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WITHANOLIDES AND RELATED ERGOSTANE-TYPE STEROIDS AS ANTIFEEDANTS FOR LARVAE OF *EPILACHNA VARIVESTIS* (COLEOPTERA: CHRYSOMELIDAE)

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Eight steroids isolated from solanaceous plants (*Physalis, Withania* and *Nicandra* species) were investigated as to their antifeedant efficacy for L_4 larvae of *Epilachna varivestis* Muls. (Coleoptera). Nicalbin A (VII) was a potent antifeedant, but at high concentrations it was also toxic to this insect. Other active compounds were withanolide E (I), 4β-hydroxywithanolide E (II), 5β,6β-epoxy-1β,14α,17β, 20α-tetrahydroxywith-24-enolide (III), Nic-1 (nicandrenone, VI) and nicalbin B (VIII). These results were quite different from the spectrum of activity of the withanolides against larvae of *Spodoptera littoralis* (Boisd.) (Lepidoptera) found in a previous study.

KEY WORDS: Antifeedants from Solanaceae; *Epilachna varivestis*; ergostane-type steroids; withanolides.

INTRODUCTION

In a recent study (1) it was shown that certain ergostane-type steroids isolated from the leaves of several solanaceous plants, as well as some closely related products (5), have a considerable antifeedant effect for larvae of a lepidopterous insect, the Egyptian cotton leafworm, *Spodoptera littoralis* (Boisd.). The most active compounds were withanolide E (I) and its 2,3-dihydro-1β-hydroxy derivative (III). In view of these results, it was deemed of interest to investigate the antifeedant properties of these and of some related compounds for a coleopterous larva, namely, the fourth-instar larva of the Mexican bean beetle, *Epilachna varivestis* Muls., the severest pest of all kinds of snap and lima beans in North America, with a distribution from Central America to Canada (2, 8).

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MATERIALS AND METHODS

The test insects

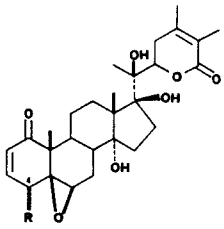
Epilachna varivestis larvae were reared on *Phaseolus vulgaris* (var. 'Saxa') beans at 25±1°C as described by Steets (12). For the mass-breeding, pieces of leaves with egg masses were laid on filter paper disks kept constantly wet in petri dishes. On hatching of the neonates the leaf pieces were transferred onto leaves on bean plants that had been grown in the glasshouse in "Bellaplast" plastic containers (17 x 12 x 6 cm height). The breeding cages were assembled on a base consisting of a flat, rectangular plastic dish (58 x 35 cm), on the broad rim of which a wooden frame (58 x 35 x 7.5 cm height) was placed; then, upon this, another frame of similar length and breadth, but 16 cm in height and with sides consisting of fine Saran netting, was stacked. This cage was covered with a glass plate, with the areas of contact between the glass and the upper frame being rendered tight by a layer of foam rubber ("Tesamoll") glued to the edge of the frame. Another type of cage used consisted of plastic crates (used to transfer bread in W. Germany) of a size similar to that of the cages described above, with all the open parts on the sides of the crates being covered by glued-on Saran netting, and with the top being covered again with a glass plate. During larval development the leaves were devoured rapidly and the plants had to be replaced frequently by fresh ones. Pupation took place after the fourth larval instar, on the lower sides of the leaves or on the Saran netting on the cages. The newly emerged adult beetles were transferred to fresh plants in other cages, in which copulation and oviposition took place. The leaves on which the egg masses had been laid were removed from the cages daily, leaf area free of eggs was cut off with scissors and the egg masses were stored until hatching as described above. Fourth-instar larvae (average weight, 18.0 mg) were used for the bioassays.

The compounds

No.	*Name*	*Source*	*Reference*
I	Withanolide E	*Withania somnifera* (L.) Dun., chemotype *III*	(4)
II	4β-Hydroxywithanolide E	*Physalis peruviana* L.	(6)
III	5β,6β-Epoxy-1β,14α,17β,20α-tetrahydoxywith-24-enolide	Obtained by reduction of I	(4)
IV	Withaferin A	*Withania somnifera* (L.) Dun., chemotype *I*	(9)
V	Withanolide D	*Withania somnifera* (L.) Dun., chemotype *I*	(10)
VI	Nic-1 (nicandrenone)	*Nicandra physaloides* (L.) Gaertn.	(3)
VII	Nicalbin A	*Nicandra physaloides* (L.) Gaertn. var. *albiflora*	(7)
VIII	Nicalbin B	*Nicandra physaloides* (L.) Gaertn. var. *albiflora*	(7)

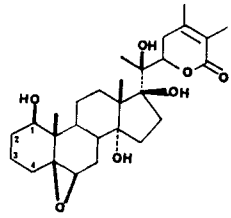
These eight compounds were divided into three groups according to their structural features. In the first group, there are three ergostane-type lactones (I, II and III), in which the side chain has the unusual α-orientation. The second group contains two lactones of the same type (IV and V), but with the side chain in the normal β-orientation. With the exception of this major difference, the compounds I–V are characterized by the number and positions of the hydroxy groups, as shown in the corresponding formulae.

The three compounds of the third group (VI–VIII) are also ergostane-type steroids; their common feature is the substitution pattern of rings A and B and the

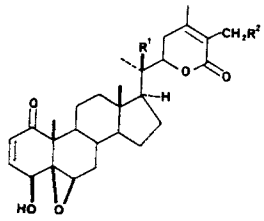


I R=H; Withanolide E

II R=OH; 4β-Hydroxywithanolide E

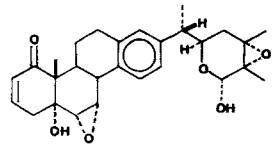


III 5β,6β-Epoxy-1β,14α,17β,20α--tetrahydroxywith-24-enolide

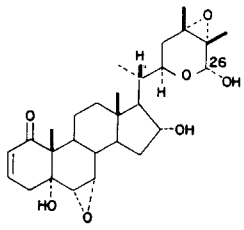


IV R^1=H; R^2=OH; Withaferin A

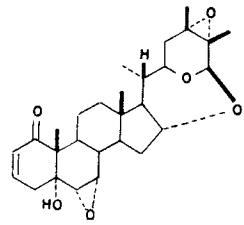
V R^1=OH; R^2=H; Withanolide D



VI Nic-1 (nicandrenone)



VII Nicalbin A



VIII Nicalbin B

normal β-orientation of the side chain. Compounds VI and VII have a cyclic epoxy-hemiacetal type side chain, whereas compound VIII has a bicyclic epoxy-acetal type side chain. In contrast to all other compounds investigated in this work, compound VI possesses a modified carbocyclic skeleton in which ring D is enlarged and aromatized. Such a structure is, so far, unique for several compounds biosynthesized in *Nicandra* spp. plants.

Antifeedant assay method

The test device is that described by Rembold *et al.* (11), slightly modified, and consists of a combination of plastic petri-dish halves as shown in Fig. 1. Thin squares of cotton wool (B), cut to such a size as to fit well into the cover of 9-cm petri dishes, were laid in inverted covers (A), and an 8-cm filter paper disk (C) was placed on top of the cotton wool squares. The cotton wool and the filter paper disk were wetted with water and a bean leaf (D) (var. 'Saxa') of sufficient size (see below) was laid, with its lower side up, on the wetted filter paper. A petri-dish bottom with a central 4.5-cm diam. hole (E) punched with a hot metal die was inserted on top of the leaf. 0.2 ml of methanolic ("Methanol reinst Merck") solutions of the test compounds was pipetted on the leaf with an Eppendorf pipette and then spread over the whole

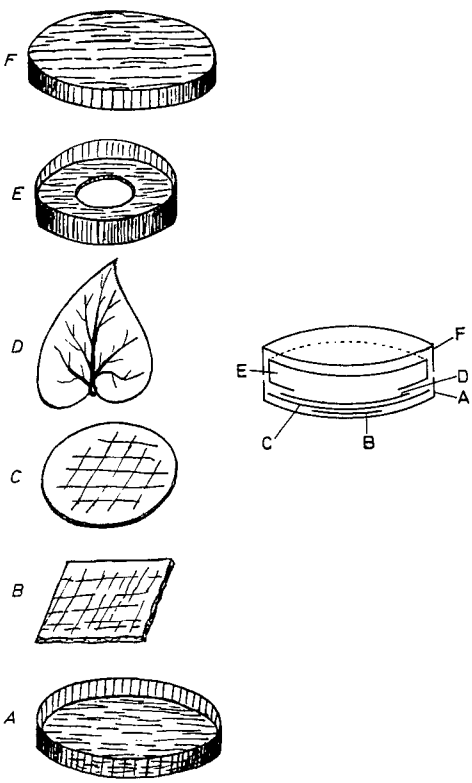


Fig. 1.

Epilachna varivestis larvae antifeedant test device composed of petri dish halves. Left: *A* – inverted petri dish cover; *B* – thin square of cotton wool; *C* – filter paper disk; *D* – bean leaf; *E* – petri dish bottom with central hole; *F* – petri dish cover. Right: assembled device (schematically).

exposed leaf area (15.9 cm^2) with a fine paintbrush. The residue was allowed to dry for half an hour* and the device was closed with a petri dish cover (F). The residue thus obtained on the exposed leaf area was ~125 mg/m^2 with a 0.1% solution and proportionally less with the lower concentrations. Two types of control runs were conducted with each experiment: methanol-only treated control, and untreated control.

A weighed L_4 *Epilachna* larva was introduced into each dish, with 20 larvae being employed per treatment. The closed dishes were then laid out singly (not in piles) on a large laboratory bench and illuminated daily for 16 h with six light tubes, two of each of the types Radium NL 20W/25 "weiss," Phillips TL 20W/25 "weiss," and Osram-L-Fluora 20W/77. The experiments were conducted at 21–23°C. The larvae were allowed to feed on the treated and control leaves for 48 h and then reweighed. After the 48-h feeding period all injured, moribund or dead insects were recorded and discarded; only survivors were weighed. For each treatment, three replicates were done on different days.

It is of great importance to use the right grade of methanol in these experiments. The results of the methanol leaf controls with various grades of methanol are given in Table 1. Whereas "Methanol reinst Merck" was innocuous, "Methanol für Analyse Riedel de Haen" was toxic under our test conditions. This toxicity disappeared, however, when the evaporation period was prolonged for several hours. One of us (Ascher, unpublished data) had a similar experience with alfalfa or cotton dipped in "methanol analytical Frutarom" *vs.* "methanol analytical reagent AnalaR B.D.H." Whereas after dipping in the first (Frutarom) grade, high toxicity for *Spodoptera littoralis* larvae persisted for 2 and 4 h, and disappeared only after 24 h of drying, the second grade (AnalaR B.D.H.) was innocuous already after 2 h.

TABLE 1

TESTING VARIOUS GRADES OF METHANOL APPLIED ON BEAN LEAVES IN THE PETRI DISH DEVICE AS TO THE WEIGHT GAIN (ΔW±S.E.) OF L_4 *EPILACHNA VARIVESTIS* LARVAE AND AS TO POSSIBLE TOXICITY

Grade and make of methanol	*Manufacturer's code no.*	*ΔW±S.E. (mg)*		*Injured and moribund larvae (%)*	
		24 h	48 h	24 h	48 h
"Methanol für Analyse Riedel de Haen"	32213	−4.2±0.36	−5.5±0.53	45	80
"Methanol reinst Merck"	6008	+10.3±0.88	+13.3±0.95	None	
"Methanol zur Analyse Merck"	6009	+6.2±1.05	+9.7±1.32	None	
Untreated control		+15.1±0.71	+19.2±0.89	None	

*There is no danger of oxidation of the test compounds with this procedure: when methanolic solutions of the compounds were evaporated to dryness at room temperature, analytical chromatoplates (HPTLC) showed definitely one spot, corresponding to the respective parent compound.

TABLE 2

THE WEIGHT GAIN (ΔW±S.E.) OF L_4 *EPILACHNA VARIVESTIS* LARVAE DUE TO 48-H FEEDING ON TREATED BEAN LEAVES
[Numbers in brackets denote ΔW±S.E. in the methanol control accompanying every experiment]

	Concentration of compound in leaf painting solution in methanol (%)				
Compound	0.1	0.05	0.025	0.01	0.005
	Average weight gain (ΔW±S.E.)/larva during 48 h (mg)				
I	−2.24±0.40	−1.28±0.59	+4.47±0.83	+11.29±0.88	
	[+13.97±0.89]	[+12.86±0.74]	[+12.56±0.53]	[+13.39±0.82]	
II	−3.23±0.28	−3.50±0.26	−1.20±0.71	+6.66±1.14	
	[+12.24±0.62]	[+11.59±0.55]	[+12.56±0.53]	[+14.72±0.84]	
III	−1.22±0.56	−1.76±0.71	+0.54±0.69	+0.41±1.03	+7.99±1.21
	[+12.10±0.64]	[+11.49±0.47]	[+12.42±0.55]	[+13.35±0.69]	[+10.58±0.78]
IV	+0.06±0.26	+3.62±0.23	+6.35±0.57		
	[+10.41±0.98]	[+13.51±0.51]	[+10.52±0.60]		
V	−1.30±0.26	+1.79±0.36	+6.58±0.49	+8.69±0.73	
	[+10.41±0.98]	[+13.51±0.51]	[+11.95±0.72]	[+10.52±0.60]	
VI	−3.74±0.37	−3.05±0.31	+1.04±1.21	+4.70±0.54	
	[+12.10±0.64]	[+12.42±0.55]	[+12.42±0.55]	[+13.35±0.69]	
VII*	−8.91±0.31	−7.71±1.05	−5.16±0.34	−1.52±0.47	+3.85±0.50
	[+10.41±0.98]	[+16.53±1.05]	[+16.53±1.05]	[+14.23±0.82]	[+13.76±0.80]
VIII	−6.58±0.60	−4.38±0.29	+3.16±0.56	+11.42±0.94	
	[+13.97±0.89]	[+12.86±0.74]	[+12.56±0.53]	[+13.99±0.82]	

Average ΔW of all untreated controls = +17.50±0.64 mg.

*Percentage of dead and moribund larvae on VII after 48-h feeding: on 0.1%, 50%; on 0.05%, 57.5%; and on 0.025%, 5%. There was no mortality in any of the other experiments.

RESULTS

The results of the feeding experiments are summarized in Table 2. An arbitrary criterion was used to compare the antifeedant efficacy of the compounds, namely, the lowest concentration at which there is no weight gain at all or an increase in weight of no more than 1 mg/larva within 48 h of feeding. In the first group of compounds, no. III, which was obtained by reduction of withanolide E (I), was active at a lower concentration (0.01%) than 4β-hydroxy-withanolide E (II) (0.025%). The latter was, in turn, slightly more active than I (0.05%). It should be noted, however, that compound II caused a greater reduction in weight than III at the 0.1% and 0.05% concentrations.

In the second group, both withaferin A (IV) and withanolide D (V) were only slightly active according to the criterion established above. Activity was apparent only at 0.1%, with compound V being somewhat more active than IV on the basis of weight gains.

In the third group, nicalbin A (VII) was by far the most active compound according to our criterion (down to 0.01%). Its range of activity is comparable with that of compound III; the weight reductions induced by VII at all concentrations above 0.01% were, however, much stronger than those obtained with III. It has to be stressed that nicalbin A (VII) was lethal to many of the insects at the two highest concentrations, apart from being a potent antifeedant for the survivors. Nicalbin B (VIII) was active only at the high concentrations (0.05 and 0.1%), but, similarly to VII, it caused a much stronger reduction in weight than all other compounds. Nic-1 (nicandrenone, VI) was active down to 0.025%, although it was less efficient in reducing weight gain than nicalbin B (VIII) at the higher concentrations (0.05 and 0.1%).

DISCUSSION

The present study was carried out with *E. varivestis* larvae which were offered treated bean leaves; the previous work (1) was done with 170-190 mg *S. littoralis* larvae, to which the compounds were offered on lamellae of an inert carrier ("Styropor," which is foamed polystyrene) treated with sucrose as a phagostimulant.

Several conclusions can be drawn by comparing the results of these two studies. In *S. littoralis*, the order of activity in the first group was I > III > II, whereas in *E. varivestis* (the present work) it was III > II > I.

In the second group, IV (withaferin A) and V (withanolide D) were inactive against *S. littoralis* at the screening dose of 0.01% used in the previous study (1). In the present work, these two compounds were not active according to our criterion of activity below the highest concentration tested, 0.1%.

In the third group, Nic-1 (VI) was inactive in *Spodoptera* at 0.01% and slightly active at 0.1%, whereas its activity in *E. varivestis* reached as low as 0.025%. Nicalbin A (VII) and B (VIII) had both been inactive at the 0.01% screening concentration in *S. littoralis* (unpublished data), whereas for *E. varivestis* nicalbin A was a highly active antifeedant and, in addition, quite toxic at high concentrations. We can not say at

present whether the activity of nicalbin A at the low concentrations is due only to the antifeedant effect *per se*, or whether abstention from feeding is not partly a manifestation of the compound's toxic effects at sublethal concentrations. Nicalbin B remained inactive in the *Epilachna* test down to 0.05%.

Although the test methods to assay antifeedant efficacy against *S. littoralis* and *E. varivestis* were different, the difference between the two species in their response to some of the compounds was too conspicuous to be explained by changes in test methodology. On the one hand, *Epilachna* larvae were, in general, much more sensitive than *Spodoptera* larvae to the antifeedants tested. On the other hand, there was a different order of activity of the compounds against the two species, *e.g.* in the first group. Of much more significance, however, was the finding that compounds inactive against *Spodoptera* were highly active against *Epilachna, e.g.* nicalbin A (VII).

Such selective activity against pest species may perhaps also permit selection of compounds innocuous to beneficial insects.

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