Title: Mechanism of Dicofol Resistance in Spider Mites II: Thin Layer Chromatographic Identification of Dicofol Metabolites in Citrus Red Mite, Panonychus citri McGREGOR

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**Introduction**

Dicofol (Kelthane®), a hydroxy analog of DDT, is one of the most effective compounds against phytophagous mites but not insect pests. While the use of dicofol has rapidly increased to control not only citrus red mite (*Panonychus citri* McGregor) but also other injurious mites, the dicofol resistance in *P. citri* McGregor presently has become a major problem on control in citrus orchards in Japan. However, little attention has been paid to studies on dicofol metabolism in mites, although these would be valuable in relation to investigations of the mite resistance.

Chlorinated hydrocarbon insecticides have been used extensively during the past two decades in controlling various insect pests. In relation to DDT and its related compounds, there have been a number of detailed studies of their metabolisms in insects. In many insects DDT-dehydrochlorinase, a glutathione dependent enzyme, catalyzed the degradation of *p,p'-DDT* to *p,p'-DDE*. An alternate biotransformation in *Drosophila melanogaster* gave rise to the hydroxy analog of **DDT**. Similarly, when third-instar larvae of *Triatoma infestans* were topically applied with **DDT**, two metabolites were detected, dicofol and a compound that behaved like 4,4'-dichlorobenzohydrol. The latter also appeared after treatment with dicofol. Furthermore, investigation showed that a kind of soil microorganism was capable of degrading DDT to a dicofol-like compound.

The previous study, therefore, sought to determine the fate of dicofol after topical application to both dicofol susceptible and resistant strains of *P. citri* McGregor in vivo. As a result, it was found that resistant strains have higher ability than susceptible strains to metabolize dicofol to the water soluble metabolites and that no significant difference in cuticle permeability between susceptible and resistant strains was found. From these results, the authors concluded that difference in dicofol metabolism is one of factors responsible for dicofol resistance.

Basing on the facts described above, tentative identifications of the chloroform extractable

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metabolites and the water soluble metabolites from dicofol susceptible and resistant mites applied topically with \(^{3}H\)-dicofol were carried out by thin-layer chromatography technique.

**Materials and Methods**

**Radio-labeled compound**: \(^{3}H\)-ring labeled dicofol was supplied by Sanyo trading Co., Ltd, via Loam and Haas Co., U.S.A. with a specific activity 0.96 mCi per mg and had a radiochemical purity of ca. 94% by thin-layer chromatography (tlc) using system B as shown in Table 1.

**Materials and Methods**

**Mites**: Dicofol susceptible strain (WSS) and resistant strain (WRS) of *P. citri* McGREGOR came from Wakayama citrus experiment station in 1969 were reared on citrus plant in green house at the Nagoya University for 3 years and adult female mites were used throughout this experiment.

WSS : A dicofol susceptible strain obtained from citrus orchards where dicofol has not been sprayed. The LD-50 by topical application was approximately 0.001 \(\mu g/\text{mite}\) \(^{3}\).

WRS : Highly resistant to dicofol, originally collected from citrus orchards being sprayed twice per annum in 1965-1969. The topical LD-50 was approximately 0.366 \(\mu g/\text{mite}\) \(^{3}\).

**Treatment**: Twenty \(m\mu g\) of \(^{3}H\)-dicofol in 2 \(m\mu l\) of furfuryl alcohol solution applied on the idiosoma of adult female of *P. citri* McGREGOR (WSS and WRS) and subsequently held on cover glass at 25\(^\circ\)C for 32 hours in plastic cage with ca. 74\% of relative humidity without food.

**Radioassay**: Tritium labeled compounds in *P. citri* McGREGOR topically applied with \(^{3}H\)-dicofol were assayed by a liquid scintillation spectrometer (Aloka LSC-502). Two counting formulations were used; solution A for counting the water soluble metabolites, consisting of 6.0 grams of PPO (2,5-diphenyloxazole), 112 grams of naphthalene, 270 mg of POPOP (1,4-bis-(5-diphenyloxazolyl)-benzene), and enough reagent grade dioxane to bring the volume to 1 liter and solution B for counting the chloroform extractable metabolites, consisting of 4 grams of PPO and 5mg of POPOP per liter of reagent grade toluene. All counts were corrected for quenching (external standard) and background.

**Extraction of radioactive materials from treated mites**: Thirty two hours after topical application with \(^{3}H\)-dicofol (0.02 \(\mu g/2 m\mu l/\text{mite}\)), 300 mites were washed on their cuticle with \(n\)-hexane twice. The mites were transferred to 10 \(m\)l of centrifuge tube and 2 \(m\)l of extraction solvent, chloroform-water (1 to 1), was added and homogenized by the grass bar. The homogenate was partitioned three times with equal volume (1 \(m\)l) of chloroform, which yielded the chloroform extractable metabolites and water soluble metabolites (Figure 1). The radioactivities of the chloroform extractable metabolites from *P. citri* McGREGOR (WSS, WRS) female adult 300 mites

- applied topically (0.02 \(\mu g/2 m\mu l/\text{mite}\))
- washed with \(n\)-hexane

\(n\)-hexane

- homogenized and centrifuged (830xg)

chloroform

water

**Fig. 1. Flow diagram for the standard extraction procedure on the female adult of citrus red mite applied topically with \(^{3}H\)-dicofol.**

dicofol susceptible and resistant mites and the water soluble metabolites from dicofol resistant mites were ca. 18,500, 17,000 and 1,630 cpm per \(m\)l respectively.

**Chromatography**: The chloroform extractable metabolites and water soluble metabolites isolated as described above were chromatographed in solvent system A, B, C and D given in Table 1.
The chromatographic techniques were carried out on the thickness of 0.25 mm plates (20×20 cm) of the silica gel with a fluorescent indicator (Silica gel F254, Merck) using the solvent systems described in Table 1. The plates were activated at 110°C for one hour and then cooled to the room temperature prior to use. Tentative identifications were based on comparisons of the chromatographic behaviour with that authentic reference compounds. The positions of the radioactivity were compared with the positions of the reference spots detected by ultra-violet light. The areas containing the radioactivity were scraped and counted by the scintillation spectrometer.

**Results and Discussion**

In previous experiments with the fate of topically applied ¹⁰⁻H-dicofol in citrus red mite, dicofol was absorbed rapidly and was not

<table>
<thead>
<tr>
<th>Compound</th>
<th>Solvent system</th>
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<tbody>
<tr>
<td></td>
<td>system A</td>
</tr>
<tr>
<td>p,p′-DDT (I)</td>
<td>0.35-0.42</td>
</tr>
<tr>
<td>Dicofol (DCF)  (II)</td>
<td>0.03-0.09</td>
</tr>
<tr>
<td>DDE            (III)</td>
<td>0.57-0.63</td>
</tr>
<tr>
<td>DBP            (IV)</td>
<td>0.01-0.05</td>
</tr>
<tr>
<td>DDMV           (V)</td>
<td>0.51-0.56</td>
</tr>
<tr>
<td>FW-152         (VI)</td>
<td>0.01-0.03</td>
</tr>
<tr>
<td>DBH            (VII)</td>
<td>0.00-0.01</td>
</tr>
<tr>
<td>DDD            (VIII)</td>
<td>0.21-0.25</td>
</tr>
<tr>
<td>DMC            (IX)</td>
<td>0.03-0.07</td>
</tr>
<tr>
<td>C_{6}H_{5}-DDT  (X)</td>
<td>0.15-0.20</td>
</tr>
<tr>
<td>H-DDT          (XI)</td>
<td>0.28-0.32</td>
</tr>
<tr>
<td>CH_{2}-DDT     (XII)</td>
<td>0.33-0.40</td>
</tr>
<tr>
<td>Bh             (XIII)</td>
<td>0.00-0.02</td>
</tr>
<tr>
<td>Bp             (XIV)</td>
<td>0.00-0.04</td>
</tr>
<tr>
<td>CBA            (XV)</td>
<td>0.00-0.00</td>
</tr>
</tbody>
</table>

**Table 2. Rf-Values of dicofol and its related compounds.**
extensively degraded, that is, about 40 to 50% of treated dicofol was penetrated within 32 hours after topical application in WSS and WRS strains. Approximately 20% of absorbed 3H-dicofol was found as water soluble metabolites in WRS but only small amount in WSS, which was considered as one of the factors responsible for resistance.

The thin-layer chromatographic identifications of dicofol metabolites from the resistant and susceptible of *P. citri* McGregor topically applied with 3H-dicofol (0.02 µg/2 µl/mite) were investigated by means of the extraction procedure as shown in Figure 1.

The chromatographic behaviours of dicofol and its related compounds in several solvent systems are shown in Table 2. The n-heptane-acetone system (system B) was useful for separating dicofol and 4,4'-dichlorobenzophenone (DBP), and n-butanol-acetic acid-water system (system D) was used principally to separate the water soluble metabolites.

Only one internal metabolite was detected in the chloroform extract of the mites intoxicated with 3H-dicofol by the thin-layer chromatography. This compound has the same chromatographic mobility as authentic dicofol having Rr 0.03-0.06 on tlc system A, Rr 0.47-0.52 on tlc system B, Rr 0.81-0.86 on tlc system C, and Rr 0.91-0.95 on tlc system D. Therefore, it should be noticed that more than ca. 85% of 3H-dicofol absorbed in WSS and ca. 30% in WRS were found as internal radioactivity after 32 hours and were not metabolized. It is well known that DDT is dehydrochlorinated by DDT-dehydrochlorinase to yield DDE in the insect body. DDT is also converted to dicofol, an alcoholic type compound, and DBP, a ketonic compound of DDT derivatives, were discovered in several species of insects. Dicofol has been known to decompose to DBP and chloroform under alkaline condition. However, the formation of DBP and/or DDE from WSS and WRS applied topically with 3H-dicofol may not occur at least in vivo. The small amounts of activity found at Rr 0.57-0.63 on tlc system A, Rr 0.84-0.88 on tlc system B, Rr 0.90-0.92 on tlc system C and Rr 0.93-0.95 on tlc system D could be accounted for in terms of trace quantities of radioactive impurities present in the original 3H-dicofol, although this compound has the same Rr value of DDE. Further proof was obtained by comparing the Rr values of the chloroform extractable metabolites from WSS and WRS, with 3H-dicofol, nonlabeled dicofol and DDE.

The large amount of 3H-dicofol is metabolized to form the water soluble metabolites in WRS. This compound was cochromatographed identically with the authentic compounds in solvent...
system C and D as shown in Figure 3. The thin-layer chromatography on silica gel F254 plates gave a compound with $R_f$ value of 0.00-0.07 on tlc system C and also 0.6-0.7 on tlc system D. This $R_f$ value does not correspond to that obtained with 4,4'-dichlorobenzohydrol (DBH) having $R_f$ 0.48-0.54 on tlc system C and $R_f$ 0.92-0.95 on tlc system D and/or other authentic compounds used in this study, although DBH appeared after topical application with dicofol-14C to third-instar larvae of *Triatoma infestans*. Therefore, the results of this study indicated hydrolytic detoxication of dicofol in WRS was a major pathway, although the chemical nature of the water soluble metabolite is not known.

**Summary**

1) Tentative identifications of the chloroform extractable metabolites and the water soluble metabolites from dicofol susceptible (WSS) and resistant (WRS) mites applied topically with $^3$H-dicofol (0.02 pg/2 mpl/mite) were carried out by the thin-layer chromatography using several solvent systems.

2) As indicated by the thin-layer chromatography of the chloroform extractable metabolites from both dicofol susceptible and resistant mites, they were not DDE, DBP and/or other related authentic compounds but dicofol itself.

3) Approximately 20% of $^3$H-dicofol absorbed are metabolized to form the water soluble metabolite(s) in the dicofol resistant mites. This compound has 0.6-0.7 of $R_f$ value on tlc system D but this $R_f$ value does not correspond to DBH or other authentic compounds used in this experiment.

4) The results of this study indicated hydrolytic detoxication of dicofol in resistant mites was a major pathway, although the chemical nature of the water soluble metabolite is not known.

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