PRECOCENE II: STABILITY AND EFFECTS
UPON WOOD-BORING ORGANISMS

FINAL RESEARCH REPORT

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SEA GRANT PROJECT SUMMARY

NEW D CONTINUING

Precocene II: Stability and Effects Upon Wood-Boring Organisms

PERCENT OF PROPOSED FUNDING FOR ANTICIPATED BENEFIT AREAS

OBJECTIVES:

This research project was designed to collect preliminary data concerning the effect of a naturally occurring chemical, precocene II, on the wood-boring isopods Sphaeroma terebrans and Limnoria tripunctata. The experimental design included acute 96 hour bioassays and chronic 10 day bioassays. Chemical analyses were also conducted for determination of appropriate extraction techniques of precocene II from solution and for stability of precocene II over time.

ANTICIPATED BENEFITS:

Estimated annual losses to maritime industries due to wood-boring organisms may be as high as $500 million. At the present time, no completely effective method is known for protection of wooden structures exposed to seawater. The results of this research project may lead to the development of new methods of protection of wood from boring organisms in the marine environment. Savings to maritime industry and individuals could be substantial in the event that this system of control proves to be applicable and cost effective.

IDENTIFIED BENEFITS TO DATE:

KEYWORDS:
ABSTRACT

The effects of precocene II (6,7-dimethoxy-2,2-dimethyl-chromene) in seawater solution were determined for two species of wood-boring isopods, Sphaeroma terebrans and Limnoria tripunctata. Acute 96 hour bioassays and chronic 10 day bioassays were employed. A precocene II concentration of 30 ppm was sufficient to kill all S. terebrans after 10 days exposure. Molting rate in S. terebrans was higher in all concentrations of precocene II than in controls, suggesting that precocene may interfere with normal molting cycles. Limnoria tripunctata was more sensitive to precocene II in solution than S. terebrans and had a calculated 96 hour LC₅₀ value of 8.4 ppm. Wood boring activity of L. tripunctata ceased after 24 hours exposure to 30 ppm precocene and ceased after 48 hours exposure to 20 ppm precocene. No difference in molting rate was observed in L. tripunctata bioassays between controls and precocene II treatments.

Chemical analysis of precocene II in solution was undertaken in order to establish the most efficient method of extraction and recovery and to determine the stability of precocene II in solution over time. Reverse phase high performance liquid chromatography utilizing an ultraviolet detector at 254 nm was chosen as the analytical method. Analysis of precocene II concentrations over time indicated that no significant changes occurred in solutions with or without experimental animals present for at least 72 hours.

The results of this study indicate that precocene II may be an effective agent in deterring the wood boring activities of S. terebrans and L. tripunctata. Further studies are needed to establish the method of action of precocene on marine isopods and to develop a method for treating wood with precocene in order to inhibit the activity of boring organisms.
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I. Introduction

This project was undertaken to study the response of two wood-boring isopods to various concentrations of precocene II in solution and to establish the stability of this chemical in seawater.

The activities of wood-boring marine organisms have been a subject of concern to man since the early Greek and Roman times (Turner, 1959). Practically any wooden structure placed in the marine environment for a period of time becomes a target for attack by one or more groups of wood-boring organisms. Estimates of annual losses by maritime industries due to wood-boring organisms range from $50 to $500 million (Ray, 1958; Hochman, 1973). Most marine wood-boring organisms fall into one of two major groups, the isopod crustaceans or the teredinid molluscs. In Florida, two species of wood-boring isopods (Limnoria tripunctata and Sphaeroma terebrans) are considered to be important contributors to wood deterioration.

Limnoria is the smaller of the isopods and uses wood as a primary food source. Sphaeroma is generally larger than Limnoria and bores into wood for protection without utilizing the wood for food. Both species are mobile as adults and capable of moving from one piece of wood to another. Development is direct in both species and growth is accomplished through a series of molts.

Growth, molting and certain aspects of reproduction have been shown to be under hormonal control in a number of crustaceans (Novales et al., 1973). The specific hormones involved and the integration of endocrine
control mechanisms in crustaceans are not fully understood. The presence of such systems, however, raises the possibility of artificial manipulation of life processes through exposure to chemical blocking agents or hormone mimics. The use of synthetic pheromones to control insect populations is well documented (Sower, 1981).

One such chemical is precocene II (6,7-dimethoxy-2,2-dimethylchromene) which was first identified as a natural plant product in 1955 by Alertson but was not considered as a biological control substance until Bowers et al. (1976) and Bowers and Martinez-Pardo (1977) described its activity as a block to juvenile hormone (JH) production in insects. It was discovered that exposure to precocene, at the appropriate life cycle stage would cause precocious metamorphosis in the milkweed bug, Oncopeltus fasciatus, and prevented development of the ovaries in female bugs by blocking JH production in the corpora allata. Precocene has thus become known as an anti-juvenile hormone, or, more correctly, as an antiallatotrophic substance. In addition to metamorphosis and ovary development, JH is also involved in diapause initiation or termination in various insects as well as production of sex pheromones and maintenance of caste systems in social insects (Bowers et al., 1976). Exposure to precocene can potentially disrupt all of these essential processes.

Preliminary work by Landau and Rao (1980) indicates that precocene II causes reduced survival, growth and reproduction in the barnacle,
Balanus eburneus and reduced survival in Artemia nauplii. These results suggest that hormonal processes in crustaceans may be affected by precocene II even though the exact mechanisms involved are unknown. The existence of a parallel system to insect JH regulation has yet to be conclusively identified within the crustaceans; however, Freeman and Costlow (1979) have found that stage-specific hormone secretion is involved in metamorphosis of crab larvae, and Gomez et al. (1973) have shown that JH analogs affect development in crustacean larvae.
II. Methods

A. Biological Investigations

The experimental animals used were Sphaeroma terebrans Bate and Limnoria tripunctata Menzies. Sphaeroma was collected from low salinity (150/oo) mangrove estuaries in Tampa Bay and Limnoria was collected on wooden blocks suspended in seawater near St. Petersburg, Florida. Sphaeroma terebrans were removed from the mangrove prop roots and placed in static aquaria with aeration prior to experiments. Powdered fish flakes (Tetra Merin) were added to these cultures weekly. Limnoria tripunctata were left in the wooden blocks and held in flow-through aquaria. Individual organisms were removed from the wood as needed for experiments.

Bioassay experiments were carried out in 400 ml (Sphaeroma) and 100 ml (Limnoria) glass containers that had been washed and acid rinsed prior to use. All experiments were run at 23-25°C under a 12:12 light:dark cycle. Light intensities were reduced during the light phase of the 24-hour cycle in order to simulate the natural conditions of the animals. In each experiment, three replicates of each treatment were run with three animals per replicate. Precocene II (6,7-dimethoxy-2,2-dimethyl-chromene, Sigma Chemical Company) was dissolved in propylene glycol to produce a stock solution of 3000 mg/l or in 95% ethanol to
produce a stock solution of 10,000 mg/l. The ethanol carrier solvent was found to be less toxic than propylene glycol to Limnoria and was thus used in all bioassays with this species. A carrier solvent control was run in each bioassay experiment and contained the equivalent amount of solvent that would be present in the highest precocene treatment for that experiment.

B. Chemical Analysis

Reverse-phase high performance liquid chromatography (HPLC) utilizing an ultra violet (uv) detector was chosen as the analytical method. The basic advantages were the ability to select the aromatic character of precocene (uv absorbance) in the presence of other compounds that may be produced by the test organisms and the ability to avoid high temperatures required by gas chromatography.

A standard stock solution of 100 mg/l precocene II was prepared in methanol (MeOH). Dilutions of the original standard were prepared for working standards. Quantitative and qualitative analyses were performed using a Varian Model 5020 HPLC with a uv-50 variable wavelength spectrophotometer as a detector. The column was a 30 cm reverse phase Micro Pak MCH-10, ODS (Varian Instruments, Sunnyvale, California).

Various mobile phases were evaluated and the most effective was a solution of methanol/water/acetic acid (90/9.9/0.1, V/V/V). Sample volumes injected ranged from 5 to 50 µl at a flow rate of 2 ml/min. The uv wavelength for detection was 254 nm.
Recovery of precocene II from aqueous solution was accomplished by liquid-liquid extraction into an organic solvent. The extraction was performed by placing a 100 ml aliquot of the aqueous precocene solution in a separatory funnel, adding 15 ml of organic solvent and shaking for 60 seconds. The phases were allowed to separate, the organic solvent recovered, and the extraction repeated twice. Combined solvent extracts were reduced in volume on a vacuum rotary evaporator at 40°C, evaporated to dryness under N₂ gas and brought to the desired volume in MeOH for HPLC analysis.

Precocene extraction efficiency was evaluated with two organic solvents, hexane and dichloromethane. Dispersion of precocene in water was enhanced by the use of a carrying solvent. A concentrated solution (1,000 mg/l or greater) of precocene was prepared in the carrying solvent and then a small volume of this stock solution was added to seawater to produce the desired precocene concentration. Three carrying solvents were evaluated, methanol, ethanol and propylene glycol. Extraction efficiency of precocene was tested in the presence of each carrier solvent.

The stability of precocene II in aqueous solution was observed by making six replicate solutions of 1 mg/l precocene in seawater (ca. 25°C/oo salinity). Duplicate samples were extracted and analyzed after 1 hour, 24 hours and 72 hours.

Analytical quality control was maintained by analysis of reagent blanks and control samples along with each set of test samples.
III. Results and Discussion

A. Biological Investigations

Standard 96 hour bioassay experiments were run separately on the two isopod species under controlled laboratory conditions. Since precocene II is highly insoluble in seawater, it was necessary to dissolve the chemical in an organic carrier solvent first and then add this solution to seawater. As a result of this procedure, an extra series of control samples had to be run in each bioassay experiment in order to determine the effect, if any, of the carrier solvent on the test organisms. Landau and Rao (1980) used acetone as a carrier solvent in their experiments with barnacles and brine shrimp; however, *Sphaeroma* was found to be highly sensitive to acetone in the present experiments.

An alternative carrier solvent that was found to be more acceptable to *Sphaeroma* in bioassay experiments was propylene glycol (1,2-Propanediol, Sigma Chemical Company). Figure 1 represents the results of a preliminary carrier solvent (propylene glycol) bioassay with *Sphaeroma terebrans*. In subsequent bioassay experiments with *Sphaeroma*, the highest concentration of carrier solvent used was 0.5% propylene glycol.

*Sphaeroma* Bioassays

The acute response of *Sphaeroma* to precocene II is presented in Figure 2. After 96 hours of exposure, only the highest concentration of precocene II (30 ppm) resulted in death of any experimental animals.
Figure 1. Survival curves for *Sphaeroma terebrans* exposed to propylene glycol in solution for 72 hours.
Figure 2. Survival curves for *Sphaeroma terebrans* exposed to precocene II in solution for 96 hours. Carrier solvent was propylene glycol (0.5% maximum).
Survival rate was 100% in all other treatments. This experiment was continued for an additional six days under the same conditions. The survival rates for the entire 10 day period are presented in Figure 3. The seawater control and 10 ppm precocene II treatments displayed 100% survival while the highest precocene concentration (30 ppm) resulted in 0% survival after 10 days. Some mortality was observed in the propylene glycol control series after the fifth day making interpretation of the 20 ppm and 30 ppm precocene II survival curves difficult. Ideally, survival in all control treatments should be 90% or better; however, mortalities were considerably higher in 20 ppm and especially 30 ppm precocene II than in the propylene glycol control. This would strongly suggest that precocene II was contributing to the death rate. These survival bioassays with Sphaeroma need to be repeated with lower concentrations of propylene glycol or with a different carrier solvent in order to establish a precise toxicity level for precocene II.

The molting rate of Sphaeroma terebrans was determined over an eleven day period in three concentrations of precocene II plus controls. These data are presented in Table 1. Sphaeroma and other isopods undergo a biphasic molting sequence in which half of the exoskeleton is shed initially, followed by the second half a short time thereafter. Each half or whole molt was recorded daily and totaled in Table 1. In the seawater and propylene glycol control series, molting activity was very low. In each concentration of precocene II however, at least one animal molted by the third day.
Figure 3. Survival curves for *Sphaeroma terebrans* exposed to precocene II in solution for 10 days. Carrier solvent was propylene glycol (0.5% maximum). Test solutions were changed on day 7.
Table 1. Molting response of *Sphaeroma terebrans* exposed to precocene II in solution. *n* = 9

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time to First Molt (days)</th>
<th>Total No. of Molts (11 days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11</td>
<td>1/2</td>
</tr>
<tr>
<td>PG Control</td>
<td>no molts</td>
<td>0</td>
</tr>
<tr>
<td>10 ppm</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>20 ppm</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>30 ppm</td>
<td>3</td>
<td>1/2*</td>
</tr>
</tbody>
</table>

*Low total number of molts was due to poor survival rate of the original 9 organisms exposed to 30 ppm precocene II.*
and in the 10 ppm treatment molting continued throughout the experiment. The low total number of molts in the 30 ppm treatment was apparently related to the low survival rate of animals in this concentration of precocene II. These data, although preliminary, would suggest that precocene II may act as a molting accelerator in *Sphaeroma*.

**Limnoria Bioassays**

Initial bioassays with *Limnoria tripunctata* utilized propylene glycol as a carrier solvent. However, this species could not tolerate even 0.3% carrier solvent for 96 hours. As an alternative carrier solvent, ethanol (95%) was used in subsequent experiments with more favorable results. Figure 4 represents the results of an acute 96 hour bioassay with three precocene II concentrations and ethanol as the carrier solvent. Survival was very low in all three precocene II treatments, reaching zero by the end of 96 hours. The 48 hour LC$_{50}$ value (concentration of precocene II that produced 50% mortality after 48 hours) was calculated to be 6.0 ppm by the log concentration versus survival method. In this experiment, test solutions were replaced on day 2.

The relatively high mortality on the last day of the above experiment for the ethanol control treatment (Figure 4) led to this experiment being repeated with the modification that test solutions were replaced daily throughout the experiment. The results of the bioassay are presented in Figure 5. As before, mortalities were higher in precocene II treatments than in controls with the 96 hour
Figure 4. Survival curves for *Limnoria tripunctata* exposed to precocene II in solution. Carrier solvent was EtOH (0.3% maximum).
LC$_{50}$ value calculated at 8.4 ppm. The 48 hour LC$_{50}$ was calculated to be 18.5 ppm. In this second experiment, conditions were more favorable for survival of experimental animals largely due to the daily water changes and thus the results of this bioassay are probably more indicative of the real effects of precocene II on Limnoria.

In addition to survival, the wood boring activity of test animals was monitored daily in three concentrations of precocene II and controls for 96 hours. Wood boring was indicated by the presence of burrows in small pieces of soft pine placed in each container and by fecal pellets on the bottom of the dishes. No wood boring activity was evident after 24 hours in 30 ppm precocene II, while wood boring ceased after 48 hours in 20 ppm precocene II. Boring continued throughout the experiment in 10 ppm precocene II, ethanol controls and seawater controls.

In comparison to Sphaeroma, Limnoria is much more sensitive to precocene II in solution with regard to survival. Sphaeroma, however, may be more sensitive to precocene II as an endocrine system modifier. No molting was observed during 96 hour tests with Limnoria in control or precocene II treatments.

The results of these preliminary experiments suggest that precocene II may be an effective agent in controlling the wood boring activities of Sphaeroma and Limnoria. Further testing is needed in order to establish the exact mode of action of precocene II on these species and to establish procedures for treatment of wood so that wood-boring species can be deterred.
Figure 5. Survival curves for *Limnoria triquintata* exposed to precocene II in solution. Carrier solvent was EtOH (0.3% maximum) and solutions were replaced daily.
B. Chemical Investigations

Analytical Methods

A series of precocene II standard solutions in MeOH were analyzed by HPLC as described above to establish the linear range of detection, retention time and response factor. Retention time was 2.5 min with a 2 ml min⁻¹ flow rate, and 1 μg of precocene produced a response of 6 cm (30% full scale) at 254 nm.

Extraction Efficiency

Percent recovery for the organic solvents, mean ± standard deviation, were 81 ± 4% for hexane and 91 ± 4% for dichloromethane. From these tests, dichloromethane was chosen as the extraction solvent to be used for test samples. Extraction efficiency was also evaluated in the presence of each carrying solvent. No significant carrying solvent effect was observed.

Precocene Stability

Results of the 72 hour precocene stability experiment are given in Table 2. These results show no significant difference among the samples taken at 1 hr, 24 hr and 72 hr, indicating that precocene was stable in the seawater solution for at least 72 hr.

Monitoring Organism Exposure

Precocene was monitored in the test media during toxicity studies to verify exposure concentrations. Results of two toxicity studies (Table 3) show some differences due to experimental variation, but no significant changes occurred throughout the toxicity experiments.
Table 2. Precocene II stability in water. Precocene study - Chemistry.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Precocene Concentration µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard</td>
<td></td>
</tr>
<tr>
<td>Precocene II</td>
<td>100</td>
</tr>
<tr>
<td>Control A</td>
<td>0</td>
</tr>
<tr>
<td>Control B</td>
<td>0</td>
</tr>
<tr>
<td>1 hr A</td>
<td>77.5</td>
</tr>
<tr>
<td>1 hr B</td>
<td>100.2</td>
</tr>
<tr>
<td>24 hr A</td>
<td>85.5</td>
</tr>
<tr>
<td>24 hr B</td>
<td>90.0</td>
</tr>
<tr>
<td>72 hr A</td>
<td>86.8</td>
</tr>
<tr>
<td>72 hr B</td>
<td>98.4</td>
</tr>
</tbody>
</table>
Table 3. Precocene II concentration in toxicity studies. Precocene study - Chemistry.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Precocene Concentration µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Propyleneglycol Carrier</td>
</tr>
<tr>
<td>Initial concentration - 10</td>
<td>11.2</td>
</tr>
<tr>
<td>- 20</td>
<td>18.3</td>
</tr>
<tr>
<td>- 30</td>
<td>26.7</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
</tr>
<tr>
<td>24 hr</td>
<td>- 10</td>
</tr>
<tr>
<td>- 20</td>
<td>14.1</td>
</tr>
<tr>
<td>- 30</td>
<td>28.4</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
</tr>
<tr>
<td>48 hr</td>
<td>- 10</td>
</tr>
<tr>
<td>- 20</td>
<td>16.3</td>
</tr>
<tr>
<td>- 30</td>
<td>23.9</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
</tr>
</tbody>
</table>

¹Composite of triplicate samples.

²Carrier solvent for dispersing precocene in seawater.
IV. Recommendations

Results of this investigation indicate that precocene II is toxic to adult wood-boring isopods. *Limnoria tripunctata* appears to be more sensitive to precocene II than *Sphaeroma terebrans*. For the protection of wooden structures in the marine environment, it is not always necessary to kill potential boring organisms, but rather to deter them by making the wood unacceptable as a habitat and food source. Further studies with precocene should concentrate on determining sublethal exposure concentrations that would still be effective in preventing wood boring activity. In addition, other chemicals similar to precocene II but more active biologically have recently been identified and should be tested in similar acute and chronic bioassay experiments with *Sphaeroma* and *Limnoria*.

Methods need to be developed and tested with regard to treatment of wood with precocene. The water insoluble nature of precocene may be an advantage for its use since the chemical may be less likely than a water soluble compound to leach out of treated wood.

Finally, the effects of precocene in the marine environment on non-target marine organisms need to be determined, along with the physiological basis for the observed effects. The fact that precocene II disrupts hormonal regulation in insects suggests that it may have a similar effect upon marine crustaceans. The observed molting response in *Sphaeroma* exposed to precocene II would support this contention. The use of precocene in laboratory studies may be useful in deciphering the mechanisms of hormonal regulation in crustaceans.
V. Literature Cited


