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NAGILACTONES FROM *PODOCARPUS NAGI* AND THEIR EFFECTS ON THE FEEDING AND GROWTH OF TOBACCO BUDWORM

MINLI ZHANG, BAI-PING YING, and ISAO KUBO*

Division of Entomology and Parasitology, College of Natural Resources, University of California, Berkeley, California 94720

ABSTRACT.—In our continuing search for new insect control agents from plant sources, eight nagilactones were isolated from *Podocarpus nagi* (Podocarpaceae). The effects of the four most abundant on the feeding and growth of larvae of the tobacco budworm, *Heliothis virescens* (Lepidoptera: Noctuidae) were investigated. The growth rate and the pupation of *H. virescens* fed on diets containing nagilactone D [**4**] were lowered and mortality was occasionally observed. The data strongly suggest that growth inhibition is caused by feeding deterrence of these norditerpene dilactones and that nagilactone D is the most potent in inhibiting the mouthpart sensory receptors.

Species of the gymnosperm *Podocarpus* species have been reported to be resistant to many insects (1–4), and nor- and bisnorditerpene dilactones such as nagilactones from these plants have been shown to be responsible, at least in part, for this resistance (5). These terpenoids have been previously reported to be toxic to the housefly, *Musca domestica* (6), the codling moth, *Laspeyresia pomonella* (6), and several other important agricultural pests (7). One of the most abundant nagilactones, nagilactone D, was demonstrated to suppress pupation and adult emergence in *M. domestica* (6), the cotton bollworm, *Heliothis zea*, and the fall armyworm, *Spodoptera frugiperda* (4). Insect growth regulation and insecticidal activity of these nagilactones could result from feeding inhibition caused by these compounds (4); however, more evidence is required to prove this hypothesis.

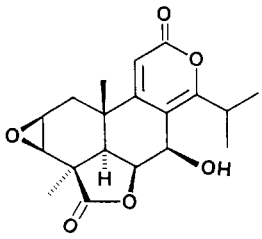
In order to carry out further biological studies, we needed to isolate greater quantities of the compounds. For our previous experiments (4), nagilactones were isolated from *Podocarpus nagi* (Thunberg) Pilger (Podocarpaceae) collected in Nara, Japan. These plants have been studied most extensively (8). However, we faced difficulty collecting plant materials from that location because *P. nagi* is protected from lumbering on the grounds of the Kasuga Shrine for religious reasons. Therefore, we collected *P. nagi* from the Kochi and Kagoshima regions, where the nagilactones were different both in quality and quantity.

In the present paper, we analyzed distributions of nagilactones in different parts of *P. nagi*, especially in the fruit. The feeding deterrence and growth inhibition of the four most abundant nagilactones of *P. nagi* on first and fifth instar larvae of the tobacco budworm, *H. virescens*, were tested. In addition, we have attempted to examine the effects of nagilactones on mouthpart sensory receptors and other probable modes of action.

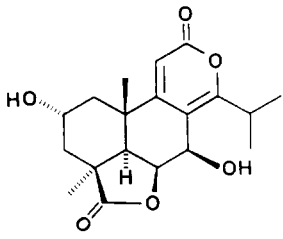
EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Mp's were uncorrected. Uv spectra were acquired on a Hitachi model 100-80 spectrometer in MeOH. Ir were obtained on a Perkin-Elmer model 1310 spectrometer. Nmr spectra were recorded on a JEOL model GSX-500 (500 MHz for ^{1}H, 125 MHz for ^{13}C) spectrometer with signals reported in ppm from internal TMS. Fabms were taken on a JEOL DX-303HF using a glycerol matrix.

CHEMICALS.—Solvents used were chemically pure and purchased from Fisher Scientific (Santa Clara, CA). The insect diet ingredients were purchased from ICN Biomedicals (Costa Mesa, CA). The authentic 1-deoxy-2β,3β-epoxynagilactone A [**1**] and 1-deoxy-2α-hydroxynagilactone A [**2**] were gifts from Professor Y. Hayashi, Osaka City University, and nagilactone C [**3**] and nagilactone D [**4**] were from our previous study (4).



1



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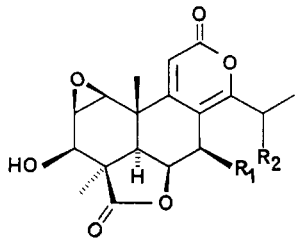

3 R_1=OH, R_2=Me
4 R_1=R_2=H

PLANTS.—The various parts of *P. nagi* were purchased in Kochi, Japan, in July 1986. The plant was identified by Prof. K. Goto, Kyoto College of Pharmacy, where a voucher specimen was deposited.

ISOLATION OF NAGILACTONES.—Nagilctones **1–4** used for the experiments were isolated from *P. nagi* as follows. Seed rind (29 g) and endosperm (410 g) were extracted three times in EtOH at room temperature, and the extracts were evaporated to dryness to give 5.3 g of seed rind and 69 g of endosperm extracts.

The seed rind extract was suspended in MeOH and partitioned with *n*-hexane. The MeOH layer was diluted with H_2O and partitioned with $CHCl_3$. The $CHCl_3$ phase was evaporated to dryness and redissolved in MeOH to give 0.6 g of crude crystal, which was chromatographed on Si gel (40–63 μm) and eluted with EtOAc-$CHCl_3$ (2:1) to give 224 mg of long white needles of 1-deoxy-2β,3β-epoxynagilactone A [**1**], mp 283–285°, and 50 mg of prisms of nagilactone C [**3**], mp 315–318°.

The endosperm EtOH extract was suspended in H_2O and fractionated into *n*-hexane (7.0 g), $CHCl_3$ (4.7 g), EtOAc (1.3 g), and H_2O (56 g) fractions. The $CHCl_3$ fraction was separated by repeated Si gel cc [$CHCl_3$-Me_2CO (6:1), C_6H_6-Me_2CO (4:1 and 1:1)] and crystallized from MeOH to give 633 mg of nagilactone D [**4**], mp 262–264°; 100 mg of 1-deoxy-2α-hydroxynagilactone A [**2**], mp 283–285°; 210 mg of **3**; and four other minor compounds. The structures of the purified compounds were identified based on spectroscopic data (uv, ir, fabms, and nmr), in particular, ^{1}H- and ^{13}C-nmr spectral data, and by direct comparison with those of the authentic samples.

INSECTS.—*H. virescens* eggs were kindly provided by the Dow Chemical Research Center (Walnut Creek, CA). Larvae were reared individually in 30-ml plastic cups in an incubator at 28 ± 1° and LD 16:8 h. The diet was prepared as described by Chan *et al.* (9). Newly hatched larvae and the fifth instar larvae in slender phase (second day) were selected for the experiment. They were identified according to the morphological characters described by Webb and Dahlman (10).

MORTALITY AND GROWTH INHIBITION TEST.—Compounds to be tested were dissolved in MeOH-$CHCl_3$ (1:1) and added to Alphacel (equal to 1% of the diet). This mixture was allowed to air dry and mixed with the diet ingredients (9,11). Control diet was prepared in the same way using only solvent.

First instar larval growth and mortality test.—A newly hatched larva was transferred into a 5-ml disposable scintillation vial with a diet containing test compounds. The growth inhibitory effect of the compounds was evaluated by determining the percentage of larvae reaching the third instar and the number of the days to do so. Thirty to forty larvae were used for each treatment.

Fifth instar larval growth test.—Weighed fifth instar larvae (240–260 mg) were placed into 30-ml plastic cups containing treated diet. After 4 days, the larvae were weighed and the relative growth rate (RGR) was obtained from $(\ln W_2 - \ln W_1)/(t_2 - t_1)$, where W_1 and W_2 are larval body wt at day 1 (t_1) and day 4 (t_2), respectively, (12). The growth inhibitory rate was expressed as follows:

$$\text{Growth inhibitory rate (\%)} = \frac{RGR_{(control)} - RGR_{(treated)}}{RGR_{(control)}} \times 100$$

After measuring the RGR, observations continued throughout the fifth instar, and the pupal weights were measured.

Oral injection of nagilactones.—The test compounds were dissolved in MeOH-$CHCl_3$ (1:1). Polyvinylpyrrolidone (PVP) was added (7 parts to 1 part test compound) as an emulsifier to the solution and it was dried in vacuo. The dry mixture was dissolved in H_2O to 1000 ppm. This solution (25 μl) was injected per os into the crop of each weighed larva (300–320 mg) through a 0.2-ml micrometer syringe (Gilmont In-

struments). The controls were injected with solution containing only PVP. Larval RGR was obtained as described above.

Feeding inhibition assay.—In order to determine whether **3** and **4** actually caused reduction of larval feeding, we measured the effects of these compounds on the fifth instar larval food consumption index (C.I.) by allowing the fifth instar larvae to feed on diet treated with **3** or **4**, or by injecting the compounds per os. The fresh wt of the diet consumed was measured, and the C.I. was calculated as described by Waldbauer (12) using the larval weight at the beginning of the experiment as the average body wt. Feeding inhibition was calculated the same way as the calcuation for growth inhibitory rate. The effect of H_2O loss on the fresh diet weight during the experiment was taken into account by measuring the H_2O loss of the control diet in the same condition as the bioassay but with no larvae feeding on it. Data were analyzed using Duncan's Multiple Range Test (13).

RESULTS AND DISCUSSION

Eight nagilactones were isolated by chromatographic methods from various parts of *P. nagi* obtained from Kochi, Japan. Chemical analysis indicated that there was a geographical variation in the type, quality, and quantity of nagilactones isolated from *P. nagi* obtained from different locations such as Kochi, Kagoshima, and Nara. All the aforesaid eight nagilactones isolated from the *P. nagi* collected in Kochi belonged to the 8:14,9:11-dienolide (α-pyrone) type, while there are three different types of norditerpene dilactones isolated from plants obtained from either Nara (15) or Kagoshima (14,16). Nagilactone D [**4**] was the most abundant nagilactone found in the seed (0.14% fresh wt) obtained from Kochi; it was detected only in the endosperm. Nagilactone C [**3**] is also found in large quantities in the endosperm as well as in seed rinds, and the whole seed contains it up to 0.06% (fresh wt). In addition, two nagilactone A derivatives **1** and **2** were found in smaller quantities (0.05% and 0.02% fresh wt, respectively). Interestingly, nagilactone E found in large quantities in the root bark of *P. nagi* collected in Nara (17) was not detected at all in any parts of plants obtained from Kochi.

Of the eight compounds, only the four most abundant, **1–4**, were studied for their effects on feeding and growth of *H. virescens* larvae. The remaining four diterpenoids were identified as nagilactones A and B and 15*S*- and 15*R*-methoxycarbonylnagilactone D, but they could not be tested because of their limited availability.

In the artificial diet feeding assay (9,11), nagilactones C [**3**] and D [**4**] showed strong inhibition to the growth of first instar larvae (Table 1). When fed on a diet containing 166 ppm of **4**, none of the larvae developed to the third instar and all eventually died. Some larvae fed on a diet containing 168 ppm of **3** reached the third larval instar, but growth was delayed. Most first instar larvae fed on diets containing **1** or **2** reached the third larval instar but took longer periods of time than normal larvae did (Table 1).

When the fifth instar larvae were fed a diet containing **4** at 160 ppm, most of the

TABLE 1. The Effect of Ingested Nagilactones on the Development of the First Instar Larvae of *Heliothis virescens*.

Compound	Conc. (ppm)	Number of larvae treated	Larvae developing to 3rd instar (%)	Number of days to develop to 3rd instar[a]
1	173	40	87.50	7.1 A
2	163	30	96.67	6.0 B
3	168	30	53.33	6.1 B
4	166	30	0.00	—
Control	—	30	100.00	4.3 C

[a]Different letters indicate significant difference ($p<0.05$) in Duncan's Multiple Range Test analysis.

larvae could not pupate. The few successful pupation events yielded small, malformed pupae (Table 2), and none of the pupae developed further. Compounds **1**, **2**, and **3** were much less active than **4** against the *H. virescens* fifth instar larvae, but their effects on larval RGR and pupal weight were still significant (Table 2).

TABLE 2. Growth Inhibitory Effect of Ingested Nagilactones on the Fifth Instar Larvae of *Heliothis virescens*.[a]

Compound	RGR[b] (±SD)	Growth inhibition (%)	Pupal weight[b] (mg ± SD)	Pupation rate (%)[c]
1	0.31 ± 0.31 C	32.61	264.32 ± 23.39 B	100
2	0.36 ± 0.05 BC	21.74	259.27 ± 26.35 B	100
3	0.39 ± 0.09 B	15.22	267.04 ± 22.93 B	100
4	0.16 ± 0.14 D	65.22	135.22 ± 22.26 C	30 (100)
Control	0.46 ± 0.08 A	—	321.05 ± 25.63 A	100

[a]Twenty larvae were used for each treatment. The concentration of the test compounds was 160 ppm.
[b]Relative growth rate (see Experimental). Different letters in the same column indicate significant difference ($p<0.05$) in Duncan's Multiple Range Test analysis.
[c]The number in the parentheses represents the percentage of abnormal pupae.

When 25 μg of **4** was injected per os into a fifth instar larval crop, both larval growth rate and pupal weight were significantly reduced (Table 3). Although the RGR reduction of the larvae treated per os with **3** was not significant, the pupal weight was significantly reduced. Orally injected **1** and **2** showed no obvious effect on larval growth or on pupation (Table 3).

Our preliminary observation suggests that the feeding deterrence of the nagilactones is the major cause for the reduction of larval RGR and pupation, because, except for larval developmental time course and pupal weight being affected, no other abnormal effects were observed. The malformed pupae caused by the treatment of **4** (Table 2) might be due to the fact that the starved fifth instar larvae could not reach the critical weight for pupation. We have found that the critical weight for pupation in *H. virescens* is 260–280 mg (to be published).

TABLE 3. Effect of Nagilactones Applied per os Injection on the Fifth Instar Larvae of *Heliothis virescens*.[a]

Compound	RGR[b] (± SD)	Pupal weight[b] (mg ± SD)
1	0.14 ± 0.08 ab	312.51 ± 37.77 A
2	0.17 ± 0.05 a	294.03 ± 28.27 AB
3	0.15 ± 0.05 ab	283.67 ± 28.37 BC
4	0.11 ± 0.05 b	266.91 ± 39.74 C
Control	0.17 ± 0.05 a	315.60 ± 25.53 A

[a]Dose: 25 μg compound/larva, 15 larvae were used for each treatment (see Experimental).
[b]Relative growth rate (see Experimental). Different letters in the same column indicate significant difference ($p<0.05$) in Duncan's Multiple Range Test analysis.

When **3** and **4** were injected per os without the compounds contacting the mouthpart sensory receptors, their effects on C.I. were similar. However, when they were fed in diets to the larvae, **4** showed more potent feeding inhibition than **3** (Table 4) indicat-

TABLE 4. Effects of Nagilactones C and D on the Food Consumption of Fifth Instar Larvae of *Heliothis virescens*.[a]

Treatment	Dose	Food consumption index[b]	Feeding inhibition (%)
Feeding assay			
3	230 ppm	0.87 ± 0.27 a	67
4	60 ppm	0.79 ± 0.22 a	70
Control	—	2.63 ± 0.79 b	—
Oral injection			
3	25 μg/larva	2.07 ± 0.79 A	34
4	25 μg/larva	2.01 ± 0.46 A	36
Control	—	3.16 ± 0.51 B	—

[a]Fifteen larvae were used for each treatment (see Experimental).
[b]Different letters indicate significant difference ($p<0.05$) in Duncan's Multiple Range Test analysis.

ing that different mechanisms might be involved in the feeding inhibition shown by these compounds.

The effect of these compounds on the larval digestive system might account for one of the mechanisms; when the compounds were applied directly into the larval crop, the deterrent effect of the compounds on the mouthpart sensory receptors was minimized. But the inhibitory effect of these compounds on food consumption was still significant, suggesting that food digestion, absorption, or other unknown factors controlling food intake were affected. In this form of administration, the effects of **3** and **4** on larval food consumption were quite similar (Tables 3 and 4).

When **3** and especially **4** were administered with diet to the larvae, feeding was greatly reduced (Table 4), suggesting that mouthpart sensory receptors could account for another important mechanism of feeding inhibition. The differences in the potency of feeding inhibition between the two nagilactones administered in this way (Table 4) was significant and might explain the difference in the activity of these nagilactones.

Though feeding inhibition was the major effect of these compounds, other mechanisms affecting larval growth and pupation could not be excluded. For example, orally injected **3** caused no significant change in larval feeding but resulted in significant reduction in pupal weight (Table 3). Furthermore, it should be noted that most compounds showing feeding deterrence, such as quassinoids (18), ajugarins (19), and limonoids (20), have strong bitter tastes to humans. However, this is not the case for nagilactones.

The RGRs of the fifth instar larvae treated per os injection were relatively low (Table 3), compared with the feeding assay (Table 2), because the larvae we used at the beginning of this treatment were at late slender and early digging phase (these phases were easier to handle for oral injection) and the body weights at these phases were greater than those used for feeding assay. However, the final weight of the larvae at the end of both assays was at around the maximum of 480–520 mg for the control larvae. Therefore, the RGRs were different in the above two treatments due to the differences in the larval weights used for the treatment.

It is obvious that nagilactones, especially nagilactone D [**4**], are responsible for the resistance of *P. nagi* to insects, but we have not yet looked for other types of compounds in this plant which might have anti-insect activities. Three different types of compounds isolated from *Podocarpus gracilior* (4) showed different types of activities. Norditerpene dilactones had insect-feeding-deterrent activity, biflavones had growth-in-

hibitory activity and a phytoecdysone had ecdysis-inhibitory activity. This multichemical resistance to insect attack is probably also true for *P. nagi*. In fact, *P. nagi* root has been found to contain a large amount of catechin (21) that had a growth-inhibitory effect on *H. virescens* larvae (22).

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