Mode of Action of Herbicidal N-Benzyl-4-chloro-N-isobutyl-2-pentenamides

Masaki HIRAKI, Kenji MATSUNARI*, Toshio FUJITA** and Ko WAKABAYASHI*

Graduate School of Agricultural Science, Tamagawa University, Tamagawa-gakuen, Machida, Tokyo 194-8610, Japan * Overseas Department, Kumiai Chemical Industry Co., Ltd., 4-26 Ikenohata 1-chome, Taito-ku, Tokyo 110-8782, Japan ** EMIL Project, #305 Heights Kyogokusho, Fuyachō-Nishikikouji-agaru, Nakagyo-ku, Kyoto 604-8057, Japan

(Received July 30, 2001; Accepted February 18, 2002)

Key words: N-benzyl-4-chloro-N-isobutyl-2-pentenamides, phytotoxic mechanism of action, protox inhibition, ethane formation, photosynthetic pigments degrees.

INTRODUCTION

N-Benzyl-4-chloro-N-isobutyl-2-pentenamides (Fig. 1) exhibited a light-dependent herbicidal activity similar to that of peroxidizing herbicides such as oxyfluorfen and chlorphthalim. However, the phytotoxic mechanism of action of the pentenamides has yet to be completely clarified. In the present study, this mechanism was investigated with the protoporphyrinogen-IX oxidase (Protox) assay at the enzyme-level and by phytotoxicity assays using intact cells of unicellular Scenedesmus acutus.

MATERIALS AND METHODS

1. Chemicals

The herbicidal activity of the compounds (1-4 in Fig. 1) in barnyard grass was reported previously. Oxyfluorfen, chlorphthalim, norflurazon and methoxyphenone were used as control chemicals. Protoporphyrinogen-IX (Protogen) was prepared by the reduction of protoporphyrin-IX (Proto-IX) with sodium amalgam under a nitrogen stream in the dark. Analytical grade chemicals for Scenedesmus acutus cultivation such as NH₄VO₃ and (NH₄)₆Mo₇O₄·4H₂O were purchased from Kanto Chemical Co. (Tokyo). All other fine chemicals and buffers were from Sigma Chemical Co. (St. Louis, Mo).

2. Measurement of Protox Inhibition

Protox inhibition was measured according to Nicolaus et al. Corn (Zea mays L. cv. DK212MF) seeds were soaked in water for 6 hr and germinated on vermiculite for 6 days in the dark at 30°C. Seedlings were harvested after exposure to light (300 μE m⁻² sec⁻¹) for 2 hr and the roots were removed. After homogenization, purified plastids containing Protox were prepared by three steps of centrifugation. The Protox activity was measured based on Proto-IX formation for 5 min at 30°C in a 3 ml assay volume, containing 0.1 M tris(hydroxymethyl)aminomethane (Tris-HCl, pH 7.3), 1 mM ethylenediaminetetraacetic acid, 5 mM dithiothreitol, 0.03% Tween 80 (w/v), 0.3-0.6 mg of etioplast protein and 2-5 μM Protogen. Fluorescence at 633 nm with excitation at 405 nm was detected by a fluorescence spectrophotometer (Hitachi F2000, Japan) with a thermostatted cell holder. Non-enzymatic oxidation of Protogen, measured with a heat-denatured and sonicated protein, was subtracted from the observed enzymatic activity.

The Protox inhibition was estimated 5 min after the addition of various amounts of test compound formulated using DMSO to the assay mixture mentioned above. The molar I₅₀ (Protox) value was calculated by the Probit method. The log value of 1/I₅₀ was used as the potency index.

3. Measurements of Growth Inhibition, Chlorophyll Decrease and Ethane Formation of Scenedesmus acutus

The phytotoxicity assays using autotrophic Scenedesmus were carried out according to Watanabe et al. Growth inhibition was estimated from the packed cell volume (pcv) in a graduated microcentrifuge tube 20 hr after incubation with the test compounds. The chlorophyll content of the extraction mixture (methanol: tetrahydrofuran: 5 mM aq. trifluoroacetic acid, 30:16:5, v/v/v) was measured spectrophotically. Indices of the growth inhibition and chlorophyll decrease were presented as molar pI₅₀, the I₅₀-values being calculated by the Probit method. The volume of ethane was measured using a gas chromatograph equipped with a flame ionization detector. The I₅₀ (Ethane), defined as being the molar concentration of the compound tested giving half the maximum of light-induced ethane formation produced by Scenedesmus during a 20 hr incubation period, was estimated through the use of a double reciprocal plot.

4. Inhibition of Carotenoid Biosynthesis in Scenedesmus acutus Cells

Autotrophic Scenedesmus cells were grown for 48 hr after the addition of various amounts of test compounds. The extraction of carotenoids was carried out under a dim light to avoid photo-degradation. The algal suspension (10 ml) was centrifuged for 10 min at 10,000×g. After decantation, the pellet was resuspended in methanol (20 ml), and 2 ml of 10 N KOH was added. Carotenoids were allowed to flow out of the algal cells at 65°C in darkness for 20 min. The mixture was shaken with 25 ml of petroleum ether-diethyl ether (90:10 mixtures to a graduated microcentrifuge tube 20 hr after incubation with the test compounds. The chlorophyll content of the extraction mixture (methanol: tetrahydrofuran: 5 mM aq. trifluoroacetic acid, 30:16:5, v/v/v) was measured spectrophotically. Indices of the growth inhibition and chlorophyll decrease were presented as molar pI₅₀, the I₅₀-values being calculated by the Probit method. The volume of ethane was measured using a gas chromatograph equipped with a flame ionization detector. The I₅₀ (Ethane), defined as being the molar concentration of the compound tested giving half the maximum of light-induced ethane formation produced by Scenedesmus during a 20 hr incubation period, was estimated through the use of a double reciprocal plot.
v/v) and a saturated sodium chloride solution (10 ml). The UV spectrum of the organic layer was recorded in a range of 250–520 nm and the absorbance at 445 nm was used to evaluate the molar I₅₀ (Carotenoid) concentration of the total colored carotenoids. The pI₅₀ (Carotenoid) was calculated by the probit method.

RESULTS AND DISCUSSION

The phytotoxic indices of N-benzyl-4-chloro-N-isobutyl-2-pentenamides (1-4) and control compounds, in terms of pI₅₀ values for Protox inhibition, ethane formation, chlorophyll decrease, carotenoid decrease and growth inhibition as well as the grades for the accumulation of phytoene and ζ-carotene, are shown in Table 1. All the pentenamides tested (1-4) exhibited the characteristics of peroxidizing herbicides, although the pI₅₀ values were about 1/10–1/100 those of the control peroxidizing herbicides, oxyfluorfen and chlorphthalim. In general, the immediate physiological response of plants to peroxidizing herbicides is to halt chlorophyll biosynthesis in chloroplasts by the specific inhibition of Protox. This inhibition is accompanied by an abnormal accumulation of Proto-IX, which acts as a photosensitizer in the light and induces the formation of ethane with the subsequent destruction of cellular constituents. Among the four pentenamides, 1 and 2 can be regarded as being moderately active peroxidizers, but 3 and 4 are not active. The order of activity corresponds well to that of the herbicidal activity indicated by the ED₉₀ value.

The phytotoxic activity of the peroxidizing herbicides is due to their interference with chlorophyll biosynthesis as a primary mechanism of action, which in turn inhibits carotenoid formation. Thus, the pI₅₀-values for chlorophyll and carotenoid formation are similar for the typical peroxidizers, oxyfluorfen and chlorphthalim, as shown in Table 1. The 50% inhibition of 1–4 for carotenoid synthesis is two to four times stronger than those for chlorophyll (compare the pI₅₀-values of 1–4). The effect of these compounds on the biosynthesis was examined by determining levels of the precursors phytoene and ζ-carotene in Scenedesmus cells. As shown in Table 1 and Fig. 2, neither phytoene nor ζ-carotene accumulates in the cells cultured with 1–4 or with oxyfluorfen and chlorphthalim. On the other hand, norflurazon and methoxyphenone, known inhibitors of carotenoid biosynthesis, caused phytoene and ζ-carotene, respectively, to accumulate at a concentration of 10⁻⁶ M. The UV spectra of the total carotenoid extract are shown in Fig. 2. The spectra of extracts from cells cultured in the presence or absence of compound 1 showed a similar pattern, but their intensity differed. These findings suggest that, most likely, the decrease in carotenoid is not due to the inhibition of carotenoid biosynthesis but a post-biosynthetic degradation. This degradation may be caused by the peroxidation of N-benzyl-4-chloro-N-isobutyl-2-pentenamides.

A deficiency of carotenoids may cause the degradation of

![Fig. 1 Chemical structures of pentenamide compounds (1, R = Cl; 2, R = CN; 3, R = SO₂CH₃; 4, R = CF₃).](image)

Table 1 Phytotoxic activities of N-benzyl-4-chloro-N-isobutyl-2-pentenamides.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Structure</th>
<th>pI₅₀</th>
<th>Ethane</th>
<th>Chlorophyll</th>
<th>Growth</th>
<th>Carotenoid</th>
<th>Phytoene</th>
<th>ζ-Carotene</th>
<th>ED₉₀*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><img src="image" alt="Structure" /></td>
<td>6.19</td>
<td>4.66</td>
<td>6.26</td>
<td>6.14</td>
<td>6.58</td>
<td>n.d.</td>
<td>n.d.</td>
<td>16</td>
</tr>
<tr>
<td>2</td>
<td><img src="image" alt="Structure" /></td>
<td>6.22</td>
<td>4.82</td>
<td>5.81</td>
<td>6.06</td>
<td>6.40</td>
<td>n.d.</td>
<td>n.d.</td>
<td>16</td>
</tr>
<tr>
<td>3</td>
<td><img src="image" alt="Structure" /></td>
<td>5.06</td>
<td>4.07</td>
<td>5.67</td>
<td>5.51</td>
<td>5.85</td>
<td>n.d.</td>
<td>n.d.</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>4</td>
<td><img src="image" alt="Structure" /></td>
<td>5.87</td>
<td>4.31</td>
<td>6.18</td>
<td>5.58</td>
<td>6.38</td>
<td>n.d.</td>
<td>n.d.</td>
<td>31-62</td>
</tr>
<tr>
<td>Oxyfluorfen</td>
<td><img src="image" alt="Structure" /></td>
<td>8.73</td>
<td>7.68</td>
<td>8.15</td>
<td>7.28</td>
<td>8.10</td>
<td>n.d.</td>
<td>n.d.</td>
<td>-</td>
</tr>
<tr>
<td>Chlorphthalim</td>
<td><img src="image" alt="Structure" /></td>
<td>7.41</td>
<td>6.43</td>
<td>7.10</td>
<td>7.00</td>
<td>7.15</td>
<td>n.d.</td>
<td>n.d.</td>
<td>-</td>
</tr>
<tr>
<td>Norflurazon</td>
<td><img src="image" alt="Structure" /></td>
<td>n.d.</td>
<td>n.d.</td>
<td>6.73</td>
<td>6.68</td>
<td>6.68</td>
<td>+</td>
<td>n.d.</td>
<td>-</td>
</tr>
<tr>
<td>Methoxyphenone</td>
<td><img src="image" alt="Structure" /></td>
<td>n.d.</td>
<td>n.d.</td>
<td>5.62</td>
<td>5.51</td>
<td>6.02</td>
<td>n.d.</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

a) +: positive accumulation. n.d.: not detected.
b) The ED₉₀ is the dosage in terms of g a.i./ha required for the 90% damage of barnyard grass seedlings. −: no data.
chlorophyll. The formation of neither phytoene nor ζ-carotene was inhibited by the pentenamides tested in this study, although the total amount of carotenoids decreased without any remarkable accumulation of phytoene. The bleaching herbicide inhibiting 4-hydroxyphenylpyruvate dioxygenase (HPPD), e.g. isoxaflutole or sulcotrione, is coupled with an accumulation of phytoene but does not inhibit phytoene desaturase activity directly. Therefore, the mechanism behind the overall decrease in carotenoids caused by the pentenamides is different from that for known inhibitors of carotenoid synthesis and may be due to the degradation of carotenoids. We have suggested previously that the phytotoxicity of the pentenamides is due not only to Protox inhibition but also to other mechanism(s) related to the regulation of chlorophyll levels. The present results seem to support this hypothesis.

ACKNOWLEDGMENTS

The authors are indebted to Professor Dr. Peter Büger, Universität Konstanz, for many helpful discussions on this work. They also appreciate the valuable technical suggestions made by Professor Dr. Yukiharu Sato, Tamagawa University.

REFERENCES


要約

N-Benzyl-4-chloro-N-isobutyl-2-pentenamide 系化合物の除草作用機構

平木真幸, 松成健二, 藤田恒夫, 若林 攻

光要求性除草活性を示した4種のN-benzyl-4-chloro-N-isobutyl-2-pentenamide系化合物の作用機構を明らかにするために, トウモロコシ発芽黄化葉由来 protoporphyrinogen-IX oxidase (Protopx) の阻害活性試験, 単細胞緑藻 Scenedesmus acutus を用いた生育阻害, クロロフィル減少効果, エタノール生成量およびカロテノイド減少効果を測定した. その結果, Protopx 阻害活性とエタノールの生成が確認された. しかし, 4種のペンテンアミド系化合物はいずれも, クロロフィル減少効果に対してよりも, カロテノイド減少効果に対してより強い活性を示した. カロテノイド生成合成経路に及ぼす影響を確認するために, ケフェンアミド系化合物添加48時間後の Scenedesmus acutus 細胞よりカロテノイドを抽出して, 其の吸収スペクトルを測定した. その結果, カロテノイドの減少は確認されたが, 中間物質の蓄積は認められなかった. 以上の結果より, ケフェンアミド系化合物はperoxidizing効果によって, いったん生成したカロテノイドが分解されるタイプの機構を併せて示することが示唆された.