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INHIBITION OF SPIRACLE AND CROCHET FORMATION AND JUVENILE HORMONE ACTIVITY OF ISOTHIOCYANATE DERIVATIVES IN THE TOBACCO HORNWORM, *Manduca sexta*

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Abstract

The structure-activity relationship of various aliphatic, alicyclic, and aromatic mono- and bisisothiocyanates affecting the growth and development of the tobacco hornworm, *Manduca sexta*, larva was studied. While topical application of short chain aliphatic monoisothiocyanates to 3rd instar *M. sexta* larvae did not affect larval development, aromatic mono- and diisothiocyanates were toxic within the dose range tested. Short chain alkylene and alicyclic 1,3- or 1,4-bisisothiocyanate derivatives, however, prevented the formation of abdominal spiracles and of crochets on the abdominal prolegs at molts following treatment. Their ED_{50} values for causing spiracular abnormalities ranged from 25 to 208 nmol/larva. Moreover, long chain mono or bisisothiocyanates with structural resemblance to known juvenoids had juvenile hormone activity in the *black M. sexta* assay (their ED_{50} range from 0.12 to 4.8 nmol/larva) and, in some cases, the formation of supernumerary 6th instar larvae was observed. Surprisingly, non-juvenoidlike bisisothiocyanates derived from α-amino acids ornithine and lysine also showed characteristic juvenile hormone effect in this test system (the ED_{50} values are 0.11 and 0.34 nmol/larva, respectively). The structural requirements for the two different types of activities as well as hypotheses about the possible mode of actions are also discussed.

INTRODUCTION

Insect development and reproduction, both involving a specific series of events, are regulated by a complex interplay of ecdysteroids and juvenile hormones (JHs)[1]. The possibility that analogues of these natural hormones, especially those of JHs, can be used as insect control agents has initiated intensive research in both academe and industry resulting in well over 10,000 JH analogs (juvenoids) synthesized in research laboratories worldwide in search for safe and selective insecticides. The principal chemical structure of almost all juvenoids resemble or mimic the sesquiterpenoid backbone of the natural JHs (for general reviews, see 1-3). Parallel to the research on JHs, there has been a search for anti-JH agents, which could either disrupt JH biosynthesis or JH action (4). One of the few non-terpenoid juvenoids is a series of aliphatic bisthiolcarbamate derivatives reported by Pallos et al. (5). Among these compounds, bisthiolcarbamate **1** showed mixed JH agonist/antagonist activity when tested on the tobacco hornworm, *Manduca sexta*, while a related thiolcarbamate herbicide **2** (thiobencarb) was shown to possess JH antagonist activity in the same assay (6,7).

Dithiocarbamates and thioureas are also known to inhibit tyrosinase and dopamine β-hydroxylase enzymes of the catecholamine biosynthesis in vertebrates (8,9) and could possibly interfere with the formation of dopamine derivatives involved in sclerotization of insect cuticle (10-12).

Based on these considerations, we prepared and assayed on third instar *M. sexta* larvae a series of new thiol-, thion-, and dithiocarbamic acid derivatives, including isothiocyanates (NCSs), of lipophilic mono- and diamines expected to interfere with either the hormonal regulation of metamorphosis or cuticle synthesis. We found that certain alkylene-bisNCSs had a novel effect on preventing the formation of abdominal spiracles and of the hooks (crochets) on the abdominal prolegs at the following molt to the fourth instar (13,14). Some of these larvae died in the molt; others grew slowly and died before the next molt with a few surviving the molt to the 5th instar.

In the present paper, we describe the results of a detailed structure-activity relationship study of these compounds. Furthermore, we show that some *bis*NCS derivatives possess JH activity and discuss the structural requirements for characteristic JH-like effects as observed in the *black* larval *M. sexta* assays.

MATERIALS AND METHODS

Compounds. The structures of all the compounds tested are given in Figure 2. Thiourea (**13**) and phenylisothiocyanate (**19**) were obtained from Reanal (Hungary), and Aldrich (USA), respectively. The dihydrochloride salt of L-2,4-diamino-butanoic acid was obtained from Aldrich, while those of L-ornithine and L-lysine were from Reanal. The amino acids were esterified by standard methods (15). Isophorone diisocyanate (IPDI) was a gift from NUODEX-Hüls (USA). Starting material diamines, 2-aminomethyl-3,3-5-trimethyl-cyclopentanamine (TMPCD) and isophorone diamine (IPD), as mixtures of geometric isomers, were kindly provided by Hüls AG (FRG). Thiadiazinethion (**12**) was synthesized by Dr. I. Bélai (Plant Protection Institute, Budapest). The synthesis of (thio)carbamates **3**, **4**, and **5** was accomplished by acylating TMPCD with the appropriate chloroformate derivative. Cyclic thiourea **6** was prepared by usual methods from TMPCD. Adamantyl derivatives **17** and **18** were prepared from the corresponding 1-bromo- or 1,3-dibromoadamantane using the methods of Stetter and Wulff (16,17).

For the synthesis of the other NCSs two general procedures were used: *A*) *Kaluza* method: reaction of the (di)amine with CS_2 in the presence of a base, then reaction of the resulting (bis)dithiocarbamic acid salt with ethyl chloroformate to form a dithiocarbamic acid - carbonic acid mixed anhydride which was then thermally decomposed to the corresponding (bis)isothiocyanate ; *B*) The (di)amine was allowed to react with thiophosgene in the presence of a base to give directly the appropriate isothiocyanate.

The following isothiocyanates are known and were prepared by method *A*): **14** and **25**; **16** (18), **20** (19), **22**, **23**, and **24** (20), **29** and **30** (21). Compounds **8**, **9**, **10**, **26**, **27**, and **32** are new and were prepared by from the corresponding precursor diamines. 1,3-Diamines, the

starting materials for compounds **26, 27,** and **32,** were synthesized using the method of Fleischer et al. (22). The following NCSs are known and were prepared by method *B*): **15**; **21** (23) and **28** (21). Compound **31** is new and its precursor amine was prepared according to Fischer et al. (24).

The crude products were purified by either distillation or chromatography using benzene or chloroform as eluent to give the pure isothiocyanates in 45-75% overall yield. The structure of the products was proven by elemental analysis, as well as by IR, ^{1}H-, and ^{13}C-NMR spectra. In cases of cyclic compounds **3-11** no attempts were made to separate the stereoisomers and the mixture of these isomers was used in the assays.

A) A representative procedure for the preparation of NCSs from amines and CS_2 using the *Kaluza*-method is as follows.

Compounds **7** and **8**. To an ice-cold mixture of TMPCD (15.6 g, 0.10 mol), chloroform (75 ml), water (45 ml), and NaOH (8.4 g, 0.21 mol) was added CS_2 (15 ml, 0.25 mol) during 2 min. After vigorous stirring at room temperature for 4 hr, the mixture was diluted with chloroform (75 ml) then cooled to 0 ^{0}C and ethyl chloroformate (21.8 g, 0.20 mol) was added dropwise during 2 min. The reaction mixture was then stirred at room temperature for 4 hr, the phases were separated, the aqueous layer extracted with chloroform, the organic phases were combined, washed with cold water and brine, dried ($MgSO_4$), and concentrated *in vacuo* at 30 ^{0}C. A two-gram sample of the crude mixed anhydride was purified by chromatography using chloroform as eluent to give 1.2 g pure **7** which solidified under 20 ^{0}C.

Elemental analysis for $C_{17}H_{28}N_2O_4S_4$ (%): C 45.10 calcd., 44.89 found; H 6.23 calcd., 6.21 found; N 6.19 calcd., 6.18 found; S 28.33 calcd., 28.80 found.

IR(neat): 3250, 3200, 2960, 1780, 1690, 1530, 1465, 1390, 1180, 1105, 1010, 920, and 850 cm^{-1}.

^{1}H-NMR($CDCl_3$): δ 10.4(m,2H), 4.3(m,4H), 3.7-4.0(m,3H), 1.8(m,1H), 1.3(m,7H), 0.95-1.14(m,11H).

The rest of the crude **7** was then stirred and heated at 110-120 ^{0}C *in vacuo* for 3 hr, then distilled to give 16.3 g (68 %) of pure **8** as a yellow, pungent oil. Bp.: 198-210 ^{0}C/1 mmHg.

Elemental analysis for $C_{11}H_{16}N_2S_2$ (%): C 54.95 calcd., 53.12 found; H 6.71 calcd.,

6.68 found; N 11.66 calcd., 11.45 found; S 26.68 calcd., 26.39 found.

IR(neat): ν 2960, 2930, 2869, 2100, 1460, 1391, 1371, 1345, 1243, and 690 cm^{-1}.

^{1}H-NMR($CDCl_3$): δ 3.4-3.7(m,3H), 1.9-2.2(m,2H), 1.65(m,2H), and 0.95-1.13(m,9H).

B) A representative procedure for the preparation of NCSs from amines and thiophosgene is as follows.

Compound **31**: Thiophosgene (63 µl, 0.82 mmol) was added to an ice-cooled mixture of 2-(4-phenoxyphenoxy)ethylamine (24) (150 mg, 0.65 mmol), chloroform (20 ml), water (10 ml), and $NaHCO_3$ (220 mg, 2.62 mmol). After stirring at room temperature for 1.5 h, the reaction mixture was poured onto ice, the phases were separated, the aqueous layer was extracted with chloroform, the organic extracts were combined, washed with cold water and brine, dried ($MgSO_4$), concentrated, and the residue was purified by chromatography (eluent: 10% ethyl acetate in hexane) to give 109 mg (62%) of **31** as white crystals. Mp.: 57-58 ^{0}C.

Elemental analysis for $C_{15}H_{13}NO_2S$ (%): C 66.39 calcd., 65.22 found; H 4.83 calcd., 4.76 found; N 5.16 calcd., 5.15 found; S 11.18 calcd., 11.35 found.

IR(CCl_4): ν 2105, 1503, 1489, 1349, 1223, 792, 762, and 691 cm^{-1}.

^{1}H-NMR($CDCl_3$): δ 3.85(2H,t,*J*=5.3Hz), 4.12(2H,t,*J*=5.3Hz), 6.8-7.0(m,5H), 7.30(m,4H).

(±)-α-Difluoromethylornithine (DFMO) was a generous gift from Dr. P.P. McCann, Merrel Dow Research Institute, Cincinnati, OH.

Animals

Tobacco hornworm larvae, *Manduca sexta*, were reared on artificial diet in a 12L:12D photoperiod at 25 ^{0}C at 60% relative humidity as described by Bell and Joachim (25).

Assay procedures

Spiracular and crochet assays. Third instar larvae were selected at the time of ecdysis. Four to 6 hr later after the cuticle had stabilized, the compounds were topically applied in 1 µl acetone (Aldrich, HPLC grade) along the dorsal midline. Compounds insoluble in acetone were dissolved in water and 1 µl was injected laterally between the 2nd and 3rd abdominal segments. Larvae were checked daily. After ecdysis to the 4th instar, the larvae were carefully observed for cuticular, spiracular and crochet abnormalities. They were then

observed after ecdysis to the 5th instar and to the pupa.

Spiracular abnormalities were classified according to the scoring system outlined in Table 1. Each spiracle (prothoracic and 8 abdominal) was scored individually, then the average score per treated larva was obtained. For a particular dose the scores for each larva were then averaged over the number treated (N=10-50). The ED_{50} value of the most active compounds was determined from the dose-response curve obtained from the calculated average scores.

Control animals (5-10 in each series of experiment) were treated with acetone (2 µl).

Black larval assay for juvenile hormone activity

Compounds were tested for JH activity using the *black Manduca* larval assay (26). Each compound was topically applied in 1 µl acetone (Aldrich, HPLC grade) laterally between the 2nd and 3rd abdominal segment. Animals were scored for degree of melanization 2 days later after the molt to the 5th instar.

Pupal assay for juvenile hormone activity

Compounds were dissolved in light mineral oil and 25 µl injected into *M. sexta* pupae 29 hr after ecdysis (27). Animals were scored at the time of the next ecdysis.

Oncopeltus assay

Compounds were dissolved in acetone (Aldrich, HPLC grade) and 1 µl applied to the abdomen of 5th (final) instar nymphs of the milkweed bug, *Oncopeltus fasciatus*, within 6-8 hr after ecdysis. The animals were scored for larval characteristics after the following ecdysis (28).

RESULTS

Activity of isothiocyanates and related compounds on larval spiracle and crochet formation

Our previous study showed that alkylene *bis*NCSs (**7, 8, 10,** and **22** in Figure 2) applied to third instar *Manduca* larvae selectively blocked spiracle and crochet formation in the following molt (13). The *mono*NCSs (**14** and **17**) were inactive at 100 µg per larva. To determine the structural requirements necessary for this activity, we have prepared additional

mono- and *bis*NCSs and assayed them for this activity. A preliminary report on the activity of some of these compounds has appeared (14).

Some of the compounds were found to be cytotoxic to the cells in the area of the topical application. This cytotoxicity was manifest by the loss of epidermal pigment within 24 hr and the absence of cuticular papillae and other marking elements in the new 4th instar cuticle. In extreme cases no or very weak 4th instar cuticle would be produced in the region at the next molt indicating the loss of a substantial number of epidermal cells. On the following tables, this cytotoxicity is indicated and in most cases was found to be dose-dependent.

As previously noted, when a compound caused the loss of either spiracles or crochets in the 4th instar, no regeneration of these structures was seen after the molt to the 5th instar (13). However, in the assays described below, we noted that when there was intermediate damage (particularly scores 2 and 3) in the 4th instar, the spiracle was able to recover to some extent during the subsequent molt, indicating some epidermal repair. No regeneration of crochets was observed.

Carbamate derivatives and monoisothiocyanates. The carbamate derivatives **3**, **4**, **5** (14), thiourea (**13**), and cyclic thiourea **6** at 100 µg/larva had no noticeable effects on growth and development. Also, dazomet (**12**), a known methylisothiocyanate precursor, the short chain monoNCS **14** and the bulky adamantyl derivative **17** were inactive (14).

By contrast, 100 µg of the long chain decylisothiocyanate (**15**) killed all larvae within 24 hr, whereas a 10-fold lower dose had no effect on growth and development (Table 2). Similarly, the undecanoic acid NCS derivative **16** was lethal at the high dose, but this showed local cytotoxicity and adversely affected growth and development at the lower doses as well (Table 2). One of the 15 larvae treated with 10 µg underwent a supernumerary larval molt after forming an undersized 5th instar larva below the critical size required for metamorphosis (29). No specific action on either the spiracles or the crochets was observed. Phenylisothiocyanate (**19**) was also ineffective on the spiracles but was quite toxic at 50 µg/larva (Table 2). By contrast, phenylethylisothiocyanate (**21**), a naturally occurring insecticide in some cruciferous crops (23), was not only cytotoxic at the site of application and lethal at 100 µg/larva (Table 2), but also showed selective dose-dependent effects on the

spiracles (Figure 3a) with an ED_{50} of 208 nmol/larva (Table 5). This compound also caused crochet loss at doses of 25 µg or above.

The phenoxyphenoxy NCS derivative **31**, in which the carbamate functionality of the juvenoid fenoxycarb (30) was replaced by an NCS group, was only slightly active on the spiracles (Figure 3b). At the highest dose 25% of the treated larvae underwent a supernumerary molt from a normal size 5th instar larva (Table 2), indicating that the juvenoid activity was retained.

Bisisothiocyanates. Unlike the monoNCSs, aliphatic compounds containing two NCS groups showed selective activity on the spiracles and crochets as characterized earlier (13). The simple 1,3-bisNCS **22**, the 1,4-bisNCS **23**, and the isopropyl-substituted 1,3-bisNCS **25** were the most active in causing spiracular malformations (Figure 3a) with ED_{50} values of 25-37 nmol/larva (Table 5). Consequently, mortality was high, both in the 4th and 5th instars (Tables 3 and 4). Interestingly, the 1,3-disubstituted compounds were cytotoxic and adversely affected crochet formation, whereas the 1,4-bisNCS derivative **23** had neither effect at doses below the lethal dose (Tables 3 and 4). The 1,6-bisNCS **24** was about twofold less active on the spiracles (Figure 3a; Table 5), but was both cytotoxic and prevented crochet formation (Table 4).

The octyl (**26**) and alkoxyalkyl (**27**) 1,3-bisNCS derivatives showed only local spiracular action near the site of application with very little dose-dependency (Figure 3b). Yet both were cytotoxic and effectively inhibited development of the larvae in the 4th and 5th instars so that few treated with doses greater than 10 µg metamorphosed (Table 3). Three of the 12 treated with 100 µg of compound **27** were undersized 5th instar larvae which underwent an extra larval molt, then succumbed. The phenoxyphenoxy derivative **32** had little activity on spiracle formation or on development (Table 3).

Among the cyclic bisNCSs, the cyclopentane derivative **8** was effective in disrupting spiracular development in a dose-dependent manner (Figure 3a) with an ED_{50} of 154 nmol/larva (Table 5). This compound also interfered with crochet formation and severely curtailed larval development (Table 3). The less rigid cyclohexane derivatives **9** and **10** had moderate to slight effects on the spiracles which were not dose-responsive (Figure 3b). The

1,3-substituted adamantane derivative **18** had no effect on spiracular development. Importantly, the cyclic bis*isocyanate* derivative **11** (Figure 3a) was more active than the corresponding bisNCS **10** (Figure 3b) on the spiracles with an ED_{50} of 58 nmol/larva, but had no effects on crochet development (Table 3).

The aromatic diNCS **20** was extremely toxic killing nearly all treated larvae at doses higher than 0.01 µg. Even at 0.005 µg/larva, only 76% of the larvae was able to molt to the 4th instar (Table 3). No selective action on spiracle formation was seen.

To examine the influence of another functionality of the aliphatic chain on the activity of 1,3-, 1,4- and 1,6-bisNCSs **22**, **23**, and **24**, related α-amino acid derivatives **28**, **29**, and **30** were synthesized and assayed (Table 4). The lysine derivative **30** was slightly cytotoxic but inactive on the spiracles. The other two compounds had slight effects on the spiracles (Figure 3b) with ED_{50} values greater than 100 µg/larva. Thus, the introduction of the alkoxycarbonyl group resulted in a substantial drop in the spiracular activity of these bisNCSs.

JH-like activity of isothiocyanates and related compounds

In the assay for anti-spiracular activity, compounds **29** and **31** caused a true supernumerary larval molt in a few of the treated larvae (Tables 2 and 4). Therefore, these compounds as well as the others were tested for JH activity using the *M. sexta black* larval assay (26). Table 6 shows the ED_{50} values for the seven compounds showing any significant activity in this assay. The bisNCS amino acid derivatives **29** and **30**, the adamantyl derivative **18** and the phenoxyphenoxy monoNCS derivative **31** are the most active compounds with ED_{50} values of 0.11-0.76 nmol/larva. These values are similar to the effect of (10*R*)-JH-III (ED_{50}=0.6 nmol/larva) and about 100-fold less than that for (10*R*,11*S*)-JH-I (31). The long chain bisNCSs **26** and **32** have 5-60-fold lower JH activity (Table 6). Interestingly, the cyclic bisNCS **8** was completely inactive in the *black* assay, but its possible precursor, the mixed anhydride **7**, had slight JH activity (ED_{50}=3.5 nmol/larva).

To further confirm that the bisNCS amino acid derivatives were acting as JHs, we also tested them in the *Manduca* pupal assay (27) and in the *Oncopeltus* assay (28). In the

Manduca pupal assay 100 µg of either **29** or **30** produced pupal cuticle at the site of injection indicating a weak JH activity. Yet these two compounds were effective JH mimics in the *Oncopeltus* assay showing ED_{50} values of 11.5 and 6.4 nmoles, respectively (N=9-15 per dose; 4 doses assayed between 1 and 40 nmoles). In this assay JH-III was found to have only weak activity at 0.4 nmol but higher doses were not tested (28).

DISCUSSION

These studies show that 1,3- and 1,4-alkenyl and cycloalkenyl bisNCS derivatives have a novel selective cytotoxic action on the spiracular epidermis of *Manduca* larvae. When these larvae molt, the lack of sufficient oxygenation of the tissues due to the absent or severely deformed spiracles effectively retards or prevents growth so that few metamorphose. The 1,3-alkenyl and -cycloalkenyl derivatives also selectively damage the crochet epidermis so that some of the crochets on the prolegs are not formed in the subsequent molts which could be detrimental under field conditions since the crochets are used to grip the plant. The overall effect is to prevent metamorphosis and thus these compounds are acting as insect growth regulators. Surprisingly, the amino acid derivatives of the 1,4- and 1,5-bisNCSs showed little of this activity but acted as juvenile hormone mimics with activities about 5-fold higher than JH-III in the *black* larval assay.

This structure-activity analysis of the bisNCSs shows clearly that for optimal effectiveness on spiracular epidermis, the -N=C=S groups should be separated by three or four carbon atoms. Introduction of a long side-chain, as in **26**, **27**, and **32**, was detrimental to this particular activity although the first two compounds were cytotoxic at the site of application. The introduction of a cycloalkane ring decreased activity with the cyclopentanyl bisNCSs being considerably more active than the cyclohexanyl derivatives. Furthermore, the cylohexanyl derivatives showed no dose-responsiveness, which might be due to the fact that these substances were mixtures of geometric isomers in which the two NCS groups are in either *cis* or *trans* positions. The isomers may have different activities and/or different

metabolic fates that could account for their variable action on each treated larva resulting in large standard deviations in the average scores. The rigid **18** had no effect on spiracles, indicating that some flexibility provided by the cycloalkane ring is necessary for this activity.

These changes in the side chain affect not only the shape and presumed active conformation of the molecule at the target site, but also determine the lipophilicity, and consequently the transport and distribution of the molecules in the organism. For instance, the introduction of an oxygen into the side chain of compound **26** decreased lipophilicity and caused a loss of JH activity, but a slight increase in cytotoxicity and growth inhibition of the resulting **27**. Also, the metabolic fate of these different derivatives may not be the same although insects usually effectively metabolize NCS allelochemicals (32).

The insecticidal properties of some NCSs of natural origin (mustard oils) found in many cruciferous plants is well documented (33,34). Recently, the larvicidal activity of several aromatic and short-chain aliphatic NCSs has been described (35; for a general review on the chemical and biological properties of NCSs, see 36). The NCS groups are capable of carbamoylating nucleophiles (e.g., amino, sulfhydryl, guanidino groups) of biopolymers. Therefore, these bisNCSs might act as bifunctional cross-linking agents. Indeed, some aromatic isocyanates and iso*thio*cyanates have been used as cross-linking reagents in protein analysis (37,38). Moreover, the cross-linking action of bifunctional alkylating agents is known to disrupt the replication of DNA and thus to confer cytotoxicity (for an overview, see 39). The chemical reactivity and spacing of the NCS groups in these compounds is thus responsible for their cytotoxic properties, but why some should be specific for the spiracular or crochet epidermis remains a mystery. Interestingly, the replacement of the -N=C=S groups in **10** by the more reactive -N=C=O groups to give bisisocyanate **11** caused a considerable increase in selective spiracular cytotoxicity without any indication of cytotoxicity to the general epidermal cells.

The crochet epidermis seems to be particularly sensitive to many different types of compounds such as azadirachtin (40), benzyl-1,3-benzodioxoles (41,42), and colchicine (42). Since colchicine is known to inhibit tubulin polymerization and thus cellular events that depend on microtubules such as mitosis (43,44) and elongation of setae and bristles (45) such

as occurs during crochet formation, it is possible that these NCS compounds act similarly. Although DNA synthesis appears to be an early response of the spiracular epidermis to ecdysteroids in the pupal molt (46), the role of mitoses in spiracular formation has not been investigated.

Our original rationale for designing thiocarbamate related compounds as potential insect growth regulators was based on the consideration that (di)thiocarbamates and thioureas, acting as strong copper-chelators, might inhibit oxidase enzymes such as tyrosinase (*o*-diphenol oxidase) and dopamine β-hydroxylase involved in catecholamine metabolism (8,9). These sulfur-containing compounds were thought to disturb the biosynthesis of catecholamine derivatives important for cuticular sclerotization (10-12). Although related N,N-diethyldithiocarbamate and phenylthiourea have been shown to be strong inhibitors of tyrosinase of *Manduca sexta* pupal cuticle *in vitro* (47), neither the carbamate **3-6** nor the isothiocyanate derivatives **7-32** tested disrupted general larval cuticular sclerotization and stabilization after ecdysis except for the local cytotoxicity effects which appeared to result from the death of the epidermal cells directly contacted by the compounds. Thiourea (**13**) had no adverse effect in *Manduca* although it has been found to disrupt growth and molting and therefore to be lethal to nymphs of the cockroach, *Periplanata americana* (48). Similarly, the bisdithiocarbamate fungicide maneb delayed molting and was lethal to nymphs of the milkweed bug, *Oncopeltus fasciatus* (49). Cytotoxicity was evident in the autolysis of cells of the midgut epithelium and of the Malphigian tubes in this case. Maneb also prevented mitosis of cells of the midgut epithelium of this insect. Presumably the lethality at the high doses and local cytotoxicity seen at lower doses of some of the long chain and aromatic monoNCSs in our study reflects a similar mode of action of these compounds.

Most compounds that act as JH agonists have similar chemical structures to the natural sesquiterpenoid hormones (for a critical review, see 2). Yet a few compounds that lack the terpenoid skeleton act also as JH mimics such as the bisthiocarbamate **1** (5) and certain aromatic thiolcarbamates (50,51). Citronellylisothiocyanate, to our knowledge the only NCS tested as candidate juvenoid, had no JH activity in *Tenebrio molitor* (52). In our assays on

Manduca the two NCS derivatives of fenoxycarb (**31** and **32**) retained some JH activity with the mono derivative being 40 times more active than the *bis*-derivative, but it was 2,000-fold less active than the parent compound. The adamantyl bisNCS **18** was as active as JH-III whereas the octyl bisNCS **26** was 3 times less active.

The surprising finding in this study was that two bisNCS derivatives of the amino acids ornithine (**29**) and lysine (**30**) were about 5 times more active than JH-III in the *black Manduca* larval bioassay. They were also active in the pupal *Manduca* and the *Oncopeltus* larval bioassays confirming that they act as JH mimics rather than simply interfere somehow with the melanization process itself such as with the action of the granular phenoloxidase (53-55). These lipophilic compounds have very little structural similarity to JH except for the ester group shown to be necessary for this activity.

One possibility is that these compounds are not mimicking the JH at the target site, but somehow disrupting subsequent events initiated by the JH-receptor complex. Since compound **29** is a 1,4-bisNCS derivative of ornithine, it is possible that it could be mimicking JH action by interfering with the activity of ornithine decarboxylase (ODC), an enzyme necessary for synthesis of polyamines which are critical for cell proliferation and division (56,57). This enzyme increases in response to ecdysone at the initiation of adult development (58), and the phorbol ester-induced increase in ODC activity in bovine lymphocytes can be inhibited by JH-III (59). Therefore, Willis (60) proposed that JH prevents metamorphosis by interfering with the production of polyamines. To test this hypothesis, we injected up to 2 mg/larva of α-difluoromethylornithine (DFMO), a known mechanism based irreversible inhibitor of ODC activity (61) into *black Manduca* larvae at different times during the critical period for JH action in the prevention of melanization. All but 2 of the 97 treated larvae melanized normally, irrespective whether the DMFO was given immediately before or during the most sensitive period for JH action or up to the time of the premelanin granule and phenoloxidase deposition (53-55). These findings are similar to those of Willis (60) who found that DFMO inhibited ODC activity in developing adult wings of *Hyalophora cecropia in vitro* but did not mimic JH in causing pupal cuticle synthesis. Although this inhibitor has a short half-life *in vivo* (P.P. McCann, personal communication), these experiments indicate

that the JH-mimicking activity of these amino acid derivatives is not likely due to an interference with ODC activity. However, other mechanism(s) by which these bisNCSs derange the complex polyamine metabolic network is also possible.

Importantly, the bisNCSs that are most active as JHs have little or no selective action on spiracles. Conversely, those that showed strong activity in the spiracular assay had no JH activity, clearly indicating the separation of these two effects. In both cases, however, they are bifunctional compounds in which the two reactive NCS moieties are optimally separated by three or four methylene groups.

These studies show that 1,3- and 1,4-bisNCS derivatives can disrupt insect growth and development through either a selective destruction of the spiracular epidermis or through action as a JH mimic depending on the overall structure of the molecule. The bifunctional carbamoylating ability of these bisNCSs is essential for the two different types of effects. Clarification of their unique mode of action and identification of the target site(s) of these compounds should help in understanding the hormonal regulation of insect metamorphosis and may serve as a starting point for the rational design of novel anti-insect agents.

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[1]Abbreviations used: JH, juvenile hormone; NCS: isothiocyanate; IPDI: isophorone diisocyanate; TMPCD: 2-aminomethyl-3,3,5-trimethylcyclopentaneamine; DFMO: α-difluoromethylornithine; ODC: ornithine decarboxylase.

TABLE 1

Scoring system for assessing spiracular damage in *Manduca sexta* larvae

Score	Description
0	Normal spiracle.
1	Slight irregularities in shape and appearance.
2	Irregularities with abnormal size openings; cuticular lining abnormal; pigmentation often abnormal; usually reduced in size.
3	Size reduced to less than half; cuticular and pigment abnormalities; often hole with very little cuticular lining.
4	Severely reduced spiracles with no cuticular lining.
4.5	No apparent spiracle, but melanized spot where tracheae attach.
5	No spiracular opening.

TABLE 2

Activity of monoisothiocyanates on *Manduca sexta* larvae

Compound No.	Dose µg/larva	Number of larvae tested	% molting to 4th (% 4th with missing crochets)	% to 5th instar larvae	% to pupae (% 6th)
Acetone	2 µl	57	100	100	100
15	100	10	0	0	0
	10	10	100	100	[a]
16	100	15	0[b]	0	0
	50	15	67[b]	60	40
	25	15	60[b]	60	60
	10	15	80[b]	80	73(7)[c]
	1	10	100	100	100
19	100	8	0	0	0
	50	18	22	6	0
	30	10	100	100	100
21	100	10	10[bd](100)	0	0
	50	10	100[bd](100)	70	70
	25	10	100[d](40)	100	100
	10	10	100	100	[a]
31	100	12	100[bd]	100	83(25)
	50	12	100[d]	100	92
	25	12	100[d]	100	100

[a] Not observed.
[b] Local cytotoxicity as described in text.
[c] Formation of a supernumerary larval instar due to limited growth during the 4th instar so that the newly molted 5th instar larva was below the critical size for metamorphosis (29).
[d] Spiracular anomalies in the 4th instar; see Figure 3 and Table 5.

TABLE 3

Activity of bisisothiocyanates on spiracle and crochet formation of *Manduca sexta* larvae

Compound No.	Dose µg/larva	Number of larvae tested	% molting to 4th	(% 4th with missing crochets)	% to 5th instar larvae	% to pupae (% 6th)
7	100	20	90[bd]	(40)	70	70
	50	20	100[bd]	(40)	95	95
	25	20	100[bd]	(30)	90	90
	10	20	100[d]	(15)	100	100
	5	10	100[d]	(0)	100	100
8	100	32	100[d]	(31)	72	28
	50	20	100[d]	(25)	75	65
	25	34	100[d]	(12)	82	74
	10	43	100[d]	(9)	93	88
	5	13	100[d]	(0)	100	100
	2.5	10	100[d]		100	[a]
	1.0	21	100		100	100
9	100	32	94[d]		94	90[e]
	50	45	100[d]		98	80[e]
	25	50	100[d]		98	60[e]
	10	49	96[d]		100	88[e]
	5	30	100[d]		90	87[e]
	2.5	15	100[d]		100	100
	1.0	11	100[d]		100	[a]
	0.1	12	100[d]		100	[a]
10	100	20	70[d]	(25)	50	30
	50	20	70[d]	(25)	65	60
	25	20	100[d]	(15)	70	70
	10	20	100[d]	(5)	100	100
	5	10	100[d]	(10)	100	100
	2.5	10	100[d]	(0)	100	100
	1.0	10	100[d]		100	100
11	100	15	93[d]		13	0
	50	15	100[d]		7	0
	25	15	100[d]		40	0
	10	15	100[d]		80	73
	5	15	100[d]		93	87
	2.5	15	100[d]		100	100
	1.0	15	100[d]		100	100
18	100	23	96		96	96
20	0.1	20	0		0	0
	0.01	20	10		10	10
	0.005	21	76		76	76
	0.0025	10	100		100	100

25	100	10	60[bd](20)	10	0
	50	10	90[bd](20)	30	0
	25	20	100[bd](40)	25	0[e]
	10	20	100[bd](15)	55	10[e](5[c])
	5	10	100[d](0)	70	40
	2.5	10	100[d]	100	100
	1.0	20	100[d]	100	100[e]
26	100	20	80[bd]	45	0[e]
	50	20	100[bd]	40	0[e]
	25	20	100[bd]	60	30[e]
	10	20	100[bd]	85	60[e]
	1.0	10	100	100	100[e]
27	100	12	75[bd](42)	33	0(25[c])
	25	15	73[bd](0)	67	[a]
	10	27	100[bd](3)	70	25[e]
	5	15	100[bd](13)	100	[a]
	2.5	15	100[d](0)	100	[a]
	1.0	27	100[d](0)	100	100[e]
32	100	12	100	100	100

[a] Not observed.
[b] Local cytotoxicity as described in text.
[c] Formation of a supernumerary larval instar due to limited growth during the 4th instar so that the newly molted 5th instar larva was below the critical size for metamorphosis (29).
[d] Spiracular anomalies in the 4th instar; see Figure 3 and Table 5.
[e] Based on 10-25 animals observed until pupation.

TABLE 4
Activity of amino acid related bisisothiocyanates on spiracle and crochet formation of *Manduca sexta* larvae

Compound No.	Dose µg/larva	Number of larvae tested	% to molting to 4th (% 4th with missing crochets)	% to 5th instar larvae	% to pupae (% 6th)
22	50	8	0	0	0
	25	22	36[bd](88)	9	0
	10	18	78[bd](57)	50	17
	5	21	100[bd](14)	76	57(5[c])
	2.5	20	100[d](15)	100	90(10[c])
	1.0	17	100[d](6)	100	100
23	10	10	70[d]	0	0
	3	29	100[d]	90	67[e]
	2	15	100[d]	100	[a]
	1.5	15	100[d]	100	[a]
	1.0	25	100[d]	100	100[e]
	0.1	10	100	100	100
24	100	10	10[d]	10	0
	50	20	55[bd](27)	20	20
	25	20	85[bd](18)	50	35
	10	20	80[bd](12)	65	60
	5	20	100[bd](5)	100	100
	2.5	20	100[bd](0)	100	100
	1.0	10	100[bd]	100	100
28	100	11	82[bd]	82	[a]
	30	10	100[bd]	100	[a]
	10	10	100[d]	100	[a]
	3	10	100[d]	100	[a]
	1	10	100	100	[a]
29	100	27	100[bd]	96	44(33)
	50	12	100[bd]	50	17
	25	42	100[bd]	88	52[e](2)
	10	42	100[bd]	100	76(2)
	5	15	100	100	[a]
30	100	15	87[b]	80	80
	10	10	100	100	100

[a] Not observed.
[b] Local cytotoxicity as described in text.
[c] Formation of a supernumerary larval instar due to limited growth during the 4th instar so that the newly molted 5th instar larva was below the critical size for metamorphosis (29).
[d] Spiracular anomalies in the 4th instar; see Figure 3 and Table 5.
[e] Based on 10-25 animals observed until pupation.

TABLE 5

Effectiveness of compounds on spiracles of *Manduca sexta* 4th instar larvae[a]

Compound	ED_{50} nmol/larva	Cytotoxicity µg
25	25	≥25
22	30	≥5
23	32	≥10
11	58	≥25
24	70	≥5
7	88	≥25
8	154	≥50
21	208	≥50
31	>370	~100
26	>370[b]	≥10
27	>380[b]	≥25
10	>390	≥50
29	>430[b]	≥10
28	>460	≥30
15	>500	none

[a] The ED_{50} values were determined from the dose-response curves plotted as in Figure 3.

[b] Cytotoxic at indicated level at site of application and this often included spiracles nearest the major application site.

TABLE 6

JH-mimic activity of isothiocyanates in the *Manduca sexta black* larval assay[a]

Compound	ED_{50} nmol/larva
29	0.11
31	0.12
30	0.34
18	0.76
26	2.0
7	3.5
32	4.8
JH-III	0.6
JH-I[b]	0.0015

[a] The ED_{50} values were determined as described in the Materials and Methods section.

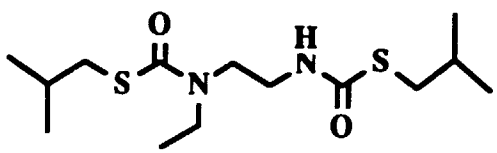
[b] Data shown for comparison, the ED_{50} value for JH-I is taken from Ref. 31.

LEGENDS

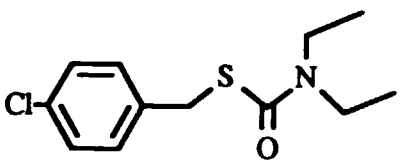
FIGURE 1. Chemical structures of bisthiolcarbamate **1** and thiobencarb **2**.

FIGURE 2. Chemical structures of isothiocyanates and related compounds tested in this study. For synthetic details, see text in the Materials and Methods section.

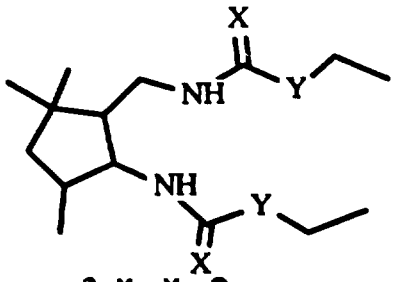
FIGURE 3. Dose-response curves for spiracular activity of bisisothiocyanates and related compounds. Each dose was tested on at least 10-20 larvae, and the average spiracular score of each larva was then averaged over the number treated yielding an average score per dosage with a range of standard deviations (SDs) from 0.13-0.92. The higher SDs (>0.5) were generally observed with the less active compounds in Figure 3b.



1



2



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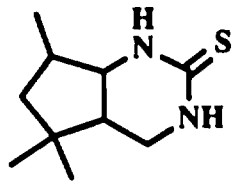
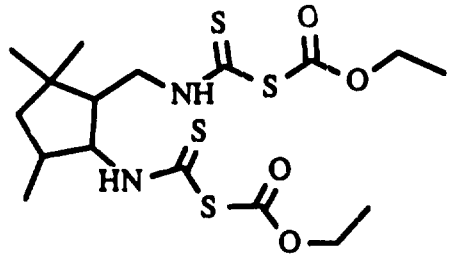
4 X = O, Y = S
5 X = Y = S



6



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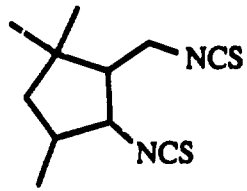

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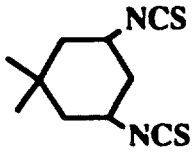

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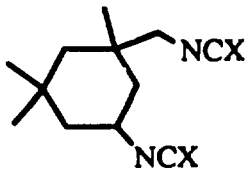
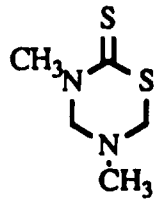


10 X = S
11 X = O



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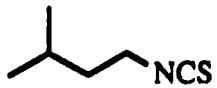
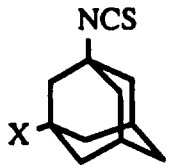
13



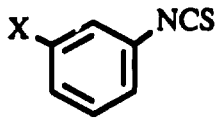
14

X – CH_2 $(CH_2)_9$– NCS

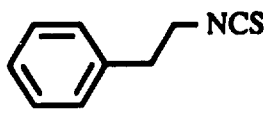
15 X = H
16 X = CO_2CH_3



17 X = H
18 X = NCS



19 X = H
20 X = NCS



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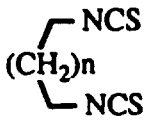
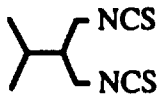


22 n = 1
23 n = 2
24 n = 4



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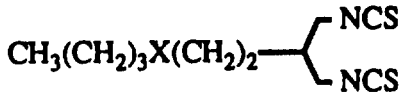
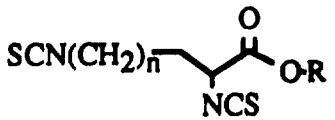
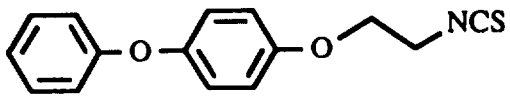


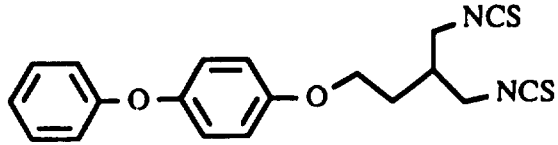
26 X = CH_2CH_2
27 X = O



28 n = 1, R = CH_3
29 n = 2, R = CH_3
30 n = 3, R = CH_2CH_3



31



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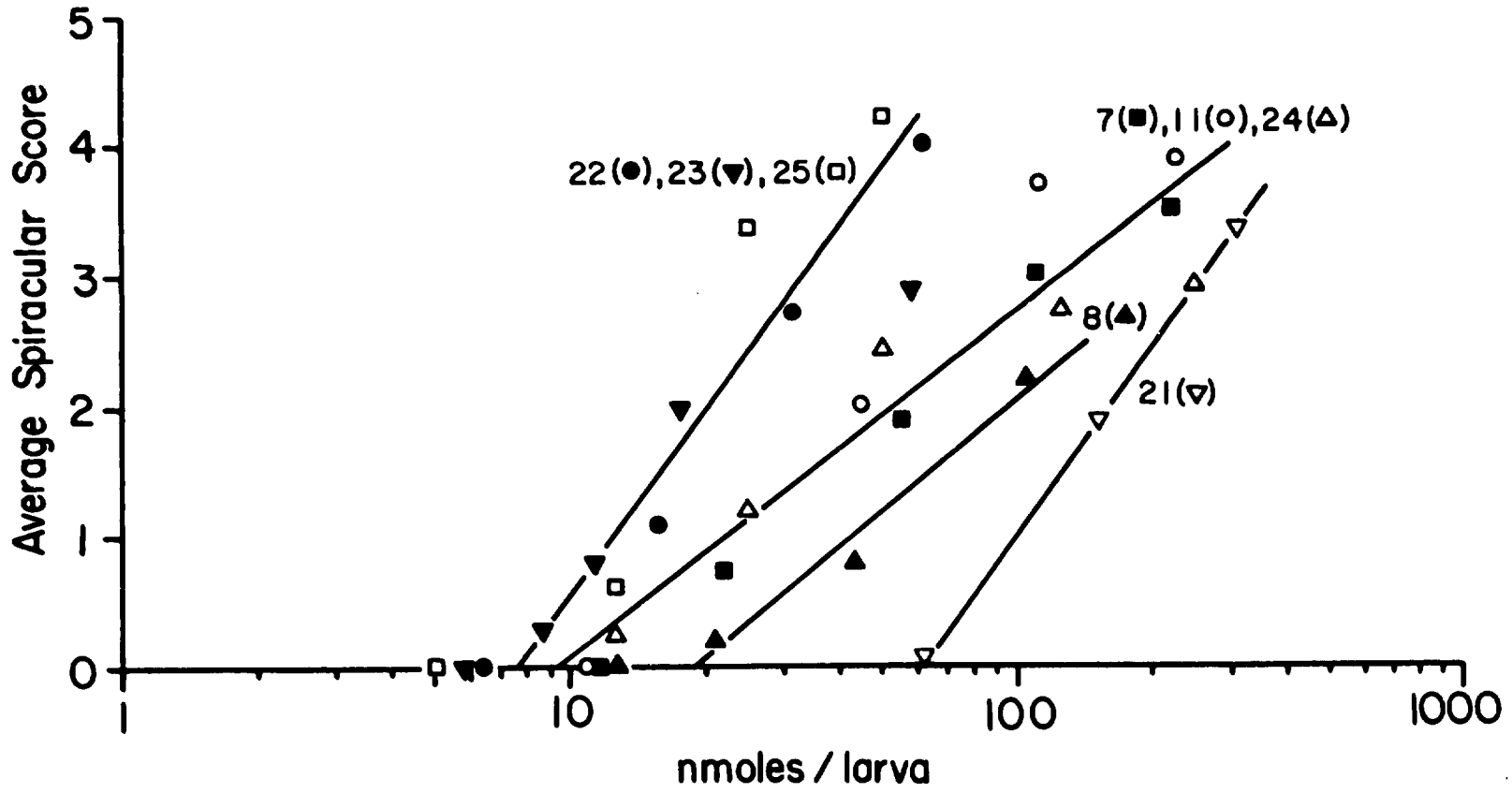
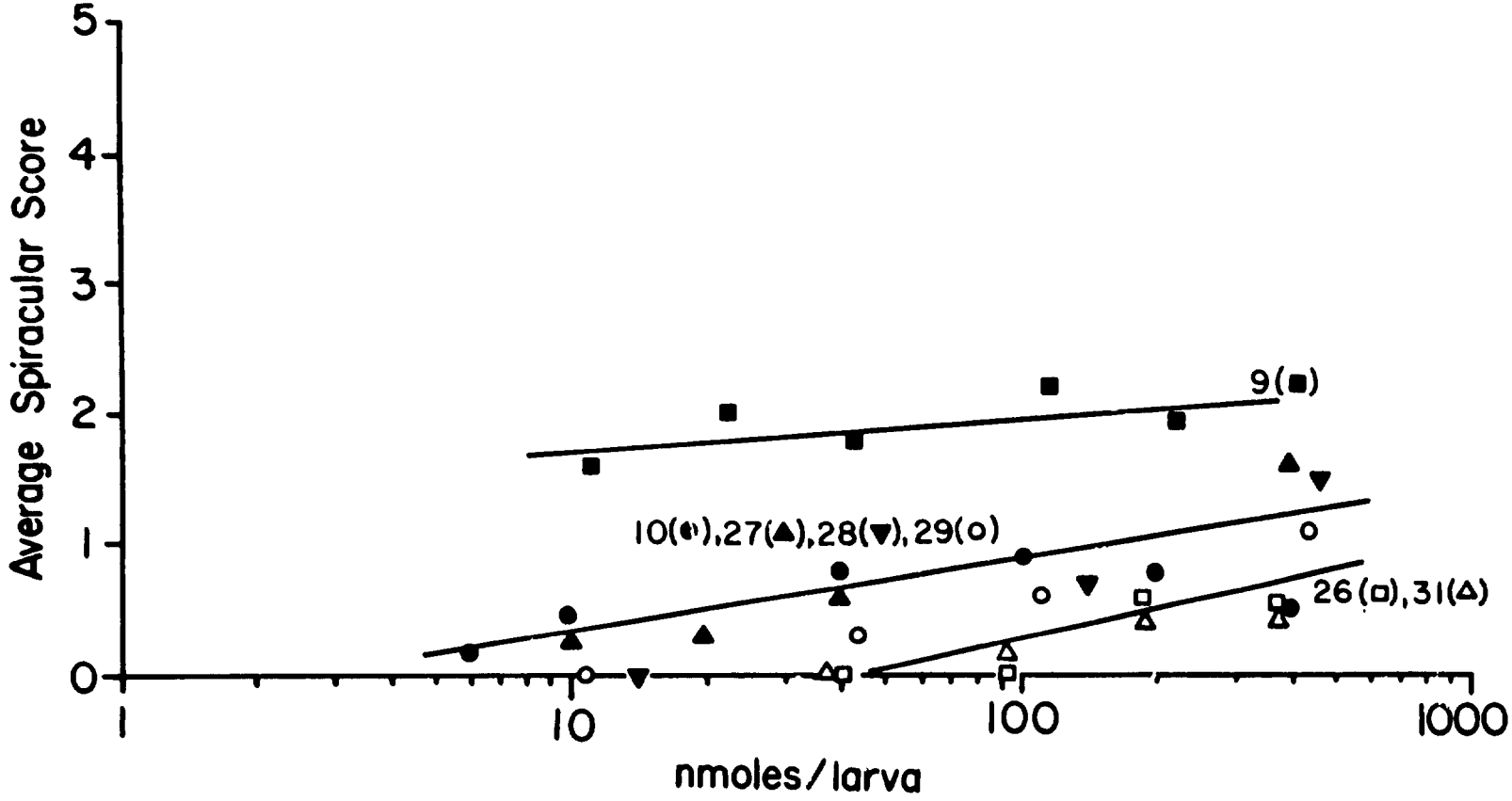

Average Spiracular Score
0
1
2
3
4
5
1
10
100
1000
nmoles / larva
22(●),23(▼),25(□)
7(■),11(○),24(△)
8(▲)
21(▽)


Average Spiracular Score
5
4
3
2
1
0
1
10
100
1000
nmoles/larva
9(■)
10(●),27(▲),28(▼),29(o)
26(□),31(△)