The location of GRF-LI neurons in the preoptic nucleus in rainbow trout was consistent with that found in codfish (*Gadus morhua*)[12]. The relation between these two GRF-containing nuclei (NLT, NPO) in fish is unclear. However, immunohistochemical evidence from rats showed that GRF-LI neuronal processes from ARC, VMN and other areas of the hypothalamus reach anteriorly to the preoptic area (POA)[22]. Moreover, the POA is known to be the area containing somatostatinergic neurons[29] and in association with the inhibitory control of GH secretion in rats[22]. This information implies the existence of a complex relationship among hypothalamic nuclei in regulating GH release.

Acknowledgment. The antiserum against rGRF (1–37), carp GRF (1–45), and carp GRF (1–29) were generous gifts from Dr J. Rivier, The Salk Institute, California, and the antiserum against hpGRF (1–44) was kindly supplied by Dr N. Sherwood, University of Victoria, Canada. A special note of thanks to Dr R. C. Fargher for his advice in this work and Dr R. J. Snyder for his comments on the manuscript. This research was supported by a grant to BAM from the National Science and Engineering Research Council of Canada.

1 Rivier, J., Spiess, J., Thorner, M., and Vale, W., Nature *300* (1982) 276.
2 Frohman, L. A., and Jansson, J.-O., Endocr. Rev. *7* (1986) 223.
3 Lechan, R. M., Lin, H. D., Ling, N., Jackson, I. M. D., Jacobson, S., and Reichlin, S., Brain Res. *309* (1984) 55.
4 Bruhn, T. O., Anthony, E. L. P., Wu, P., and Jackson, I. M. D., Brain Res. *424* (1987) 290.
5 Lin, H. D., Bollinger, J., Ling, N., and Reichlin, S., J. clin. Endocr. Metab. *58* (1984) 1197.
6 Merchenthaler, I., Vigh, S., Schally, A. V., and Petrusz, P., Endocrinology *114* (1984) 1082.
7 Leidy, J. W. Jr, and Robbins, R. J., J. clin. Endocr. Metab. *62* (1986) 372.
8 Shibasaki, T., Kiyosawa, Y., Masuda, A., Nakahara, M., Imaki, T., Wakabayashi, I., Demura, H., Shizume, K., and Ling, N., J. clin. Endocr. Metab. *59* (1984) 263.
9 Jozsa, R., Korf, H.-W., and Merchenthaler, I., Cell Tissue Res. *247* (1987) 441.
10 Bosman, F. T., and Assche, C. V., J. Histochem. Cytochem. *32* (1984) 1139.
11 Bruhn, T. O., Mason, R. T., and Vale, W. W., Endocrinology *117* (1985) 1710.
12 Pan, J. X., Lechan, R. M., Lin, H. D., Sohn, J., Reichlin, S., and Jackson, I. M. D., Endocrinology *116* (1985) 1663.
13 Luo, D., and McKeown, B. A., Comp. Biochem. Physiol. (1989) in press
14 Hinton, D. E., J. Fish Res. Board Can. *32* (1975) 416.
15 Weller, T. H., and Coons, A. H., Proc. Soc. exp. Biol. *86* (1954) 789.
16 Karp, G., in: Cell Biology, pp. 744. McGraw-Hill Book Company, New York 1984.
17 Wilson, L., and Bryan, J., Adv. cell. molec. Biol. *3* (1974) 21.
18 Garland, D. L., Biochemistry *17* (1978) 4266.
19 Andreu, J. M., and Timasheff, S. N., Proc. natl Acad. Sci. USA *79* (1982) 6753.
20 Daikoku, S., Kawano, H., Noguchi, M., Nakanishi, J., Tokuzen, M., Chihara, K., and Nagatsu, I., Brain Res. *399* (1986) 250.
21 Bloch, B., Brazeau, P., Ling, N., Bohlen, P., Esch, F., Wehrenberg, W. B., Benoit, R., Bloom, F., and Guillemin, R., Nature *301* (1983) 607.
22 Muller, E. E., Physiol. Rev. *67* (1987) 962.
23 Jacobowitz, D. M., Schulte, H., Chrousos, G. P., and Loriaux, D. L., Peptides *4* (1983) 521.
24 Fellmann, D., Bugnon, C., and Lavry, G. N., Neurosci. Lett. *58* (1985) 91.
25 Billard, R., and Peter, R. E., Reprod. Nutr. Develop. *22* (1982) 1.
26 Mill, P. J., in: Comparative Neurobiology, p. 193. Edward Arnold, London 1982.
27 Gorbman, A., Dickhoff, W. W., Vigna, S. R., Clark, N. B., and Ralph, C. L., in: Comparative Endocrinology, p. 45. John Wiley and Sons Inc., New York 1983.
28 Wagner, G. F., and McKeown, B. A., Cell Tissue Res. *231* (1983) 693.
29 Day, T. A., Oliver, J. R., Menadue, M. F., Davies, B., and Willoughby, J. O., Brain Res. *238* (1982) 55.

0014-4754/89/060577-04$1.50 + 0.20/0

Arylpyridyl-thiosemicarbazones: A new class of anti-juvenile hormones active against Lepidoptera

A. E. Barton, K. D. Wing, D. P. Le, R. A. Slawecki and R. Feyereisen*

*Research Labs, Rohm and Haas Co., 727 Norristown Road, Spring House (Pennsylvania 19477, USA), and *Dept of Entomology and Agricultural Chemistry, Oregon State University, Corvallis (Oregon 97331, USA)*
Received 2 August 1988; accepted 14 March 1989

Summary. A new class of anti-juvenile hormone agents is described. Active anti-juvenile hormone compounds were either diazine thiosemicarbazones or aryl substituted pyridyl thiosemicarbazones, synthesized from substituted benzaldehydes. While many analogs in these classes showed feeding and growth inhibition in a variety of insects, a select group caused formation of precocious pupal characteristics in *Agrotis ipsilon* (black cutworm) and *Heliothis virescens* (tobacco budworm) and black cuticle and precocious pupae in *Manduca sexta* (tobacco hornworm). They were active only by diet incorporation. The symptoms of precocious development could be reversed by co-administration of a juvenoid. One of the active compounds was shown to inhibit juvenile hormone biosynthesis in vitro by corpora allata of the cockroach *Diploptera punctata*. However, none of the compounds were active inhibitors of purified chicken liver prenyl transferase.
Key words. Thiosemicarbazones; anti-juvenile hormone; insect growth regulator; Lepidoptera; juvenile hormone biosynthesis inhibitor.

The search for new classes of insect growth regulators has been hampered by our lack of knowledge about the insect endocrine system and the paucity of different classes of compounds active in agronomic situations. At present only the benzoylphenylureas, exemplified by diflubenzuron and the newer more potent analog CGA-112913[1],

and the juvenile hormone analogs such as methoprene and fenoxycarb[2] are used to control agricultural pests. The juvenile hormone system of insects has been often quoted as being a desirable site of attack for novel pesticide synthesis. In theory, compounds which would accelerate metamorphosis and thus shorten the insect's destructive larval stage would be most suitable for agricultural situations. Unfortunately only a few chemical classes have been shown to have anti-juvenile hormone activity against the Lepidoptera, the most important order of pests, and in no case has the activity been of commercial level[3]. In this report we reveal a novel class of anti-juvenile hormones which acts against Lepidoptera, some of the structure-activity relationships within this class, and evidence supporting juvenile hormone biosynthesis inhibition as their possible mode of action.

Materials and methods

Synthetic methods. The pyridine and pyrazine thiosemicarbazones were prepared from the corresponding carbonyl compound by condensation with a carbodithioate followed by displacement of methanethiol with pyrrolidine[4] or by condensation of the carbonyl compound with pyrrolidine thiosemicarbazide in methanol[4].

Some of the aryl pyridine carbonyl compounds were known[5]. The synthetic routes previously utilized were not regio-specific, producing several isomeric aryl-picolines. As only the 6-aryl pyridine thiosemicarbazones were active, a regio-specific synthesis was required. A general and efficient route[6] afforded the 6-aryl picolines in good overall yield. Selenium dioxide oxidation[5] gave the desired carbonyl compounds.

Biology. Larvae of the tobacco budworm *Heliothis virescens* F. (U.S.D.A), tobacco hornworm *Manduca sexta* L. (Carolina Biological, USA) and black cutworm *Agrotis ipsilon* (Rohm and Haas culture) were all raised from the egg stage on commercial black cutworm diet (Bioserv, USA) at 26 °C, 16 L:8 D photoperiod. Newly ecdysed penultimate instar budworms (4th instar), hornworms (4th instar) or cutworms (5th instar) were placed on diet into which had been incorporated the compound by admixing a solution of 0.5 ml DMSO/acetone (1:1) containing the compound into 100 ml of diet while it was fluid (65 °C). The test larvae were kept in individual containers. The budworms were observed after 10 days and the hornworms and cutworms after 14 days for morphological outcome, and especially for growth inhibition and/or precocious pupal cuticle formation. EC_{50}'s were the dosages wherein 50% of the larvae displayed precocious pupal cuticle or other anti-JH signs, based on eye-fitted log dose-response curves. When the juvenoid fenoxycarb[7] was used, it was mixed directly into the same diet as the anti-juvenile hormone compound. Inhibition of juvenile hormone biosynthesis in isolated corpora allata was performed as described previously[8]. Inhibition of purified chicken liver prenyl transferase was performed by a modification of the methods of Rilling[9]. The enzyme was kindly donated by Prof. C. D. Poulter (Univ. of Utah, Dept. of Chemistry). The candidate thiosemicarbazone was preincubated with the enzyme for 10 min by addition of 1 µl of DMSO solution to 90 µl of the enzyme, before adding substrate. The reaction was allowed to proceed for 10 min, before termination by addition of 200 µl methanol-conc. HCl (4:1). Isooctane (1 ml, Burdick and Jackson, USA) was used for extracting the 14C-labelled farnesol. A 500-µl aliquot was removed for liquid scintillation counting.

Results and discussion

Figure 1 shows the anti-juvenile hormone effects of these compounds on three lepidopteran species. Tobacco budworm larvae gave the most consistent dose-dependent responses, ranging from larviform pupae to almost perfect tiny premature pupae. The responsive black cutworm showed heavily sclerotized pupal patches but never produced a perfect premature pupa, even at high doses. The tobacco hornworm larvae characteristically responded with a gradient of morphologies even at a single dose, ranging from larvae with blackened head capsules to black fifth instars to larviform pupae, all indications of juvenile hormone deficiency in this insect. The compounds were ineffective by topical administration or when sprayed leaves were fed to larvae. The reasons for this are currently unknown.

The structure-activity relationships displayed by the thiosemicarbazone anti-juvenile hormones are shown in table 1. It is immediately apparent that compounds active against the tobacco budworm require an ortho-substituted phenyl in the 6-position of the pyridine. The best substituents are Cl, Br, F and methyl. Ortho CF_3 and 2,4-dichloro are moderately active, while compounds substituted in the meta or para position were completely inactive as anti-juvenile hormones. Thus it appears that steric effects are more important than electronic effects in eliciting anti-juvenile hormone symptoms. Interestingly, the ortho-substituted compounds active against the budworm were merely growth inhibitors against the black cutworm; however the pyrazine derivative (compound **17**[4]) led to growth inhibition and pupal patches in the final larval instar of the cutworm. This is the first known report of anti-juvenile hormone effects on the black cutworm. Analogs containing unsubstituted phenyl, compounds without the phenyl, or alkyl or aryl substituents at other positions in the pyridine ring were completely inactive as anti-juvenile hormones. Likewise, major modification of the thiosemicarbazone moiety lead to a complete abolishment of anti-JH activity, though the reduced form (compound **8**) is as active as compound **1**. Many of the thiosemicarbazones were powerful antifeedants. Strong growth inhibition and delayed development are always associated with observed anti-JH effects in these

compounds. While controls show the usual dramatic weight gain, animals undergoing precocious development remained stunted. While it is commonly observed that poor nutrition or starvation can lead to formation of supernumerary larval instars [10], we observed exactly the opposite effect in these small larvae. As expected, the intoxicated tobacco budworms and tobacco hornworms initiated premature wandering and burrowing behavior before the precocious pupation. No premature development was observed in instars before the penultimate to final instar molt. When the juvenoid fenoxycarb was admixed with compound **1** at different concentrations and given to 4th instar tobacco budworm larvae, there was a dose-dependent increase in the number of final instar larvae produced, as opposed to precocious or larviform pupae (table 2). This clearly shows that the anti-juvenile hormone effects of compound **1** can be eliminated by a juvenoid. Additional experiments show that this

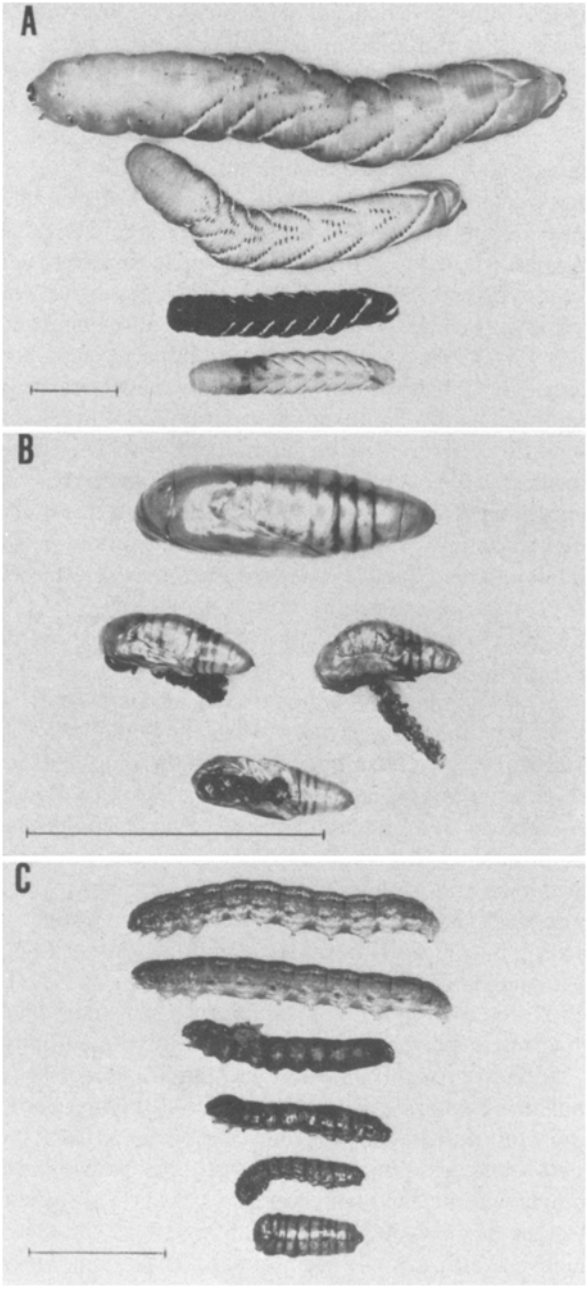
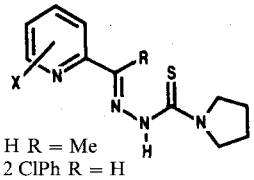
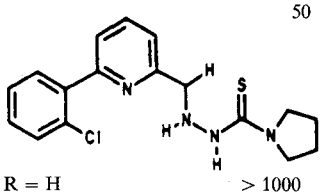
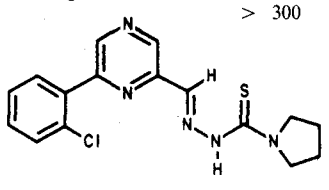
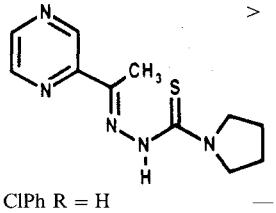


Figure 1. Anti-juvenile hormone effects of thiosemicarbazones on 3 lepidopteran species. *A Manduca sexta.* From top to bottom; control wandering fifth instar, control day 1 fifth instar, compound **1**-treated black fifth, compound **1**-treated precocious wanderer. *B Heliothis virescens.* Top control pupa, lower 3 compound **1**-treated precocious pupae. *C Agrotis ipsilon.* Top 2 control sixth instar larvae, lower 4 compound **17**-treated larvae possessing pupal cuticle. Marker in all figures = 2 cm.

Table 1. Anti-juvenile hormone activity of thiosemicarbazones towards penultimate instar Lepidoptera larvae*

Compound	Structure	EC_{50} (ppm in diet) *H. virescens*[a]	*A. ipsilon*[b]
			
**A	X = H R = Me	> 1000	> 1000
1	X = 2 ClPh R = H	50	> 100
2	X = 2 FPh R = H	70	> 100
3	X = 2 MePh R = H	70	> 100
4	X = 2 BrPh R = H	70	> 100
5	X = 2,4 ClPh R = H	90	—
6	X = 2 CF_3Ph R = H	100	> 100
7	X = 2 Cl,3,4 dioxolanoPh R = H	150	> 300
8		50	—
9	X = Ph R = H	> 1000	> 1000
10	X = 4 BrPh R = H	> 1000	> 1000
11	X = 4 SMePh R = H	> 1000	> 1000
12	X = 20 MePh R = H	> 1000	> 300
13	X = 2 Cl, 5 NO_2Ph R = H	> 1000	> 100
14		> 300	> 300
15	X = 2,5 MePh R = H	> 100	> 100
16	X = 4 FPh R = H	> 100	> 100
17		> 100	30
18	X = 3 ClPh R = H	—	> 1000
19	X = 3 BrPh R = H	—	> 1000
20	X = 1-naphthyl R = H	—	> 100

* Expressed as dosage where 50% of larvae demonstrate precocious pupal cuticle or other anti-juvenile hormone signs after incorporation of compound into the artificial diet; n ≥ 10 per dose, with at least 4 doses per treatment. Experiments where response was observed were replicated at least 3 times. [a] Responding animals were either precocious pupae or larviform pupae. [b] Responding animals were last instar larvae possessing pupal patches. ** The known 2-acetylpyridine thiosemicarbazone **A** [4] exhibits only growth inhibition but no anti-juvenile hormone activity against any of the test species.

Table 2. Inhibition of thiosemicarbazone-induced precocious development in L4 *H. virescens* larvae by co-administration of fenoxycarb in artificial diet*

ppm compd **1**	ppm fenoxycarb	Normal L5	L5 w/tan pupal patches	Precocious larviform pupa	Precocious pupa
100	0.0	0	0	50	50
100	0.01	0	0	60	40
100	0.1	0	10	90	0
100	1.0	0	100	0	0
100	10.0	100	0	0	0
0	10.0	100	0	0	0

* n = 10 or more larvae per treatment.

'rescue effect' can be elicited if the animal is fed compound **1** for less than 3 days; after this time the animal is apparently committed to precocious development. In an attempt to determine the reversibility of the inhibitory effect, corpora allata of precocious wandering larvae were removed and implanted into black 4th instar *Manduca sexta* larvae (a JH-deficient strain)[11], before spiracle apolysis. When the hosts were allowed to molt to 5ths, there was not a consistent ability to maintain black host pigmentation. Many of the hosts molted to green 5ths, indicating that the implanted corpora allata had become active again. Thus, presuming that the thiosemicarbazones were affecting JH biosynthesis, the effect seemed to be reversible (note that normal 5th instar wandering larvae have corpora allata which are inactive in this assay, resulting in hosts which molt to a black 5th). Compound **1** does not inhibit purified chicken liver prenyl transferase enzyme at concentrations up to 1×10^{-4} M. All assays were performed at enzyme concentrations and times which were well within the linear portion of the respective curves. Other anti-juvenile hormones such as the allyl alcohol derivatives[13] are thought to act on this enzyme. Incubation of compound **1** with isolated corpora allata of the cockroach *Diploptera punctata* led to the dose-response relationship seen in figure 2. Thus the EC_{50} of compound **1** in this system is 50 µM. These are levels of toxicant which could be achieved in vivo on

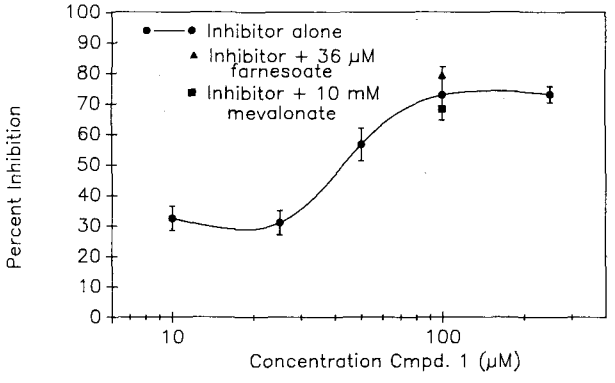


Figure 2. Inhibition of *Diploptera punctata* corpora allata juvenile hormone biosynthesis by compound **1**. All incubations were in 0.1 ml TC 199 medium with 0.05 mM [^{3}H-methyl] methionine for 3 h at 30 °C. Compound **1** was added in 0.5% DMSO; controls had 0.5% DMSO alone. Control release rate was 68.1 ± 2.7 pmol JH III/gland − h. All data are X ± SEM (n = 7).

poisoning with compound **1**. Thus compound **1** may be a JH biosynthesis inhibitor, though it could have other actions as well. The most active compound previously observed in this system was an acetylene-containing juvenoid, with a EC_{50} of 20 µM[12]. However, this compound also had measurable juvenile hormone agonist activity in vivo, while compound **1** has no JH activity whatsoever. In addition, when one attempts to 'rescue' the inhibition of corpora allata held in vitro by addition of farnesoate or mevalonate, there is no recovery of JH biosynthesis. From these data one may presume that the site of the inhibition is either at the epoxidation or methyl transfer step. Compound **1** and its analogs did not cause precocious metamorphosis in cabbage loopers, milkweed bugs, southern armyworms, boll weevils, Southern corn rootworms or immature cockroaches.

Thiosemicarbazones have been tested as anticancer agents[14] and antimalarials[15]. Inhibition of the DNA synthesizing enzyme ribonucleotide diphosphate reductase has been strongly implicated as the basis for these activities[14]. In addition, it has been known for some time that compounds of this class have strong growth inhibition properties in insects[4, 16]. This is the first report of thiosemicarbazones possessing anti-JH properties. While they appear to be of limited commercial potential, these compounds may prove to be useful models for the study of JH biosynthesis and its inhibition.

Acknowledgments. The authors thank Mei-Ann Liu (Harvard Univ.) and B. A. Sames (Rohm and Haas) for technical assistance and Drs G. R. Carlson, L. Safranek and Prof. Carroll Williams for helpful discussions.

1 Retnakaran, A., Granett, J., and Ennis, T., Comp. Insect Physiol. *12* (1985) 529; Ishaaya, I., Nemny, N. E., and Ascher, K. R. S., Phytoparasitica *12* (1984) 193.
2 Staal, G. B., et al., in: Bioregulators for Pest Control, p. 201. ACS Sympos. series 276 (1985).
3 Staal, G. B., A. Rev. Ent. *31* (1986) 391.
4 deMilo, A. B., Redfern, R. E., and Borkovec, A. B., J. Agric. Food Chem. *31* (1983) 713.
5 Chen, C.-T., Chen, S.-F., and Lee, S.-J., Heterocycles *5* (1976) 239.
6 Barton, A. E., and Le, D. P., Synthetic Commun., submitted for publication.
7 Fischer, U., Schneider, F., and Zurfluh, R., Chem. Abstr. *92* (1980) 643. (58473X) Eur. Pat. Specn. 4334F. Hoffmann-La Roche.
8 Feyereisen, R., Meth. Enzym. *111* (1985) 530.
9 Rilling, H. C., Meth. Enzym. *110* (1984) 145.
10 Jones, D., Jones, G., and Bhaskaran, G., Ent. exp. appl. *28* (1980) 259.
11 Safranek, L., and Riddiford, L. M., J. Insect. Physiol. *21* (1975) 1931.
12 Brooks, G. T., Pratt, G. E., Mace, D. W., and Cocks, J. A., Pest. Sci. *16* (1985) 132.
13 Quistad, G., Cerf, D. C., Kramer, S. J., Bergot, B. J., and Schooley, D. A., J. Agric. Food Chem. *33* (1985) 47.
14 Sartorelli, A. C., Adv. Enzym. Reg. *15* (1976) 117.
15 Klayman, D. L., Bartosevich, J. F., Griffin, T. S., Mason, C. J., and Scovill, J. P., J. med. Chem. *22* (1979) 855.
16 Pedersen, L. E. K., Svendsen, A., and Per, D., Klemmensen Pest. Sci. *25* (1984) 462.

0014-4754/89/060580-04$1.50 + 0.20/0