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Naturally Occurring Steroids from Solanaceous Plants as Potential Insect Antifeedants

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Abstract

In an effort to discover new plant-derived antifeedants, numerous solanaceous plants were investigated as to their palatability by larvae of Spodoptera littoralis. Physalis peruviana and Withania somnifera chemotype III strongly deterred larvae from feeding. In the latter plant, a good correlation between the presence of withanolide E and feeding detergency of S. littoralis could be established.

Extracts of a great number of other solanaceous plants were bioassayed for antifeedant effect against a series of phytophagous insects (S. littoralis, S. frugiperda, Boarmia selenaria, Epilachna varivestis, Acalymma vittata, Diabrotica undecimpunctata howardi, Tribolium castaneum, T. confusum, Sitophilus oryzae, Callosobruchus maculatus, Oryzaephilus surinamensis).

Promising plants (P. peruviana and W. somnifera) were cultivated on a larger scale. A good supply of plant material is thus available for future work.

In view of the high antifeeding activity of withanolide E against larvae of S. littoralis, we tried to establish correlations between the chemical structure and the above activity. To this end, synthetically modified analogs were prepared in order to assess the importance of the various structural features of the original molecule for the overall activity of the system. The modifications refer to the substitution pattern of rings A, B, A and B, D and D including the side chain.

The compounds active against Lepidoptera are characterized by a α oriented side chain; conversely, the compounds active against Coleoptera (e.g. nicalbin A) possess a β oriented side chain.

The immediate benefit of the work is the demonstration that a series of powerful plant-derived insect antifeedants are present in selected solanaceous plants from which they can be isolated in order to be used as such or as suitably modified analogs, for future field trials.

Objectives of the original research proposal

The objectives of this research project were as follows (vide pp. 4 - 5 of original proposal):

(a) To find naturally occurring compounds from solanaceous plants which possess insect antifeeding properties and can be used in the control of insects harmful to agricultural crops. The behavior of Spodoptera littoralis towards potential antifeedants from this group will be investigated in the Toxicology Laboratory at Bet Dagan by primary screening, followed by scrutinizing the effects on feeding behaviour and on metamorphosis. In the laboratories of the U.S. counterpart, bioassays of extracts, fractions and some promising compounds will be conducted against Lepidoptera, Coleoptera and Homoptera insect species.

(b) To study the relationship between chemical structure and antifeeding properties within this group of compounds, in order to learn the contribution of each functional group and each structural moiety to the overall activity of the molecule. The larger number of structurally related compounds which were isolated during our previous work and which will be isolated within the framework of the present study, makes such an investigation feasible.

Practically, all listed objectives have been realized. Additionally, many of the plants extracts and fractions thereof, prepared and bioassayed by the U.S. member of the team were also investigated biologically in Israel.

LIST OF COMPOUNDS

<u>1</u> Withanolide skeleton	<u>27</u> Physapubenolide
<u>2</u> Nicandrenone (Nic-1)	<u>28</u> Pubescenin
<u>3</u> Physalin B	<u>29</u> Withanolide J
<u>4</u> Withaphysalin C	<u>30</u> Withanolide H
<u>5</u> Acnistin	<u>31</u> 5 β ,6 β -Epoxy-1 β ,14 α ,17 β ,20-tetrahydroxywith-24-enolide
<u>6</u> Withaferin A	<u>32</u> 14-Deoxywithanolide S
<u>7</u> Jaborosalactone A	<u>33</u> 14,20-Oxidewithanolide S
<u>8</u> Withanone	<u>34</u> 5-O-Methylwithanolide S
<u>9</u> Withanolide D	<u>35</u> 5 α -chloro-5-deoxywithanolide S
<u>10</u> Withanolide G	<u>36</u> Withanolide S 6-mesylate
<u>11</u> Withanolide E	<u>37</u> 5 α ,6 α -Epoxy isomer of <u>11</u>
<u>12</u> Nic-2	<u>38</u> 6 β ,14 α ,17 β ,20-Tetrahydroxy-1-oxowitha-2,4,24-trienolide
<u>13</u> 2,3-Dihydrowithanolide D	<u>39</u> 4 β ,5 β -Epoxy-6 β ,14 α ,17 β ,20-Tetrahydroxy-1-oxowitha-2,24-dienolide
<u>14</u> 2,3-Dihydrowithaferin A	<u>40</u> 6 α ,14 α ,17 β ,20-Tetrahydroxy-1-oxowitha-2,4,24-trienolide
<u>15</u> 4 β -Hydroxywithanolide E	<u>41</u> 4 β ,5 β , -Epoxy-6 α ,14 α ,17 β ,20-tetrahydroxy-1-oxowitha-2,24-dienolide
<u>16</u> Physalin A	<u>42</u> 17-Isowithanolide E
<u>17</u> 2,3-Dihydrowithanolide E	<u>43</u> 5 α ,6 α -Epoxywithanolide J
<u>18</u> Withanolide S	<u>44</u> 5 β ,6 β -Epoxywithanolide J
<u>19</u> Withaperuvine	<u>45</u> 14,15-Epoxy-14-deoxywithanolide S
<u>20</u> 4-Deoxyphysalolactone	<u>46</u> 5-Deoxy-14,20-oxidewithanolide S diacetate
<u>21</u> Physalolactone	<u>47</u> 4 β ,5 β -Epoxy-5-deoxy-14,20-oxidewithanolide S diacetate
<u>22</u> <u>Withanicandrin</u>	<u>48</u> 5 α -Chloro-5,14-Dideoxywithanolide S
<u>23</u> Daturalactone A	
<u>24</u> Nicalbin A	
<u>25</u> Nicalbin B	
<u>26</u> Physapubescine	

- 49 5 α -Chloro-5-deoxy-14,20-oxidewithanolide S
- 50 14-Deoxywithanolide E
- 51 14,20-Oxidewithanolide E
- 52 5 β ,6 β -Epoxy-14 α -hydroxy-1,17-dioxoandrost-2-ene
- 53 Acetyl- δ -lactone

1. PLANT-DERIVED INSECT ANTIFEEDANTS

Antifeedants are compounds which deter insects from feeding on treated plants without killing them by intoxication or repelling them from the plant. The insects stay on the plant and by refusing to feed, die of starvation. The approach was reviewed recently by Schoonhoven (1982), Jermy (1983) and Bernays (1983). The antifeedant approach to insect control is not new. Thiram was used in the U.S.A. as an antifeedant against the Japanese beetle already in the 1930's. Metzger and Grant (1932) assayed the antifeedant activity of 500 plant extracts, and Eger (1937) tested feeding deterrents by offering them to lepidopterous larvae in their drinking water.

Industrial products, such as the fentin (triphenyltin) fungicides, have attained importance as antifeedants since 1963 when Murbach and Corbaz (1963) reported on the discovery that Brestan (fentin acetate) "induced in an unknown way, a strong reduction of the number of egg batches and larvae" of the Colorado beetle, Leptinotarsa decemlineata, in artificially infested potato plots. Solel (1964) noted in a field trial against Cercospora leaf spot on sugar beet that zineb-treated and control plots were highly infested with larvae of the Egyptian cotton leafworm, Spodoptera littoralis, while those treated with Brestan were free from damage by this pest. It was demonstrated (Ascher and Rones 1964; Ascher and Nissim 1965) that these were antifeedant effects: losses in weights of larvae kept for 48 hr on sugar beet leaves treated with 0.1% fentin acetate were nearly the same as in starved larvae. Though even on leaves treated

with adequate concentrations of fentin acetate larvae executed "test bites", i.e. consumed tiny bits of leaf, they soon stopped feeding. Fentin acetate seemed to be a true antifeedant, not a repellent, since the larvae remained on the treated leaves without feeding on them; even prestarving the larvae for several days would not induce them to feed on fentin acetate treated leaves.

The organotin fungicides Brestan and Du-Ter (fentin hydroxide) are used in East Africa on a practical scale as antifeedants against larvae of the giant looper, Boarmia (Ascotis) selenaria reciprocaria and the leaf skeletonizer, Leucopplema dohertyi in coffee plantations for many years (Bardner and Mathenge 1974; Smith 1980). The area of organotin antifeedants has been reviewed recently by Ascher (1986).

In recent years, however, researchers have paid much more attention to plant-derived, naturally-occurring antifeedants. The spectrum of naturally-occurring antifeedants must be impressive. Although only about 10,000 secondary plant metabolites have been chemically defined, their estimated number is 400,000 or more, of which only several hundreds have been assayed as antifeedants. In the reviews of Vigneron (1978) and Koul (1982), 83 and approximately 130 well-defined antifeedants, respectively, were listed as belonging to the classes of glycosides, quinones and phenols, terpenes (mono-, sesqui-, di- and triterpenes and tetranortriterpenoids), coumarins, furanocoumarins, lignanes, alkaloids, steroids, polyacetylenes and various other products. Some of the most active natural antifeedants are terpenoids. Special interest has been paid to

three triterpenoid antifeedants isolated from the trees of Indian lilac (Azadirachta indica A. Juss) and Persian lilac (Melia azedarach L.), viz., azadirachtin, meliantriol and salannin (Schmutterer et al. 1981; Schmutterer and Ascher 1984).

Antifeedants of natural or synthetic origin either inhibit gustatory receptors, e.g. sugar receptors, or stimulate specialized deterrent receptors; the situation is complicated by the fact that an antifeedant may stimulate either a few specialized deterrent receptors or a larger number of deterrent receptors of low specificity.

Until recently, relatively little work has been done on steroids as antifeedants. Ecdysterone, inokosterone and ponasterone, insect moulting hormones, present also in plants (Nakanishi et al. 1966) have antifeedant properties for larvae of Pieris brassicae at concentrations superior to $0.4 \times 10^{-4}M$; complete antifeeding was obtained at concentrations of $3 \times 10^{-4}M$. Mamestra brassicae larvae were less susceptible (Ma 1972). Ecdysterone reduces water intake when offered to Dysdercus spp. in their drinking water (Schoonhoven and Derksen-Koppers 1973).

Within the group of steroids whose chemistry is being investigated at the Faculty of Agriculture in Rehovot, the only earlier study of antifeeding properties was done on nicandrenone (2). This compound was isolated by von Gizycki and Kotitschke (1951) from Nicandra physaloides, a plant which was later shown to be unacceptable to larvae of the tobacco hornworm, Manduca sexta (Yamamoto and Fraenkel 1960). For the chemical aspects of the problem, see Ch. 2.6.

Nicandrenone has been tested (Ascher et al. 1980) by the Styropor method (Ascher and Meisner 1973) on larvae of S.littoralis. The compound was inactive at our screening concentration of 0.01%. On the other hand, some biogenetically related steroids, isolated from solanaceous plants and known under the generic name of withanolides (see Ch. 2.1) have been found (Ascher et al. 1980) to have considerable antifeeding activity. One of these compounds (withanolide E) (11) totally inhibited feeding at a concentration of 0.0025%, whereas another compound (5 β ,6 β -epoxy-1 β ,14 α ,17 β ,20-tetrahydroxywith-24-enolide) (31), obtained by a synthetic modification of the former, showed a similar activity at a concentration of 0.005%.

It was quite clear that we have here some compounds with antifeedant properties, which, in contradistinction to perhaps more potent other naturally occurring antifeedants, are stable enough to be of practical value.

2. CHEMICAL INVESTIGATIONS; NATURAL PRODUCTS

2.1 Naturally occurring ergostane-type steroids

The withanolides are a group of naturally occurring steroids built on an ergostane-type skeleton in which C-22 and C-26 are appropriately oxidized for forming a δ -lactone ring. For convenience, this basic structure was designated as the "withanolide" skeleton (1). The compounds were isolated from various solanaceous plants belonging to the genera Withania, Acnistus (Dunalia), Physalis, Nicandra, Datura, etc., where they occur mainly in the leaves.

Further investigation in this area led to the discovery of several groups of compounds biogenetically related to the withanolides, but with a modified carbocyclic skeleton:

- a) ring D aromatic steroids, represented mainly by Nic-1 (nicandrenone) (2);
- b) 13,14-seco-steroids, represented mainly by physalin B (3) and withaphysalin C (4);
- c) acnistins, possessing a bicyclic system in the side chain (5).

2 Nic-1 (nicandrenone)

3 Physalin B

4 Withaphysalin C

5a R = α -H, β -OH Acnistin E

5b R = H₂ Acnistin A

The withaphysalins (compound 4 for instance) represent intermediate stages in the process leading from the regular withanolides to the highly oxidized physalins (compound 3 for instance).

Thus far there are about 100 known representatives of this group of compounds, the structures of which were elucidated.

The major structural feature, common to all the withanolides and related ergostane derivatives is oxidation at positions 1, 22, and 26. In most compounds C₁ is oxidized to the ketone and in a few of them, only to the alcohol level; with only one exception, C₂₂ is oxidized to the alcohol level and always has the 22 β _F configuration; C₂₆ is oxidized to either the carboxylic acid, or the aldehyde level. Additional oxidations at almost all

other positions of the carbocyclic system or the side chain differentiate between the various compounds.

In an early review (Glötter et al. 1978) the withanolides (1), which are the largest group of highly oxidized ergostane-type steroids with an unmodified cyclopentanoperhydrophenanthrene skeleton were sub-divided according to the following structural features:

20-H Compounds with the whole side chain β -oriented, for instance:

withaferin A
6

jaborosalactone A
7

withanone
8

20-OH Compounds with the whole side chain β -oriented, for instance:

withanolide D
9

withanolide G
10

20-H or 20-OH compounds with the whole side chain α -oriented, for instance:

withanolide E
11

12 nic-2

These subdivisions are important for understanding the biogenetic aspects of the problem. With very few exceptions, the plants which synthesize 20-H withanolides are unable to produce the 20-OH counterparts, and vice versa (Kirson et al. 1970). The withanolides are the only group of natural steroids in which the side chain, possessing all carbon atoms, is α -oriented. The few previously known natural compounds of this type have has only 2 carbon atoms in the side chain (pregnane derivatives) (Kirson and Glotter 1981).

Ergostane type steroids having structures of the types described above were first isolated from Withania somnifera. During the work leading to the characterization of these compounds, it became evident (Abraham et al. 1968) that W. somnifera growing in Israel is divided into several chemotypes according to their content in variously substituted withanolides.

Plants containing 20-H withanolides (chemotype I) do not produce 20-OH compounds (chemotype II) and vice-versa. Plants

producing withanolides with an α -oriented side chain belong to a special type (chemotype III), different from the previous two. For the distribution of these chemotypes, see Chs.2.3 and 4.4.

2.2 Supply of plant material

Most of the plants which were necessary for the present project were raised at the experimental farm of the Faculty of Agriculture (see Table I, Ch. 4.1)

2.3 Distribution of the chemotypes of *Withania somnifera* in Israel

The geographical distribution of *W. somnifera* in Israel was investigated as early as 1968 (Abraham et al. 1968). It was reinvestigated within the framework of this project (Ascher et al. 1984) (see Ch. 4.4).

The natural habitat of *W. somnifera* chemotype II is in the northern part of the country. 2,3-Dihydrowithanolide D (13) was also found along with withanolide D (9), the major constituent of this plant. This is the first instance when this compound was isolated from natural sources. In addition to plants belonging to this chemotype, several plants belonging to chemotype III were also found. In addition to the chemical analysis, these plants were recognized by the antifeedant response of *Spodoptera littoralis*; in chemotype III, withanolide E (11) is the major constituent (see Ch. 2.8).

In the southern part of the country, the natural habitat of chemotype I, whose major constituent is withaferin A (6), several plants were found to contain 2,3-dihydrowithaferin A (14) in addition to withaferin A (the ratio was 1:1 in one of the

plants).

2.4 General procedure for work-up of plant material

Previous studies led to the conclusion that withanolides accumulate mainly in the leaves and not in the roots or other parts of the plant. The only exception, thus far, seems to be that of withaperuvin which was isolated from the roots of Physalis peruviana (Frolow^Y et al. 1981).

Leaves are dried at ambient temperature in a shadowed area during several days and the process is then continued in a ventilated oven at 50-55°C. The dried leaves are then crushed and stored in well-closed plastic or metal containers.

The extraction is done continuously (Soxhlet apparatus) with methanol in batches of ca. 300 g of dry leaves until the extracting solutions are almost colorless. Usually, 4-5 l of methanol are used per batch. The solution is concentrated at atmospheric pressure to a volume of ca. 0.5 l; water (1 l) is then slowly added, while stirring, in order to precipitate pigments. After 24 h at room temperature, the aqueous-methanol suspension is filtered to give a brown-colored solution. The dark green residue is discarded.

The clear solution is extracted with petroleum ether (60 - 80°) (3 x 300 ml) in order to remove residual pigments. The solution is then thoroughly re-extracted with diethyl ether; the ether extract is washed with brine, dried over Na_2SO_4 and evaporate to dryness. The residue containing the steroidal components which is then obtained is further submitted to chromatographic fractionation.

Chloroform can be successfully used instead of diethyl ether for extraction. Chromatography is done in one or more stages as mentioned for each of the individual isolation procedures described in the present report.

2.5 Chemical investigations of *Physalis peruviana*

P. peruviana is a perennial herb which is also cultivated in some regions of India (Uttar Pradesh, for instance) for its edible fruit. A first investigation was done with leaves of *P. peruviana* collected in Southern India (Ooty Hills near Pondicherry). Seeds of the plant were subsequently secured and plants were raised by the Department of Introduction of the Volcani Institute, Bet Dagan, and later at the Experimental Farm of the Faculty of Agriculture.

The extraction of the plant material (1 kg) received from India was done according to the general procedure outlined above (2.4). The crude residue obtained after extraction with diethyl ether was chromatographed on silica gel H (Merck) (500 g). The column was eluted with mixtures of methylene chloride and ethyl acetate. CH_2Cl_2 - EtOAc (7:3) gave physalin A (16) (0.1 g); CH_2Cl_2 - EtOAc (1:1) gave dihydrowithanolide E (17) (0.1 g) followed by withanolide E (11) (2 g). Further elution with EtOAc-MeOH (98:2) afforded 4 β -hydroxywithanolide E (15) (2 g).

The plants which were later raised in Israel from seeds received from Pondicherry most probably belonged to a different strain of *P. peruviana*. They contained 4 β -hydroxywithanolide E (15) but none of the first three compounds mentioned above.

The characterization of 4 β -hydroxywithanolide E (15) is

based on spectral analysis, mainly ^1H n.m.r., and comparison with withanolide E (11). Since the structure of the latter was unequivocally established by crystallographic analysis, the comparison of the spectral properties could lead to reliable conclusions concerning the structure of 4β -hydroxywithanolide E (Kirson et al.1976).

11 Withanolide E

R=H 15a 4β -Hydroxywithanolide E
R=Ac 15b 4β -Acetoxywithanolide E

In view of the low solubility of 4β -hydroxywithanolide E (15a) in CDCl_3 , the n.m.r. assignments rely on the following data: a) comparison of the signals of relevant protons of withanolide E (11) and 4β -hydroxywithanolide E acetate (15b) in CDCl_3 solution; b) comparison of the same signals in withanolide E and 4β -hydroxywithanolide E in $\text{C}_5\text{D}_5\text{N}$ solution.

The only significant differences are in the signals of 2-H, 3-H and 4-H: they are as expected for the introduction of a OH group at C_4 .

Further work on P. peruviana was done with plants growing in the outskirts of Varanasi: (Uttar Pradesh, India).

The mixture of ergostane type steroids from the leaves of this strain was much more complex than that obtained from the previous one. The most important observation was that 4β -hydroxywithanolide E is practically absent in this strain.

Table 1. NMR Data for withanolide E and 4 hydroxywithanolide E

Cpd	Methyl Groups							
	2-H	3-H	4-H	6-H	22-H	19-H	18-H	21-H 27 & 28-H
<u>11</u>	6.03 dq (10)	6.87 dd		3.20 [3.18]	4.88 m [5.16]	1.25 s [1.30]	1.10 s [1.37]	1.42 s [1.76] 1.93; 1.88 [1.92; 1.66]
<u>15a</u>	[6.50 d] (10)	[7.33 dd] (10;6)	[4.08 d] (6)	[3.42] (w $\frac{1}{2}$ 4)	[5.4 m]	[1.87]	[1.36]	[1.78] [1.83; 1.95]
<u>15b</u>	6.30 d (10) [6.48]	7.08 dd (10;6) [7.18]	4.66 d (6) [5.00]	3.31 (w $\frac{1}{2}$ 4) [3.47]	4.78 dd [5.15]	1.41 s [1.72]	1.08 s [1.32]	1.41 s [1.73] 1.89; 1.95 [1.82; 1.91]

Spectra are recorded at 60 MHz in CDCl₃ soln. Chemical shifts are in units. Data for pyridine solutions are in brackets. Coupling constants (in Hz) are in parentheses.

According to the structures of the compounds which were obtained, it seems that both withanolide E (11) and 4 β -hydroxywithanolide E (15a) did exist in earlier stages of the biogenetic development in this strain, but the epoxide ring in both compounds was opened to give the corresponding glycols and chlorohydrins.

Hydrolytic opening of the 5 β ,6 β -epoxide in withanolide E (11) takes place with cleavage of the C₅-O bond and leads to the formation of a trans diaxial glycol (withanolide S 18). Conversely, in the opening of this epoxide in 4 β -hydroxywithanolide E (15a), the C₆-O bond is cleaved and a trans diequatorial glycol (withaperuvin 19) is obtained. Whereas formation of chlorohydrin from 15a follows the same cleavage pattern, the formation of chlorohydrin from withanolide E (11) is subsequent to cleavage of the C₆-O bond and not of the C₅-O as in the hydrolytic process. 4-Deoxyphysalolactone (20), resulting by such a cleavage of withanolide E in nature, is indeed a diequatorial 5 β -hydroxy, 6 α -chloro-derivative. This special cleavage could not be reproduced under laboratory conditions. Treatment of withanolide E with HCl in tetrahydrofuran solution resulted in the quantitative formation of the diaxial 5 α -chloro, 6 β -hydroxy-derivative (see Ch. 3.2.2).

The structure of withaperuvine (19) which was isolated from the roots of the Varanasi variety of *P. peruviana* was confirmed by crystallographic analysis. This analysis was necessary in view of apparent discrepancies in the ^1H and ^{13}C n.m.r spectra of this compound (Frolow et al. 1981).

2.6 Chemical investigation of *Nicandra physaloides*

Two varieties and several strains of this plant were raised from seeds at the Experimental Farm of the Faculty of Agriculture. Earlier chemical investigations were published in 1951 (von Gizycky and Kotitsche 1951) without any significant findings concerning the identity of the chemical constituents. They were followed by several publications, the last in 1964

(Nalbandov et al.), in which the isolation of a crude product with weak antifeedant activity against Manduca sexta was reported. Although a name (nicandrenone) was given to this product, no significant research was done in order to determine its structure.

Our first investigations were done with a strain of N. physaloides whose natural habitat is in southern India (Ooty Hills); the plant material was received from Pondicherry in the form of a crude extract obtained according to the general procedure described in Ch. 2.4.

The crude steroid mixture was chromatographed on a silica gel H (Merck) column (500 g). Elution was done with mixtures of methylene chloride and ethyl acetate. Elution with a 4:1 mixture afforded nicandrenone (2) (100 mg); it was followed (1:1 mixture of the above eluants) by withanicandrin (22) which was characterized as a 12-oxowithanolide (6 α ,7 α -epoxy-5 α -hydroxy-1,12-dioxo-22R-witha-2-24-dienolide). Our work for the characterization of nicandrenone (2) was discontinued in view of two publications reporting the structure of this compound.

2 Nicandrenone

22 Withanicandrin

The crude mixture of steroids isolated by Begley and co-workers (1976) from N. physaloides raised from commercial seeds of unspecified geographic origin, contained nicandrenone (Nic-1) and related compounds (Nic-10, Nic-12 and Nic-17) having the same rearranged skeleton as well as several compounds with a normal unmodified carbocyclic skeleton (Nic-3, Nic-7, Nic-11). Although the authors noted that "the composition of the natural product mixture varied with each succeeding crop and it seems likely that there was genotypic variation in the commercial seed used", the origin of the various compounds was not investigated.

Bates and co-workers (1972, 1974) obtained only Nic-1 (2) and Nic-2 (12) from N. physaloides, of Peruvian origin. The plant material collected by Subramanian and Sethi in southern India contained nicandrenonolactone (Glötter et al. 1976) and Nic-2, along with nicandrenone and withanicandrin (22) (Kirson et al. 1972); the latter was not present in the strains investigated by previous authors.

In view of the reported antifeedant activity of nicandrenone (2) a re-investigation of this plant was undertaken. According to the chemical constituents, the two varieties which were studied (see Ch. 4.1, Table I) are characterized in the following manner.

(A). The variety containing ergostane type steroids of the nicandrenone type, i.e. compounds with a rearranged carbocyclic skeleton, in which ring D is enlarged and aromatized, along with a few other ergostanes possessing a normal carbocyclic skeleton. All strains belonging to this variety have violet flowers.

(B). The variety with white flowers (N. physaloides var.

albiflora). Seeds of this plant were received from the botanical garden of Kosice (Czechoslovakia). This variety did not contain any compounds of the nicandrenone type, but only ergostane derivatives with a normal skeleton.

Extraction and fractionation of the crude extract obtained from seeds of the variety with violet flowers received from India and raised in Israel, afforded withanicanthin (22) which was, however, accompanied by daturalactone A (23). The latter was originally isolated in pure state from Datura quercifolia (Kalla et al. 1979) and it was characterized as the epoxy-lactone analogue of withanicanthin (22).

23 Daturalactone A

Attempted separation of the mixture of daturalactone A and withanicanthin proved to be so difficult that we decided to test the antifeedant activity of the mixture and not that of the pure compounds.

Extraction and fractionation of leaves of N. physaloides var. albiflora (1.2 kg) was done as described in Ch. 2.4. Chromatographic fractionation was done on silica gel 60 (Merck)

70-230 mesh (400 g). Elution with a mixture of methylene chloride and ethyl acetate (7:3) afforded nicalbin B (25) (1.2 g); further elution with ethyl acetate only gave nicalbin A (24) (1.1 g).

24 Nicalbin A

25 Nicalbin B

Nicalbins A and B (Kirson et al. 1980) possess the same substitution pattern of rings A and B as nicandrenone (2) and withanicandrin (22). Nicalbin A has an epoxy-lactol in the side chain (hemiacetal structure) and a secondary hydroxy-group at C₁₆ (16 α -OH). The presence of this OH group enabled an intramolecular attack on the hemiacetal type carbon (C₂₆) leading to the displacement of the 26-OH and formation of the bicyclic structure present in the side chain of nicalbin B (25). As expected, this displacement takes place with inversion of configuration at C₂₆.

Both nicalbin A and B were inactive in the S. littoralis test. In contrast to nicalbin B, nicalbin A is a strong antifeedant for Epilachna varivestis; this leads to the supposition that the free hemiacetal in nicalbin A is instrumental for this activity.

2.7 Chemical investigation of *Physalis pubescens*

An investigation of the steroidal constituents of *Physalis pubescens* L. conducted a few years ago at the Faculty of Agriculture resulted in the isolation and characterization of physapubescin (26). The plants were raised at the experimental farm of the Faculty from seeds received from the Botanical Garden of Dijon, France. In connection with the present investigation on the antifeedant properties of several *Physalis* species, *P. pubescens* plants were raised again in 1981, this time from seeds collected in the outskirts of Waltair, India. There were small morphological differences between the Dijon and Waltair varieties and, much to our surprise, not even a trace of physapubescin (26) could be isolated from the leaves of the latter variety. Instead of compound (26), two new ergostane-type steroids, never encountered before, were isolated and characterized: an unsaturated lactone which was given the name physapubenolide (27) and the monoglucoside of an epoxy lactone which was named pubescenin (28). The identity of the plant material was confirmed by Professor S.K. Roy of the Banaras Hindu University, who kindly analysed a herbarium specimen of *P. pubescens* raised from seeds collected in India by Dr. S.S. Nittala.

As can be seen from the structure of physapubescin (26) and physapubenolide (27), the difference between the two compounds, as far as the side chain is concerned, is much alike to that between daturalactone A (23) and withanicandrin (22). Whereas in 27 and 22 the side chain is an unsaturated lactone, in 23 it is an epoxy-lactone which results from oxidation of an epoxy-lactol similar to that present in 26.

The difference in the substitution pattern of the carbocyclic skeleton in physapubescin (26) and physapubenolide (22) is very significant. Whereas the former is a 14-H compound, the latter is a 14 α -OH derivative. Usually, withanolides are either 14 α -H or 14 α -OH compounds. The ecdysterols (the insect moulting hormones) are the only other group of natural steroids having a 14 α -OH function. The 14-OH function is β -oriented in bufadienolides and cardenolides, two largely distributed groups of natural steroids. Thus far, physapubenolide is the second withanolide type compound with a 14 β -OH functional group.

The difference between the side chains of physapubescin and physapubenolide points towards one difference in their biogenesis: in the former, the oxidation at C₂₆ stops at the aldehyde level, thus leading to an unsaturated lactol which is easily transformed by an epoxidizing enzyme present in the plant into the corresponding epoxy-lactol.

Epoxidation of an unsaturated alcohol (allylic alcohol) takes place under different conditions than that of an unsaturated ketone. The first are epoxidized by peroxy-acids, whereas the latter require hydrogen peroxide or hydroperoxides under basic conditions. Similar differences exist between the epoxidation of unsaturated lactols and unsaturated lactones.

The epoxidizing enzymes present in Withania, Physalis and other related solanaceous plants can epoxidize isolated double bonds and allylic alcohols, but cannot epoxidize either unsaturated ketones and unsaturated lactones.

In the few instances when epoxy-lactones are found in such plants (mainly in Datura spp.) they result, most probably by oxidation of a previously formed epoxy-alcohol and not by epoxidation of a previously formed unsaturated lactone.

Pubescenin (28), which possesses an epoxy-lactone in the side chain is an example of this kind. In this compound, however, the carbocyclic ring system remained in one of the first stages of the biogenetic oxidative process: the C₁-OH was not further oxidized to the corresponding ketone thus precluding elimination. Furthermore, the original 3 β -OH was transformed into the corresponding glucoside. There is no evidence whatsoever as to whether glucosidation occurred before or after hydroxylation at C₁.

2.8 Chemical investigation of Withania somnifera, chemotype III

Of the three chemotypes of W. somnifera growing in Israel (see Ch. 2.3) the only one containing compounds which act as antifeedants for Spodoptera littoralis is chemotype III. Plants of this type were raised from seeds at the Experimental Farm of the Faculty of Agriculture. The procedure for isolation of the crude mixture of steroids was similar to that described (see Ch. 2.4).

The crude mixture obtained from 2 kg of dry leaves was fractionated by chromatography on silica gel H (800 g). Elution with mixtures of methylene chloride - ethyl acetate afforded the following major components.

CH ₂ Cl ₂ :	EtOAc	9 :1	withanolide J	<u>29</u>	(300 mg)
"	"	8.5:0.5	withanolide G	<u>10</u>	(450 mg)
"	"	1:1	withanolide E	<u>11</u>	(7 g)
"	"	1:1	withanolide H	<u>30</u>	(200 mg)

The antifeedant properties of crude fractions from this chromatography are summarised in Table III, see Ch. 4.4.

It is worth mentioning that in withanolides J, G and H which do not possess antifeedant properties, the side chain is β -oriented.

3. CHEMICAL INVESTIGATIONS: SYNTHETIC MODIFICATIONS OF WITHANOLIDE E

In view of the results obtained by testing the antifeedant activity of naturally occurring withanolides on Spodoptera littoralis, according to which the most active compound was withanolide E, we decided to study the relationship between the chemical structure of this compound and its activity. The contribution of each functional group and of each steric feature of the molecule was analyzed. The chemical modifications of withanolide E and the procedures used for their implementation will be presented and discussed in this chapter.

Withanolide E 11

3.1. Modifications of ring A.

3.1.1 Catalytic hydrogenation of withanolide E afforded 2,3-dihydrowithanolide E (17)

The reaction was done at room temperature under a slight positive pressure of hydrogen, in the presence of Pd on CaCO_3 as catalyst. Absolute alcohol was used as solvent. The reaction ceased after absorption of one equivalent of hydrogen (ca. 1 hr). After filtration of the catalyst, the solvent was removed in vacuum and the product was obtained as a white powder. It possessed the expected n.m.r. spectrum and showed one spot on a chromatoplate.

3.1.2 NaBH_4 reduction of withanolide E resulted in the 2,3-dihydroxy-derivative ($5\beta,6\beta$ -epoxy- $1\beta,14\alpha,17\beta,20$ -tetrahydroxywith-24-enolide) (31).

The conditions of the reaction and the stereochemical assignment of C_1 are based on a study done with model compounds (Weissenberg and Glotter, 1977).

According to the above cited work, a sharp difference exists between the behaviour of 2-en-1-ones in AB trans and AB cis steroids in the presence of NaBH_4 . The reaction with the former takes place by 1,2-addition of the hydride ion to give a mixture of the stereoisomeric allylic alcohols. The reaction with AB cis steroids takes place by 1,4 addition of the hydride ions to first

give the saturated ketone which is further reduced to the corresponding saturated alcohol.

To a solution of withanolide E (100 mg) in methanol (25 ml) NaBH_4 (50 mg) was added in several portions at room temperature. After stirring for 2 h, the solution was brought to pH ~ 7 . The solvent was removed and the product precipitated with water. It showed one spot on a chromatoplate and possessed the expected n.m.r. spectrum.

3.1.3 Epoxidation of the double bond in the 2-en-1-one system

The last modification of ring A which was investigated is the transformation of the 2-en-1-one into an epoxy-ketone. A new method which was devised to this end (Glötter and Zviely 1986) is highly effective for unsaturated ketones having a hydroxy group in the proximity. In the absence of such a group, no epoxidation takes place.

From the point of view of the present project, the disadvantage of the method is that epoxidation of the double bond is accompanied by cleavage of the 17-20 bond. Both reactions take place at practically the same rate. This cleavage is analyzed in Ch. 3.6.

3.2. Modifications of ring B

3.2.1 Transformation of withanolide E into the corresponding trans diaxial glycol (withanolide S)

Withanolide S (18) seems to be absent in chemotype III of Withania somnifera. It was isolated from an artificial type obtained by cross pollination experiments (chemotypes I by III) (Glötter et al. 1977). The compound was first obtained in the laboratory by opening of the epoxide ring in withanolide E (11) with H_2SO_4 in acetone solution. Under very mild conditions (-10°C) this reaction results in a mixture of three compounds: Withanolide S (18), 14-desoxy-withanolide S (32) and 14,20-oxido-withanolide S (33) (for the last two compounds, see Ch. 3.5).

Better results were obtained by using HClO_4 as the source of H^+ (yields up to 55%). A new procedure which was developed (HIO_4 in tetrahydrofuran solution) afforded withanolide **5** almost quantitatively.

To a solution of withanolide **E** (50 mg) in tetrahydrofuran (1 ml), a solution of HIO_4 (25 mg) in the same solvent (5 ml) was added. The solution was kept overnight at room temperature. The reaction was monitored by TLC. Water was then added and the THF was removed in vacuum. The residue was filtered and the product (42 mg) was washed with water. The product was identified by comparison with an authentic sample.

3.2.2 5-O-Methylwithanolide **5** (**34**)

The compound was first obtained as an artifact during extraction of leaves of Withania somnifera chemotype III (see Ch. 2.8) with methanol recovered from previous extractions. In order to confirm that this compound indeed possesses the structure **34**, it was prepared by opening the epoxide ring in withanolide **E**.

A solution of withanolide E (50 mg) in methanol (10 ml) containing ca. 2 mg of HIO_4 was left overnight at room temperature. After neutralization (KHCO_3), the solvent was removed, the residue was filtered, washed with water, and dried. It showed one spot on TLC and had the same n.m.r. spectrum as the artifact.

3.2.3 Transformation of withanolide E into the corresponding trans diaxial chlorohydrin (5α -chloro- 6β , 14α , 17β -20-tetrahydroxy-1-oxo-witha-2,24-dienolide) (35).

Our major interest in this chlorohydrin is as a protecting group for performing further reactions while preserving the possibility of again closing an epoxide ring with the same configuration as in the original compound (11). The chlorohydrin was obtained by treatment of withanolide E with HCl in a solvent which could not behave as a competing nucleophile. The chlorohydrin could be re-transformed into the starting epoxide in the presence of alumina. The last experiment was done in order to learn the conditions under which similar reactions must be done on other chlorohydrins to be prepared in the future from derivatives of withanolide E.

To a solution of withanolide E (500 mg), in tetrahydrofuran (25 ml), cold 36% HCl (3 ml) was added dropwise at 0°C with constant stirring. The reaction was monitored by TLC; the spot

of the starting material disappeared after 1 h. The solution was neutralized with aq. NaHCO_3 solution, most of the solvent was removed at room temperature and the product was isolated with chloroform. It showed only one spot on a chromatoplate and had the expected n.m.r. spectrum.

The above chlorohydrin (35) (15 mg) was dissolved in chloroform and the solution was adsorbed onto neutral alumina (10 g) (Woelm activity II) in a 100 ml round-bottomed flask. After thorough homogenization, the solvent was removed in vacuum; a free flying powder was thus obtained. The time necessary for the reaction to occur on the surface of adsorbent was determined by extraction of small aliquots at given intervals, until the TLC spot of 35 disappeared. Three hours were enough in order to retransform the chlorohydrin into the $5\beta,6\beta$ -epoxide (11). The adsorbed product was introduced in a small column and eluted with ethyl acetate. The product was identified by TLC and by n.m.r.

3.2.4 Transformation of withanolide E into the $5\alpha,6\alpha$ -epoxy stereoisomer ($5\alpha,6\alpha$ -epoxy-14 α ,17 β ,20-trihydroxy-1-oxowitha-2,24-dienolide).

Withanolide S (1 g) (18) (see paragraph 3.2.1) was dissolved in pyridine (7 ml). Freshly distilled mesyl chloride (1.5 ml) was slowly added to the cooled solution (0°C) and the resulting mixture was held at +4°C for 10 h. It was then poured onto crushed ice and the residue was filtered to give 1.05 g of a mesylate which was submitted to the next step without purification; it was only dried in a dessicator under vacuum.

The crude product had the expected signals in the n.m.r. spectrum.

The crude mesylate (36) (500 mg) was dissolved in chloroform (10 ml) and adsorbed onto silica gel (230-400 mesh) (15 g) in a round-bottomed flask. The mixture was homogenized by thoroughly and then heated on a water bath for 4 h at 90°C (bath temperature) in a rotating Rotavapor. The mixture was then transferred into a small column and the product was eluted with ethyl acetate to give an amorphous powder (37) (300 mg) which could not be induced to crystallize. It had the expected n.m.r. spectrum.

3.3 Modifications of rings A and B

The second most important natural withanolide with insect antifeedant activity (for S. littoralis) is 4 β -hydroxywithanolide E (15). This compound is characterized by the presence of an α -epoxy-alcohol (4 β -hydroxy-5 β ,6 β -epoxide) system.

15 4 β -hydroxywithanolide E

Withanolide E (11) possesses a 5 β ,6 β -epoxide which is homoallylic with respect to the ring A double bond; the allylic position (C₄) in this compound is unsubstituted. In 4 β -hydroxywithanolide E (15), the epoxide has the same position with respect to the double bond, but the allylic position is substituted (4 β -OH). We therefore decided to attempt the preparation of the additional isomeric epoxy-alcohols, 4 β ,5 β -epoxy,6 β -hydroxy; 4 β ,5 β -epoxy-6 α -hydroxy; 4 α ,5 α -epoxy-6 β -hydroxy; 4 α ,5 α -epoxy-6 α -hydroxy and 5 β ,6 β -epoxy-4 α -hydroxy. Thus far, we have succeeded in preparing only the first two isomers.

3.3.1 Preparation of 4 β ,5 β -epoxy-6 β ,14 α -17 β ,20-tetrahydroxy-1-oxowitha-2,24-dienolide

A. Formation of the 6 β -hydroxy-dienone (6 β ,14 α ,17 β ,20-tetrahydroxy-1-oxowitha-2,4,24-trienolide)

In the first procedure which was devised, the acetate of 4 β -hydroxywithanolide E (15) was used as starting material.

4 β -Hydroxywithanolide E acetate (550 mg) in tetrahydrofuran (5 ml) was stirred with $\text{Pd(PPh}_3)_4$ (230 mg) for two days under nitrogen. The solvent was then removed with a stream of nitrogen and the residue was introduced in a column of silica gel (70-140) (~ 20 g) made up with chloroform. The column was washed with

chloroform to elute the triphenylphosphine oxide which was formed in this reaction. Further elution with CHCl_3 -EtOAc (1:1) yielded pure hydroxy-dienone (38) (410 mg). It was fully characterized.

The only disadvantage of this procedure is that the starting material is much more difficult to obtain than withanolide E (11). 4β -Hydroxywithanolide E (15) exists in Physalis peruviana in relatively small amounts and its separation involves a much more elaborate chromatographic procedure than that required for the purification of withanolide E.

In the second procedure for preparation of the dienone, withanolide E (11) was used as starting material.

Five capillary drops of dry triethylamine were added to a solution of withanolide E (30 mg) in methanol (3.5 ml). The solution was heated to reflux and the progress of the reaction was monitored by TLC; 24 h were necessary to bring it to completion. The solvent was removed and the residue was evaporated several times in the presence of small amounts of chloroform in order to remove all traces of the reagent. The product was identified by TLC and n.m.r. and by direct comparison with the 6β -hydroxy-dienone (38) obtained in the Pd mediated reaction.

8. Epoxidation of the dienone (38). Previous studies on the epoxidation of steroidal 4-en-1-ones led to the conclusion that the reagent approaches the molecule from the top to give the $4\beta,5\beta$ -epoxide without any trace of the stereoisomeric $4\alpha,5\alpha$ -epoxide.

Although the presence of the neighboring 6β -hydroxy-group may assist in the control of the epoxidation, preferentially from the same side as that group, the stereoselective β -attack seems to be intrinsic in electrophilic reactions of the 2,4-dien-1-one system. Thus, the 6α -hydroxy analogue (see section 3.3.2) as well as cholesta-2,4-dien-1-one without any neighboring hydroxy-group showed the same stereoselectivity.

To a solution of the 6β -hydroxy-dienone (38) (100 mg) in chloroform (10 ml) *m*-chloroperbenzoic acid (40 mg) was added. The mixture was stirred for 6 h and the progress of the reaction was monitored by TLC. The reaction mixture was washed with 5% NH_3 solution (3 x 5 ml), then with water; the organic layer was dried over Na_2SO_4 and the solvent was removed. The crude product (39) showed one spot on a chromatoplate and had the expected n.m.r. spectrum.

3.3.2 Preparation of $4\beta,5\beta$ -epoxy- $6\alpha,14\alpha,17\beta,20$ -tetrahydroxy-1-oxowitha-2,24-dienolide

Treatment with sodium hydroxide of the mesylate (36) obtained from withanolide S (18) affords the 6 α -hydroxydienone (40). The reaction proceeds through the intermediate formation of the 5 α ,6 α -epoxide (37) which reacts with the base in excess in the same manner as it reacts with Et₃N, i.e. by abstraction of a proton^{from} the allylic C₄.

To the above crude mesylate (36) (500 mg), 5% NaOH in methanol (20 ml) was added and the mixture was heated to reflux for 4 h. Water (30 ml) was then added and the solution was acidified (pH $\sim$ 3) with 1N HCl. The acidic solution was extracted with chloroform (3 x 25 ml). The combined chloroform extracts were washed with water, dried and evaporated. The 6 α -hydroxydienone (40) had the expected n.m.r. spectrum. It showed one spot on a chromatoplate and was used as such for the epoxidation step.

Alternatively, the 5 α ,6 α -epoxide (37) (100 mg) prepared as described (3.2.3) was dissolved in methanol (10 ml) and triethylamine (15 capillary drops) was added. The reaction proceeded as described for the similar reaction which was done with withanolide E (11) (see paragraph 3.3.1). The product was identical with that obtained from the mesylate (36).

There is a sharp difference between the behaviour of the epoxide rings in withanolide E and in the stereoisomeric 5 α ,6 α -epoxy analogue, in the presence of alcoholic NaOH. Whereas the latter gave easily the 6 α -hydroxydienone, the former led only to intractable mixtures. The reaction with triethylamine as basic reagent for this purpose is by far superior to the MeOH/NaOH reagent which was first used.

Epoxidation of the 6 α -hydroxydienone (40) (100 mg) was done as described for the 6 β -stereoisomer, to give the 4 β ,5 β -epoxy-6 α -hydroxy derivative (41). The product had the expected n.m.r. spectrum.

3.4 Modifications at C-17

One of the most relevant aspects of the withanolides is the unusual orientation of the C-17 side chain. In naturally occurring steroids of plant and animal origin, this side chain is β -oriented. The withanolides and the related, highly oxidized ergostane-type steroids occurring in solanaceous plants are the only group of steroids in which compounds with a C-17 α -oriented side chain were found.

From the point of view of the antifeedant properties against Spodoptera littoralis, it is quite clear that the configuration at C-17 is a critical factor. With the exception of withanolides with an α -side chain, weak antifeedant activity on Spodoptera littoralis was found only in nicandrenone (2), a compound in which the side chain is β -oriented but the general shape of the molecule is different than in other steroids with a β side chain; this is due to the shape of ring D in nicandrenone which is enlarged and aromatized. All other natural withanolides with a β -side chain that we investigated proved to be inactive.

One of the most active compounds was withanolide E (11). In order to substantiate the observation that 17 β compounds behave differently than their counterparts belonging to the 17 α series, we prepared a compound (42) which is the C₁₇-epimer of withanolide E (17-iso-withanolide E).

11 Withanolide E

42 17-Isowithanolide E

Two types of withanolides were isolated from Withania somnifera chemotype III (see Ch. 2.8). The major fraction contained withanolide E (11). A minor fraction consisted of withanolides with a β -oriented side chain. Of the latter, two compounds are especially noteworthy: withanolide G (10) and withanolide J (29), the first unsubstituted at C-17 (17α -H) and the latter with a hydroxy group at this position (17α -OH).

10 Withanolide G

29 Withanolide J

On treatment of withanolide J with m-CPBA, a mixture of stereoisomeric 5 α ,6 α and 5 β ,6 β -epoxides was obtained.

43 5 α ,6 α -Epoxywithanolide J

44 5 β ,6 β -Epoxywithanolide J

Both compounds were inactive in the S. littoralis test. If we correlate this finding with the results obtained by testing withanolide S (18) in which the side chain is α -oriented, but the 5,6 epoxide is transformed into a trans diol (5 α ,6 β), it seems obvious that two structural features are necessary in order to induce antifeeding properties against S. littoralis, in this series of compounds: an epoxide at 5,6 and an α -oriented side chain at C-17.

The 5 α ,6 α -stereoisomer (37) of withanolide E gave the possibility to test the role of the stereochemistry at C₅ and C₆; 5 β ,6 β -epoxywithanolide J (44) was prepared in order to assess the role of the configuration at C₁₇.

3.5 The position of the side chain with respect to ring D

The structure of withanolide E (11) was unequivocally determined by crystallographic analysis as (20S,22R)-5 β ,6 β -

14 α ,17 β -20-trihydroxy-1-oxowitha-2,24-dienolide, with a boat-like conformation for ring A, a distorted half chair for ring B and a normal chair for ring C; ring D has an envelope form in which C-14 is out-of-plane. The compound crystallizes with two molecules of water; the C-14 and C-20 hydroxy-groups are hydrogen bonded. As a result, the 17 β -OH---C-13 CH₃ and 17 β -OH---22-H distances are decreased and the 20 α -OH---C-13 CH₃ distance is increased.

We were interested in learning whether changes in the relation between 20-OH and 14 α -OH may induce changes in the anti-feeding properties of compounds in this series. Two major possibilities were open for such an investigation.

a) Elimination of the 14-OH group would cancel the hydrogen bond present in withanolide E and would allow a higher degree of flexibility to the side chain.

b) Formation of a covalent bond between C₁₄ and C₂₀ by the intermediary of an oxide bridge would increase the degree of bending of the side chain towards ring D and would freeze the system without any possibility of conformational flexibility.

Treatment of withanolide E with H₂SO₄ in acetone solution leads to opening of the epoxide ring to the corresponding 5 α ,6 β diol as well as to elimination or nucleophilic displacement of the 14-OH.

The opening of the epoxide ring is faster than the reaction in ring D and therefore, the epoxide ring cannot be preserved while performing the above changes in ring D under acidic conditions. For experiments directed towards the formation of withanolide S as the only or at least as the major component of the reaction, see Ch. 3.2. The experiments discussed in the present chapter (3.5) are focused on the preparation of compounds 32 and 33 (Kirson et al. 1980).

Treatment of withanolide E (11) with acetone containing a larger amount of 8N-aqueous sulphuric acid for 4 h at room temperature took place with concomitant opening of the epoxide ring to give a mixture of (17S,20S,22R)-5 α ,6 β ,17 β ,20-tetrahydroxy-1-oxowitha-2,14,24-trienolide 32 (75% yield) and an isomeric compound 33 (20% yield) which was shown to be the oxide bridged derivative. The ratio between these two compounds changed to 3 : 7 by performing the reaction with 98% sulphuric acid in acetone solution.

Formation of these compounds by acid treatment of withanolide E can be rationalized by assuming selective protonation of 14-OH with subsequent formation of a 14-carbonium ion, or alternatively, non-selective protonation of either 14- or 20-OH to give a 14- or a 20-carbonium ion. According to the first alternative, elimination of a proton from C-15 leads to double bond formation, whereas internal nucleophilic attack by 20-OH leads to closure of the 14,20 oxide bridge. Based on steric considerations (the accurate stereochemistry of withanolide E was determined by crystallographic analysis), such an attack can take place only with retention of configuration at

C-14 and therefore the configuration of the bridgehead carbons should be 14R,20S. According to the second alternative, the C-14 carbonium ion should be responsible for formation of compound (32), whereas closure of the oxide bridge (compound 33a) should be brought about by attack of the 14 α -OH on a C-20 carbonium ion. In this case, retention of configuration at C-20 should lead to the 14R,20S stereochemistry, the same as above, whereas rotation about the C-17 - C-20 bond should facilitate back-side attack at C-20 to give the 14R,20R stereoisomer, in which the 21-methyl group is endo with respect to the oxabicyclo[2.2.1]heptane fragment.

Acetylation of the bridged compound 33a with acetic anhydride in pyridine, overnight at room temperature, resulted in the 6-monoacetate (33b) whereas at higher temperature increasing amounts of the 6,17-diacetate (33c) were obtained. At 95°C, the reaction leads to the exclusive formation of the diacetate 33c.

Experimental data. Transformation of withanolide E 11 into 5 α ,6 β ,17 β ,20-tetrahydroxy-1-oxowitha-2,14,24-trienolide (32) and 14 α ,20-epoxy,5 α ,6 β ,17 β -trihydroxy-1-oxowitha-2,24-dienolide (33a). 8N-Sulphuric acid (3 ml) was added to a solution of withanolide E

(11) (600 mg) in acetone (400 ml). After 4 h at room temperature, the solution was neutralized with aqueous sodium hydrogen carbonate, water was added and the product was extracted with ether (3 x 300 ml). The extract was washed with aqueous 10% sodium chloride, dried (Na_2SO_4) evaporated, and the residue was separated by p.l.c. [benzene - ethyl acetate - ethanol (2 : 18 : 1)]. The upper band was extracted with chloroform-methanol (6 : 4) to give compound 32 (450 mg), which could not be induced to crystallize; similarly, the lower band gave compound 33a (120 mg), which could not be induced to crystallize;

98% Sulphuric acid was slowly added to a stirred solution of 1 (950 mg) in acetone. After 4 h at room temperature the solution was worked up as described above, to yield compounds 32 (250 mg) and 33a (685 mg).

Compound 33a (100 mg) was acetylated with acetic anhydride (1.5 ml) and pyridine (1.5 ml) overnight at room temperature to yield 33b only. When the acetylation was performed at 50°C for 5 h, a mixture of 33b and 33c was obtained in the ratio 4 : 1. After 18 h at 95°C, the diacetate (33c) was obtained, 80% yield.

All these compounds were fully characterized by their n.m.r. spectra as well as by mass spectrometric determinations.

Epoxidation of Δ^{14} in compound 32 to give 14,15-epoxy-14-desoxy-withanolide S (45).

The reaction led to the formation of an inseparable mixture of the two stereoisomeric $14\alpha,15\alpha$ - and $14\beta,15\beta$ -epoxides in roughly equal amounts. The non-stereoselective epoxidation is due to two opposite directing effects: the 20-OH group directs the epoxidizing agent from the rear of the molecule whereas the 17β -OH facilitates the top side attack.

To a solution of compound 32 (50 mg) in chloroform (10 ml), m-chloroperbenzoic acid (20 mg) was added. The reaction was complete after 4 h (TLC control). The solution was then washed with 5% aq. ammonia (2 x 5 ml), then with water and dried. The product obtained after removal of the solvent showed one spot on a chromatoplate: the n.m.r. spectrum pointed to the presence of the mixture of epoxides.

The availability of compound 33 gave the possibility to prepare derivatives modified in rings AB.

Preparation of compound 46.

To a solution of the diacetate (33c) (50 mg) in dry pyridine (5 ml) at -12°C (ice-salt mixture), cold, freshly distilled thionyl chloride (1 ml) was slowly added. After 5 min the mixture was poured onto ice and the yellow solid which separated was filtered off, washed, and dried. Preparative t.l.c. (ethyl acetate-benzene, 4 : 1) gave the dienone (2) (40 mg), m.p. $270\text{--}274^{\circ}\text{C}$ (decomp.) (from ethanol).

Epoxidation of the dienone 46 to the epoxy derivative 47

The reaction was done as described for compound 32. The product (one spot on a chromatoplate) was identified by its n.m.r. spectrum.

Attempted preparation of Δ^{14} withanolide E and 14,20-oxido-withanolide E.

The high rate of the reaction leading to the opening of the 5 β ,6 β -epoxide in withanolide E in the presence of H₂SO₄ (see beginning of Ch. 3.5, reactions 11 $\rightarrow$ 32 + 33), renders difficult ^{the} modifications in ring D and the side chain, while preserving the 5 β ,6 β -epoxide. A way of circumventing this difficulty was therefore devised. As shown, the chlorohydrin 35 can be prepared without any secondary reaction in ring D; furthermore, it can be re-transformed into the original epoxy-derivative 11.

The chlorohydrin was therefore prepared as described (Ch. 3.2.3), and was subsequently submitted to the reaction with H₂SO₄ in acetone to give compounds 48 and 49 which were separated by chromatography. The re-closure of the epoxide ring, leading to compounds 50 and 51, is being investigated in order to find the best experimental conditions. Thus far, these compounds were obtained only in low yields.

To a suspension of the chlorohydrin 35 (100 mg) in acetone (75 ml) at -10°C, 6 capillary drops of conc. H₂SO₄ were added while stirring. The progress of the reaction was monitored by TLC and no change was noticed after 2 h at -10°C. The temperature was raised at $\sim$ 15°C. The reaction was complete after 72 h. After the usual work-up, the product was separated on preparative chromatoplates. The upper band was identified by n.m.r. as the Δ^{14} derivative 48; the lower band was identified as the oxido-derivative 49.

3.6. Cleavage of the C₁₇-C₂₀ bond.

Cleavage of this bond should give an opportunity to divide the molecule of withanolide E into two moieties: the carbocyclic system possessing the same functions as the natural compound and the side chain, for instance:

No satisfactory method was available for cleavage of the ditertiary 17-20 glycol in withanolide E without inducing changes in other functions present in the molecule.

It is well known that periodic acid and periodate salts are the reagent of choice for the smooth cleavage of primary-primary, primary-secondary, and secondary-secondary glycols. It is less effective with secondary-tertiary and ineffective with tertiary-tertiary glycols. Other agents, such as $\text{Pb}(\text{OAc})_4$, which could cleave such glycols, bring about other undesired secondary reactions.

We have now developed a new procedure for cleavage of ditertiary glycols which operates under exceedingly mild conditions. The reagent is vanadyl acetylacetonate which can function in the following three versions:

- 1 - In stoichiometric amounts;
- 2 - As catalyst in the presence of *t*-butyl hydroperoxide;
- 3 - As catalyst in the presence of *m*-chloroperbenzoic acid.

All these conditions are operative with 11 leading to compounds 52 and 53 in excellent yields.

The reaction with the stereoisomeric isowithanolide E (42) (β -oriented side chain) required 24 h at 70°C in order to bring about the quantitative cleavage of the 17,20-glycol. This was performed in benzene-dichloromethane solution with a catalytic amount of $[\text{VO}(\text{acac})_2]$, in the presence of *t*-butyl hydroperoxide as the oxidant.

The different behaviour of compounds (11) and (42) can easily be rationalized by taking into account the preferred conformations of the corresponding molecules along the C-17 - C-20 bond. In (11) (see A) the two hydroxy groups are in a gauche relationship and can easily form a cyclic complex with the reagent (after a slight rotation in order to achieve a syn relationship). In (42) (see B), these groups are in an anti relationship and rotation to the syn position, although possible, requires more energy due to steric interference between the 18-methyl group and the lactone ring.

Experimental. To a solution of (11) (0.1 mmol) in dry benzene (10 ml), a solution of $[VO(acac)_2]$ (0.1 mmol) in the same solvent (5 ml) was added. The starting material (11) completely disappeared after 1 h at room temperature (ca. 25°C, t.l.c. evidence). The solution was filtered through Florisil (100 - 200 mesh; 3 g) and elution was completed with dichloromethane-ethyl acetate (7:3). The n.m.r. spectrum of the crude mixture pointed to the total disappearance of the 22-H signal (multiplet, δ 4.88) in the starting material and the appearance of a triplet, δ 4.67 (J 7 Hz), due to the above proton in lactone 53. This signal had the same intensity as that of the vinylic 2-H and 3-H. The integration was done on expanded spectra (20 Hz cm^{-1}) recorded at 300 MHz. The crude product (two spots on t.l.c.) gave, after separation on a preparative chromatoplate, 52 (26 mg) and 53 (15 mg).

4. BIOLOGICAL INVESTIGATIONS

4.1 General outline

The purpose of this project was to investigate alternative ways of crop protection by using the insect antifeeding properties of secondary metabolites produced by certain solanaceous plants. The major target insect in our study was Spodoptera littoralis, the Egyptian cotton leafworm, one of the most harmful pests of agricultural crops in the Middle East. The investigation dealt also with some stored product pests, such as Tribolium castaneum, the red flour beetle, Sitophilus oryzae, the rice weevil, Callosobruchus maculatus, the cowpea weevil, and Oryzaephilus surinamensis, the saw-toothed grain beetle, as well as with the antifeeding activity against several other insect pests of U.S. agriculture, such as Spodoptera frugiperda, the fall armyworm; Epilachna varivestis, the Mexican bean beetle; Diabrotica undecimpunctata howardi, the spotted cucumber beetle; D. balteata, the banded cucumber beetle; and Acalymma vittata, the striped cucumber beetle.

Our approach was to locate solanaceous plants not fed upon by pest insects and to find the antifeedants in them, if any, which we thought would be responsible for feeding deterrence.

4.2. Agrotechnical preparatory work and primary screening of plants for antifeedants by feeding them to insects

A series of plants containing withanolides and related ergostane-type steroids were raised from seeds at the experimental farm of the Faculty of Agriculture at

Rehovot (Table I).

Table I. Plants raised from seeds at the experimental farm of the Faculty of Agriculture at Rehovot, Israel

Name	Details
<u>Physalis peruviana</u> ,	raised from seeds received from Pondicherry, India
<u>P. pubescens</u>	raised from seeds received from the Botanical Garden of Dijon, France
<u>P. alkekengi</u>	
<u>P. nicandroides</u>	
<u>P. lanceifolia</u>	
<u>P. angulata</u>	
<u>P. ixocarpa</u>	
<u>P. viscosa</u>	Plants raised from seeds received under these names were identical with <u>P. ixocarpa</u>
<u>P. philadelphica</u> ,	
<u>P. curasavica</u>	
<u>P. minima</u>	
<u>P. minima indica</u>	
<u>P. pubescens</u>	raised from seeds received from India, (Dr. S. Nittala); it was found to be a strain of the previously investigated <u>P. pubescens</u> Dijon, see Ch. 2.7)
<u>Nicandra physaloides</u>	10 strains

<u>N. physaloides</u> , <u>var. albiflora</u>	2 strains
<u>Withania somnifera</u>	chemotype III

Several plant species (Nicandra and Physalis spp.) of those listed in Table I were fed for 48 h to 80-100 and 170-190 mg Spodoptera littoralis larvae. The results are given in Table II which shows that of all the plants assayed, only Physalis peruviana, which has a relatively high 4 β -hydroxywithanolide E (15) content, consistently caused a weight loss to both weight ranges of the larvae. P. angulata caused a weight reduction in the 170-190, but not in the 80-100 mg larvae. All other plants allowed an increase in larval weight, although much lower than alfalfa, the control plant.

Table II. Weight gain of Spodoptera littoralis larvae feeding for 48 h on different solanaceous plants (Nicandra and Physalis species) in comparison with alfalfa (27°C).

Average weight gain (ΔW , mg) during 48 h									
Nicandra physaloides					Physalis				
	30771 ^a Nepal	30323 ^a	21225 ^a Kew gardens	19116 ^a Ooty Hills	peruviana ^b	nicandroides	minima indica	angulata	pubescens ^c
	80 - 100 mg larvae								
Plant tested	+25.6	+41.2	+43.9	+25.9	<u>-25.0</u>	+86.2	+60.5	+23.6	+125.2
Control	+266.3	+266.3	+217.7	+217.7	+266.3	+266.3	+266.3	+266.3	+266.3
Starved larvae	-26.9	-26.9	-26.6	-26.6	-26.9	-26.9	-26.9	-26.9	-26.9
	170 - 190 mg larvae								
Plant tested	+69.3	+48.3	+13.6	<u>+28.6</u>	<u>-18.0</u>	+150.8	+57.5	<u>-23.1</u>	+177.4
Control	+270.2	+270.3	+207.3	+207.3	+304.9	+304.9	+304.9	+304.9	+304.9
Starved larvae	-53.1	-53.1	-69.6	-69.6					

- ^a Introduction number, Dept. of Introduction, Volcani Institute, Bet Dagan, Israel.
^b Raised from seeds received from Pondicherry, India
^c The Indian strain (see Ch. 2.7 and Table I in Ch. 4.2).

4.5 Location of the antifeedant activity in fractions obtained from Withania somnifera chemotype III and Physalis peruviana.

The antifeedant effects of fractions obtained through chromatography of Withania somnifera chemotype III plants are given in Table III. Activity was evidently dependent on withanolide E content of the fraction.

Table III. Antifeedant properties of 0.01% solutions of fractions obtained through chromatography of Withania somnifera chemotype III extract against 170-190 mg Spodoptera littoralis larvae. There were 40 one-larva replicates per fraction; $t = 27^{\circ}\text{C}$.

Fraction	48-h 'Styropor' consumption (mg/larva)
1* upper spots	33.6 $\pm$ 2.5
2 crystalline fractions containing withanolide E (<u>11</u>)	7.3 $\pm$ 1.0
3 wax containing withanolide E	9.9 $\pm$ 2.6
4 mixture of withanolides E (<u>11</u>) and H (<u>30</u>)	20.8 $\pm$ 3.6
5 nearly pure withanolide H	22.0 $\pm$ 2.3
6**lower spots	16.2 $\pm$ 2.0
7**lower spots	27.0 $\pm$ 3.3
Control (5% sucrose only)	29.0 $\pm$ 3.4

All antifeedant solutions contained also 5% sucrose.

* Major components of this fraction are withanolides G (10) and J (29).

**The lower spots contain small amounts of unidentified compounds.

Table IV shows the activity of variously treated extracts of the strain of Physalis peruviana raised in Rehovot, another source of withanolides. The total steroids (entry 1 in Table IV) isolated from the plant extract were phagodeterrent as demonstrated by their overcoming the phagostimulation exerted by 5% added sucrose (sucrose control alone, see entry 6). The unprocessed P. peruviana extract (entry 2) did not cause any feeding.

Table IV. Antifeedant properties of 0.4% solutions in H₂O:MeOH (1:1) of different extracts of Physalis peruviana against 170-190 mg Spodoptera littoralis larvae. There were 20 one-larva replicates per test; $t = 27^{\circ}\text{C}$.

No.	Solutions tested	48-h 'Styropor' consumption (mg/larva)
1.	0.4% of the dried total steroids isolated from the <u>Physalis peruviana</u> extract, + 5% sucrose	0
2.	0.4% Unprocessed <u>P. peruviana</u> total extract	0
3.	Ditto, + 5% sucrose	0.9 $\pm$ 0.3
4.	0.4% <u>P. peruviana</u> extract after removal of steroids	3.6 $\pm$ 0.7
5.	Ditto, + sucrose	15.4 $\pm$ 2.0
6.	Control (5% sucrose alone)	20.6 $\pm$ 1.4

At this stage, we deemed this could be ascribed to either deterrence or total lack of phagostimulants in the extract. The next experiment (entry 3) showed, however, that the P. peruviana extract neutralized the phagostimulant effect of sucrose, i.e.,

that it contained deterrents. These are steroids, since although the extract after removal of the steroids (entry 4) was only slightly attractive (lack of phagostimulants), it no longer neutralized the phagostimulant effect of sucrose (entry 5).

5. SCREENING FOR ANTIFEEDANT ACTIVITY OF SOLANACEAE

5.1 Plant material

The air-dried parts of the following 10 solanaceous plants collected at various world locations and received through the National Cancer Institute Natural Products Program were processed in the U.S.A. They were finely ground in a Wiley mill and extracted in Sohlet extractors first with ethyl ether and then with 95% ethanol:

<u>Acnistus arborescens</u>	r + s	(Brazil)
<u>Capsicum fuscoviolaceum</u>	r + s	(Costa Rica)
<u>Cestrum lanatum</u>	s	(Costa Rica)
<u>Cestrum nocturnum</u>	t + l	(New Zealand)
<u>Cestrum parqui</u>	r	(India)
<u>Datura discolor</u>	r + s + l + f	(Mexico)
<u>D. inoxia</u>	l	(Pakistan)
<u>D. metel</u>	l	(West Africa)
<u>Hyosciamus aureus</u>	r + s + l	(Israel)
<u>Nicotiana glauca</u>	ws + sb	(Mexico)
<u>Physalis longifolia</u>	whole	Utah (USA)
<u>Solanum aviculare</u>	s + l	(New Zealand)
<u>Solanum brachystachys</u>	-	- -
<u>Solanum ramulosum</u>	r	(Brazil)
<u>Solanum umbellata</u>	s	(Peru)
<u>Solanum verbascifolium</u>	r + s + l	(Peru)
<u>Solanum verbascifolium</u>	rb + sb	(Thailand)
<u>Withania coagulans</u>	s	(Pakistan)

<u>Withania somnifera</u>	r	(Tanzania)
<u>Withania somnifera</u>	whole	(South Africa)

r = roots; s = stems; l = leaves; f = fruit; rb = root bark;
sb = stem bark; ws = woody stem; t = twigs

The following extracts were prepared in Israel and put at the disposal of the U.S.D.A. group which gave it the following Beltsville code (AI3) numbers:

Extract of <u>Physalis ixocarpa</u>	AI3-42947
Extract of <u>Physalis pubescens</u>	AI3-42948
Extract of <u>Physalis lanceifolia</u>	AI3-42959
Extract of <u>Nicandra physaloides</u> var. <u>albiflora</u>	AI3-42950

5.2 Bioassay methods

Spodoptera littoralis (Egyptian cotton leafworm)

To assess antifeeding activity with S. littoralis larvae, the "Styropor method" was employed: thin (0.6 mm) lamellae (6 x 4 cm) of "Styropor" (foamed polystyrene) of density 0.016 (P₁₆) were dipped into the solutions of pure compounds or extracts dissolved in ethanol:water (7:3) and 5% sucrose was added to each test solution. The lamellae were left to dry for 24 h and then weighed individually. They were then offered singly, in large, 15-cm-diam, petri dishes, to S. littoralis larvae weighing 170 - 190 mg, together with water absorbed in cotton wads in small plastic containers. The weight of "Styropor" consumed per larva and the weight of fecal pellets voided (not included in this report) were recorded after 48 h and served as criteria of antifeedant activity.

Spodoptera frugiperda (fall armyworm)

Larvae of S. frugiperda from a laboratory stock culture, reared on an artificial diet, were used for the antifeedant test. The desired concentration of the compound or extract was incorporated into 100 g of hot diet by using a 220-ml jelly jar fitted with an Osterizer^R blender cutter head. After the diet was

blended for approximately 1 min, it was poured into 28 ml clear plastic cups (approximately 8 g/cup), and allowed to cool to room temperature. Control diets were untreated. One newly emerged 1st-instar larva was placed in each cup (ten cups were used for each concentration/replicate). The cups were then capped with pre-punched lids and placed in a holding room at $27 \pm 1^{\circ}\text{C}$ and $50 \pm 5\%$ R.H. The larvae were observed for mortality and weighed daily beginning on the 7th day (or as noted). The desired concentration of a compound or an extract was dissolved in acetone, and applied topically to 3rd- and 4th-instar larvae in the dorsal thorax region with a calibrated 1 μl glass micropipette. Controls received only 1 μl acetone. Treated larvae were observed and weighed daily until death or pupation occurred. These experiments were done at Beltsville, MD.

Boarmia selenaria (giant looper)

For this test, young leaves of avocado were treated with methanolic solutions of the compounds and exposed for 48 h to larvae of B. selenaria weighing 4-7 mg each, after which the larvae were reweighed and their weight gain compared to that of the control larvae.

Epilachna varivestis (Mexican bean beetle)

The test device consisted of a combination of plastic petri dish halves. Thin squares of cotton wool, cut to such a size as to fit well into the cover of 9-cm petri dishes, were laid in

inverted covers, and an 8-cm filter paper disk 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 (cv. "Brittle wax") of sufficient size was laid, with its lower side up, on the wetted filter paper. A petri dish bottom with a central 4.5-cm diam. hole punched with a hot metal die was inserted on top of the leaf; 0.2 ml of methanolic solutions of the test compounds was pipetted on the leaf with an Eppendorf pipette and then spread over the whole exposed leaf area (15.9 cm^2) with a fine paintbrush. The residue was allowed to dry for at least one hour and the device was closed with a petri dish cover. 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. Controls consisted of methanol-only treatments. A weighed L_4 Epilachna larva was introduced into each dish, with 20 larvae being employed per treatment; each treatment was replicated 1 - 5 times. The closed dishes were then laid out singly (not stocked in piles) on a large laboratory bench. The experiments were conducted at 23°C . The larvae were allowed to feed on the treated and control leaves for 48 h and were then reweighed. After the 48-h feeding period, injured, moribund or dead insects, if any, were recorded and discarded; only survivors were weighed.

Adult female Diabrotica undecimpunctata howardi Barber (spotted cucumber beetle), reared on corn, and adult female Acalymma vittata (F.) (striped cucumber beetle) reared on squash at $27^\circ\text{C} \pm 1^\circ\text{C}$, and $60 \pm 5\%$ RH, were collected soon after emergence and starved 24 h before testing. Leaf discs about 20 mm in

diameter were cut with a cork borer from young leaves of Burpee cantaloupe plants grown in flats in a greenhouse. Test leaf discs were dipped into suspensions, emulsified with a Brinkman Polytron (PCU-2), of the plant extracts in acetone or ethanol with 0.01 percent Tween 20 in water. Untreated leaf discs were dipped into a blank of acetone and Tween 20. After air drying, two treated and two untreated discs were placed in the bottom of a polyethylene dish (93 mm diameter x 73 mm deep) and five female beetles were introduced. Two replicates were carried out for each treatment.

Preliminary investigations indicated that some fractions were toxic in closed dishes, so all tests were conducted using a loose-weave muslin cover, which was atomized periodically with water during the first 6 h of observation at 3, 6, and 22 h by the same observer. Results are presented as percentage eaten ($\text{percent consumed-treated} \times 100 / \text{percent consumed-treated} + \text{untreated}$), which gives a total of 50 percent when treated and untreated discs are consumed in equal amounts. The lower the percentage, the more active the antifeedant. These experiments were done at Vincennes, IN.

Tribolium castaneum

In the antifeedant and insecticidal bioassay method wheat flour containing 5% Torula yeast (Waldhof-Aschaffenburg, Mannheim, W. Germany) was used as a basic diet. For a bioassay, 10 g diet was mixed with 10 ml acetone containing the test compounds, or with 10 ml acetone alone as control. Following evaporation of the

solvent and thorough mixing, the diet was distributed in 2-g portions in test vials 2.5-cm in diameter. Twelve white larvae, 0-3 h after hatching, were placed in each test vial and kept at 27°C. Larval weight was determined at the age of 14 days and subsequently, data on pupation and adult emergence were recorded.

Furthermore, test materials investigated in the U.S.A. were submitted to the USDA Laboratory at Savannah, GA, to determine toxicity by topical application to adults of the rice weevil (Sitophilus oryzae) and the cowpea weevil (Callosobruchus maculatus). Acetone solutions were prepared containing 50 µg/ml of compounds or the solvent-free plant extracts. Treatments were applied by anesthetizing adult weevils briefly with CO₂, picking them up individually with a vacuum tweezer, and applying 0.5 µl (25 µg test candidate) of the solution to the dorsal thorax with a microapplicator. Control insects were anesthetized and treated with 0.5 µl of the solvent. Twenty insects (10 males and 10 females) were treated with each dose. After treatment, the insects were held in 100-mm-diameter petri dishes (5 males and 5 females/dish) in a room maintained at 27°C and 60% RH. The dishes were examined for dead insects every 24 h for 7 days. Insects that did not move when touched gently with a small pointed object were considered dead.

Finally, some further tests were conducted to determine attractancy, repellency, and toxicity to stored product insects. The test was conducted separately with 2 species of stored grain insects, namely: confused flour beetle (Tribolium confusum) and the sawtoothed grain beetle (Oryzaephilus surinamensis). A total of 200 adult insects (mixed sexes) of each species is exposed in a choice olfactometer in the laboratory and allowed free access to a dish of 125 grams of wheat into which 400 mg of the test material has been incorporated; exposure time is 72 h, after which the number of insects accumulated in the wheat (number attracted) and the number of dead insects is counted. A check (control) dish of untreated wheat is exposed to the insects at the same time. These experiments were done at Beltsville, MD.

5.3 Results with extracts of various Solanaceae

Results of feeding deterrency against larvae of Spodoptera frugiperda are given in Table V. The standard, nicalbin A at 100 ppm incorporated in the diet of fall armyworm larvae, showed a very high degree of deterrency. Some deterrency was also shown by the ethanol extract of Cestrum parqui roots and by the ether extracts of Datura innoxia leaves and Solanum verbascifolium mixed roots and stems.

Table V - Bioassay against 1st instar fall armyworm (Spodoptera frugiperda) larvae by incorporation in the larval diet at 100 ppm (U.S.A.)

Test Material	Avg. wt. (mg)	
	after 7 days	% Mortality
Nicalbin A	10.1	0
<u>Acnistus arborescens</u> rt, st, ether extract	41.2	0
" " " " , ethanol extract	40.6	0
<u>Capsicum fuscoviolaceum</u> rt, st, ether extract	53.4	0
" " " " , ethanol extract	42.3	10
<u>Cestrum lanatum</u> st, ether extract	32.0	0
" " " , ethanol extract	34.0	0
" <u>nocturnum</u> tw, lf, ether extract	34.5	0
" " " " , ethanol extract	41.2	0
" <u>parqui</u> rt, ether extract	31.5	0
" " " , ethanol extract	22.6	10
<u>Datura inoxia</u> lf, ether extract	20.4	0
" " " , ethanol extract	41.3	0
<u>Solanum aviculare</u> st, lf, ether extract	40.6	0
" " " " , ethanol extract	28.4	0
" <u>ramulosum</u> rt, ether extract	68.6	0
" " " , ethanol extract	37.2	0
" <u>umbellata</u> st, ether extract	36.4	0
" " " , ethanol extract	37.2	0
" <u>verbascifolium</u> rt, st, ether extract	18.8	0
" " " " , ethanol extract	29.2	0
Check	40.4	0

Code (plant part): lf = leaves, rt = roots, st = stems, tw = twigs

Against Acalymma vittata, nicalbin A was very effective at 0.5% but not at 0.1%. Considerable deterrency was shown at both concentrations by the ether extract of mixed roots and stems of Capsicum fuscoviolaceum and D. inoxia, by the ether extract of C. parqui at 0.5%, and by both concentrations of the ethanol extract

of Cestrum nocturnum mixed leaves and twigs. The ether extract of D. inoxia was the only one which at 0.5% was not consumed at all after 22-h exposure.

Table VI - Laboratory bioassay against adult striped cucumber (Acalymma vittata) beetles with treated leaf discs (U.S.A.)

Test Material	Dosage (%)	Percentage of disc consumed in		
		3 hr.	6 hr.	22 hr.
Nicalbin A	0.1	25	26	38
	0.5	0	0	6
Check (untreated)				62
<u>Acnistus arborescens</u> rt, st, ether extract	0.1	24	30	51
" " " " " "	0.5	38	42	50
" " " " " " , ethanol extract	0.1	20	22	57
" " " " " " " "	0.5	17	36	46
<u>Capsicum fuscoviolaceum</u> rt, st, ether extract	0.1	0	0	14
" " " " " " " "	0.5	0	0	14
" " " " " " , ethanol extract	0.1	10	11	44
" " " " " " " "	0.5	0	0	46
<u>Cestrum lanatum</u> st, ether extract	0.1	33	20	39
" " " " " " " "	0.5	0	0	33
" " " " " " , ethanol extract	0.1	57	70	56
" " " " " " " "	0.5	63	50	42
<u>Cestrum nocturnum</u> tw, lf, ether extract	0.1	38	41	52
" " " " " " " "	0.5	58	63	62
" " " " " " , ethanol extract	0.1	10	6	19
" " " " " " " "	0.5	0	0	10
<u>Cestrum parqui</u> rt, ether extract	0.1	0	0	40
" " " " " " " "	0.5	0	0	17
" " " " " " , ethanol extract	0.1	0	0	42
" " " " " " " "	0.5	7	26	38
<u>Datura inoxia</u> lf, ether extract	0.1	0	7	28
" " " " " " " "	0.5	0	0	0
" " " " " " , ethanol extract	0.1	20	24	52
" " " " " " " "	0.5	17	34	37
<u>Solanum aviculare</u> st, lf, ether extract	0.1	39	45	48
" " " " " " " "	0.5	20	19	44
" " " " " " , ethanol extract	0.1	30	39	39
" " " " " " " "	0.5	37	41	41
<u>Solanum ramulosum</u> rt, ether extract	0.1	25	13	26
" " " " " " " "	0.5	6	4	41
" " " " " " , ethanol extract	0.1	42	44	40
" " " " " " " "	0.5	45	50	46
<u>Solanum umbellata</u> st, ether extract	0.1	33	38	43
" " " " " " " "	0.5	6	3	37
" " " " " " , ethanol extract	0.1	10	18	50
" " " " " " " "	0.5	24	28	38
<u>Solanum verbascifolium</u> rt, st, ether extract	0.1	18	28	40
" " " " " " " "	0.5	13	7	32
" " " " " " , ethanol extract	0.1	48	40	49
" " " " " " " "	0.5	41	32	48
Check (untreated)				50

Code (plant part): lf - leaves, rt = roots, st = stems, tw = twigs

On testing these extracts against stored product insects it was found that for the most part, the materials toxic to the rice weevil by topical application were also toxic to the cowpea weevil (Table VII). This was true of nicalbin A, which was very toxic to both species, the ethanol extracts of Acnistus arborescens and Cestrum nocturnum and the ether extract of C. lanatum. The ether extract of C. parqui and the ethanol extract of Capsicum fascioides were somewhat less toxic to both species of insects. All other extracts appeared to have little effect on the insects.

Table VII - Toxicity of nicalbin A and crude extracts of solanaceous plants applied topically (25 ug) to adult rice weevils (Sitophilus oryzae) and cowpea weevils (Callosobruchus maculatus) (U.S.A.).

Test Material	Avg. % mortality at indicated days after treatment					
	Rice weevil			Cowpea weevil		
	3	5	7	3	5	7
Nicalbin A	92	95	100	94	95	95
<u>Acnistus arborescens</u> rt, st, ether extract	8	10	10	0	7	9
" " " " " " ethanol extract	55	60	75	72	79	83
<u>Capsicum fuscoviolaceum</u> rt, st, ether extract	4	5	10	0	0	5
" " " " " " ethanol extract	35	48	57	58	65	79
<u>Cestrum lanatum</u> st, ether extract	65	80	91	73	82	90
" " " " " " ethanol extract	0	5	10	2	6	6
<u>Cestrum nocturnum</u> tw, lf, ether extract	0	2	3	1	1	3
" " " " " " ethanol extract	32	44	59	58	67	83
<u>Cestrum parqui</u> rt, ether extract	37	51	62	32	41	48
" " " " " " ethanol extract	1	2	4	6	9	10
<u>Datura innoxia</u> lf, ether extract	0	0	1	4	6	9
" " " " " " ethanol extract	8	8	9	0	5	10
<u>Solanum aviculare</u> st, lf, ether extract	0	1	1	2	7	8
" " " " " " ethanol extract	5	7	10	8	9	15
<u>Scianum ramulosum</u> rt, ether extract	0	0	5	0	3	4
" " " " " " ethanol extract	4	6	7	2	3	5
<u>Solanum umbellata</u> st, ether extract	0	0	2	0	2	3
" " " " " " ethanol extract	1	3	4	5	6	6
<u>Solanum verbascifolium</u> rt, st, ether extract	2	4	5	0	0	4
" " " " " " ethanol extract	4	7	10	4	5	7
Control (acetone only)	0	0	0	0	0	2

Code (plant part): lf = leaves, rt = roots, st = stems, tw = twigs

The extracts found active in any of the insect species tested at the U.S.D.A. were assayed in Israel against Spodoptera littoralis larvae with the "Styropor" method (Table VIII). None of them showed activity in this test.

Table VIII - Assaying for antifeedant properties of 0.1% solutions of extracts prepared in the U.S.A of various Solanaceae; Spodoptera littoralis larvae, 170-190 mg; 20 replicates per extract; t=27°C.

ENT-Number	Extract	Solution prepared in	48-h 'Styropor' consumption (mg/larva)
43034	<u>Capsicum fuscoviolaceum</u> , roots, stems (ether extract)	methanol	12.8 ± 1.8 (15.8 ± 1.7)*
43049	<u>Datura innoxia</u> , leaf (ether extract)	methanol	11.8 ± 1.6 (15.8 ± 1.7)
43068	<u>Cestrum lanatum</u> , stems (ether extract)	methanol	14.1 ± 2.2 (15.8 ± 1.7)
43122	<u>Cestrum nocturnum</u> twigs, leaves (ethanol extract)	ethanol	15.0 ± 1.6 (13.5 ± 2.2)
43127	<u>Solanum verbascifolium</u> , roots (ether extract)	methanol	12.1 ± 1.7 (15.8 ± 1.7)
43132	<u>Acnistus arborescens</u> , roots, stems (ethanol extract)	ethanol	11.3 ± 1.5 (13.5 ± 2.2)
43136	<u>Cestrum parqui</u> , roots (ethanol extract)	ethanol	9.7 ± 1.5 (13.5 ± 2.2)

* Numbers in parentheses denote feeding on lamellae treated with 5% sucrose only (controls). All the antifeedant solutions contained also 5% sucrose.

The same extracts were also assayed against Tribolium castaneum neonate larvae by incorporation at 0.1% in their diet (Table IX). Only the Datura innoxia ether extract (ENT-43049) was highly active in as far as it reduced weight gain after 14 days to 24% of that of the control, whereas the other extracts were inactive in this test (cf. results in Table VI).

Table IX - The effect of extracts of various Solanaceae prepared in the U.S.A., incorporated at 0.1% into the diet of neonate Tribolium castaneum larvae, on their weight after 14 days. Each experiment consisted of 5 replicates of 5 larvae each; $t=27^{\circ}\text{C}$. The extracts are the same as in Table VIII, where full details are given.

Extract ENT-number	Mean weight of larvae $\pm$ S.E. in mg
43034	1.9 $\pm$ 0.1
43049	0.5 $\pm$ 0.04
43068	2.0 $\pm$ 0.07
43122	2.0 $\pm$ 0.1
43127	2.0 $\pm$ 0.08
43132	1.7 $\pm$ 0.1
43136	1.7 $\pm$ 0.07
Control	2.1 $\pm$ 0.08

The ethanol extracts of this series were assayed also against Epilachna varivestis larvae (Table X) and two of them showed some activity.

Table X - Antifeedant properties of 0.1% solutions of some extracts of Solanaceae against fourth-instar larvae of Epilachna varivestis on treated bean leaves. Twenty replicates for each extract; $t=27^{\circ}\text{C}$.

Extract ENT-number	Average weight gain ($\Delta W \pm$ S.E.) during 48 h (mg)
43122	5.7 $\pm$ 1.9
43132	1.3 $\pm$ 2.5
43136	0.9 $\pm$ 1.8
control	7.3 $\pm$ 2.0

An ether extract code (No. CH 381.4) of leaves of another Datura species, Datura metel from West Africa, followed by the ethanol extract of the marc (CH 374.7) was prepared in the U.S.A. In the usual feeding tests with larvae of S. frugiperda, the ether extract showed no antifeedant effect, but the ethanol extract reduced feeding by 80%; similar results were obtained in tests conducted with adults of the banded cucumber beetle, Diabrotica balteata. Analogous results were obtained with Spodoptera littoralis larvae (Table XI; ethanol extract active, ether extract inactive).

Table XI - Antifeedant properties of two Datura metel extracts at 0.25% in 70% ethanol containing 5% sucrose against 170-190 mg Spodoptera littoralis larvae.

Extract	48-h 'Styropor' consumption (mg/larva)
<hr/>	
CH 374.7	3.5 $\pm$ 1.2 (19.1 $\pm$ 1.8)
CH 381.4	16.7 $\pm$ 1.7 (19.1 $\pm$ 1.8)

Numbers in parentheses denote feeding on lamella treated with 5% sucrose only (controls).

Against Epilachna varivestis larvae, both extracts showed activity (Table XII), with the ethanol extract (CH 347.4) being more active than the ether extract. (CH 381.4).

Table XII - Antifeedant properties of two Datura metel extracts against fourth-instar larvae of Epilachna varivestis on treated bean leaves.

Extract	Concentration of extract in leaf-painting solution (X)			
	0.1	0.05	0.01	0.005
	Average weight gain ($\Delta W \pm S.E.$ /larva) during 48 h (mg)			
CH 374.7	-4.4 $\pm$ 0.4 (8.7 $\pm$ 0.7)	-1.9 $\pm$ 0.4 (12.1 $\pm$ 0.7)	3.8 $\pm$ 0.8 (4.9 $\pm$ 1.1)	6.1 $\pm$ 1.1 (4.9 $\pm$ 1.1)
CH 381.4	-3.8 $\pm$ 0.5 (8.7 $\pm$ 0.7)	0.4 $\pm$ 1.0 (12.1 $\pm$ 0.7)	10.2 $\pm$ 1.2 (4.9 $\pm$ 1.1)	5.7 $\pm$ 0.6 (4.9 $\pm$ 1.1)

Numbers in parentheses denote average weight gain ($\Delta W \pm S.E.$)/larva in control leaf discs painted with ethanol only.

Finally, in the U.S.A. 11 further solanaceous plant samples were extracted with 95% ethanol and tested for their antifeedant activity on Spodoptera frugiperda larvae (Table XIII). Of these, six were considered effective: these are the extracts of Withania coagulans stems from Pakistan, W. somnifera whole plant from South Africa, Nicotiana glauca woody stems with bark from Mexico, W. somnifera root from Tanzania, Datura discolor root from Mexico, and Solanum verbascifolium leaves from Peru.

Table XIII - Bioassay against 1st instar fall armyworm (Spodoptera frugiperda) larvae by incorporation in the larval diet at 100 ppm (U.S.A.).

Ethanol Extract of	Collected	Avg. wt. (mg) after 7 days	% Mortality
<u>Datura discolor</u> rt.	Mexico	67	10
" " st-lf-fr	Mexico	90	0
<u>Hyoscyamus aureus</u> rt-st-lf	Israel	88	0
<u>Nicotiana glauca</u> ws-sb	Mexico	48	0
<u>Physalis longifolia</u> whole	Utah (U.S.A.)	102	0
<u>Solanum verbascifolium</u> lf	Peru	72	0
" " rb	Thailand	91	0
" " sb	Thailand	97	0
<u>Withania coagulans</u> st	Pakistan	44	0
<u>Withania somnifera</u> rt	Tanzania	63	0
" " whole	South Africa	51	0
Check		114	0

Code (plant part): fr=fruits, lf=leaves, rb=root bark, rt=roots, sb=stem bark
st=stems, ws=woody stems

The extracts found active in the U.S.A. were further tested in Israel. They were found to be inactive against S. littoralis larvae (Table XIV).

Table XIV - Bioassay against 170-190 mg Spodoptera littoralis larvae with the Styropor method; all extracts tested at 0.1%.

Ethanol extract of	Aqueous solvent for preparation of Styropor dipping solution, containing also 5% sucrose	Avg. wt of Styropor consumed per larva within 48 h. mg Treatment	Control
<u>Datura discolor</u> , roots	Water	20.4	23.6
<u>Nicotiana glauca</u> , stems and bark	Water	11.3	23.6
<u>Solanum verbascifolium</u> , leaves	70% EtOH	27.2	23.6
<u>Withania coagulans</u> , stems	70% EtOH	18.7	23.6
<u>Withania somnifera</u> , roots	70% EtOH	14.2	23.6
<u>Withania somnifera</u> , whole plant	70% EtOH	20.4	23.6

The same held true for Epilachna varivestis, against which these extracts were inactive (Table XV).

Table XV - Bioassay against L₄ Epilachna varivestis larvae on bean leaves treated with 0.1% solutions of extracts.

Ethanol extract of	Solvent for painting solutions on leaves	Avg. wt gain of larva within 48 h. mg	
		Treatment	Control
<u>Datura discolor</u> , roots	Water	6.7	8.1
<u>Nicotiana glauca</u> , stems and bark	Water	9.8	12.1
<u>Solanum verbascifolium</u> , leaves	EtOH	10.7	12.1
<u>Withania coagulans</u> , stems	EtOH	14.7	12.1
<u>Withania somnifera</u> , roots	EtOH	12.4	12.1
<u>Withania somnifera</u> , whole plant	EtOH	13.2	12.1

The extracts of Physalis species and of Nicandra physaloides prepared in Israel were assayed at Beltsville against two species of stored product insects, with withanolide E serving as standard (Table XVI). All the extracts were repellent to Oryzaephilus surinamensis and the Nicandra physaloides extract was strongly repellent to Tribolium confusum.

Table XVI - Effect of exposure of 2 species of stored grain insects to wheat treated with candidate test materials ^a (U.S.A)

	Number of insects attracted and number of dead insects ^b			
	<u>Tribolium confusum</u>		<u>Oryzaephilus surinamensis</u>	
	Attracted	Dead	Attracted	Dead
Withanolide E	24	0	15	7
Check ^c	86	12	39	32
.....				
<u>Physalis ixocarpa</u> extract	36	5	9	16
<u>Physalis pubescens</u> extract	58	10	9	15
<u>Physalis lanceifolia</u> extract	18	4	1	7
<u>Nicandra physaloides</u> extract	3	1	1	8
Check ^c	69	0	95	9

^a 125 grams of wheat + 400 mg of test material

^b 200 insects of each species per test exposed for 72 hours

^c untreated wheat

The same extracts were assayed against Spodoptera frugiperda larvae. There was no activity at all with the extracts (Table XVII) and marginal activity only of withanolide E.

Table XVII - Bioassay against 1st-instar fall armyworm larvae by incorporation in the larval diets at 100 ppm (U.S.A.)

Our Number	Test Material	Avg. wt (mg) After 7 days	% Mortality
42946	Withanolide E	102.4	0
	Check	137.4	0
.....			
42947	<u>Physalis ixocarpa</u> extract	139.8	0
42948	<u>Physalis pubescens</u> extract	127.6	0
42949	<u>Physalis lanceifolia</u> extract	144.2	0
42950	<u>Nicandra physaloides</u> extract	133.0	0
	Check	148.0	10

5.4 Results obtained with extracts of Solanum brachystachys

Aerial parts of this plant (stems + leaves + flowers + fruits) were extracted with ether. The crude extract was divided into basic, acidic and neutral fractions.

Only the crude neutral fraction showed slight activity when tested on 1st instar fall armyworm larvae. Activity of the neutral fraction was maintained when tested on Spodoptera littoralis larvae (Styropor method), one-third Styropor consumption of that in control.

A crude acidic fraction showed also some activity; this could not be maintained by further chromatographic fractionation of the neutral portion of the total ether extract.

Weak activity of one of the fractions (hexane eluate) was found when tested on L₄ Epilachna varivestis larvae.

In view of these results, the work with S. brachystachys was discontinued.

6. EVALUATION OF THE ANTIFEEDING PROPERTIES OF WITHANOLIDES AND
RELATED ERGOSTANE-TYPE STEROIDS (PURE COMPOUNDS)

6.1 Results obtained with *Spodoptera littoralis*, *Epilachna varivestis* and *Tribolium castaneum*. Research done following the preliminary studies (see Ch. 4.3) is detailed in the following paper.

THE ANTIFEEDANT EFFECT OF SOME NEW WITHANOLIDES AGAINST THREE INSECT
SPECIES; SPODOPTERA LITTORALIS, EPILACHNA VARIVESTIS AND TRIBOLIUM
CASTANEUM

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Abstract

The antifeedant effect of several new withanolides on larvae of Spodoptera littoralis, Epilachna varivestis and Tribolium castaneum was investigated. 2,3-Dihydrowithanolide E (II) was an antifeedant for all three species; nicalbin A (XIV), and a mixture of withanicandrin (IX) and daturalactone A (X) were active against E. varivestis and T. castaneum. Some activity was shown by 6 β ,14 α ,17 β ,20 α_F -tetrahydroxy-1-oxowitha-2,4,24-trienolide(V) against S. littoralis, 4 β ,5 β -epoxy-6 α ,14 α ,17 β ,20 α_F -tetrahydroxy-1-oxowitha-2,24-dienolide (VII) against E. varivestis and T. castaneum, 5 α ,6 α , -epoxy-14 α ,17 β ,20 α_F -trihydroxy-1-oxowitha-2,24-dienolide (VI) against E. varivestis and nicalbin B (XV) against T. castaneum.

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INTRODUCTION

In recent studies it was shown that withanolides and related ergostane-type steroids are antifeedants for larvae of the Egyptian cotton leafworm, Spodoptera littoralis (Boisd.) (1,5) and of the Mexican bean beetle, Epilachna varivestis Muls. (6). The most active compounds against S. littoralis were withanolide E (I) and 5 β , 6 β -epoxy-1 β ,14 α ,17 β ,20 α _F-tetrahydroxywith-24-enolide (III). The compounds most active against E. varivestis were nicalbin A (XIV), withanolide E (I) and 4 β -hydroxywithanolide E (IV).

The present study was undertaken with the purpose to assay further, new compounds of this series, and to include effects against larvae of a stored product pest, the red flour beetle, Tribolium castaneum (Herbst).

MATERIALS AND METHODS

The insects

The following insects were grown as described previously: Spodoptera littoralis on alfalfa (2, 4); Epilachna varivestis on beans (Phaseolus vulgaris var. 'Brittle wax') (6) and Tribolium castaneum on wheat flour containing 5% Torula yeast (11,12).

The Compounds

(Table of compounds)

I R=H; Withanolide E (8)

IV R=OH; 4 β -Hydroxywithanolide E (14)

II R=O; 2,3-Dihydrowithanolide E (8)

III R= β -OH; α -H;5 β ,6 β -Epoxy-1 β ,14 α ,17 β ,
20 α -tetrahydroxywith-24-enolide (8)

V Δ^4 ; 6 β -OH (10)

VI 5 α ,6 α -epoxy (10)

VII 4 β ,5 β -epoxy; 6 α -OH (10)

VIII 4 β ,5 β -epoxy; 6 β -OH (10)

IX Δ^{24} ; Withanicandrin (16)

X 24,25-epoxy; Daturalactone A (13)

XI Δ^2 ; Withaferin A (17)

XII 2,3-Dihydrowithaferin A (17)

XIII Epoxywithanolide G (9)

(Mixt. of $5\alpha,6\alpha$ and $5\beta,6\beta$ -epoxides)

XIV Nicalbin A (15)

XV Nicalbin B (15)

XVI Nic-1 (Nicandrenone) (7)

The antifeedant bioassays

Spodoptera littoralis

To assess antifeeding activity with S. littoralis larvae, the "Styropor method" (3) was employed: thin (0.6 mm) lamellae (6 x 4 cm) of 'Styropor' (foamed polystyrene) of density 0.016 (P_{16}) were dipped into a solution of the compounds in ethanol:water (7:3) and 5% sucrose was added to each test solution. The lamellae were left to dry for 24 h and then weighed individually. They were then offered singly, in large 15-cm-diam petri-dishes, to S. littoralis larvae weighing 170-190 mg, together with water absorbed in cotton wads in small plastic containers. The weight of 'Styropor' consumed per larva and the weight of fecal pellets voided (not included in this report) were recorded after 48 h and served as criteria of antifeedant activity.

Epilachna varivestis

The test device for E. varivestis (6) consisted of a combination of plastic petri-dish halves. Thin squares of cotton wool, cut to such a size as to fit well into the cover of 9-cm^{-diam} petri dishes, were laid in inverted covers, and an 8-cm filter paper disk 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 (var. 'Brittle wax') of sufficient size was laid, with its lower side up, on the wetted filter paper. A petri-dish bottom with a central

4.5-cm-diam hole punched with a hot metal die was inserted on top of the leaf; 0.2 ml of methanolic solutions of the test compounds was pipetted on the leaf with an Eppendorf pipette and then spread over the whole exposed leaf area (15.9 cm²) with a fine paintbrush. The residue was allowed to dry for at least one hour and the device was closed with a petri-dish cover. The residue thus obtained on the exposed leaf area was 125 mg/m² with a 0.1% solution and proportionally less with the lower concentrations. Controls consisted of methanol-only treatments.

A weighed L₄ Epilachna larva was introduced into each dish, with 20 larvae being employed per treatment; each treatment was replicated 1-5 times. The closed dishes were then laid out singly (not stocked in piles) on a large laboratory bench. The experiments were conducted at 23°C. The larvae were allowed to feed on the treated and control leaves for 48 h and were then reweighed. After the 48-h feeding period, injured, moribund or dead insects, if any, were recorded and discarded; only survivors were weighed.

Tribolium castaneum

In the biological assay for T. castaneum (11,12) wheat flour containing 5% Torula yeast (Waldhof-Aschaffenburg, Mannheim, W. Germany) was used as a basic diet. For a bioassay, 10 g diet was mixed with 10 ml acetone containing the test compounds, or with 10 ml acetone alone as control. Following evaporation of the solvent and thorough mixing, the diet was distributed in 2-g portions in

test vials 2.5-cm in diameter. Twelve white larvae, 0-3 h after hatching, were placed in each test vial and kept at 27°C. Larval weight was determined at the age of 14 days and, subsequently, data on pupation and adult emergence were recorded.

RESULTS AND DISCUSSION

Spodoptera littoralis

Table 1 confirms the antifeedant activity of withanolide E (I), compound III and 4^β-hydroxywithanolide E (IV) for S. littoralis, which was already reported (5) previously in a preliminary notice. We demonstrated now that the newly available compound 2,3-dihydro-withanolide E (II) is a highly active antifeedant. Marginal activity at a high concentration (0.1%) only, was shown by 6^β,14^α, 17^β,20^α_F-tetrahydroxy-1-oxowitha-2,4,24-trienolide (V). All the rest of the compounds in Table 1 (VI-XIII) were inactive.

(Table 1)

Epilachna varivestis

The high antifeedant effectivity of nicalbin A (compound XIV) against E. varivestis (this compound was inactive against S. littoralis) and the somewhat lesser activity of withanolide E (I) (Table 2), which had been demonstrated in a previous work conducted by this group (6), was confirmed.

It was now found that the inseparable mixture* of withanicandrin (IX) and daturalactone A (X) (inactive against S. littoralis, see Table 1) was a highly active antifeedant against E. varivestis. Three new compounds showing activity against E. varivestis were 5 α ,6 α -epoxy-14 α ,17 β ,20 α -trihydroxy-1-oxowitha-2,24-dienolide (VI), 2,3-dihydrowithanolide E (II) and 4 β ,5 β -epoxy-6 α ,14 α ,17 β ,20 α -tetrahydroxy-1-oxowitha-2,24-dienolide (VII).

(Table 2)

Tribolium castaneum

Three of the compounds tested (Table 3) were antifeedants against T. castaneum in descending order of activity: nicalbin A (XIV) with practically no survival at 0.05% and sizable effect (arbitrary criterion - weight gain less than half of that in controls) at as low as 0.005% in the diet; the mixture of withanicandrin (IX) and daturalactone (X), active at the three

* Withanicandrin (IX) was isolated (16) in 1970 from a variety of Nicandra physaloides which did not contain daturalactone A (X) (13). Extraction of a new batch of plant material (1982) which, evidently, was of a different chemotype, resulted in the above inseparable mixture. Pure daturalactone A (X) was isolated from Datura spp. [see (16) and references cited therein]. Neither of the pure compounds IX and X were available for the present study.

highest concentrations tested (0.05%, 0.025%, 0.01%); and nicalbin B (XV), active at 0.05% and 0.025% only. Of the remaining compounds tested only VII showed some activity at the highest concentration tested, 0.05%. The rest of the compounds in Table 3 had no antifeedant effect in this insect.

(Table 3)

The effect of the two compounds found most active according to Table 3 (XIV; IX+X) on subsequent (following the lapse of 14 days) larval development, pupation and emergence of T. castaneum is given in Table 4.

(Table 4)

Comparison of the results for nicalbin A (XIV) - Tables 3 and 4 - shows that antifeeding does not necessarily imply mortality. For instance, though the larvae on 0.025% nicalbin A reached an average weight of only 0.01 mg after 14 days (Table 3), many of them pupated eventually (Table 4).

In our experiments, nicalbin A was a conspicuous antifeedant for Coleoptera (Epilachna, Tribolium), but had no effect against the lepidopteran S. littoralis (5). On the other hand, one of us (M. Jacobson, unpublished ^{results} ^) found that nicalbin A was a strong antifeedant for larvae of the fall armyworm, Spodoptera frugiperda.

when incorporated at a concentration of 0.01% in the larval diet. In Coleoptera, Jacobson found nicalbin A to be very effective as antifeedant, when offered on treated leaf disks to adults of the striped cucumber beetle, Acalymma vittata, and to be very toxic to the two stored products pests, the rice weevil, Sitophilus oryzae, and the cowpea weevil, Callosobruchus maculatus, on topical application. This is in accordance with our findings (Table 3) on the high toxicity of nicalbin A, incorporated at 0.05% into the diet, to neonate T. castaneum larvae.

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Table 1. Antifeedant properties of pure compounds against 170-190 mg *Spodoptera littoralis* larvae. There were 20-100 one-larva replicates for each concentration of every compound; T=27°C.

Compound	Concentration of compound in dipping liquid (%)				
	0.2	0.1	0.05	0.01	0.005
			48-h 'Styropor' consumption (mg/larva)		
Withanolide E (I)			1.4±0.2 (28.7±1.7)**	6.4±0.8 (28.6±1.8)	2.3±0.5* (20.6±1.0)
2,3-Dihydrowithanolide E (II)			0.4±0.1 (18.0±1.1)	1.3±0.3 (19.4±1.1)	2.4±0.4 (20.7±1.1)
Compound III			2.2±0.6* (24.6±1.1)	4.9±0.8 (17.7±1.5)	9.8±1.6* (21.5±1.6)
4S-Hydroxywithanolide E (IV)			3.9±0.7 (22.9±1.5)	18.2±2.3* (34.4±2.6)	
Compound V	6.7±1.6 (23.4±1.7)	12.0±1.4 (23.4±1.7)	12.9±1.3 (20.0±1.4)		
Compound VI			27.7±1.6 (24.9±1.5)	21.1±2.2 (24.6±2.7)	
Compound VII			30.2±2.2 (35.6±3.3)	37.7±1.8 (35.6±3.3)	
Compound VIII			17.4±2.8 (17.2±1.9)		
Withanicandrin + Daturalactone A (inseparable mixture; IX+X)			22.1±2.8 (24.9±1.5)	26.4±1.4 (24.6±2.7)	
Withaferin A (XI)	25.9±2.6 (30.2±2.1)	24.7±1.1 (30.2±2.1)	25.7±2.7 (33.3±2.4)	24.7±2.1 (33.3±2.4)	
2,3-Dihydrowithaferin A (XII)	32.3±2.1 (30.2±2.1)	26.9±2.5 (30.2±2.1)	26.7±1.7 (33.3±2.4)	27.5±2.2 (33.3±2.4)	
Epoxywithanolide G (XIII)		16.5±1.5 (19.3±1.5)			

* Results taken from Ascher et al. (11).

** Numbers in parentheses denote feeding on lamellae treated with 5% sucrose only (controls). All the antifeedant solutions contained also 5% sucrose.

Table 2. Antifeedant properties of pure compounds against fourth-instar larvae of *Epilachna varivestis* on treated bean leaves. There were 20-100 one-larva replicates for each concentration of every compound; $T=27^{\circ}\text{C}$.

Compound	Concentration of compound in leaf-painting solution (%)				
	0.1	0.05	0.025	0.01	0.005
	Average weight gain ($\pm$ S.E./larva) during 48 h (mg)				
Withanolide E (I)	-2.1 $\pm$ 0.7 (+13.5 $\pm$ 0.9)	-3.9 $\pm$ 0.5 (-10.8 $\pm$ 0.9)	-5.6 $\pm$ 0.8 (+12.4 $\pm$ 0.9)	+1.5 $\pm$ 1.0 (-12.4 $\pm$ 0.9)	
2,3-Dihydrowithanolide E (II)	-1.5 $\pm$ 0.8 (+11.7 $\pm$ 0.9)	0 $\pm$ 0.6 (+9.8 $\pm$ 0.7)	+1.3 $\pm$ 0.4 (+12.0 $\pm$ 0.6)	+8.6 $\pm$ 0.7 (+10.6 $\pm$ 0.8)	+12.1 $\pm$ 1.1 (+11.4 $\pm$ 1.4)
Compound V	-1.1 $\pm$ 1.0 (+16.1 $\pm$ 0.7)			+1.5 $\pm$ 1.0 (+16.1 $\pm$ 0.7)	
Compound VI	-3.1 $\pm$ 0.6 (+10.5 $\pm$ 0.9)	-3.1 $\pm$ 0.5 (+7.8 $\pm$ 0.8)	-2.6 $\pm$ 0.5 (+11.1 $\pm$ 0.7)	-1.7 $\pm$ 0.8 (+6.7 $\pm$ 0.9)	+0.7 $\pm$ 0.9 (+12.0 $\pm$ 0.9)
Compound VII	+0.8 $\pm$ 0.9 (+12.4 $\pm$ 0.8)	-0.5 $\pm$ 0.7 (+10.8 $\pm$ 0.8)	0 $\pm$ 0.6 (+8.7 $\pm$ 0.9)	0.9 $\pm$ 1.2 (+6.5 $\pm$ 0.9)	-0.6 $\pm$ 1.0 (+6.5 $\pm$ 0.9)
Compound VIII	+4.2 $\pm$ 0.7 (+16.1 $\pm$ 0.7)			+8.9 $\pm$ 0.3 (+16.1 $\pm$ 0.7)	
Withanicandrin + Dacturalactone A (IX+X)	-8.2 $\pm$ 0.5 (+11.2 $\pm$ 1.3)	-7.2 $\pm$ 0.5 (+7.8 $\pm$ 0.9)	-5.3 $\pm$ 0.5 (+10.2 $\pm$ 0.6)	-0.5 $\pm$ 0.5 (+8.9 $\pm$ 1.2)	-0.1 $\pm$ 0.5 (+8.8 $\pm$ 0.6)
Niceibin A (XIV)	-11.2 $\pm$ 0.6 (+11.2 $\pm$ 1.3)	-12.6 $\pm$ 0.7 (+7.8 $\pm$ 0.9)	7.0 $\pm$ 0.5 (+11.5 $\pm$ 1.0)	-3.7 $\pm$ 0.7 (+6.9 $\pm$ 0.5)	-2.8 $\pm$ 0.6 (+9.8 $\pm$ 0.6)
				-3.1 $\pm$ 1.1 (+6.6 $\pm$ 0.9)	+1.7 $\pm$ 1.1 (+5.9 $\pm$ 1.3)

* Numbers in parentheses denote average weight gain ($\pm$ S.E.) / larva in control leaf discs painted with methanol only. The average $\pm$ S.E./larva in larvae starved for 48 h was -7.9 $\pm$ 0.5 mg.

Table 3. The effect of pure compounds incorporated into the diet of neonate *Tribolium castaneum* larvae on their weight after 14 days. Each experiment consisted of 5 replicates of 12 larvae each and was repeated 1-5 times on different days; Numbers in parentheses denote weight $\pm$ S.E. in acetone only-treated controls accompanying each experiment. $T=27^{\circ}\text{C}$.

Compound	Concentration of compound in diet (%)				
	0.05	0.025	0.01	0.005	0.001
		Mean weight of larva $\pm$ S.E. in mg			
Withanolide E (I)	0.7 $\pm$ 0.06 ^c (1.4 $\pm$ 0.09)	1.0 $\pm$ 0.0 (1.4 $\pm$ 0.09)	1.2 $\pm$ 0.06 (1.4 $\pm$ 0.05)	1.2 $\pm$ 0.08 (1.4 $\pm$ 0.09)	1.2 $\pm$ 0.05 (1.4 $\pm$ 0.06)
2,3-dihydrowithanolide E (II)	1.2 $\pm$ 0.08 ^c (1.4 $\pm$ 0.09)	1.3 $\pm$ 0.07 (1.4 $\pm$ 0.09)	1.5 $\pm$ 0.08 (1.4 $\pm$ 0.09)		
Compound III	1.7 $\pm$ 0.05 (1.5 $\pm$ 0.0)		1.8 $\pm$ 0.1 (1.5 $\pm$ 0.0)		
4 β -Hydroxywithanolide E (IV)	1.3 $\pm$ 0.05 ^c (1.4 $\pm$ 0.09)	1.5 $\pm$ 0.1 (1.4 $\pm$ 0.09)	1.7 $\pm$ 0.2 (1.4 $\pm$ 0.06)		1.6 $\pm$ 0.09 (1.4 $\pm$ 0.06)
Compound VI		1.6 $\pm$ 0.09 (1.8 $\pm$ 0.07)	1.5 $\pm$ 0.1 (1.8 $\pm$ 0.07)		
Compound VII	0.9 $\pm$ 0.03 ^c (2.4 $\pm$ 0.02)		1.9 $\pm$ 0.08 (2.4 $\pm$ 0.02)		
Withanicandrin + Daturalactone A (IX+X)	0.2 $\pm$ 0.06 ^d (2.1 $\pm$ 0.07)	0.4 $\pm$ 0.1 ^e (2.1 $\pm$ 0.07)	0.8 $\pm$ 0.1 ^c (2.1 $\pm$ 0.07)	1.1 $\pm$ 0.09 (2.2 $\pm$ 0.08)	1.9 $\pm$ 0.1 (2.5 $\pm$ 0.06) 2.3 $\pm$ 0.1 (2.7 $\pm$ 0.1)
Nicalbin A (XIV)	All but 2, out of 240, dead	0.01 $\pm$ 0.00 ^{a,b} (1.8 $\pm$ 0.1)	0.2 $\pm$ 0.1 ^c (1.7 $\pm$ 0.1)	0.6 $\pm$ 0.06 (1.8 $\pm$ 0.1)	0.7 $\pm$ 0.04 (1.9 $\pm$ 0.06)
Nicalbin B (XV)	0.2 $\pm$ 0.02 ^c (1.3 $\pm$ 0.07)	0.5 $\pm$ 0.06 (1.3 $\pm$ 0.07)	1.2 $\pm$ 0.1 (1.3 $\pm$ 0.07)		1.9 $\pm$ 0.1 (1.8 $\pm$ 0.1)
Nic-I (XVI)	0.5 $\pm$ 0.007 ^c (1.5 $\pm$ 0.0)		1.5 $\pm$ 0.07 (1.5 $\pm$ 0.0)		

^aThe larvae were too small to be weighed in all replicates except two (out of twenty). Larvae which could not be weighed because they were too small (weight of 12 larvae <0.1 mg), were assigned weight 0 and included in calculating the mean weight of larvae.

^b147 out of 240 larvae were found at weighing time.

^cAt this and lower concentrations, practically all larvae were found at weighing time and could be weighed.

^dLarvae too small to be weighed in 10 out of 25 replicates; 141 larvae out of 300 found at weighing time.

^eLarvae too small to be weighed in 10 out of 25 replicates; 229 larvae out of 300 found at weighing time.

Table 4. The toxic effect on larvae and pupae of Tribolium castaneum kept from the neonate stage in treated diets (animals from experiments in Table 7); T=27°C.

I, percentage of larvae dying before the prepupal stage; II, cumulative percentage of larvae dying before and during the prepupal stage; III, cumulative percentage mortality up to pupation; IV, percentage mortality up to the adult stage plus percentage of pupal-adult intermediates and abnormal adults. All results corrected for control mortality according to Abbott's formula.

		Concentration of compound in diet (%)				
Compound	Criterion	0.05	0.025	0.01	0.005	0.0025
Percentage of insects affected according to criteria I-IV						
Withanicandrin + daturalactone (IX+X))	I	67.9	40.1	10.0	1.4	0
	II	67.9	40.1	10.0	1.4	0
	III	67.9	40.1	10.0	1.4	0
	IV	78.6	41.7	11.1	2.9	0
Nicalbin A (XIV)	I	99.2	46.9			
	II	99.2	46.9			
	III	99.2	48.4			
	IV	99.2	53.1			

6.2 Antifeedant activity of selected withanolides against *Boarmia selenaria*

Table XXII shows that only the two highest concentrations (0.1%) and 0.075%) of nicalbin A (24) and the highest concentration (0.1%) of withanicandrin (22) and to a lesser extent of physalin B, showed some antifeedant activity. The rest of the compounds were inactive in this insect. It has to be stressed that even in the three compounds showing some activity, it disappeared if initial larval weight exceeded 10 mg.

Table XXII - Antifeedant properties of pure compounds against 5-10 mg *Boarmia selenaria* larvae. There were 1-4 20-larva replicates for each concentration of every compound; t=27°C.

Compound	Concentration of compound in dipping liquid (%)				
	0.1	0.075	0.05	0.025	0.01
	Average weight gain/larva during 48 h (mg)				
Nicalbin A (24)	1.9(6.3)*	0.4(3.1)	3.7(3.1)	2.7(3.1)	6.0(5.8)
Withanicandrin (22)	0.8(6.3)	2.0(3.0)	2.7(3.8)	2.3(3.0)	4.4(4.8)
4 β -Hydroxywithanolide E (15)	3.7(4.4)	3.8(4.0)			
Withanolide E (11)	5.2(6.6)				
α -Withanolide E (37)	6.1(5.4)				
1-Hydroxy-2,3-dihydrowithanolide E (31)	4.9(5.4)				
Nicalbin B (24)	5.8(5.4)				
2,3-Dihydrowithanolide E (17)	4.8(5.4)				
Nic-1 (Nican-drenone) (2)	7.6(6.6)				
Withaferin A (6)	9.6(7.8)				
Withanolide D (9)	7.0(7.8)				
6 α -isowithanolide E (41)	5.9(6.1)				

*The number in parentheses denotes weight gain in untreated controls.

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Description of cooperation

Extracts of solanaceous plants prepared in the U.S.A. and pure compounds isolated in Israel from other solanaceous species, were bioassayed for antifeedant effects against species of insects characteristic for each of the two countries. Details of these experiments are given in Ch. 5.

There was thus a continuous exchange of materials and information between the Israeli and the U.S. laboratories.

E. Glotter and K.R.S. Ascher visited the Agricultural Research Center at Beltsville and M. Jacobson visited the Faculty of Agriculture at Rehovot and the Volcani Center at Bet Dagan. These mutual visits contributed to the coordination of our research efforts.

Evaluation of the research achievements

This evaluation is done with respect to the original research proposal (vide there, pp. 14 - 19)

The acceptability by S. littoralis larvae of the solanaceous plants grown in experimental plots at the Faculty of Agriculture of the Hebrew University at Rehovot was determined. Also, many of these plants were extracted and the extracts bioassayed for antifeedant activity. Promising plants which have been shown to contain antifeedants, especially those producing withanolide E and 4 β -hydroxywithanolide E, were cultivated on a larger scale.

An extensive screening of extracts of numerous solanaceous plant species and fractions obtained thereof for antifeedant activity was conducted in both the U.S.A. and Israel.

Compounds present in crude extracts of individual plant species and chemotypes were determined by HPLC and other advanced methods and their bioassays conducted as detailed in the research proposal.

A number of new compounds were isolated from plants, and further, numerous synthetic analogs were prepared (see Ch. 3).

Elucidation of chemical structures of new compounds isolated was achieved by classical chemical procedures as well as by IR and UV spectroscopy, circular dichroism, n.m.r. and mass spectroscopy. Structure-activity relationships (SAR) were determined as far as possible.

The time schedule of the work plan was as follows:

Time Schedule of Work Plan

Israel, Hebrew University of Jerusalem (HUJ), Faculty of Agriculture

- 1st year: Raising additional plants from seeds.
Preparation of extracts as outlined in Ch. 5-a
(general scheme)
Preparation of larger quantities of the compounds
described in our earlier works; many of them are now
available only as analytical samples for comparison
purposes.
- 2nd year: Structural elucidation of new compounds.
Preparation of degradation products and derivatives.
- 3rd year: Preparation of synthetic analogs, according to the
results of structure-activity studies.

Israel, Agricultural Research Organization (ARO) Institute of Plant Protection

- 1st year: Screening of feeding-inhibition of plants.
Testing extracts
Primary screening of steroids on 'Styropor' and
screening of selected compounds on plants.
- 2nd year: Primary screening of steroids on 'Styropor'
(continued)
Screening promising compounds on plants in the
laboratory.
Primary screening of degradation products and
derivatives.

3rd year: Continuation of the screening.

U.S.A. Beltsville Agricultural Research Center (BARC)

- 1st year: Preparation of extracts of species of Withania, Physalis, Dunalia and Capsicum, which are available in the U.S. Laboratory evaluation of these extracts, and of samples received from Israeli cooperators, as feeding deterrents and growth regulators at Beltsville, Maryland; Vincennes, Indiana; and Wooster, Ohio.
- 2nd year: Continuation of laboratory (and greenhouse) biological evaluation of samples received from Israeli cooperators.
- 3rd year: Continuation of biological evaluation of materials from U.S.A. and Israel at Beltsville, Vincennes and Wooster, including field evaluation.

According to the above, practically everything (except field screening in the U.S.A.) outlined in the original time schedule was accomplished.

Benefit to agriculture

The time schedule of work which eventually crystallized from this research proposal did not envisage field trials in Israel. The immediate benefit of the work is the demonstration that a whole spectrum of powerful plant-derived insect antifeedants can be isolated from some selected solanaceous plant species. Interestingly enough, some of these compounds are specific for Coleoptera, and others, mainly for Lepidoptera. This renders powerful support to the plant-derived insect antifeedant approach.

List of publications under BARD sponsorship

1. Ascher, K.R.S., Eliyahu, M. Glotter, E., Kirson, I. and Abraham, A. Distribution of the chemotypes of Withania somnifera in some areas of Israel: feeding studies with Spodoptera littoralis larvae and chemical examination of withanolide content (1984) *Phytoparasitica* 12(3-4):147-155.
2. Glotter, E., Goldman, A., Kirson, I. and Zviely, M. Selective cleavage of ditertiary glycols under mild conditions, with bisacetylacetonato-oxovanadium (1985) *J. chem. Soc. Perkin I*, 229-231.
3. Glotter E., Sahai, M., Kirson, I. and Gottlieb, H.E. Physapubenolide and pubescenin, two new ergostane-type steroids from Physalis pubescens (Solanaceae) (1985) *J. chem. Soc. Perkin I*, 2241-2245.
4. Glotter, E. and Zviely, M. Stereoselective vanadium-catalysed epoxidation of $\alpha\beta$ -unsaturated ketones possessing a neighbouring hydroxy-group (1986) *J. chem. Soc. Perkin I*, 327-331.
5. Ascher, K.R.S., Eliyahu, M., Glotter, E., Goldman, A., Kirson, I., Abraham, A., Jacobson, M. and Schmutterer. The antifeedant effect of some new withanolides against three insect species: Spodoptera littoralis, Epilachna varivestis and Tribolium castaneum (1986) *Phytoparasitica*, submitted.
6. Ascher, K.R.S. and Glotter, E. The antifeedant effect of steroids isolated from solanaceae on different insect species (1986). Sixth intern. Congress of Pesticide Chemistry, Ottawa, Canada, 2D/E-19 (Communication).