Protoporphyrinogen-IX Oxidase Inhibition by
$N$-(2,4,5-Trisubstituted phenyl)-3,4,5,6-
tetrahydrophthalimides

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A quantitative correlation between protoporphyrinogen-IX oxidase (Protox) inhibition and peroxidizing activity of $N$-(2,4,5-trisubstituted phenyl)-3,4,5,6-tetrahydrophthalimides was examined. Analogues with five types of $N$-aryl moieties were synthesized. Type I: $N$-phenyl-3,4,5,6-tetrahydrophthalimide and $N$-(2-fluorophenyl)-3,4,5,6-tetrahydrophthalimide; Type II: $N$-(4-chlorophenyl)-3,4,5,6-tetrahydrophthalimide and $N$-(4-chloro-2-fluorophenyl)-3,4,5,6-tetrahydrophthalimide; Type III: $N$-(4-chloro-3-propargyloxyphenyl)-3,4,5,6-tetrahydrophthalimide and $N$-(4-chloro-2-fluoro-5-propargyloxyphenyl)-3,4,5,6-tetrahydrophthalimide; and Type IV: isopropyl 2-chloro-5-(3,4,5,6-tetrahydrophthalimido)benzoate and isopropyl 2-chloro-4-fluoro-5-(3,4,5,6-tetrahydrophthalimido)benzoate; as well as Type V: $N$-(3,4-dihydro-3-oxo-4-propargyl-2H-1,4-benzoxazin-6-yl)-3,4,5,6-tetrahydrophthalimide and $N$-(3,4-dihydro-7-fluoro-3-oxo-4-propargyl-2H-1,4-benzoxazin-6-yl)-3,4,5,6-tetrahydrophthalimide. Inhibition of Protox isolated from etioplasts of corn (Zea mays cv. Anjou) and Echinochloa utilis, and light-induced ethane formation by Scenedesmus acutus were assayed. The 2-fluorine substituted compound of Type II showed a stronger activity both by Protox inhibition and by ethane formation than the 2-non-substituted compound. However, in Types III-V, 2-non-substituted compounds exhibited stronger activity in Protox inhibition than 2-fluoro compounds, and light-induced ethane formation by 2-fluoro compounds was stronger than with 2-non-substituted compounds. Our findings indicate that a 2-fluoro substitution of the phenyl ring enhances the phytotoxic activity to the intact cells but not necessarily Protox inhibition.

INTRODUCTION

It is well known that special aniline derivatives of cyclic imide herbicides (see structures in Fig. 1), which include a 4-chloro-2-fluoro-5-substituted phenyl group, exhibit a high peroxidizing phytotoxity by inducing inhibition of chlorophyll biosynthesis and photooxidative destruction of plant membranes affecting photosynthetic pigment contents.\(^1\)\(^-\)\(^4\) The immediate physiological response of plant against these herbicides is the halt of chlorophyll biosynthesis by specific inhibition of protoporphyrinogen-IX oxidase (Protox, EC 1.3.3.4). This inhibition is accompanied by an abnormal accumulation of protoporphyrin-IX, which acts as a photosensitizer and induces radical formation with subsequent destruction of polyunsaturated fatty acids, major constituents of the acyllipids in thylakoids and cell membranes, to evolve saturated short-chain hydrocarbons.\(^5\) Such a process starting from Protox inhibition and resulting in short-chain hydrocarbon production is referred to the peroxidizing mode of action for a certain group of herbicides.

With a large number of cyclic imides including compounds in Fig. 1, it has been established that their herbicidal efficacy in greenhouse tests can be quantitatively correlated with peroxidative phytotoxic parameters, \(e.g.\) growth inhibition, decrease of chlorophyll and carotenoids contents, and light-induced ethane formation, obtained from autotrophic \textit{Scenedesmus acutus} cells.\(^1\)\(^,\)\(^5\) A Protox inhibition test with etioplasts of corn and \textit{Echinochloa utilis} can be used in combination with the other phytotoxic parameters network. A reliable quantitative correlation has been established among the parameters mentioned above.\(^1\) In this paper, special
aniline derivatives of cyclic imides were assayed for Protox inhibition and peroxidizing phytotoxic activity on intact cells (ethane formation), to examine whether a correlation could be established among Protox inhibition, peroxidizing activity and structural features.

**MATERIALS AND METHODS**

1. **Chemicals**

   N-Phenyl-3,4,5,6-tetrahydrophthalimide (1), N-(2-fluorophenyl)-3,4,5,6-tetrahydrophthalimide (2), N-(4-chlorophenyl)-3,4,5,6-tetrahydrophthalimide (3), N-(4-chloro-2-fluorophenyl)-3,4,5,6-tetrahydrophthalimide (4), N-(4-chloro-5-propargyloxyphenyl)-3,4,5,6-tetrahydrophthalimide (5), and N-(4-chloro-2-fluoro-5-propargyloxyphenyl)-3,4,5,6-tetrahydrophthalimide (6) were synthesized by the condensation reaction of 3,4,5,6-tetrahydrophthalic anhydride and the corresponding anilines in AcOH. Isopropyl 2-chloro-5-(3,4,5,6-tetrahydrophthalimido)benzoate (7), and isopropyl 2-chloro-4-fluoro-5-(3,4,5,6-tetrahydrophthalimido)benzoate (8) were prepared according to the method of Yamada et al. Isopropyl 2-chloro-5-(3,4,5,6-tetrahydrophthalimido)benzoate (7), and isopropyl 2-chloro-4-fluoro-5-(3,4,5,6-tetrahydrophthalimido)benzoate (8) were prepared according to the method of Yamada et al. Isopropyl 2-chloro-5-(3,4,5,6-tetrahydrophthalimido)benzoate (7), and isopropyl 2-chloro-4-fluoro-5-(3,4,5,6-tetrahydrophthalimido)benzoate (8) were prepared according to the method of Yamada et al. Isopropyl 2-chloro-5-(3,4,5,6-tetrahydrophthalimido)benzoate (7), and isopropyl 2-chloro-4-fluoro-5-(3,4,5,6-tetrahydrophthalimido)benzoate (8) were prepared according to the method of Yamada et al. Isopropyl 2-chloro-5-(3,4,5,6-tetrahydrophthalimido)benzoate (7), and isopropyl 2-chloro-4-fluoro-5-(3,4,5,6-tetrahydrophthalimido)benzoate (8) were prepared according to the method of Yamada et al.

The chemical structures were confirmed by melting point, IR- and NMR-spectroscopy, and elementary analysis.

Analytical chemicals for phytotoxic assays and other fine chemicals, e.g. protoporphyrin-IX and others, were purchased from Tokyo Kanto Chemical Co., Tokyo, Japan, or Sigma Chemie, München, Germany.

2. **Phytotoxic Assays of Compounds**

2.1 **Determination of protoporphyrinogen-IX oxidase (Protox) inhibition**

Determination was carried out according to the method of Nicolaus et al. Corn seeds (Zea mays cv. Anjou, Yukijirushi-neugyo Co., Tokyo) were soaked in water for 6 hr and germinated on vermiculite for 6 days in the dark at 30°C. The seedlings were harvested after exposure to light (300 µEinstein(E)/m² × sec) for 2 hr. After homogenizing the seedlings, purified plastids which contained Protox were prepared by three differential centrifugation steps. Protoporphyrinogen-IX was prepared by reduction of protoporphyrin-IX with sodium amalgam under a stream of nitrogen in the dark. Protox activity was measured at 30°C by formation of protoporphyrin-IX. Protox inhibition was determined after adding the compounds to be tested to a 3 ml assay solution containing 0.1 M tris-(hydroxymethyl)aminomethane (Tris-HCl, pH 7.3), 1 mM EDTA, 5 mM dithiothreitol (DTT), 0.03% Tween 80 (w/v), 0.3–0.6 mg of etioplast protein and 0.2–5 µM protoporphyrinogen-IX. The amount of protoporphyrinogen-IX formed was determined for 5 min by a Hitachi F 2000 fluorescence spectrophotometer with a thermostated cell holder, using an excitation and emission wavelength of 405 and 633 nm, respectively. The I₅₀ (Protox) values were determined by reciprocal plots of protoporphyrinogen-IX formation (in percent of control) vs. inhibitor concentration (Dixon plot). The I₅₀ (Protox) values were calculated from the equation, \( \text{pI}_{50} = -\log_10 \text{I}_{50} \). Protox inhibition was measured according to the methods mentioned above.

2.2 **Ethane formation using Scenedesmus acutus**

Autotrophic culture of the green microalga *Scenedesmus acutus*, was carried out according to Watanabe et al. Ethane formation induced by the cyclic imides in *S. acutus* cells was determined according to Ogino et al. The I₅₀ (Ethane) value, the molar concentration which gives half of the hypothetical maximum of light-induced ethane formation during a 20-hr light incubation period, was estimated by double-reciprocal plots of the rates of ethane formation vs. inhibitor concentrations. I₅₀ (Ethane) was used to discuss the peroxidizing effects of the compounds, wherein pI₅₀ (Ethane) = -log I₅₀ (Ethane). Protox inhibition by the cyclic imides was
measured with etioplasts of corn and *E. utiliss*, and expressed as \( p_{50} \) (Protox corn) and \( p_{50} \) (Protox *Echinochloa*), respectively.9)

**RESULTS AND DISCUSSION**

Although peroxidizing activity, exhibited by ethane formation (\( p_{50} \) (Ethane)), and Protox inhibition (\( p_{50} \) (Protox corn) and \( p_{50} \) (Protox *Echinochloa*)) were quite different in intensity among the compounds tested, all the compounds exhibited ethane formation and Protox inhibition (Table 1). Thus, they are confirmed to possess peroxidizing activities. The order of peroxidizing activity (ethane formation) is as follows; compounds 8, 6, 7, 4 and 10 are the most potent peroxidizers, compounds 5 and 9 are also active, followed by compound 3. Weaker activity was observed with compounds 1 and 2 of Type I (see \( p_{50} \) (Ethane) column in Table 1). It should be noted that most potent peroxidizers belong to the aniline derivatives with a 2-fluoro substituent.

Table 1 Phytotoxicities of \( N\)-(substituted phenyl)-3,4,5,6-tetrahydrophthalimides.

<table>
<thead>
<tr>
<th>Type No.</th>
<th>Compound</th>
<th>Melting point °C</th>
<th>( p_{50} ) (Protox)</th>
<th>( p_{50} ) (Protox <em>Echinochloa</em>)</th>
<th>( p_{50} ) (Scenedesmus)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>139-140 (139-140)</td>
<td>5.8</td>
<td>5.9</td>
<td>5.0</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>114-116</td>
<td>5.6</td>
<td>5.5</td>
<td>5.0</td>
</tr>
<tr>
<td>II</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Cl</td>
<td>166-167 (166-168)</td>
<td>7.6</td>
<td>7.9</td>
<td>6.0</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>81-82 (78.5-78.0)</td>
<td>8.4</td>
<td>8.3</td>
<td>6.6</td>
</tr>
<tr>
<td>III</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Cl</td>
<td>146-147 (145.5)</td>
<td>9.4</td>
<td>9.1</td>
<td>6.3</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>136-137 (136.4)</td>
<td>8.7</td>
<td>8.5</td>
<td>6.9</td>
</tr>
<tr>
<td>IV</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Cl</td>
<td>85-86 (85-85.5)</td>
<td>9.0</td>
<td>8.7</td>
<td>6.7</td>
</tr>
<tr>
<td>8</td>
<td>Cl</td>
<td>liquid</td>
<td>8.9</td>
<td>8.1</td>
<td>7.1</td>
</tr>
<tr>
<td>V</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>206-207 (205-206)</td>
<td>9.0</td>
<td>8.8</td>
<td>6.2</td>
</tr>
<tr>
<td>10</td>
<td>F</td>
<td>195-196 (196.0)</td>
<td>8.5</td>
<td>8.4</td>
<td>6.5</td>
</tr>
</tbody>
</table>

No or little difference in Protox inhibition was observed between the corn and *Echinochloa* etioplast preparations. The compounds without 2-fluorine at the \( N\)-phenyl moiety (5, 7 and 9) were the best Protox inhibitors. The derivatives (6 and 8) and their benzoxazine analogue (10) with a 2-fluorine atom were about 1/5 to 1/3 as active as these best inhibitors (5, 7 and 9). Introduction of a fluorine atom at the 2-position on the phenyl ring of compound 3 exhibited an approximately 7 times stronger Protox inhibition than the non-fluorine analogue (see \( p_{50} \) (Protox) data of compounds Type II, in Table 1).

This contrasting activity between ethane formation and Protox inhibition of the aniline derivates of cyclic imide compounds attracted our special attention. By structure-activity studies on a large number of the cyclic imide class of herbicides, it has been shown that, besides the 2-fluoro-4-halogeno substitution, an additional substitution at position 5 of the phenyl moiety (such as alkoxy, alkenyloxy, amino acid, carboxy-methylthio group and the like) increases the peroxidizing herbicidal activity.1,3) This is exemplified here with compounds 1 and 2 (Type I), 3 and 4 (Type II), 5 and 6 (Type III), as documented by ethane formation (last column). The Protox inhibition in cell-free level corresponds exactly with the phytotoxic activity (i.e. ethane formation) to the intact cell. The 2-fluorophenyl group plus 2,3-dihydro-4H-1,4-oxazine ring including the 4,5-position of the \( N\)-phenyl moiety (10) was also reported to produce quite good peroxidizing activity in molecular design of herbicides.1) The compounds of this line of molecular design are considered to have improved stability in the environment, better uptake by plant cells or better affinity to the target enzyme Protox. The compounds 5, 7 and 9, however, having been substituted only at phenyl ring positions 4 and 5, are more inhibitory to the isolated Protox than to the intact *Scenedesmus* cell. The corresponding compounds with an additional fluorine at position 2 (compare 5, 6 (Type III); 7, 8 (Type IV) and 9, 10 (Type V)) exhibit better activity with intact cells (higher \( p_{50} \) for ethane) than those without 2-fluoro substituent, while the 2-fluoro substitution decreases activity toward Protox. Compounds 3 and 4 (Type II), however, do not match with this finding. The evidence, existence of the outlier Type II, may be due to a species-specific effect since intact algae and a higher plant Protox are compared.

In essence, our finding indicates that the peroxidizers without a 2-fluorine group fit better to the active site of the target enzyme Protox. The 2-fluorine group of the phenyl ring may contribute to stability, and/or facilitate uptake or intracellular translocation of the peroxidizers. More experiments are needed, before we can conclude whether the finding of this paper is generally true for all members of the cyclic imide class of peroxidizing herbi-
Cides. Experiments are under way in our laboratories to further clarify this point.

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REFERENCES


要約

2,4,5-三置換フェニル基を有する環状イミド系化合物のプロトボルフィリノゲン-IXオキシダーゼ阻害

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N-(2,4,5-Trisubstituted phenyl)-3,4,5,6-tetrahydrophthalimides の Protop Oxidase 阻害と Peroxidizing 活性の相関を検討した。5 タイプの N-アリル置換[I: N-Phenyl-3,4,5,6-tetrahydrophthalimide と N-(2-fluorophenyl)-3,4,5,6-tetrahydrophthalimide; II: N-(4-chlorophenyl)-3,4,5,6-tetrahydrophthalimide と N-(4-chloro-2-fluorophenyl)-3,4,5,6-tetrahydrophthalimide; III: N-(4-chloro-3-propargyloxyphenyl)-3,4,5,6-tetrahydrophthalimide と N-(4-chloro-2-fluoro-5-propargyloxyphenyl)-3,4,5,6-tetrahydrophthalimide; IV: isopropyl 2-chloro-5-(3,4,5,6-tetrahydrophthalimido)benzoate と isopropyl 2-chloro-4-fluoro-5-(3,4,5,6-tetrahydrophthalimido)benzoate; および V: N-(3,4-dihydro-3-oxo-4-propargyly-2-H-1,4-benzoxazin-6-yl)-3,4,5,6-tetrahydrophthalimide と N-(3,4-dihydro-7-fluoro-3-oxo-4-propargyly-2-H-1,4-benzoxazin-6-yl)-3,4,5,6-tetrahydrophthalimide] を合成し，トウモロコシとヒエのエチオプラストから抽出した Protop 活性に対する阻害活性と Scenedesmus acutus によるエタンの発生を測定した。タイプ II の化合物では，2-フッ素置換化合物は無置換化合物に比べ Protop 阻害活性およびエタン発生においてより強い活性を示した。しかしながらタイプ III 〜 V の化合物では，2-フッ素置換基を持たない化合物がより強い Protop 阻害活性を示した。しかし光誘導のエタン発生は，2-フッ素置換化合物の方が大きかった。フェニル基の 2-フッ素置換は植物毒性活性（Peroxidizing 活性）を高めるが，Protop 阻害活性の向上には必ずしも必要ではない。