Generation of Reactive Oxygen Species from Hinokitiol under Near-UV Irradiation

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Near-UV irradiation caused the decomposition of hinokitiol in an aqueous solution. During the photochemical reaction, the distinct electron spin resonance signal characteristic of the adduct of 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) with the hydroxyl radical was accompanied by small signals corresponding to the adduct of DMPO with the superoxide anion radical. More than 95% of *Escherichia coli* cells were killed by the incubation with hinokitiol under near-UV irradiation by BLB fluorescent lamps. These results indicated the generation of reactive oxygen species during photochemical reaction of hinokitiol under near-UV irradiation.

Key words: bactericidal activity; hinokitiol; near UV; reactive oxygen species

Hinokitiol (4-isopropyl tropolone), a constituent of Japanese cypress and western red cedar, has been reported to have biological activities including antibacterial, anti-fungal, anti-tumor, and antioxidative actions. As hinokitiol has a characteristic cyclic 7 carbon ring (tropolone) skeleton and shows a maximum absorption peak at 244 nm with smaller absorption peaks in the near-UV region (280–380 nm), we were interested in the behavior of hinokitiol under near-UV irradiation. We report here the generation of the superoxide anion radical (O$_2^-$) and hydroxyl radical (•OH) and the bactericidal activity in the near-UV-induced reaction of hinokitiol.

Irradiation was started by illuminating 56.7 μM hinokitiol (Wako Pure Chemical Industry, Osaka, Japan) diluted in a 10 mM phosphate buffer containing 1 mM EDTA at pH 7.4, with near-UV light (2.5 mW/cm$^2$). This light was obtained by using a heat-cut filter and UV-D33S-pass-filter (Toshiba, Japan) which passed light of 295–390 nm from a 200-W xenon lamp fitted in the UV-illuminating system L-5662-01 (Hamamatsu Photonics, Hamamatsu, Japan) as described previously. Near-UV-induced photochemical decomposition of hinokitiol was confirmed by the decrease in absorption. After 8 min of irradiation, hinokitiol had been changed to compound(s) which showed no absorption in the UV region (Fig. 1).

Hydroxylamine oxidation, a method for the detection of O$_2^-$, was monitored by the formation of nitrite ion$^{10}$ with time during the photochemical reaction. The oxidation proceeded almost linearly with irradiation time, and was inhibited by superoxide dismutase (Wako Pure Chemical Industry). Under anaerobic conditions, little oxidation proceeded. The ESR spectrum appeared within 5 min of the hinokitiol photoreaction and consisted of the 1:2:2:1 quartet pattern (a$_N$=a$_H$=1.48 mT) characteristic of an adduct of 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) with •OH, as shown by unfilled circles in Fig. 2. Together with DMPO-OH signal, small signals corresponding to DMPO-OOH, the adduct of

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**Fig. 1.** Decomposition of Hinokitiol under Near-UV Irradiation.

The reaction mixture of 56.7 μM hinokitiol in 10 mM K-phosphate at pH 7.4 containing 1 mM EDTA was irradiated with near-UV light (2.5 mW/cm$^2$). At the times indicated, the absorption spectra were recorded to follow the decomposition of hinokitiol.
DMPO with \( \cdot O_2 \) (\( a_{\beta} = 1.43 \) mT, \( a_{\alpha} = 1.15 \) mT, \( a_{\gamma} = 1.52 \) mT) were detected as shown by filled circles. The different intensity of the signals derived from both adducts may have been due to the different rate constants of trapping for \( \cdot OH \) (2.1–5.7 \( \times 10^4 \) m\(^{-1}\) sec\(^{-1}\)) and \( \cdot O_2 \) (10 m\(^{-1}\) sec\(^{-1}\)), respectively.11) No signal was detected without irradiation. The test for hydroxylamine oxidation and the results shown in Fig. 2 indicate the generation of \( \cdot O_2 \) and \( \cdot OH \) during the photochemical decomposition of hinokitiol.

The test for bacterial killing activity was carried out by counting the number of colonies that had appeared after 16 h of culture on an agar plate at 37°C, according to the procedure described previously.12) Incubation of *Escherichia coli* cells (6 \( \times 10^5 \) CFU/ml) at room temperature with 50 or 100 \( \mu \)M hinokitiol in the dark respectively killed about 10% or 22% of cells (Table 1). Near-UV irradiation (0.5 mW/cm\(^2\) from BLB fluorescent lamps, Toshiba FL20S) of the cell suspension in a glass test tube for 45 min showed 16.5% killing effect. On the other hand, nearly a half and more than 95% of the cells were killed under near-UV irradiation in solutions containing 50 \( \mu \)M and 100 \( \mu \)M hinokitiol, respectively.

The results of control experiment, in which 100 \( \mu \)M hinokitiol was pre-irradiated for 15 min with high-intensity (2.5 mW/cm\(^2\) as shown in Fig. 1) of near-UV light before incubation with the cells, showed no killing effect, suggesting that the hinokitiol derivative(s) being decomposed by near-UV irradiation had no bactericidal activity (data not shown). Coombs and Trust13) have reported the decomposition of hinokitiol under normal laboratory light (a mixture of fluorescent light and sunlight) for 5 hr and a loss of antifungal activity against *Mikroccocus lysodeikticus* and *Aeromonas hydrophila*. Growth inhibition was markedly reduced after hinokitiol had been exposed to light for 5 hr, but the control solution maintained in the dark displayed no reduced antifungal activity.13)

The photochemical decrease of hinokitiol was not inhibited by the \( \cdot OH \) scavenger, mannitol (200 mM), suggesting no participation of \( \cdot OH \) in the degradation of hinokitiol. At the initiation of the photochemical reaction, hinokitiol absorbs near-UV photons then is brought to the triplet state by excitation. The well known D-1 process may be applicable to the photochemical event of hinokitiol yielding \( \cdot O_2 \) and \( \cdot OH \). The triplet state causes charge separation, generating the hinokitiol anion and cation. The hinokitiol anion will donate an electron to \( O_2 \), producing \( O_2^- \). The reaction product having no absorption in the near UV region and no bactericidal activity may be derived from the hinokitiol cation. The possible decomposition product under laboratory light has been suggested to be isopropyl-substituted 4-oxo-cyclopentane-1-acetic acid.12) Two molecules of \( O_2^- \) dispropionate to \( H_2O_2 \) and \( O_2 \), and a trace amount of \( Fe^{2+} \) will act as Fenton reagent to yield \( OH \) from \( H_2O_2 \). The \( OH \) generated in the near-UV hinokitiol system may be the bactericidal species.13)

### Table 1. Bactericidal Activity of Hinokitiol under Near-UV Irradiation

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CFU' x 10^4/ml</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>58.2</td>
<td>100</td>
</tr>
<tr>
<td>Near UV</td>
<td>48.6</td>
<td>83.5</td>
</tr>
<tr>
<td>Hinokitiol (50 ( \mu )M)</td>
<td>52.8</td>
<td>90.7</td>
</tr>
<tr>
<td>Hinokitiol (100 ( \mu )M)</td>
<td>45.6</td>
<td>78.4</td>
</tr>
<tr>
<td>Hinokitiol (50 ( \mu )M) + near UV</td>
<td>27.8</td>
<td>47.8</td>
</tr>
<tr>
<td>Hinokitiol (100 ( \mu )M) + near UV</td>
<td>2.76</td>
<td>4.7</td>
</tr>
</tbody>
</table>

* Escherichia coli DH cells (6 \( \times 10^8 \) CFU/ml) in the late log phase grown at 37°C in an LB medium were washed twice with physiological saline and then incubated for 45 min at room temperature in a medium (1 ml) supplemented with or without hinokitiol either in the dark or near-UV irradiation (0.5 mW/cm\(^2\)) with fluorescent lamps (Toshiba FL20S-BLB). After this treatment the mixture was diluted 100-fold with sterile 0.9% NaCl and plated on to nutrient agar as described previously.12) CFU values are shown as the average of three different culture plates after 16 hr of growth at 37°C.

### References


