# Naturally Occurring Insect Control Chemicals

## Isoboldine, a Feeding Inhibitor, and Cocculolidine, an Insecticide in the Leaves of *Cocculus trilobus* DC.

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A feeding inhibitor and a new insecticidal alkaloid were found in the leaves of *Cocculus trilobus* DC. One of the feeding inhibitory constituents in this plant, present at a concentration of about 0.007% on a fresh weight basis, was isolated and identified as the aporphine alkaloid, isoboldine. The threshold concentrations of inhibitory activity against *Trimeresia miranda* Butler and *Prodenia litura* Fabricus were about 200 p.p.m. for both insects. The insecticidal constituent, present at a concentration of about 0.03% on a fresh weight basis, was isolated and identified as cocculolidine, a recently described erythrina alkaloid containing a five-membered lactone. However, these constituents are inactive against an insect which feeds on *C. trilobus*, *Oraesia excavata* Butler, suggesting an interesting aspect of the host-insect interrelationship.

Investigations carried out during recent years (Thorsteinson, 1960) on the host selection of phytophagous insects showed that in both polyphagous and oligophagous species host specialization is governed by the botanical distribution of feeding stimulants and inhibitors. Much work has been done to find specific plant substances evoking feeding responses, while considerably less is known about plant substances having feeding inhibitory activity. Buhr *et al.* (1958) have shown that some of the alkaloid glycosides in solanaceous plants affected the larvae of the Colorado potato beetle, *Leptinotarsa decemlineata* Say, mainly by the repellent action of the food. Moreover, in corn plants, one of the resistance factors to the European corn borer infestation was identified as 6-methoxy-benzoxazolinone (Beck and Stauffer, 1957; Loomis *et al.*, 1957; Smissman *et al.*, 1957). From the standpoint of pesticide chemistry, the possibility of protecting plants against insect pests by treating the leaves of the host plant with feeding inhibitors contained in other plants is worthy of further investigation. *Cocculus trilobus* DC., which is well known as the host plant of Japanese fruit-piercing moths, was noticed to be rarely attacked by other insects in the field. Therefore, it seems reasonable to assume that this plant contains feeding inhibitors or insecticidal principles. In tests conducted at Nagoya University, an alkaloid having feeding inhibitory activity and an insecticidal one were found in the leaves of *C. trilobus*, which also have been used as Chinese medicine in Japan.

### EXPERIMENTAL

**Materials.** The insects used were *Trimeresia miranda* Butler, *Prodenia litura* Fabricus, and *Oraesia excavata* Butler, an insect which feeds on *C. trilobus*. The first were collected from the hedges of *Euonymus japonica* Thunb., which is their natural habitat, just prior to the beginning of the experiments; hence no previous artificial conditioning influenced the host selection behavior. The third instar larvae of the others, which were laboratory cultures maintained on the leaves of their host plants, were donated by the Laboratory of Applied Entomology, Nagoya University.

The plants which were used for the leaf-disk tests, *E. japonica* and *C. trilobus*, were collected in the Mikawa area of Aichi prefecture, while the leaves of *Ipomoea batatas* Lam. var. *edulis* Makino, which grew in the greenhouse, were used for the test against *P. litura*.

**Leaf-Disk Test.** In preliminary experiments, two leaf disks, 16 mm. in diameter, were punched out with a cork-borer from the leaves of *E. japonica*. One disk was immersed in an acetone solution of sample for 2 minutes and the other in pure acetone, which was used as the control disk. After air drying, these disks were placed symmetrically in a polyethylene case (100 mm. in diameter and 45 mm. in depth), and then 10 larvae of *T. miranda* were introduced into the case. About half the area of the control disk was eaten within 2 hours. The consumed areas of the disks were measured by Dethier's method (1947). When the consumed areas of the sample disks were less than 20% of those of the control disks, the samples were considered to have strong feeding inhibitory activity. The leaf-disk tests were repeated twice.

In the experiments against *P. litura*, the above method was used. However, the feeding of *O. excavata* was too slow to consume the disks within several hours, and the disks dried up. Therefore, the halves of leaves cut along the main vein were used in place of disks, and the results were checked after 12 hours.

**Extraction.** *C. trilobus* (Menispermaceae) was collected in the Mikawa area of Aichi prefecture. Fresh leaves of *C. trilobus* were cut into thin pieces with a meat slicer. Fifteen kilograms of the cut leaves were then placed in an enameled iron tank which was fitted with a three-necked lid, and 60 liters of methanol were added. After 1 hour of refluxing, the hot methanol extract was decanted. The extraction with methanol was repeated twice. The combined extracts were then evaporated on a water bath (temperature about 50° C.) under reduced pressure. The residual solution (15 liters) was acidified with dilute hydrochloric acid and extracted three times with 3 liters of ether. The ether extracts were dried over anhydrous sodium sulfate and evaporated in a water bath to give 19 grams of the ether-soluble fraction. The acidic aqueous

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layer was made basic with ammonium hydroxide and extracted four times with 3 liters of chloroform. The chloroform extracts were then dried over anhydrous sodium sulfate and evaporated under reduced pressure to give 15 grams of the basic fraction. These fractions were used for the leaf-disk tests. The disks treated with a 1.1% acetone solution of the ether-soluble fraction were consumed with almost the same ratio as the control disks, while the disks treated wth a 0.4% acetone solution of the basic fraction were not consumed. Apparently, the basic fraction of *C. trilobus* contains substances having feeding inhibitory activity.

**Isolation of Feeding Inhibitor.** Further fractionation of the basic fraction was accomplished on an alumina (Merck standard) chromatographic column using a mixture of chloroform and methanol as the elution solvent. The chloroform solution of the basic fraction (15 grams) was poured onto an alumina chromatographic column (3 × 30 cm.) and eluted with 650 ml. of chloroform, 500 ml. of 10% methanol in chloroform, 450 ml. of 50% methanol in chloroform, and 250 ml. of methanol. The eluates were collected in 200- or 150-ml. fractions as shown in Table I. After the solvents in each fraction had been evaporated, oily materials remained. These materials were next subjected to thin-layer chromatography (Figure 1) and the antifeeding tests were performed (Table I). The thin-layer chromatograms were dried and sprayed with a 0.5% potassium permanganate solution. The fractions eluted with chloroform (fractions 1 to 4) showed only one spot ($R_f$ 0.64) and had no feeding inhibitory activity at 0.45% concentration. Fraction 5 showed two spots ($R_f$ 0.64 and 0.44) and had feeding inhibitory activity at 0.45% concentration. Fraction 6 showed a main spot at $R_f$ 0.44 and had feeding inhibitory activity at 0.45 and 0.12% concentrations. Fraction 9 showed three spots in the region below $R_f$ 0.10 and had feeding inhibitory activity at 0.45 and 0.12% concentrations. Therefore, both the component having $R_f$ 0.45 and the component in the region below $R_f$ 0.10 might have activity. Fraction 6 and 9 were then further purified. Fraction 6 consisted essentially of a single component, as shown in the thin-layer chromatogram in Figure 1, and the main spot gave a positive ferric chloride test, suggesting the presence of a phenolic group. Fraction 6 was dissolved in 30 ml. of 5% hydrochloric acid. The acidic solution was made

**Table I. Chromatographic Purification of the Basic Fraction**

| Fraction No. | | Elution Solvent, Ml. | Eluent, Grams |
|---|---|---|---|
| 1 | $CHCl_3$ | 200 | 1.184 |
| 2 | $CHCl_3$ | 200 | 2.503 |
| 3 | $CHCl_3$ | 150 | |
| 4 | $CHCl_3$ | 100 | 0.711 |
| 5 | 10% MeOH–$CHCl_3$ | 100 | 0.398 |
| 6 | 10% MeOH–$CHCl_3$ | 150 | 0.893 |
| 7 | 10% MeOH–$CHCl_3$ | 150 | 0.393 |
| 8 | 10% MeOH–$CHCl_3$ | 100 | 0.172 |
| 9 | 50% MeOH–$CHCl_3$ | 150 | 0.438 |
| 10 | 50% MeOH–$CHCl_3$ | 150 | Trace |
| 11 | 50% MeOH–$CHCl_3$ | 150 | Trace |
| 12 | MeOH | 250 | Trace |

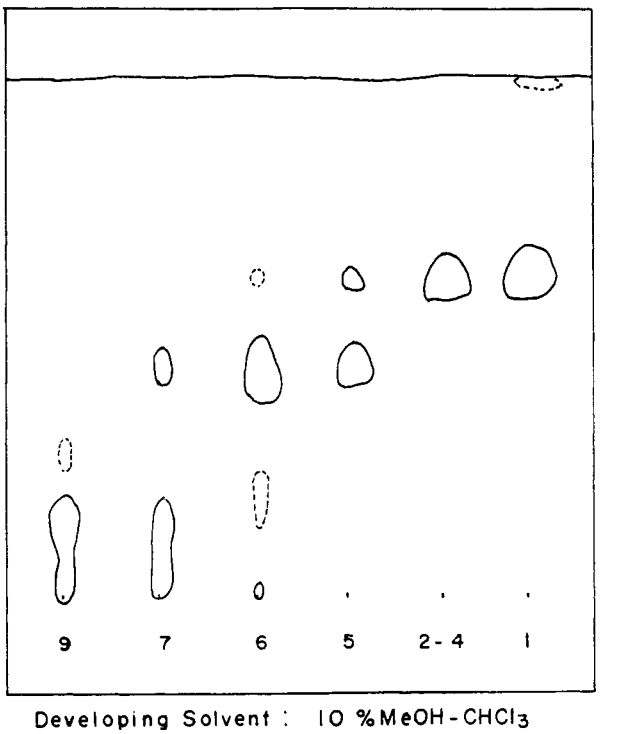
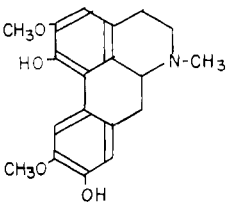
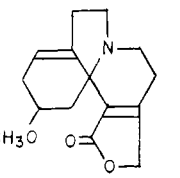



**Figure 1. Thin-layer chromatograms of the basic fractions**
Numbers represent fraction numbers in Table I

basic with 20% sodium hydroxide and extracted with chloroform to remove trace amounts of the nonphenolic alkaloids. When an excess amount of ammonium chloride was added to the aqueous alkaline solution, a white precipitate appeared. It was extracted with chloroform, and the chloroform was dried over anhydrous sodium sulfate and evaporated under reduced pressure. The residue (700 mg.) was dissolved in benzene and stored in a refrigerator overnight to give 100 mg. of prismatic crystals, m.p. 99° C. The concentration of this phenolic alkaloid in fresh leaves of *C. trilobus* was approximately 0.007%. The leaf-disk test against *T. miranda* showed that this crystalline alkaloid has feeding inhibitory activity. Fraction 9, which consisted of three or more components, was further chromatographed on alumina column; however, these components could not be separated in a pure state. The phenolic alkaloid isolated from the active fraction was identified as isoboldine from its chemical and physical properties as shown below.



Isoboldine



Cocculolidine

**Feeding Inhibitory Activity of Isoboldine.** To *T. miranda*. To determine the threshold concentration of feeding inhibitory activity, serial dilutions of pure isoboldine were used for the leaf-disk test against *T. miranda*. The feeding ratios (the consumed amount of the sample disk expressed as a percentage of that of the control

disk) were near zero at concentrations of 200 p.p.m. or over. At concentrations of 100 and 10 p.p.m., the feeding ratios were 40 to 59 and 48 to 126%, respectively (Table II). From the above results, one may conclude that isoboldine has a feeding inhibitory activity to *T. miranda* at 200 p.p.m. or over—namely, the threshold concentration is 200 p.p.m.

To *P. litura*. The leaf-disk test against *P. litura* was conducted with 200- and 100-p.p.m. acetone solutions of pure isoboldine, in which the leaves of the host plant of this insect, the sweet potato, *Ipomoea batatas* Lam. var. *edulis* Makino, were used for feeding disks. Isoboldine also showed feeding inhibitory activity at 200 p.p.m. against this polyphagous insect.

To *O. excavata*. Test against *O. excavata* was conducted with 1000- and 500-p.p.m. acetone solutions of pure isoboldine. As shown in Table III, isoboldine had no activity, even at 1000 p.p.m.

**Identification of Isoboldine as a Feeding Inhibitory Alkaloid.** Recrystallization of the feeding inhibiting alkaloid from chloroform yielded prismatic crystals, m.p. 127° C., $(\alpha)_D^{31}$ + 60° (c., 0.5 $CHCl_3$). Elemental analysis of this material was as follows: C, 53.55; H, 4.97; and N, 3.26%. This analysis suggested $C_{19}H_{21}O_4N \cdot CHCl_3$: C, 53.74; H, 4.96; and N, 3.17%. The ultraviolet spectrum ($\lambda_{max}^{MeOH}$ 306 mμ, log ε = 4.11 and 281 mμ, log ε = 4.03) indicated that the alkaloid might be an aporphine. The mass spectrum had the parent peak at *m/e* 327, supporting the molecular formula, $C_{19}H_{21}O_4N$, suggested by the elemental analysis. The spectrum also had *M*-1 and *M*-43 peaks as shown in Figure 2, which are the most characteristic features of the mass spectra of aporphine alkaloids possessing an *N*-methyl function (Budzikiewicz *et al.*, 1964). From the above data, the alkaloid was considered to be the aporphine alkaloid isoboldine. Acetylation of this alkaloid with acetic anhydride and sodium acetate, and recrystallization of the product from methanol yielded prismatic crystals of triacetate, m.p. 170–2° C. (m.p. reported for isoboldine triacetate, which is a degradation product having phenan-

**Table II. The Feeding Inhibitory Activity of the Basic Fractions**

| Fraction No. | Concentration, % | Amount Consumed: Control *A*, % | Amount Consumed: Sample *B*, % | Feeding Ratio, $(B)/(A) \times 100$ | Activity[a] |
|---|---|---|---|---|---|
| 1 | 0.45 | 20 | 15 | 75 | − |
| | | 50 | 15 | 30 | + |
| 2–3 | 0.45 | 18 | 13 | 68 | − |
| 4 | 0.45 | 13 | 12 | 92 | − |
| | | 32 | 19 | 59 | − |
| 5 | 0.45 | 27 | 6 | 22 | + |
| | | 18 | 0 | 0 | ++ |
| 6 | 0.48 | 18 | 0 | 0 | ++ |
| | | 20 | 0 | 0 | ++ |
| | 0.12 | 19 | 4 | 21 | + |
| | | 22 | 1 | 5 | ++ |
| 7 | 0.47 | 29 | 0 | 0 | ++ |
| | | 54 | 0 | 0 | ++ |
| 9 | 0.47 | 14 | 0 | 0 | ++ |
| | | 43 | 0 | 6 | ++ |
| | 0.12 | 41 | 4 | 10 | ++ |
| | | 25 | 0 | 0 | ++ |

[a] ++ = strong feeding inhibitory activity (*B* = 0–20% of *A*).
+ = slight feeding inhibitory activity (*B* = 20–50% of *A*).
− = no feeding inhibitory activity (*B* = 50% over of *A*).

**Table III. The Feeding Inhibitory Activity of Isoboldine**

| Insect | Concentration, P.P.M. | Amount Consumed: Control *A*, % | Amount Consumed: Sample *B*, % | Feeding Ratio $(B)/(A) \times 100$ | Activity[a] |
|---|---|---|---|---|---|
| *T. miranda* | 1000 | 15 | 0 | 0 | ++ |
| | | 35 | 4 | 11.4 | ++ |
| | 500 | 28 | 3 | 10.7 | ++ |
| | | 42 | 0 | 0 | ++ |
| | 200 | 23 | 0 | 0 | ++ |
| | | 22 | 0 | 0 | ++ |
| | 100 | 39 | 23 | 59.0 | − |
| | | 20 | 8 | 40.0 | + |
| | 10 | 23 | 11 | 47.8 | + |
| | | 27 | 34 | 126.0 | − |
| *P. litura* | 200 | 33 | 2 | 6.1 | ++ |
| | | 30 | 0 | 0 | ++ |
| | 100 | 37 | 10 | 26.4 | + |
| | | 33 | 11 | 33.4 | + |
| *O. excavata* | 1000 | 38 | 38 | 100.0 | − |
| | | 29 | 29 | 100.0 | − |
| | 500 | 65 | 35 | 54.0 | − |
| | | 66 | 66 | 100.0 | − |

[a] See Table II for activity designations.

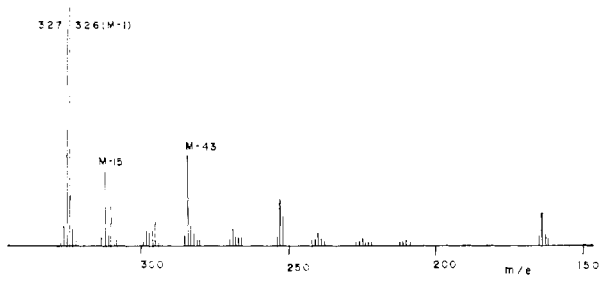



**Figure 2. Mass spectrum of isoboldine**

threne chromophore, 168–9° C.) (Chikamatsu *et al.*, 1961). Elemental analysis (C, 66.27; H, 6.10; and N, 3.18%) suggested $C_{25}H_{27}O_7N$ (C, 66.21; H, 6.00; and N, 3.09%). The infrared spectrum of the triacetate showed absorptions at 1750 and 1620 cm.$^{-1}$, which were assigned to phenol-acetate and *N*-acetyl carbonyl groups. The ultraviolet spectrum ($\lambda_{max}^{MeOH}$ 252 sh. 259, 282, 303, 315, 351, and 368 mμ; log ε = 4.71, 4.80, 4.30, 4.21, 4.27, 3.32, and 3.34) indicated that the triacetate was a phenanthrene derivative, which was produced by cleavage of the C—N bond followed by the acetylation of the nitrogen as shown by Chikamatsu *et al.* (1961). The ultraviolet and infrared spectra of this alkaloid were identical to those of authentic isoboldine (Figure 3), which was kindly provided by M. Tomita, University of Kyoto. From the above data, the alkaloid was concluded to be isoboldine.

**Insecticidal Component, Cocculolidine.** Fraction 1 to 4 obtained by the alumina column chromatography showed only one spot on thin-layer chromatography (Figure 2). Recrystallization of this material from carbon tetrachloride gave prismatic crystals, $C_{15}H_{19}O_3N$ (by elemental analysis and mass spectrum), m.p. 144–146°C., $(\alpha)_D^{25}$ + 237° (c., 1.0 in $CHCl_3$), which was named cocculolidine according to the scientific name of the plant, *Cocculus trilobus* DC., and had the structure shown above (Wada *et al.*, 1966, 1967). In preliminary tests, some toxicity resulted with green rice leaf hopper, *Nephotettix cincticeps* Uhler, and Azuki bean weevil, *Callosobruchus chinensis* Linne, from cocculolidine. These tests were conducted at the research laboratory of Tōa Pesticides Co., Odawara-City, Japan, and are reported in detail by Wada and Munakata (1967). In a test against *O. excavata* in the author's laboratory, the mortality was zero during a 24-hour period, even when the concentration of cocculolidine in acetone was increased to 1.0% (Wada and Munakata, 1967).

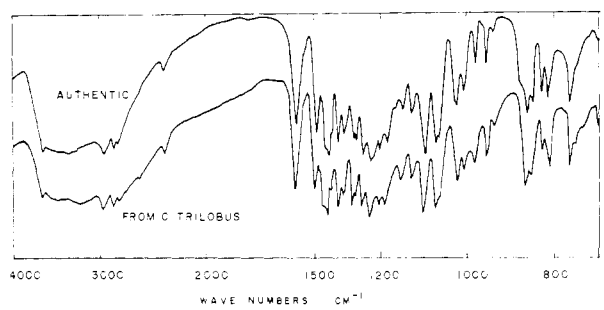



**Figure 3. Infrared absorption spectra of authentic isoboldine and from *C. trilobus* in KBr**

## DISCUSSION

Isoboldine has feeding inhibitory activity to two species of insects at 200 p.p.m. or over, as shown above. Fraction 9, which was shown to contain no isoboldine by TLC, also has feeding inhibitory activity. Therefore, it seems reasonable to assume that isoboldine would act as a resistance factor in cooperation with some other basic substances. Being an insecticidal alkaloid, cocculolidine also would act as a resistance factor against insects. Erythrina alkaloids, the group to which cocculolidine belongs, are well known to have curare-like action (Unna and Greslin, 1944; Unna *et al.*, 1944). Based on the preliminary tests, it was confirmed that cocculolidine itself has the same action, Thus, the toxicity could be related to the curare-like action. Isoboldine and cocculolidine, which act as resistance factors against various insects, have no activity to *O. excavata*, which accepts *C. trilobus* as the host plant. These facts suggest an interesting aspect of the host-insect interrelationship. There must be considerable difference in the sensitivity of insects toward these plant components.

From the standpoint of pesticide chemistry, the most important aspect in the search for new insect control chemicals is to find compounds which are selectively toxic to insects and have low toxicity to mammals. From such a standpoint, Lichtenstein *et al.* (1962, 1963) have isolated as naturally occurring insecticides myristicin from the edible parts of parsnips and 2-phenylethylisothiocyanate from the same parts of turnips. These investigations on insect resistance factors in plants contribute to the development of insect control.

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