Mechanism of the Phytotoxic Action of Herbicidal \(N\)-Isobutyl-\(N\)-(4-substituted benzyl)-4-halo-2-pentenamides

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The \(N\)-isobutyl-\(N\)-(4-substituted benzyl)-4-halo-2-pentenamides show a potent light-dependent herbicidal activity against the barnyard grasses. The herbicidal mechanism was investigated with assay systems using cotton seedlings and an enzyme preparation from them as well as cultured unicellular microalgae. Besides the protoporphyrinogen oxidase inhibition, some other mechanism(s) inhibiting step(s) involved in the chlorophyll biosynthetic pathway were suggested as the origin of the phytotoxicity.

Key words: light-dependent herbicidal activity, \(N\)-benzyl-\(N\)-isobutyl-4-halo-2-pentenamides, protoporphyrinogen oxidase inhibition, structure-activity relationships.

INTRODUCTION

We previously found that certain \(N\)-alkyl-\(N\)-(substituted benzyl)-4-halo-2-alkenamides (I) exhibit a highly potent herbicidal activity against the barnyard grass, \textit{Echinocloa oryzicola}.\textsuperscript{1)} The application rates were 15-30 g/ha at the lowest depending upon combinations of substituents.\textsuperscript{1)} Among compounds, the optimum combinations of substituents were of \(R_1=\text{Me}, X_1=\text{Cl}, R_2=\text{i-Bu}\) and \(X_2=p-\text{Cl}\) and -CN.\textsuperscript{1,2)} The herbicidal characteristics were light-dependent similar to such ortho-substituted diphenyl ethers as oxyfluorfen and \(N\)-substituted phenyl dicarboximides as chlorophthalim.\textsuperscript{1)}

Because the structural features of compounds (I) are quite different from either diphenyl ethers or substituted phenyl dicarboximides, we have examined the mode of action of compounds (I) using pentenamides (II) with seedlings of cotton, \textit{Gossypium hirsutum} L. var. Cocher and an enzyme preparation from them as well as cultured unicellular microalgae. This paper describes that their phytotoxicity is indeed dependent on the inhibition of protoporphyrinogen IX oxidase (protox) but significantly due to additional inhibitory mechanism(s) perhaps on some function(s) involved in the chlorophyll biosynthesis.

MATERIALS AND METHODS

1. Materials

The pentenamides (II) used in this paper are those synthesized previously\textsuperscript{1)} as listed in Table 1. Fluthiacet-methyl (III), a powerful protox inhibiting herbicide (after isomerization), was from the same sample as used before.\textsuperscript{3)} Oxadiazone (IV), nitrofen (V), oxyfluorfen (VI), and chlorophthalim (VII) were prepared from commercial formulations. Protoporphyrin IX and the Hoagland mixture were purchased from Sigma Chemical (St. Louis, MO).
2. Plant Materials

Presterilized seeds of cotton (Gossypium hirsutum L. var. Cocher) and pea (Pisum sativum L. var. Akabana-tsurunashi) were purchased commercially. A strain, TAM C-212, of the unicellular algae, Chlorella pyrenoidosa, was obtained from the Institute of Applied Microbiology, University of Tokyo.

3. Phytotoxicity Test

The "solution" of test compounds (100 μM) was prepared by emulsifying the corresponding amount in defined volumes of the "aqueous solution" (1/2500 dilution) of KUMITEN. A surfactant, KUMITEN, which contains 20% polyoxyethylene phenyl ether and 6% sodium polynaphthylmethanesulfonate, was available from the Kumiai Chemical Ind. Co., Tokyo. The presterilized cotton seeds were sown on sufficiently wet vermiculite pads, and grown under dark conditions at 27°C. Seven days after germination, the etiolated seedlings were withdrawn and their cotyledons were immersed with the above prepared test solution for 30 sec. Then, the entire seedlings were placed in a vial and sustained by cotton pads so that the roots were immersed in the Hoagland mixture solution without including test compounds as shown in Figs. 1 and 2. The vials were kept at 27°C for 24 hr under light conditions (18,200 lux) as well as under dark conditions. The phytotoxic effects were judged from symptoms of seedlings.

4. Effect on the Chlorophyll Biosynthesis of Cotton Cotyledons

The cotton seedlings were kept under dark conditions for 5-7 days after germination at 27°C. After being placed for approximately 1 hr under the room light, a certain weight of the cotyledons (0.5-1.0 g fresh weight in total) was excised and incubated in 25 ml of distilled water, in which various amounts of test compounds were dispersed as an acetone solution (final concentration of acetone was below 1% (v/v)) at 27°C for 24 hr under light (18,200 lux). After the excised cotyledons were taken out and air-dried, chlorophyll was extracted with 20 ml of 80% acetone. The chlorophyll concentration was measured with the absorbance values (A cm⁻¹) at 665 and 649 nm by a Hitachi 220A spectro-photometer according to the equation shown below.4) The same amount of excised cotyledons without any treatment was employed as the control.

Total chlorophyll concentration (μg/ml) = 6.45A 665 + 17.72A 649

The total chlorophyll content in the unit amount of cotyledons (μg/g fresh weight) was estimated from the concentration calculated as above (μg/ml), the volume of the extraction solvent (20 ml) and the fresh weight (g) of cotyledons used for the extraction.

5. Preparation of Protoporphyrinogen IX

Protoporphyrin IX (9 mg) was dissolved in 15 ml of 10 mM KOH containing 20% (v/v) ethanol and the solution was filled up to 20 ml with 10 mM KOH. While being bubbled with N₂ gas to remove O₂, the solution was reduced with 25 g of freshly prepared 3% sodium amalgam. The operation was undertaken in a vessel by mixing/crushing the amalgam with the solution well using a pestle for 15 min. The solution was filtered through a glass wool pad under the N₂ atmosphere. The filtrate, after being supplemented with 18 ml of the Tris-HCl buffer (pH 8.7) containing 1 mM EDTA and 4 mM dithiothreitol, was passed through a membrane filter (Millex-GS; Millipore, Bedford, MA) and the pH was adjusted to 8.5-8.8 with 40% H₃PO₄ which had been bubbled with N₂ gas. This protoporphyrinogen IX preparation (about 200 μM) was stable at least for a week at -80°C. All operations were conducted under dim light.

6. Measurement of the Protoporphyrinogen Oxidase Activity

Etiolated cotton cotyledons, 5-7 days after germination, were placed under the room light for 3 hr so that they were greened slightly.9) Then, the greened cotyledons (60 g) were homogenized in 300 ml of 30 mM
Hepes-KOH buffer (pH 7.8) additionally containing 0.5 M sucrose, 5 mM dithiothreitol, 1 mM EDTA, 1 mM MgCl₂, 0.5 mM KCN, 0.2% bovine serum albumin, and 15 g of polyvinylpyrrolidone. After filtration of the homogenate through nylon gauze, the filtrate was centrifuged at 300×g for 5 min to separate the debris as the precipitates. The supernatant was further centrifuged at 10,000×g for 1 min. The precipitates were suspended in an appropriate amount of a modified Hepes-KOH buffer (30 mM) of pH 7.8. The buffer was without augmentation of KCN and polyvinylpyrrolidone and with lowering the concentration of dithiothreitol from 5 mM to 1 mM, but otherwise the same as that used for the homogenization, and again centrifuged at 150×g for 5 min. The supernatant was separated and centrifuged further at 2000×g for 5 min and the precipitates were suspended in 90 ml of the modified Hepes-KOH buffer solution (pH 7.8) used just before. The suspension was centrifuged still further at 500×g for 20 min. The precipitates were suspended in 10 ml of the modified Hepes-KOH buffer solution and stored at -80 °C as the etioplast stock suspension.

The etioplast suspension (3-4 ml) was sonicated after diluting with the equivalent volume of 1% Tween 20 (final concentration: 0.5% (v/v)), just before the assay of the enzymatic activity. The enzyme reaction mixture (1 ml) consisted of 100 mM Tris-HCl (pH 8.0), 1 mM EDTA, 4 mM dithiothreitol and 60 μM of the protoporphyrinogen IX (0.3 ml of the substrate preparation). Appropriate amounts of test compounds were added to the mixture as the solution in acetone (the final concentration of acetone was below 1% (v/v)). The reaction was initiated by the addition of 0.05-0.2 ml of the above prepared etioplast suspension. After incubation for 60 min at 30°C under dark conditions, a portion of the mixture (100-25 μl) was transferred to 2.9-2.975 ml of 100 mM Tris-HCl buffer (pH 8.7) containing 1 mM EDTA, 5 mM of dithiothreitol and 1% (v/v) Tween 80. The fluorescence at 630 nm (with excitation at 410 nm) was immediately measured with a Hitachi 650-60 fluorescence spectrophotometer. Heat denatured etioplast suspension was used as the control.

7. Effect on Chlorophyll Biosynthesis and Growth of Green Microalgae
A "minimum" aliquot of a suspension of the reserved IAM strain of the chlorella was inoculated into a modified Bristol liquid medium9 and pre-cultured autotrophically for 6 days at 27°C under 18 hr illumination per a day with fluorescent lamps (9100 lux). Cells were then collected by centrifugation, washed and re-suspended in the same medium so that the absorbance at 650 nm was adjusted at 0.5. This suspension was cultured further for 3 days with various amounts of test compounds under the same conditions as those of the pre-culture. The cell proliferation was estimated by measuring the absorbance at 650 nm. The effect of DCMU (100 μM) was monitored as a negative control to show the perfect inhibition against the proliferation without varying the absorbance. Test compounds were dissolved in acetone and added in the culture medium so that the final acetone concentration was 0.1-1% (v/v).

The C. pyrenidosa was also grown heterotrophically in the dark in the modified Bristol liquid medium. An aliquot of the chlorella suspension pre-cultured autotrophically was added in the medium augmented by 0.4% (w/v) of glucose and 0.25% (w/v) of the Difco yeast extract and cultured at 27°C for 3 days in the dark with various concentrations of test compounds.

Chlorophyll was extracted from the autotrophically grown chlorella cells contained in 5 ml of the culture suspension with 3 ml of hot methanol (60°C) after being collected by centrifugation, and total chlorophyll content was estimated by measuring the absorbance values (A cm⁻¹) at 666 and 653 nm according to the equation shown below.10) The cell growth was measured on the basis of the dry weight of cells from 7 ml of the culture medium. The cell sample without culturing was employed as the control.

\[
\text{Total chlorophyll concentration (µg/ml)} = 2.57\cdot A_{666} + 23.6\cdot A_{653}
\]

8. Effects on the Mitochondrial Respiratory Chain11)
Mitochondria were prepared from etiolated pea seedlings according to Nawa and Asahi.12) Succinate-cytochrome C reductase function was assayed by measuring the rate of increase in the absorbance at 550 nm attributable to the reduction of the cytochrome C with a Hitachi 220 spectrophotometer using a sipper cell. The value of molar extinction coefficient, 1.92×10⁴, was used to calculate the amount of reduced cytochrome C. The reaction was initiated by adding the mitochondria (5-25 µg protein) to 3 ml of aq. solution containing 50 mM potassium phosphate (pH 7.2), 1 mM KCN, 27 µM cytochrome C and 20 mM sodium succinate, in addition to various concentrations of the test compounds as acetone solution so that the final concentration of acetone is below 1%.

RESULTS

1. Phytotoxic Symptom of Cotton Seedlings Treated by Protox Inhibitors and Pentenamides
Figure 1-a shows that "typical" protox inhibitors such as fluthiacet-methyl (III), oxadiazon (IV), and nitrofen (V) are phytotoxic in the sequence of III>IV>V under light conditions. At the rate of 100 μM, fluthiacet-methyl (III) "completely" kills the seedlings. While nitrofen (V) browns the almost entire seedling body, there are still 10 to 30% green spots left on the cotyledon.
As shown in Fig. 1-b, seedlings do not suffer any damage under dark conditions. Cotyledons are just etiolated with a pale yellowish color regardless of whether they have been treated with protox inhibitors.

Figure 2-a shows the light-dependent phytotoxicity of the pentenamides (II) on the cotton cotyledons. The highest toxicity is observed in compound (II-1: $X_1=\text{Cl}, X_2=\text{CN}$) which seems to correspond with that of fluthiacet-methyl (III). The lowest effects are exerted by compounds (II-4: $X_1=\text{Cl}, X_2=\text{CF}_3$ and II-5: $X_1=\text{Cl}, X_2=\text{SO}_2\text{Me}$), which are similar to that of nitrofen (V), in that about 10 to 20 and 50 to 70% green spots, respectively, are left on the cotyledons. The phytotoxicity of compounds (II-2 and -3) is similar to that of oxadiazon (IV). Under dark conditions, seedlings do not suffer any lethal effect. There is, however, a conspicuous difference in the symptom of cotyledons. Unlike compounds (III-V) in Fig. 1-b, pentenamides, in particular, compounds (II-1-4) exhibit obvious dark-browning as well as wrinkling effects on cotyledons in Fig. 2-b.

2. Effect on the Chlorophyll Biosynthesis of Cotton Cotyledons

The inhibitory effect of compounds (II-1, -2, and -3) is slightly higher than or almost equivalent with that of oxadiazon (IV) [$pI_{50} (M): 6.7$], while that of compound (II-4) is closer to that of nitrofen (V) [$pI_{50} (M): 5.5$] as shown in Fig. 3. The effect of compound (II-5) is lowest among others.

3. Inhibition of Protoporphyrinogen Oxidase of Cotton Cotyledons

As shown in Fig. 4, the pentenamides (II) exhibit certain degrees of inhibition against the protox preparation from cotton cotyledons. However, their inhibitory potency is generally lower than that of nitrofen (V). The variations in the potency do not seem to correspond with
those in the chlorophyll biosynthesis inhibition. The protox inhibitory potency of compounds (II-1 and -3) is lower than that of compounds (II-2 and -4).

4. Inhibition of Growth and Chlorophyll Biosynthesis of Unicellular Algae

Figure 5 indicates that the pentenamides (II) are inhibitive against the autotrophic growth of C. pyrenoidosa IAM C-212. But, the activity is about 1/10 or lower than that of oxadiazon (IV) and nitrofen (V). An exception is compound (II-1) which shows a higher activity in the higher concentration range. Against the heterotrophic growth, however, even compounds (II-2 and -3), which are "moderately" inhibitive against the autotrophic growth, do not show significant inhibition. The situation is similar to that of oxadiazon (IV) and nitrofen (V) as indicated by parenthesized figures in Fig. 6. The biosynthesis of total chlorophyll is also inhibited by compounds (II-2 and -3) but the activity of compound II-3 is lower than that of compound II-2 as apparent in Fig. 6. This feature does not seem to correspond with that observed against the chlorophyll biosynthesis of cotton cotyledons.

5. Effects on the Respiratory-chain Electron-Transport System

Practically no effect is observed for pentenamides (II) on the electron-transport system at least between succinic acid and cytochrome C as listed in Table 2. 

**DISCUSSION**

The above results are summarized in Table 3. Whenever possible, the inhibitory potency is shown in terms of pI₅₀ value estimated by the probit transformation method.¹⁴

Variations in the phytotoxicity among five pentenamides (II-1--5) against cotton seedlings under light conditions corresponded well with variations in the inhibition against the shoot elongation of seedlings of the barnyard grass, Echinochloa oryzicola measured previously.¹⁵ The pI₅₀ (M) value against the barnyard grass shoot growth varies between 3.88 for compound (II-1) and 1.88 for (II-5).²¹ The cotton seedlings were believed to be a sensitive plant material yielding reliable bioassay data similar to cucumber and barnyard grass seedlings which have been used often in this type of studies.¹,¹⁸--¹⁷

Because the whole boy phytotoxicity indices were only represented by potency grades, no quantitative comparison was possible with activities measured at the pI₅₀ value for each compound. Thus, comparisons were made by examining similarities in the variation patterns of toxicity values and ratings for the set of compounds. From such comparisons of the light-dependent phytotox-
icity with inhibitory potencies against other assay systems for the pentenamides (II) and known protox inhibitors (III–V) in Table 3, some structure-activity features are drawn as follows:

1) For the entire series of compounds (II–V), the potency variations in the light-dependent phytotoxicity [A] are best related, among others, to those in the inhibition against the chlorophyll biosynthesis in cotton cotyledons [C]. From compound (II-3), the phytotoxicity [A] seems lower than that expected from the [C] activity. This may be due to an effect of the difference (Br vs. Cl) in the substituent X1 on the whole body activity [A].

2) For compounds (III–V), the light-dependent phytotoxicity [A] corresponds very well not only to inhibitory activity against the chlorophyll [C] but also to that against the protox inhibition [D] using preparations from cotton cotyledons. The phytotoxicity [A] variations also “roughly” correspond to variations in the growth inhibitory activity [E] against the chlorella.

3) For compounds (II-1–5), the potency variations in the [A] activity seem to also correspond with those in the chlorella activity [E]. The potencies [E, F] are lower than those of compounds (III–V).

4) For compounds (II-1–5), the potency variation pattern in the protox inhibition [D] activity does not correspond well with that in the chlorophyll inhibition [C]. Moreover, the potency [D] is much lower than that expected from their [C] activity. In fact, the [C] activity of compounds (II-1–4) is roughly located between those of oxidiazon (IV) and nitrofen (V).

5) The dark-browning and wrinkling damage [B] caused by compounds (II) under dark conditions may or may not occur under light conditions. However, the pattern in the potency variations in the [A] and [C] activities for compounds (II–V) seems to be similar to that in the [D] activity augmented by that in the [B] effect.

Depending upon differences in the transport process to the target site of action, the bioactive potency of a certain compound is usually modified with variations in the physicochemical properties that may concern the interaction with biological systems within each of steps in the process. For a series of bioactive compounds such as compounds (II), the pattern of potency variations, in addition to the potency itself, would in general vary depending upon the assay procedures or the locations of

Table 3 Inhibitory potency in various assays of pentenamides (II) and protox inhibiting herbicides (III–V).

<table>
<thead>
<tr>
<th>Comps.</th>
<th>Phytotoxicity</th>
<th>Cotton Cotyledon</th>
<th>Chlorella</th>
</tr>
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<tbody>
<tr>
<td>II-1</td>
<td>++++++</td>
<td>+++</td>
<td>6.8</td>
</tr>
<tr>
<td>II-2</td>
<td>+++</td>
<td>+</td>
<td>6.6</td>
</tr>
<tr>
<td>II-3</td>
<td>+++</td>
<td>+++</td>
<td>7.1</td>
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<tr>
<td>II-4</td>
<td>+</td>
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<td>+</td>
<td></td>
<td>4.2</td>
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<tr>
<td>III</td>
<td>+++++</td>
<td></td>
<td>8.2</td>
</tr>
<tr>
<td>IV</td>
<td>+++++</td>
<td></td>
<td>6.7</td>
</tr>
<tr>
<td>V</td>
<td>++</td>
<td></td>
<td>5.5</td>
</tr>
</tbody>
</table>

[a] pl50 values were estimated by the probit transformation.
[b] Visually judged. ++: completely killed, ++++: cotyledons are killed, and stems are partially survived; +: cotyledons are killed, but stems are not wilted; +: cotyledons are damaged with 10–30% green spots left, +: cotyledons are damaged with 50–70% green spots left.
[d] Inhibition of chlorophyll biosynthesis (pl50, M; the 95% confidence intervals are ±0.05–0.35).
[e] Protox inhibition (pl50, M; the 95% confidence intervals are ±0.15–0.40).
[f] Inhibition of autotrophic growth (pl50, M; the 95% confidence intervals are about ±0.10).
[g] Inhibition of chlorophyll biosynthesis (pl50, M; the 95% confidence intervals are ±0.30–0.80).
[h] Not measured.
[i] From ref. 3).
[j] Protox preparation from corn cotyledons. The inhibition against that from cotton cotyledons seems to be almost equivalent.
[k] Against Scenedesmus acutus, from ref 13).
[l] pl50 value against protox preparation from corn cotyledons in ref. 3) is almost identical to that from cotton cotyledons.
the target site of action as observed among activities [C, D, E, and F]. The potency as well as the potency variation pattern of compounds (III–V) is almost equivalent in terms of pI50 (M) between activities [C] and [D]. Thus, in compounds (III–V), the chlorophyll biosynthesis inhibition [C] could be mostly due to the protox inhibition [D] and that the translocation within cotyledon tissues of these physicochemically different molecules would not be critical or not rate-limiting for their interaction with the target for the exhibition of the activity.

With the above inference in mind, the potency in the protox inhibition [D] of compounds (II) much lower than that expected from their chlorophyll biosynthesis inhibition [C] strongly suggests that the [C] inhibition would be induced not only by the protox inhibition [D] but also by other inhibitory mechanism(s) involved in the total chlorophyll biosynthesis system. This unknown toxicity mechanism(s) could correspond with the origin of the dark-browning and wrinkling effects “additionally” appearing on the etiolated cotyledons. This suggestion would not be inconsistent with the assay results with C. pyrenidosa. Against heterotrophically grown algae cells, compounds (II) did not exhibit significant growth inhibition similar to compounds (III–V).

The emerging rate of the phytotoxicity of compounds (II) was rather quick. Therefore, the possibility of the inhibition against the respiratory-chain electron transport system was examined. As shown in Table 2, however, this possibility is not approved.

According to recent measurements, pI50 (M) values against the protox preparation from corn cotyledons was 6.22 for compound (II-1), while those for oxyfluorfen (VI) and chlorophthalim (VII) were, respectively, 8.73 and 7.41.18) Compounds (II) were also observed to cause light-dependent ethane formation which is due to peroxidative degradation of photosynthetic cell membranes induced by the protox inhibition. The pK_A (M) value, K_A being the concentration giving half the maximum of the ethane formation, was 4.82 for compound (II-1) compared with 7.63 and 6.43 for oxyfluorfen (VI) and chlorophthalim (VII), respectively.18) These values indicate that the present compounds (II) are, in fact, members of peroxidizing herbicides, but the peroxidizing potency is one to two orders of magnitude lower than those of compounds VI and VII. The full account of these observations will be described elsewhere.

**CONCLUSION**

Certain N-isobutyl-N-benzyl-4-halo-pentenamides were potentially tolerable as practical herbicides with a low application rate to eradicate barnyard grasses in the paddy field. The characteristic of their phytotoxic action was that the action is light-dependent. In the present study with cotton cotyledon preparations, their light-dependent phytotoxicity was shown not only due to the protox inhibition/peroxidation but also to other inhibitory mechanism(s) perhaps against some step(s)/site(s) involved in the chlorophyll biosynthetic pathway.

The relative importance of the protox inhibition within the total phytotoxicity may, however, vary depending upon plant materials used in the assay. In our previous study, the quantitative structure-activity relationship was analyzed for a number of systematically modified analogs of compounds (II) using the phytotoxicity data against the seedlings of the barnyard grass, *Echinochloa oryzicola*. The fact that a clear-cut correlation equation for a single dependent variable for the phytotoxicity was obtained would indicate that the importance/weight of the main mechanism, perhaps the protox inhibition/peroxidation, surpasses the second, if any, in this barnyard grass assay.

Because structural feature of pentenamides (II) is markedly different from that of known protox inhibitors, it should be interesting to explore to separate the secondary effect(s) from the protox inhibition as well as to enhance the selectivity between weeds and crops even within the same family by means of elaborated structural evolutions.

**ACKNOWLEDGMENTS**

The authors would like to express their sincere thanks to Professor Ko Wakabayashi of Tamagawa University for his measuring activities for the protox inhibition with corn cotyledons and the ethane formation with *Scenedesmus acutus*.
18) K. Wakabayashi, Private communication, Dec. 2000

要　約

N-Isobutyl-N-(4-substituted benzyl)-4-halo-2-pentenamide 項の除草活性の発現機構

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N-Isobutyl-N-(4-substituted benzyl)-4-halo-2-pentenamide 項はヒエに対し顕著な光要求性の除草活性を示す。ワタの幼植物およびそれより調製した酵素標品、ならびに培養した単細胞藻類を用いたアッセイ系により、殺草のメカニズムの解明を試み、一連の供試化合物は明らかにprotoporphyrinogen oxidase(PPO)阻害活性を示したが、植物毒性の強度変化は、むしろクロロフィル生合成阻害活性の強度変化によく対応した。したがって，本系列の化合物植物毒性は，PPOの阻害以外にクロロフィル生合成のほかの段階の阻害が重なっていることが示唆された。