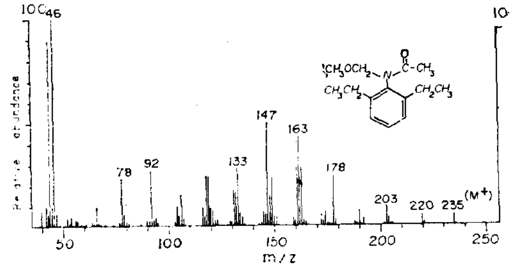

Fig. 5. Mass spectrum of 2-hydroxy-2′,6′-diethyl-N-(methoxymethyl) acetanilide

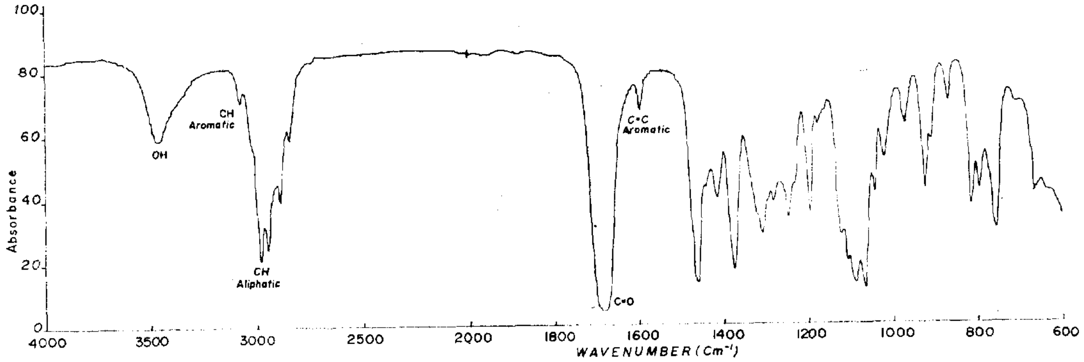

Fig. 6. IR spectrum of 2-hydroxy-2′, 6′-diethyl-N-(methoxymethyl) acetanilide

sistent with what is expected of this chemical structure. The rest fragment peaks were not followed.

For further identification, the IR spectrum of this product was obtained as seen in Fig. 6. In this spectrum, the O-H stretching vibration occurs at 3, 470 cm^{-1}. The strong C-O single bond stretching vibration is observed at 1, 070 cm^{-1}. Besides this band, an O-H in-plane bending absorption is found at 1, 375 cm^{-1} which is also the position of the C-H bending vibration for the methyl group. The C=O stretching vibration occurs at 1, 685 cm^{-1}.

Chromatographic results of the degradation products on GLC and TLC

Table 1 presents the retention times and Rf values of alachlor and its degradation products as analyzed by GLC and TLC.

Table 1. Comparison of chromatographic data of alachlor and its degradation products on GLC and TLC

Compound	Retention time (min.)	Rf Developing solvent A	Rf Developing solvent B
Alachlor	5. 2	0. 80	0. 80
Product 1	1. 7	0. 69	0. 71
Product 2	4. 4	0. 57	0. 64

Phytotoxicity of the degradation products

As a monocotyledonous species for the test, rice seedlings were used. Table 2 shows the growth of shoots and roots of rice seedlings which were grown for 8 days in petri dishes containing varying cencentration solutions of the compounds. The seeds were germinated separately beforehand by soaking in distilled water. All the figures represent the averages of 10 seedlings. Comparing the phytotoxicity of the products with each other, the product 1, 2, 6-diethyl-N-(methoxymethyl) acetanilide, lost its phytotoxic effectiveness as evidenced by Table 2 and Fig. 7. This means that if the chlorine in the chloroacetyl group of alachlor is replaced by hydrogen in one way or another, the resulting product is not so effective as the parent. On the contrary, if the chlorine of the chloroacetyl moiety is replaced by a hydroxyl group which is also a nucleophile, the effectiveness of the resulting product was almost the same as before. As can be seen in Table 2, the product 2, 2-hydroxy-2′, 6′-diethyl-N-(methoxymethyl) acetanilide exhibited almost the same phytotoxicity as alachlor.

Duke(22) conducted a study on the mode of action of chloroacetamides using propachlor which is an analog of alachlor. He showed that cell elongation and protein synthesis by normal and auxin-induced cucumber hypocotyl tissue was inhibited by propachlor at 5 ppm and that the inhibition of protein synthesis preceded the inhibition of growth and nucleic acid synthesis. Based on these results, he indicated that propachlor could be active by preventing the activation of amino acids and aminoacyl-tRNA formation or by interfering with the transfer of aminoacyl-tRNA to the polypeptide. Jaworski(8) suggested that because of the reactive nature of the α-chlorine in the α-chloroacetamides, a nucleophilic displacement might occur between the amino

Table 2. Phytotoxicity of alachlor and its degradation products to rice seedlings at varying concentrations

Treatment	Elongation (mm)[a)] 5×10⁻⁴ M Shoot[b)]	5×10⁻⁴ M Root	1×10⁻³ M Shoot	1×10⁻³ M Root
Distilled water	16.05a	42.60a	—	—
Contro l	—	—	23.20a	17.10a
Alachlor	5.85b	5.75c	4.50c	4.60c
Product 1	19.15a	15.30b	16.40b	10.30b
Product 2	5.15b	5.80c	4.10c	3.50c

a) All data represent averages of 10 seedlings in each treatment.

b) Numbers followed by the same letter within a column are not significantly different at the 5% level using Duncan's multiple range test.

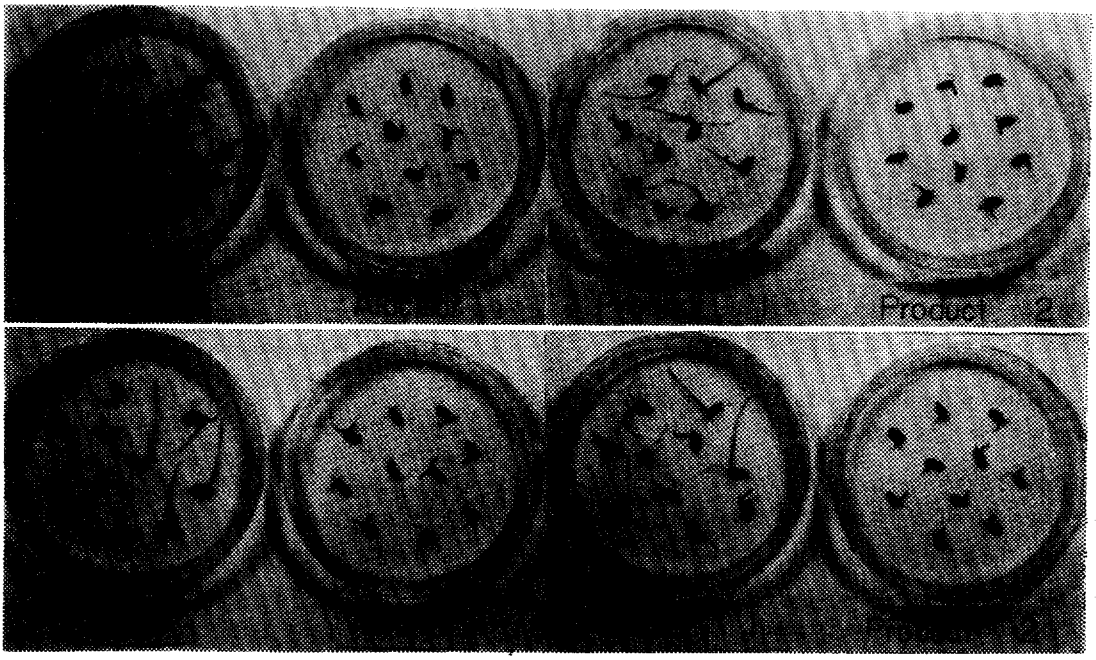

Fig. 7. Growth of rice seedlings over a period of 8 days after germination at the concentrations of 5×10^{-4}M(upper) and 1×10^{-3}M (lower) of alachlor and its degradation products

group of methionyl-tRNA and the herbicide for the interference with protein synthesis.

Considering the possible nucleophilic displacement between the amino group of the aminoacyl-tRNA and the herbicide, it can be understood that alachlor and the product 2 containing chlorine and hydroxyl group, respectively, in the chloroacetyl moiety in their chemical structures can exhibit the same effectiveness.

In the previous study by Lee,⁽²¹⁾ it was shown that some selected microorganisms produced 2-hydroxy-2′, 6′-diethyl-N-(methoxymethyl) acetanlide as their major degradation product, when alachlor was put in pure culture of the microorganisms. Fig. 8 shows one of the results obtained.

Since alachlor is readily degraded in soil mainly by microorganisms and the product 2, 2-hydroxy-2′, 6′-diethyl-N-(methoxymethyl) acetanilide can be formed as one of the major degradation products, it is thought that the product 2 can contribute to some extent to the effectiveness of alachlor as a herbicide.

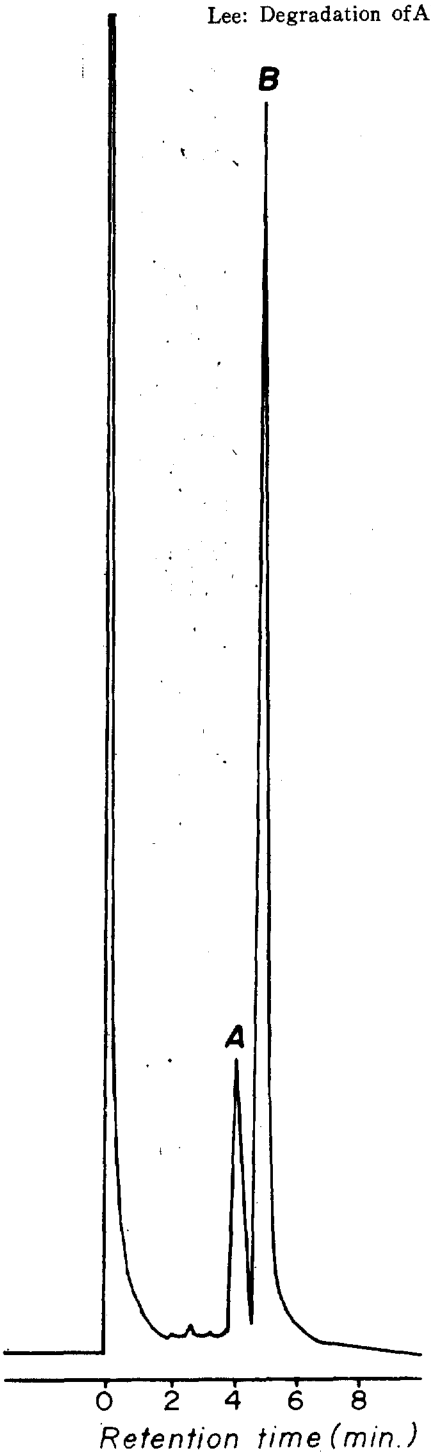

Fig. 8. Gals-iquid chromatogram showing production of product 2, 2-hydroxy-2′, 6′-diethyl-N-(methoxymethyl) acetanilide, by a microorganism, *Streptomyces coelicolor* 1023 in pure culture

A : Product 2

B : Intact alachlor

In the phytotoxicity of the degradation products to radish, a dicotyledon, at the concentration of 5×10^{-4}M, the differences in growth between the products were difficult to distinguish (Fig. 9). Meanwhile, at the concentration of 1×10^{-3}M, almost no growth was recognized except for the control.

In this case, however, product 1 and 2 showed almost the same effectiveness as alachlor. Concerning mungbean which is also a dicotyledonous species, at the concentration of 5×10^{-4}M, the same results were obtained as seen in Fig. 10. As seen in this figure, the growth in the control which is distilled water was outstanding, whereas the product 1 also showed a better growth than alachlor and the product 2. As for the alachlor uptake by plant seedlings, the shoots are known to be the major site of absorption.(23,24,25,26) However, other studies showed that root absorption of alachlor might play a part in the plant injury(27,28). Nevertheless, acetanilide herbicides are general growth inhibitors affecting shoot and root growth following seed germination.(1,3,4,5)

In conclusion, as Jaworski(8) pointed out, the observed inhibition in the growth of the sample seedlings by alachlor and its degradation product 2 may be the sum total resulting from a variety of mechanisms affected by them.

要　約

Alachlor의 토양중 분해산물인 2,6-diethyl-N-(methoxymethyl) acetanilide(분해산물 1)와 2-hydroxy-2′,6′-diethyl-N-(methoxymethyl) acetanilide (분해산물 2)를 합성하였다. 분해산물 1은 실온에서 아연분말 존재하에 alachlor를 3N-염산과 반응시켜서 얻었고 분해산물 2는 alachlor를 중탄산소다의 포화용액과 90°C 에서 78시간 반응시켜 합성하였다. 분해산물 2는 5×10^{-4}M와 1×10^{-3}M의 농도에서 특히 어린벼에 alachlor 와 거의 같은 독성을 보인 반면 분해산물 1은 그 약효를 상실하였다. 따라서 alachlor 구조중의 염소원자를 수산기로 치환해도 alachlor의 식물독성은 별 영향을 받지 않으나 수소원자로 치환할 경우는 영향을 받는 것으로 보인다.

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Fig. 9. Growth of radish over a period of 8 days after 2 day's soaking in distilled water, at the concentration of 5×10⁻⁴M of alachlor and its degradation products Control contains 0.5% ethanol and 0.25% Triton X-100 in distilled water

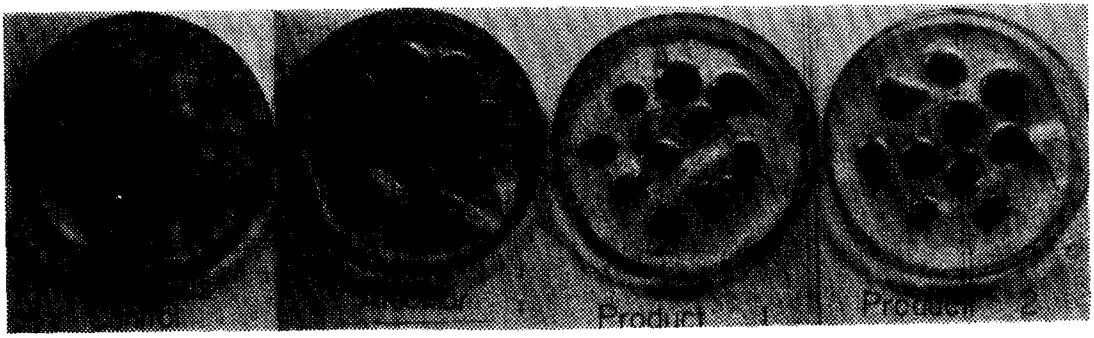

Fig. 10. Growth of mungbean over a period of 5 days after germination, at the concentration of 5×10⁻⁴M of alachlor and its degradation products Control is distilled water

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