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SUBSTITUENT EFFECT ON THE INHIBITION OF CHLOROPHYLL FORMATION BY *N*-PHENYL OXADIAZOLIDINEDIONE DERIVATIVES IN CUCUMBER AND SPECULATION ON THE HERBICIDAL ACTION

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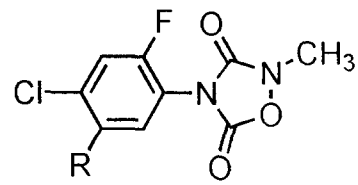
Abstract - The inhibition of chlorophyll formation in cucumber cotyledons by *N*-phenyl oxadiazolidinedione derivatives **Ia-u** showed similar trend as their herbicidal activities. In case of oxadiazolidinedione **Iq**, with a propargyloxy substituent, both the highest herbicidal activity and inhibitory action (pI_{50} = 6.37) were observed. The accumulation of protoporphyrin IX and cellular electrolyte leakage by oxadiazolidinedione **Ia**, **Ik** and **Iq** were well correlated with their inhibition of chlorophyll biosynthesis. These results suggest that the herbicidal activity of oxadiazolidine **Ia-u** is originated from the inhibition of chlorophyll biosynthesis.

INTRODUCTION

In the biosynthetic pathway of chlorophyll, the inhibition of protoporphyrinogen oxidase (Protox), involved in the last step of protoporphyrin IX (Proto IX) biosynthesis has been an attractive target site in the design and development of a herbicide. The accumulated substrate protoporphyrinogen IX (Protogen IX) is mainly oxidized to Proto IX by Protox-like enzyme in cytoplasm or on plasmamembrane. Interacting with sun light, it generates the singlet oxygen causing lipid peroxidation of the cell membrane and then eventually results in the cell death by way of the dehydration or bleaching.[1,2,3] Previously, we reported the synthesis and herbicidal activities of *N*-phenyl oxadiazolidinedione derivatives **I** in an attempt to develop a potent herbicide.[4] The analysis of structure activity relationship of oxadiazolidine **I**, however, was not sufficient to explain how the highest activity was observed in case of propargyloxy substituent (**Iq**). In a continuation of this research, we were interested in the mode of herbicidal action of oxadiazolidine **I** and its structure activity relationship. Based on the structural similarity of oxadiazolidine **I** with other known herbicides,[5,6,7] it was suggested that their herbicidal activities might be derived from the inhibition of the biosynthesis of chlorophyll. With this assumption in mind, we measured the accumulation of Proto IX and cellular electrolyte leakage in cucumber cotyledons by oxadiazolidine derivatives **Ia**, **Ik** and **Iq** as representatives. In addition, the substituent effect on the inhibition of chlorophyll formation by oxadiazolidinedione derivatives **Ia-u** was studied and compared with the herbicidal activity.



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MATERIALS AND METHODS

Chlorophyll formation inhibition. The inhibition assay of the chlorophyll formation was proceeded following a known procedure with minor modification.[8] The samples were dissolved in absolute acetone containing a non-ionic surfactant Tween 20 (Yakuri Chem.) and diluted with 10 m*M* potassium phosphate buffer (pH 6.0). The final concentrations of acetone and Tween 20 in the solution were 0.1% and 0.01%, respectively. Cucumber (Cucumis sativus L.) seeds were grown in moist vermiculite in darkness for 5 days at 25 °C. Under the green safe light, five pairs of etiolated cotyledons were placed on a sheet of filter paper (Whatman No. 2) containing 2 mL of various concentration of compounds in Petri dishes (radius 5.5

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† *Abbreviations* : Protox, protoporphyrinogen oxidase ; proto IX, protoporphyrin IX ; protogen IX, protoporphyrinogen IX.

cm). Incubation was carried out in the dark for 8 h followed by in light(white fluorescent, 30 μ mol. $m^{-2}s^{-1}$) for 10 h at 25 °C. After incubation, the fresh weight of incubated tissues was measured and soaked in 10 mL of absolute methanol for 24 h at 25 °C under dark. This process induced a complete extraction of chlorophyll. Absorbances at 665.2 nm and 652.4 nm were measured on the Beckman DU-65 spectrophotometer to quantify the amount of chlorophyll.[9] The inhibitory activity of a sample was expressed as inverse log value of molar concentration(pI_{50}) at which 50% of chlorophyll formation was inhibited compared to the untreated control.

Protoporphyrin IX accumulation. The etiolated twenty cotyledons of cucumber(about 0.95 g of fresh weight) were cut under a dim green light then placed in a 9 cm diameter Petri dish containing 5 mL of 10 m*M* potassium phosphate buffer(pH 6.0) with 100 μ*M* of the sample dissolved in acetone and Tween-20. The final concentrations of acetone and Tween-20 were 1% and 0.01%, respectively. The tissues were incubated in a growth chamber for 14 h at 25 °C in darkness then exposed to continuous white light at 120 $\mu mol.m^{-2}s^{-1}$ for 3 h. Without the light source, incubated tissues were homogenized in acetone containing 0.1N NH_4OH (v/v = 9:1) using cold mortar and pestle. The homogenate was centrifuged at 39,000g at 4 °C for 12 min and the protoporphyrin IX was extracted from the supernatant. The chlorophyll and other full esterified tetrapyrroles were extracted twice using equal and 1/3 volume of hexane. The remaining hexane-extracted acetone residue was used for the determination of protoporphyrin IX by spectrofluorometer (SFM, Kontron).[10]

Table 1. The inhibition of chlorophyll formation in cucumber cotyledon and herbicidal activities of oxazolidinedione derivatives **Ia-u**

compound **I**	substituent (R)	pI_{50}[a]	activity[b]
a	OCH_3	2.33	0
b	OH	4.38	9
c	$OCOOCH_2CH_3$	4.43	10
d	OSO_2Ph	2.96	13
e	OSO_2Me	4.57	61
f	$OCOCH_2CH_3$	4.25	3
g	OCONHMe	4.33	1
h	COOEt	5.34	69
i	COOH	4.04	10
j	COONa	3.81	11
k	H	4.20	61
l	CONHPr	4.10	23
m	NH_2	4.24	11
n	NHEt	4.66	49
o	NHCOOEt	4.31	28
p	CH_3	4.23	46
q	OCH_2CCH	6.37	97
r	OCOMe	4.32	8
s	NHCOOMe	4.06	56
t	$NHCOCF_3$	3.89	14
u	$OCH_2CH_2CH(Me)_2$	3.40	10

[a] Inverse log value of the concentration of a sample giving 50% inhibition of chlorophyll formation. [b] The inhibition of weeds at 0.25 kg/ha; all data are from the reference 4.

The excitation and emission wavelengths were set at 400 nm and 630 nm, respectively. All measurements were triplicated and compared with a reference curve of standard protoporphyrin IX(Sigma Chemical Co.) dissolved in 80% acetone.

Electrolyte leakage measurement. The seeds of cucumber were germinated in moist vermiculate in flats and grown for 5 days in the greenhouse at 35/25 °C (day/night) by watering. Forty 6 mm diameter cotyledon discs were placed in 6 cm diameter polystyrene Petri dish containing 7 mL of 1% sucrose, 1 m*M* MES buffer(pH 6.5) with or without 100 μ*M* of sample in acetone containing Tween 20. The final concentrations of acetone and Tween were 1% and 0.01%, respectively. The tissues were incubated in a growth chamber for 12 h at 25 °C in the dark and then exposed to continuous white light at 120 $\mu mol.m^{-2}s^{-1}$ up to 24 h. The electrolyte leakage into the bathing medium was determined periodically using conductivity meter(AOC-10, Denki Kagaku Keiki). All measurements were triplicated and the results were expressed as the changes in the conductivities from the initial measurement because of the background fluctuation in different treatment solutions.

RESULTS AND DISCUSSION

Structure activity relationship on the inhibition of chlorophyll biosynthesis. The experimental results for the inhibition of chlorophyll synthesis in cucumber cotyledons by oxazolidinedione derivatives **Ia-u** are summarized in the Table 1 as pI_{50} value with their herbicidal activities. The highest pI_{50} value (6.37) was observed in case of propargyloxy substituent (**Iq**). The oxadiazolidine **Ia**, containing methoxy substituent, showed the lowest inhibition as well as the lowest herbicidal activity. In case of ester group (**Ih** COOEt), relatively high inhibition was observed (pI_{50} = 5.34). There was no clear relationship between the inhibition and the physicoparameters such as electronic parameters, size and lipophilicity of substituent R of oxadiazolidinedione (**I**). As shown in the cases of **Ib** (OH), **Ii** (COOH) and **Ik** (H), pI_{50} values of those are almost same around 4.2. This means that the inhibition is not dependent on the electron donating and withdrawing effect of the R substituent on the phenyl ring of oxadiazolidine(**I**). The correlation of inhibition of chlorophyll formation and herbicidal activity of oxadiazolidine derivative **I** was well coincident. In cases of **If** (OCOEt), **Ig** (OCONHMe) and **Ir**(OCOMe), however, the herbicidal activities were very low(<10% inhibition of weeds) even though their chlorophyll inhibition was quite high (pI_{50} = ~4.3).

Assay of protoporphyrin IX. The accumulation of Proto IX was assayed with oxadiazolidines **Iq**, **Ik** and **Ia** as representative samples because they showed high, medium and poor inhibition of chlorophyll formation, respectively. As shown in the Figure 1, there was a clear correlation between the herbicidal activity of oxadiazolidine **I** and the Proto IX

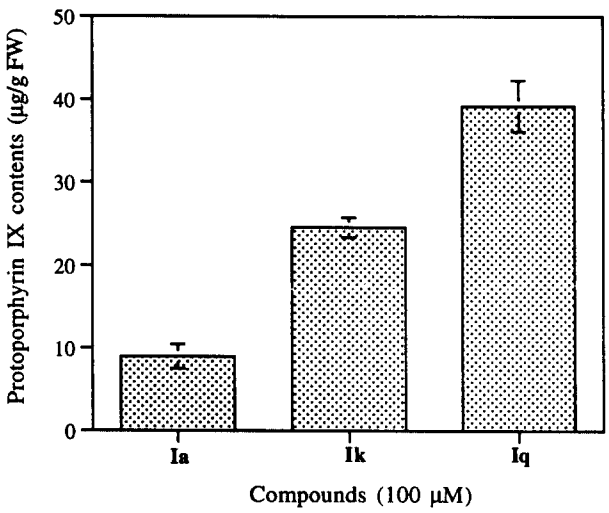

Figure 1. Effect of oxadiazolidine derivatives **Ia, Ik** and **Iq** on the accumulation of protoporphyrin IX in etiolated cucumber cotyledon. Vertical bars represent standard deviation of three replicates. Etiolaed cucumber cotyledons placed on test solutions were exposured to continuous light at 120μmol/m²/s at 25 °C for 3 h following 12 h dark incubation.

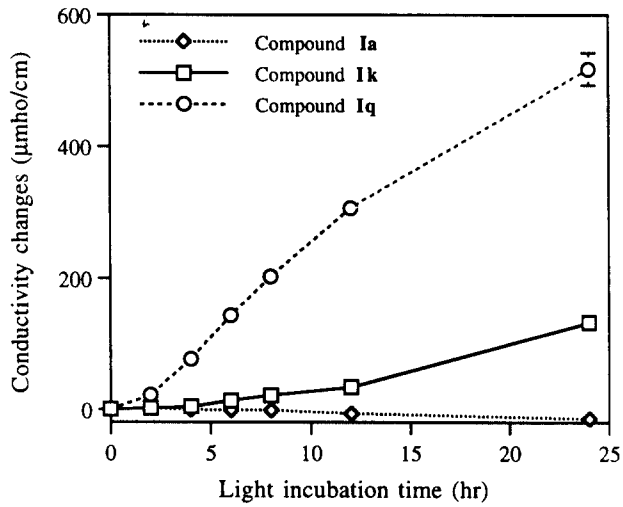

Figure 2. Effect of oxadiazolidine derivatives **Ia, Ik** and **Iq** (100 μ*M*) on cellular leakage from cucumber cotyledon discs exposured to continuous light at 120 μmol/m²/s at 25 °C following 12 h dark incubation. Vertical bars represent standard deviation errors of three replicates.

accumulation. The highest accumulation of Proto IX (40 μg/g fresh weight) was observed when the cotyledons were irradiated in the presence of oxadiazolidine (**Iq**). Compared to this, relatively low and small amount of accumulation of Proto IX was observed in cases of OCH_3 and H substituted oxadiazolidines **Ia** and **Ik** of which inhibitory activities were lower than that of propargyloxy substituent (**Iq**).

Assay of cellular leakage. The cucumber cotyledons were treated with the oxadiazolidines **Ia**, **Ik** and **Iq**, respectively. The conductivity change caused by cellular electrolyte leakage was measured by irradiation time as shown in the Figure 2. The light induced cellular leakage was linearly correlated with the inhibition of chlorophyll as well as the herbicidal activity of the oxadiazolidine(**I**). In case of propargyloxy substituent **Iq**, the conductivity change was the highest. The lowest change was observed in case of methoxy substituent **Ia** which showed also the lowest herbicidal activity.

As a conclusion, the accumulation of Proto IX and cellular leakage by the oxadiazolidinedione derivatives **Ia-u** correlated well with their herbicidal activities. These result suggest that the herbicidal activity of oxadiazolidine **I** is attributed to the inhibition of chlorophyll formation acting on the Protox. Based on the literature search, the oxadiazolidinedione **I** can be classified as an *N*-phenylimide inhibitor among the representative Protox-inhibitors such as diphenyl ether and *N*-phenylimide compounds.[7,11,12]

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