WTFRC Project AE-03-335

Project title: Feeding stimulants to increase efficacy of insecticides
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Cooperator(s): Dr. Carol A. Sheppard, Harmony Borkhard-Wier, Sarah Overbee, Shannon Reive, Scott Stewart

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Objectives: The studies previously done and published by PI and Co-PI in codling moth neonates (1,2) indicated that monosodium glutamate (MSG) stimulates feeding in neonates of this species. Therefore MSG can be successfully used for enhancement of insecticide formulations by either increasing their efficacy and/or maintaining their efficacy at reduced toxic ingredient amounts (3-6). MSG is moderately rain-fast, however (5). To solve the problem of moderate MSG rain-fastness, PI and Co-PI suggested use of trans-1-aminocyclobutane-1,3- dicarboxylic acid (*trans*-ACBD). *trans*-ACBD has superior rain-fastness, and feeding stimulatory and pesticide enhancing properties (7), however it is difficult to synthesize, and expensive because of its low-scale production for laboratory use only. The general objective of this project was to design or suggest another chemical substance that has feeding stimulatory properties similar to monosodium glutamate, but is cheaper than *trans*-ACBD.

To that end, we selected twelve chemicals that, similar to *trans*-ACBD, have amino and carboxylic acid groups attached to either cyclic or aliphatic (acyclic) hydrocarbon chain. There are three groups of such chemicals: (1) derivatives of aminocyclobutanecarboxylic acid, (2) derivatives of L-aminophosphono acids, and (3) derivatives of D-aminophopshono acids. Additionally, we selected for tests three other chemicals that are reported to have activity similar to that of *trans*-ACBD in vertebrate experimental systems. The following chemicals were tested in our study:

<u>derivatives of aminocyclobutanecarboxylic acid</u>

1. 1-Aminocyclobutane-1-carboxylic acid (ACBC)
2. *cis*-1-Aminocyclobutane-1,3-dicarboxylic acid (*cis*-ACBD)
3. *trans*-1-Aminocyclobutane-1,3-dicarboxylic acid (*trans*-ACBD)

Note: *trans*-ACBD was already tested during our previous research on codling moth feeding. The rationale for re-testing this drug in the present study was to evaluate the fidelity of our testing procedure in the new laboratory using insects that were shipped from Washington State to Kansas State.

<u>derivatives of L-aminophosphono acids</u>

1. L-(+)-2-Amino-4-phosphonobutyric acid (L-AP4)
2. L-(+)-2-Amino-5-phosphonopentanoic acid (L-AP5)
3. L-(+)-2-Amino-6-phosphonohexanoic acid (L-AP6)

<u>derivatives of D-aminophopshono acids</u>

1. D-(-)-2-Amino-4-phosphonobutyric acid (D-AP4)
2. D-(-)-2-Amino-5-phosphonopentanoic acid (D-AP5)
3. D-(-)-2-Amino-7-phosphonoheptanoic acid (D-AP7)

<u>chemicals with *trans*-ACBD activity in vertebrates</u>

1. L-Serine-O-phosphate (O-phospho-L-serine)
2. (L)-(+)-a-Amino-3,5-dioxo-1,2,4-oxadiazolidine-2-propanoic acid (quisqualate)
3. (RS)-(Tetrazol-5-yl)glycine

The effects of the aforementioned drugs on feeding initiation and intensity have been studied. Our proposal included using computer software for chemical structure analysis and comparison in the drugs tested. Because the project ends in March 2005, this task is just being undertaken during the upcoming three months. For now (December 2004), comparison of the structures of the chemicals increasing feeding behavior in codling moth has been done visually, without computer aid. However, even this tentative comparison clearly suggests a cheaper alternative to *trans*-ACBD.

There were no and will be no deviations from the original objective or schedule. The project will be finished in March 2005.

Significant findings:

- ACBC initiates and increases feeding at concentrations 0.1 mg/ml and 1 mg/ml
- Feeding stimulatory activity of the derivatives of L-aminophosphono acids increases together with decrease in length of aliphatic hydrocarbon chain of these acids
- The derivatives of L-aminophosphono acids have no effects on feeding behavior
- (RS)-(Tetrazol-5-yl)glycine initiates and increases feeding at concentrations 0.1 mg/ml and 1 mg/ml

Methods:

Insects

Codling moth adults (*C. pomonella)* originating from USDA-ARS at Yakima, WA, USA, were held at 25^{o}C, 70-80%RH, under a 16L:8D photoperiod. Wax paper was provided as an oviposition surface. The circadian hatch began approximately 6h into the photophase. The neonates used to test materials were collected on the first day of hatching, 1 h post-hatch.

Chemicals

All tested substances were purchased from Tocris Cookson, (St. Louis, MO), and dissolved in double distilled water, containing 0.02% Triton X-100.

Bioassay technique

Circular sections of uniform size (12 mm of diameter) were removed from Honeycrisp™ (U.S. patent No. 7197) foliage, avoiding the rib area. Test solutions (10 μl) were distributed evenly, using a strip of polyethylene foil, over the upper surface of the excised sections that subsequently were allowed to air dry. To prevent any loss of tested solutions throughout the drying procedure, the sections were placed, dry side down, on small plastic cubes, so the solution could form a meniscus, and surface tension prevented any run-off over the edges of the treated section. Next, the same procedure was employed for treatment of the sections' lower surfaces. Treated sections were placed in bioassay stations and each section was infested with one, 1h old neonate larva. To prevent dehydration, the bioassay stations were placed in Petri dishes with wet filter paper placed on the bottom of each dish. The surface area of each leaf disc that was consumed was determined using a stereo microscope equipped with an ocular square mesh reference scale (No. 12-561-RG2, Fisher Scientific, Pittsburgh, PA, USA). Thirty-three fragments of leaves, chosen randomly from mid-rib areas of the leaves, were also measured visually and then dried and weighed to establish a relationship between optical measurement and weight of foliage consumed. Based on this determination, consumed areas of the leaf were converted to an estimated dry weight of leaf. Remaining details of this bioassay are described elsewhere (2,5,8).

Experimental design

Every chemical was tested at the following concentrations: 0.0001, 0.001, 0.01, 0.1, and 1 mg/ml. Control leaves were treated with 0.02% Triton X-100 in double distilled water.

To evaluate the effects of tested drugs on feeding initiation, the following procedure was used. Sixteen experimental larvae were individually exposed to the aforementioned test solutions in bioassay stations. Control larvae (N=16 for each concentration of each substance) were exposed to 0.02% Triton X-100. The number of larvae feeding was monitored at 15 min intervals for 3h- period. This procedure was repeated 5 times. The results were expressed as average (mean ± SEM) length of time before commencement of feeding

To evaluate the effects of tested drugs on feeding intensity, 8 larvae were individually exposed to each concentration of each substance, and this procedure was repeated 4 times. Amounts of leaf tissue consumed by neonates were estimated 24h after infestation of each bioassay station, and expressed as average (mean ± SEM) leaf consumption.

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Statistics

No set of data on feeding induction passed normality tests ($P>0.05$). Here, mean times needed for induction of feeding were compared between groups exposed to respective sugars or sugar substitutes or to control solutions (0.02% Triton X-100) using Kruskal-Wallis test.

All data sets on leaf consumption passed tests for normality with $P<0.05$ (GraphPad InStat®, GraphPad Software Inc., San Diego, CA), and were subjected to regression analyses.

Regardless of the test used, results were regarded as significantly different at $P<0.05$.

Results and discussion:

Derivatives of aminocyclobutanecarboxylic acid

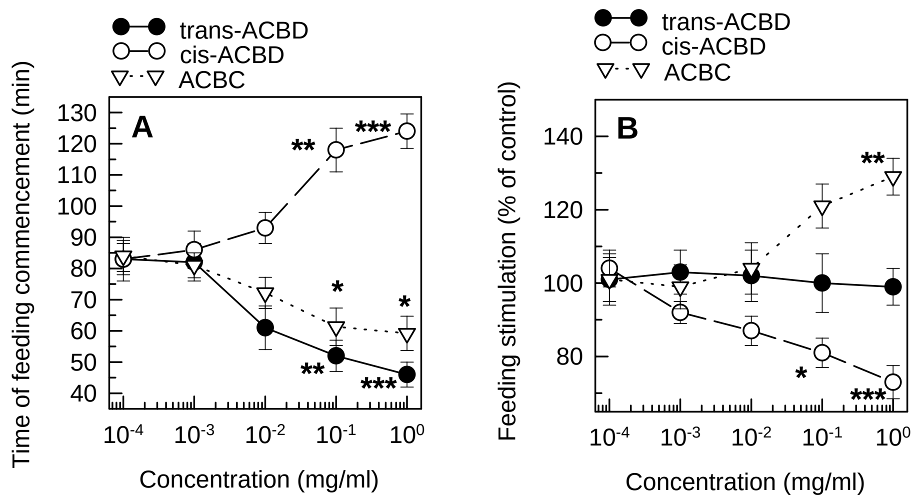


Fig. 1. The effects of aminocyclobutanecarboxylic acid derivatives on initiation (A) and intensity of feeding (B) by codling moth neonates. Each datum point refers to mean ± SEM obtained for ninety (A) or thirty-two (B) larvae. The time of feeding commencement in controls varied between 81.9 ±8.1 and 85.8±7.2 min. The amount of foliage ingested in control groups varied between 129.4 ± 11.8 and 136.6 ± 12.1 μg larva^{-1} . * P<0.05, ** P<0.01, *** P<0.001

Trans-ACBD initiated feeding, but had no effect on feeding intensity (Fig. 1A,B), a result which corroborates well with our previously published data (9).In contrast, *cis*-ACBD significantly decreased feeding by codling moth neonates (Fig. 1B), as well as delayed feeding commencement (Fig. 1A). This result is in concert with anecdotal evidence reported by other authors in exotic coleopterans (10). ACBC accelerates feeding commencement by over 30 minutes and stimulates feeding by approximately 30% in comparison to control (Fig. 1 A, and B, respectively). These findings demonstrate three facts. First, hydrogen and carboxylic acid groups have to be attached to cyclobutyl ring of aminocyclobutanecarboxylic acids in *trans*- and not in *cis*- conformation to accelerate feeding commencement in codling moth neonates. Secondly, *cis*-conformation of amino and carboxylic acid groups provides effects deterrent to codling moth neonates. Consequently, feeding stimulatory activity of cyclobutanecarboxylic acids may be modified by substitutions in the cyclobutyl ring. Thirdly, the results with ACBC show that only one pair of amino and carboxylic acid groups, on one side of the cyclobutyl ring, is needed for acceleration of feeding commencement and increasing amounts of foliage consumed (please refer to Table 1 for chemical structures of tested chemicals).

Derivatives of L-aminophosphono acids

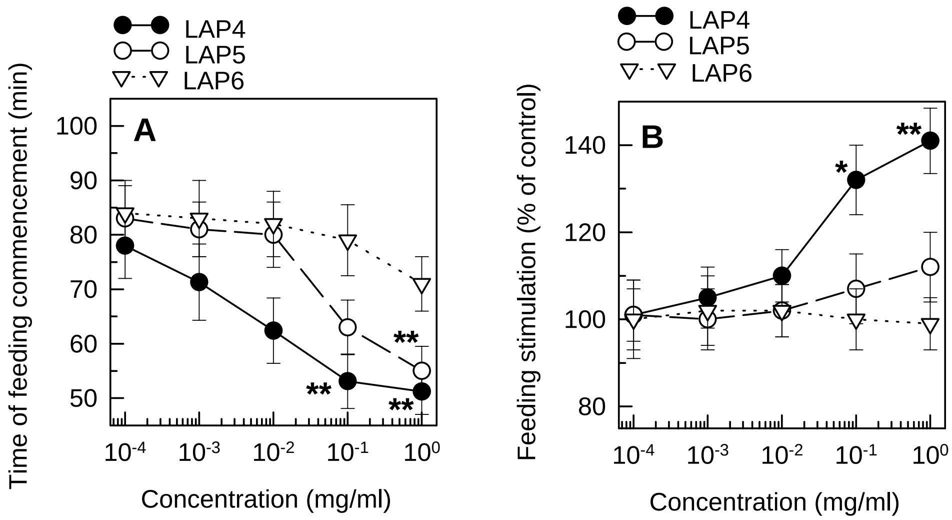


Fig. 2. The effects of L-aminophosphono acid derivatives on initiation (A) and intensity of feeding (B) by codling moth neonates. Each datum point refers to mean ± SEM obtained for ninety (A) or thirty-two (B) larvae.The time of feeding commencement in controls varied between 82.3 ±7.2 and 84.1±8.1 min. The amount of foliage ingested in control groups varied between 127.6 ± 109 and 137.5 ± 11.8 μg larva^{-1} * P<0.05, ** P<0.01

An interesting correlation between the molecular structure and effects on feeding was observed in this group of chemicals. The longer the aliphatic hydrocarbon chain of these acids is, the less feeding stimulatory activity of a given chemical is. For instance, LAP-4, which has 3-carbon aliphatic hydrocarbon chain, both accelerated feeding commencement (Fig. 2A) and significantly increased leaf consumption (Fig. 2B) at concentrations 0.1 and 1 mg/ml. However, LAP-5, which has 4-carbon aliphatic hydrocarbon chain, only accelerated feeding at 1mg/ml concentration (Fig. 2A), and had no effects on feeding intensity (Fig. 2B). LAP-6, which has an even longer aliphatic hydrocarbon chain consisting of 5 carbon molecules, had no effects on feeding (Fig. 2 A,B).

Derivatives of D-aminophopshono acids

It is noteworthy that none of these acids influenced codling moth neonates feeding behavior (Fig. 3). This finding, together with data presented on Fig.2, demonstrates that amino and carboxylic acid groups have to be attached to aliphatic hydrocarbon chain of aminophosphonoacids in L- and not in D- conformation, to initiate or stimulate feeding in codling moth neonates (Table 1 summarizes this finding in reference to chemical structures of tested drugs). Please note that in this study DAP-7 was used. There is no DAP-6 being produced and sold thus far.

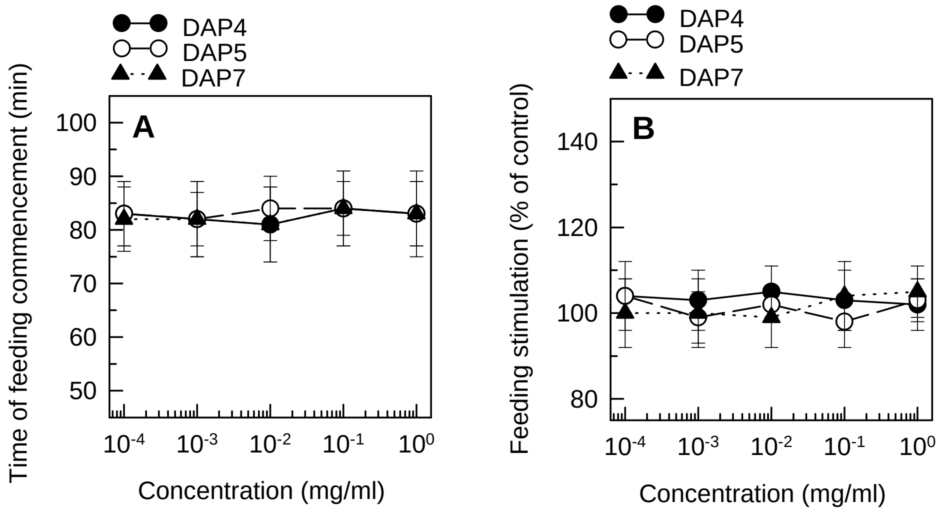


Fig. 3. The effects of D-aminophosphono acid derivatives on initiation (A) and intensity of feeding (B) by codling moth neonates. Each datum point refers to mean ± SEM obtained for ninety (A) or thirty-two (B) larvae. The time of feeding commencement in controls varied between 83.4 ±6.3 and 86.1±6.3 min. The amount of foliage ingested in control groups varied between 126.1 ± 10.3 and 129.9 ± 11.0 μg larva^{-1} . No statistically significant differences found.

Chemicals that have trans-ACBD activity in vertebrates

In this group of chemicals, only RS-tetrazol-5-yl-glycine both accelerated feeding commencement (Fig. 4A) and increased feeding intensity (Fig. 4B). O-phospho-L-serine only accelerated feeding commencement (Fig. 4A), and quisqualate had no effects. The low activity of O-phospho-L-serine and absence of activity from quisqualate, corroborates very well the data presented in Fig. 3, in that both chemicals have their amino and carboxylic acid groups in D- conformation, suggesting that this conformation does not interact with neonates' sense of taste. Stimulation of feeding behavior by RS-tertazol-5-yl-glicyne corresponds well with the fact that this chemical has very high *trans*-ACBD- like activity in vertebrate experimental systems.

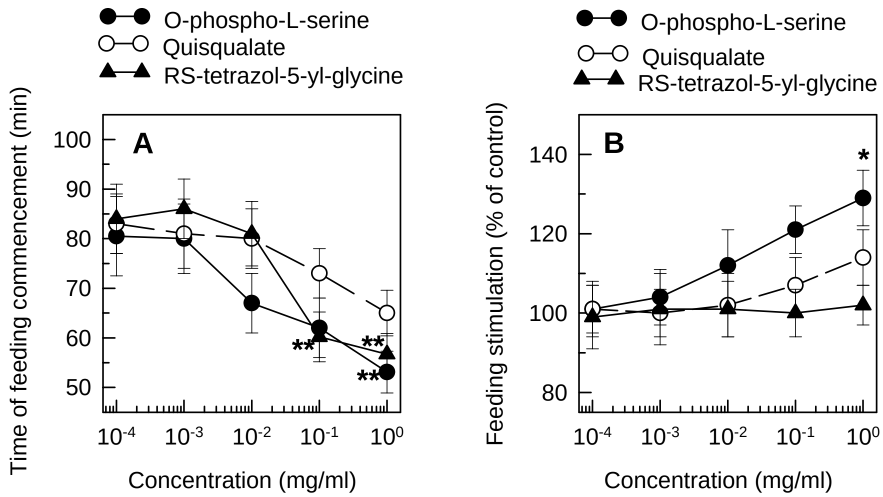
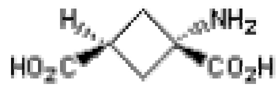
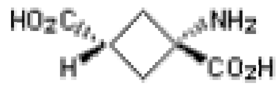
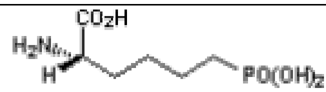
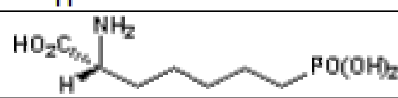
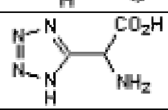
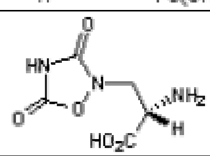


Fig. 4. The effects of chemicals that have *trans*-ACBD activity in vertebrates, on initiation (A) and intensity of feeding (B) by codling moth neonates. Each datum point refers to mean ± SEM obtained for ninety (A) or thirty-two (B) larvae. The time of feeding commencement in controls varied between 83.4 ±6.3 and 86.1±6.3 min. The amount of foliage ingested in control groups varied between 126.1 ± 10.3 and 129.9 ± 11.0 μg larva^{-1} . * P<0.05, ** P<0.01.

Modification of feeding behavior versus chemical structure of tested chemicals

The trends we observed here are illustrated with Table 1. Generally, it seems that chemicals that possess feeding stimulatory properties have amino and carboxylic acid groups either attached in L-conformation to short aliphatic hydrocarbon chain, or to cyclobutane ring with no further groups attached. These findings inform the direction of the search for cheap and effective alternatives of *trans*-ACBD. The finding that RS-tertazol-5-yl-glicyne stimulates feeding has little practical output, since this chemical is as expensive as *trans*-ACBD. One could employ ACBC as an alternative to *trans*-ACBD. ACBC is more than 3 times cheaper than *trans*-ACBD and has only 6-fold higher water solubility, equaling 12g/l. This is substantially less than MSG water solubility, which equals 789g/l. Therefore, one should expect that ACBC will prove rain-fast under field conditions. The other direction indicated by our findings is that use of a chemical with a short aliphatic hydrocarbon attached to amino and carboxylic acid groups in L-conformation should be explored further. Such a chemical, namely L-aspartic acid, has a water solubility only 2-fold higher than *trans*-ACBD. Additionally, the retail prices of L-aspartic acid are only 4 times higher than those of MSG ($60 versus $15 per kg), and over 30,000 times lower than those of *trans*-ACBD.

Table 1. Comparison of effects on feeding versus chemical structure in tested chemicals

Name	Chemical structure	Effect on feeding initiation	Effect on feeding intensity
ACBC		+	+
trans-ACBD		+	0
cis-ACBD		-	-
LAP-4		+	+
LAP-5		+	0
LAP-6		0	0
DAP-4		0	0
DAP-5		0	0
DAP-7		0	0
RS-tertazol-5-yl-glicyne		+	+
O-phospho-L-serine		+	0
Quisqualate		0	0

+ feeding behavior stimulation, - feeding behavior inhibition, 0 no effect on feeding behavior

Introductory experiments with L-aspartic acid

We have evaluated potential feeding stimulatory properties of L-aspartic acid in preliminary feeding assays. This compound accelerated feeding commencement by approximately 30 minutes, and increased leaf consumption by approximately 37% (Table 2). These values are very similar to those that were found for MSG and *trans*-ACBD in our previous studies (2,7).

Table 2. Effect of L-aspartic acid on initiation and intensity of feeding by codling moth neonates

Behavior observed	Control† (N=20)	1 mg/ml L-aspartic acid (N=24)
Feeding commencement (minutes)	84.2±6.2	49.8±3.2 **
Leaf consumption (μg larva^{-1})	124.3± 10.6	176.5±13.5 ***

† 0.02% Triton-X in water

** $P<0.01$, ***$P<0.001$

Significance to the industry and potential economic benefits

Our previous publications show LD50 analysis indicating that addition of MSG or *trans*-ACBD to Spinosad or *Bacillus thuringiensis*- based commercial pesticide formulations increases their effectiveness 2-3 fold (6,7). Our current research indicates that L-aspartic acid may serve as a potential substitute for MSG and *trans*-ACBD. Although we have no absolute numbers or values to illustrate the economic impact of our current accomplishments, our findings stand to have an enormous significance for the apple growing industry of the Northwest, since application of appropriate feeding enhancers can reduce the amounts of insecticides used for codling moth control, thereby improving the quality of fruit and safety of pesticide application. We observed the same degree of feeding stimulation in larvae exposed to MSG or *trans*-ACBD (2,7) and in larvae exposed to L-aspartic acid (Table 2). Therefore, we may expect that the latter compound will have pesticide- enhancing potential similar to that of MSG and *trans*-ACBD. We believe that L-aspartic acid should be further studied in terms of its effects on feeding, rain-fastness and potential for enhancement of orally active insecticides. A proposal of such studies will be the subject of a separate application.

References:

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9. Pszczolkowski M.A., Zahand. A, Bushman S.M, and Brown J.J (2003). Effects of calcium and glutamate receptor agonists on leaf consumption by lepidopteran neonates. Pharm. Biochem. Behav. 74:389-394
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Project title: **Feeding enhancements for insecticides targeting neonate lepidopteran larvae**
PI: Maciej Pszczolkowski
Project duration: 2004-2005 (one year)
Project total (one year): $15,000
Current year request: $ 15,000

Item	
Salaries[1]	1640
Benefits[2]	492
Wages[3]	7533
Equipment[4]	1920
Supplies[5]	2765
Travel[6]	650
Total	15000

1. Dr. Pszczolkowski's salary (training and supervision of two student workers throughout the duration of the project)
2. Benefits of Dr. Pszczolkowski, according to the policies of Kansas State University
3. Time-slip wages + benefits of two student technical assistants at average labor of 40h/month/person, average salary 7.00$/hour, throughout the project duration.
4. Economy- grade stereo dissecting microscope and illuminator
5. Costs of insect shipments, apple tree purchase and greenhouse, materials for feeding stations, small laboratory supplies, access to crystallographic database
6. 50% of coverage of domestic conference fee and travel