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EVALUATION OF NOVEL NONPEPTIDIC CHEMICAL ANALOGS OF TRYPSIN MODULATING OOSTATIC FACTOR (TMOF): A NEW BIORATIONAL APPROACH FOR MANAGING LEPIDOPTERAN PESTS OF COTTON

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Abstract

Three nonpeptidic chemical analogs (IBI-152, IBI-156 and IBI-172) of trypsin modulating oostatic factor (TMOF), an insect hormone inhibiting trypsin biosynthesis in mosquitoes, were synthesized based on the structure of the native peptide. The median lethal concentration (LC50) for TMOF and these analogs was estimated for larvae of the northern house mosquito, *Culex pipiens*, using a static 5-day bioassay. IBI-152 demonstrated the same larvicidal activity as TMOF while IBI-156 and IBI-172 were 1.2- and 1.5-fold more active than TMOF, respectively. All three TMOF analogs were more toxic by injection than TMOF in fourth instars of the tobacco hornworm (*Manduca sexta*), tobacco budworm (*Heliothis virescens*), and the cotton bollworm (*Helicoverpa zea*). Injection of TMOF into second stadium tobacco hornworms had no effect on trypsin activity or growth. Apparently the mosquito hormone is inactive in this species and at the developmental stage examined. Finally, results of a two-choice feeding bioassay with the tobacco budworm indicated that IBI-152 has anti-feedant properties.

Introduction

Trypsin modulating oostatic factor (TMOF) is an insect hormone originally isolated from the ovaries of *Aedes aegypti* (Diptera: Culicidae) that regulates trypsin biosynthesis in mosquitoes (Borovsky, 1988). This decapeptide, Tyr-Asp-Pro-Ala-Pro-Pro-Pro-Pro-Pro-Pro (Borovsky et al., 1993), actuates a specific receptor on the mosquito gut signaling the termination of biosynthesis of the major digestive enzyme trypsin (Borovsky et al., 1994). Synthetic TMOF has been shown to inhibit the growth and development of mosquito larvae feeding on this peptide, resulting in death by starvation (U.S. Patent number: 5,629,196). Insect Biotechnology Inc. (Durham, NC) is developing this technology for the control of mosquito populations.

TMOF or peptidic analogs have been shown to inhibit trypsin biosynthesis in other medically important insects including the house fly, *Musca domestica* (Diptera: Muscidae), stable fly, *Stomoxys calcitrans* (Diptera: Muscidae) and the cat flea, *Ctenocephalides felis* (Siphonaptera: Pulicidae) (Borovsky et al., 1990). Inhibition of trypsin biosynthesis by TMOF or TMOF-like factors has also been demonstrated in insects of agricultural importance including the citrus weevil, *Diaprepes abbreviatus* (Coleoptera: Curculionidae)(Yan et al., 1999) and the tobacco budworm, *Heliothis virescens* (Lepidoptera: Noctuidae)(Nauen et al., 2001). These findings suggest that TMOF-like peptides may play an important role in the regulation of digestion in a number of insect species.

In order to extend the TMOF technology into the agricultural arena, several nonpeptidic chemical analogs of TMOF (IBI-152, IBI-156 and IBI-172; chemical structures not shown, patent pending) were synthesized based on the chemical structure of the native peptide. This paper examines the toxicity of this novel chemistry in the northern house mosquito, *Culex pipiens* (Diptera: Culicidae), tobacco hornworm, *Manduca sexta* (Lepidoptera: Sphingidae), tobacco budworm, and the cotton bollworm, *Helicoverpa zea* (Lepidoptera: Noctuidae). The effects of TMOF on the trypsin activity and growth rate of second stadium tobacco hornworms were also examined. Finally, feeding experiments were conducted with the tobacco budworm in an effort to elucidate the mode of action of IBI-152.

Materials and Methods

Insect Rearing

Insects were maintained at 27±2°C, 50±10% RH, and a 14:10 (L:D) photoregime. *Culex pipiens* egg rafts were purchased from Carolina Biological Supply Co. (Burlington, NC). Mosquito larvae were allowed to emerge in covered beakers containing sterile double distilled H_2O. Tobacco budworms and cotton bollworms were reared on artificial heliothine diet (Burton, 1970). Tobacco hornworms were reared on artificial diet according to Bell and Joachim (1976).

Test Materials

Synthetic TMOF was purchased from SynPep Corp. (Dublin, CA). Nonpeptidic chemical analogs, IBI-152, IBI-156 and IBI-172 (referred to hereafter also as TMOF analogs) were synthesized by MI and RJL in the Department of Chemistry at NC State University. The design and synthesis of these compounds will be described elsewhere and in the patent literature.

Mosquito Bioassay

The median lethal concentration (LC50) for TMOF and TMOF analogs was estimated for larvae of *C. pipiens*, using a static 5-day bioassay modified from an existing protocol (mosquito larval assay, D. Borovsky, pers. comm.). Day 1 first stadium *C. pipiens* were transferred singly to individual wells of a 96-well microtiter plate in approximately 20 µL of sterile double distilled H_2O. Excess water was removed and 150 µL of a 0.14% yeast (Brewers-debittered, inactive) solution was immediately added to each well. Stock solutions of TMOF and TMOF analogs were prepared in sterile double distilled H_2O and DMSO, respectively. Dilutions from these stock solutions were then made into sterile double distilled H_2O so when 50 µL was transferred to each well of the microtiterplate, the desired final concentration was obtained in a total volume of 200 µL per well. Controls for TMOF and TMOF analogs were treated with 50 µL of H_2O and 2% (v/v) DMS0 in H_2O, respectively. Following treatment, plates were covered with plastic transparent lids and placed in a sealed plastic container with 100% humidity to prevent water evaporation. Larvae were monitored every 24 hours for five days for mortality (cessation of contraction of dorsal longitudinal muscles and failure to respond to a blunt probe in 10 seconds). Doses are presented as mM. In all LC50 estimations, treatments (5 doses) and solvent controls were replicated at least three times with 12 insects per replicate.

Injection of TMOF and TMOF Analogs

Early fourth stadium tobacco hornworms, tobacco budworms and cotton bollworms were selected for these experiments based on head capsule size and weight. TMOF and TMOF analogs were diluted in sterile double distilled H_20 and acetone, respectively. Larvae were injected with TMOF, IBI-152, IBI-156 or IBI-172 in 0.5 µL of solvent at a discriminatory dose of 306 mg/kg (milligrams compound per kilogram insect weight) in the perivisceral hemocoel with glass needles. Glass needles were manufactured on a P-2000 micropipette puller (Sutter Instrument Co., Novato, CA) using the following program: Heat = 370, Filament = 4, Velocity = 70, Delay = 200, Pull = 7. Controls for TMOF and TMOF analogs were injected with 0.5 µL of H_20 and acetone, respectively. Treated insects were monitored every 24 hours for eight days for mortality (failure to respond to a blunt probe in 10 seconds). Thirty insects were assayed per treatment and solvent control (3 replicates of 10 insects per replicate). Data are expressed as mean cumulative corrected percent mortality ± 1 SEM.

Trypsin Activity

Day zero second stadium tobacco hornworms were first weighed and then injected with 0.5 µL of sterile double distilled H_2O containing 20 µg of TMOF as previously described. Controls were injected with 0.5 µL H_2O. After feeding for 24 hours, larvae were reweighed, pooled into microcentrifuge tubes (3 insects per tube, 2 tubes per treatment) and frozen at –80°C for enzyme assays. Total body homogenates were prepared in buffer (0.1M Tris-HCl, 20mM $CaCl_2$, 0.01% PTU, pH 9.0) followed by two centrifugations steps at 10,000g at 4°C to produce a clear supernatant. Trypsin activity was measured for the supernatant using the chromogenic artificial substrate Nα-benzoyl-DL-arginine p-nitroanilide (BApNA, Sigma Chemical Co., St. Louis, MO; Erlanger et al., 1961) using a protocol modified from Lam et al. (2000). Reactions were initiated by adding 150 µL of substrate solution (0.1M Tris-HCl, 20 mM $CaCl_2$, 1.07 mM BApNA, pH 9.0) to microtiter plate wells containing 10 µL of supernatant diluted in ice-cold buffer. The final substrate concentration was 1.0 mM BApNA. Positive controls (bovine trypsin, Sigma Chemical Co., St. Louis, MO) and buffer blanks were included in all experiments. Assay plates were automixed and incubated at 30°C in a Thermomax® microplate reader (Molecular Devices, Inc., Sunnyvale, CA) for 5 minutes. Linear change in absorbance due to production of p-nitroaniline was monitored at 405 nm for 5 to 15 minutes, and the results were analyzed with Softmax® Pro (Molecular Devices, Inc., Sunnyvale, CA). Protein determinations were made according to Bradford (1976) using bovine serum albumin (Fisher Scientific, Fair Lawn, NJ) as a standard. Activity is expressed as mOD/min/mg protein, and growth rate is expressed as the change in wet weight per day (mg/day). Data are the mean of three replicates ± 1 SEM.

Two-Choice Bioassay

Dehydrated diet pellets made from artificial diet (Bailey et al., 2001) were saturated with IBI-152 diluted in acetone. Control pellets were saturated with acetone alone. Following evaporation of the acetone, pellets were rehydrated with sterile double distilled H_2O. The final concentration of IBI-152 in the test pellets was 1.5 mg/g wet weight of diet. One test and one control pellet were placed on opposite sides of a small petri plate (diameter = 60mm, height = 15mm). Early fifth stadium tobacco budworms were placed into the plates which were then covered (1 insect/dish, n = 5). Larvae were allowed to feed *ad libitum* for twenty-four hours.

Statistics

Abbot's correction was applied to all data from dose response experiments (Abbot, 1925). LC50s were determined by plotting log dose versus probit plus 5 mortality (Sokal and Rohlf, 1995; Finney, 1971; Microsoft Excel, 1997). Significance tests were conducted with Student's t tests (Microsoft Excel, 1997).

Results and Discussion

Toxicity of TMOF and TMOF Analogs in *Culex pipiens*

The results of the mosquito bioassay are shown in Table 1. The LC50s for IBI-156 and IBI-172 were 1.2- and 1.5-fold lower than the LC50 for TMOF, while no differences were found between IBI-152 and TMOF. Of the three analogs tested, IBI-172 had the greatest activity with an LC50 of 0.28 mM compared to TMOF with an LC50 of 0.43 mM. There were no observable physical differences between larvae that had survived a five-day exposure to TMOF or IBI-172 (Figure 1). Larvae surviving these two treatments were however significantly smaller and less developed than the corresponding control (Figure 1). Similar observations were made in comparisons of larvae surviving exposures to IBI-152, IBI-156 and TMOF. These results suggest that TMOF analogs may have a mode of action similar to TMOF in mosquito larvae. Further studies will be needed to validate this hypothesis.

Toxicity by Injection of TMOF and TMOF Analogs

To compare the effects of TMOF and TMOF analogs on lepidopteran larvae, early fourth stadium tobacco hornworms, tobacco budworms and cotton bollworms were injected with TMOF, IBI-152, IBI-156 or IBI-172 at a discriminatory dose of 306 mg of active ingredient/kg. TMOF did not have any effect on growth rate or molting (data not shown). TMOF was also non-toxic (Figure 2). However, all three analogs produced greater than 50% mortality eight days after injection in the three species tested. There were no significant differences between treatments for each species or between species for each analog (Student's t tests, $P > 0.05$). Larvae treated with the analogs either did not feed or feeding was minimal, producing few if any fecal pellets. Complications during molting were also observed and many of the treated insects, including survivors, were unable to completely shed the exuviae. The injections were made into the perivisceral cavity through a last abdominal proleg. Those larvae that survived treatment became darkened in appearance in the area of the injection and unresponsive to prodding in the same area. In respect to the latter, the insects remained responsive to touch at the head and thorax. These effects were different from those observed in mosquitoes that were fed TMOF (Figure 1), suggesting a possible different mode-of-action of TMOF analogs in lepidopteran larvae.

Trypsin Activity

To determine if TMOF is active on an earlier instar of the tobacco hornworm, day zero second stadium larvae were injected with TMOF, and 24 hours later the average growth rate and trypsin activity were compared to controls. The results of these experiments are shown in Figure 3. There were no significant differences in the average growth rate (Student's t test, $P = 0.95$) or trypsin activity (Student's t test, $P = 0.84$) between insects injected with 20 µg TMOF and the control. Similar results were obtained when insects were treated with 50 and 100 µg of TMOF (data not shown). Nauen et al. (2001) found that injection of second instar tobacco budworms with TMOF resulted in 50% inhibition of trypsin biosynthesis after 24 hours at a dose of 0.2 ng. Apparently, the tobacco hornworm differs from the tobacco budworm relative to its response to TMOF injections and its effect on trypsin activity.

Anti-feedant Activity of TMOF Analogs

In preliminary feeding experiments with TMOF analogs incorporated into artificial diet, second instars of the tobacco budworm demonstrated an obvious preference for control diet pellets over those treated with IBI-152 (data not shown). These analogs have a noticeable odor even when diluted to low concentrations, and therefore, we hypothesized that these compounds may have anti-feedant properties. In a two-choice bioassay with fifth instars of the tobacco budworm, diet pellets treated with solvent alone were chosen over pellets treated with IBI-152 in 5 out of 5 trials. In 4 out of the 5 trials, the diet pellets treated with solvent alone were entirely consumed within 24 hours where as no appreciable reduction in size was observed for pellets treated with IBI-152 (Figure 4). The solvent was allowed to completely evaporate before the diet was hydrated and insects were allowed to feed (see Materials and Methods). These results suggest that IBI-152 has anti-feedant activity against the tobacco budworm under these conditions. Additional experiments to characterize this activity and determine the utility of this discovery in the management of agricultural pests are in progress.

Summary

- The LC50s for IBI-156 and IBI-172 were significantly lower than the LC50 for TMOF for larvae of the northern house mosquito. There were no significant difference in the toxicities of IBI-152 and TMOF.
- There were no observable differences in the growth and development of the tobacco hornworm, tobacco budworm or cotton bollworm, when injected as early fourth instars with TMOF compared to controls.
- IBI-152, IBI-156 and IBI-172 caused larval mortality at comparable levels in the tobacco hornworm, tobacco budworm and cotton bollworm, when injected as early fourth instars with 306 mg of active ingredient/kg.
- There were no significant differences in the average growth rate or trypsin activity of second stadium tobacco hornworms injected with TMOF compared to controls.
- Results of a two-choice bioassay with fifth instars of the tobacco budworm suggest anti-feedant properties for IBI-152.
- These findings suggest that the TMOF technology may be applicable to the control of agricultural pests important to cotton through the use of novel analog chemistry.

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Table 1. Comparison of LC50s 5 days after exposure of TMOF or TMOF analogs of day 1 first stadium *Culex pipiens.*

Compound	LC50 (mM)	Regression curve	R^2	95% CI	N
TMOF	0.43	y = 8.46x + 3.08	0.99	0.38-0.49	240
IBI-152	0.51	y = 8.24x + 2.39	0.99	0.47-0.56	192
IBI-156	0.36	y = 12.16x + 5.46	0.99	0.34-0.37	204
IBI-172	0.28	y = 10.8x + 5.91	0.99	0.28-0.29	240

CI, confidence interval; N, number of individuals tested.

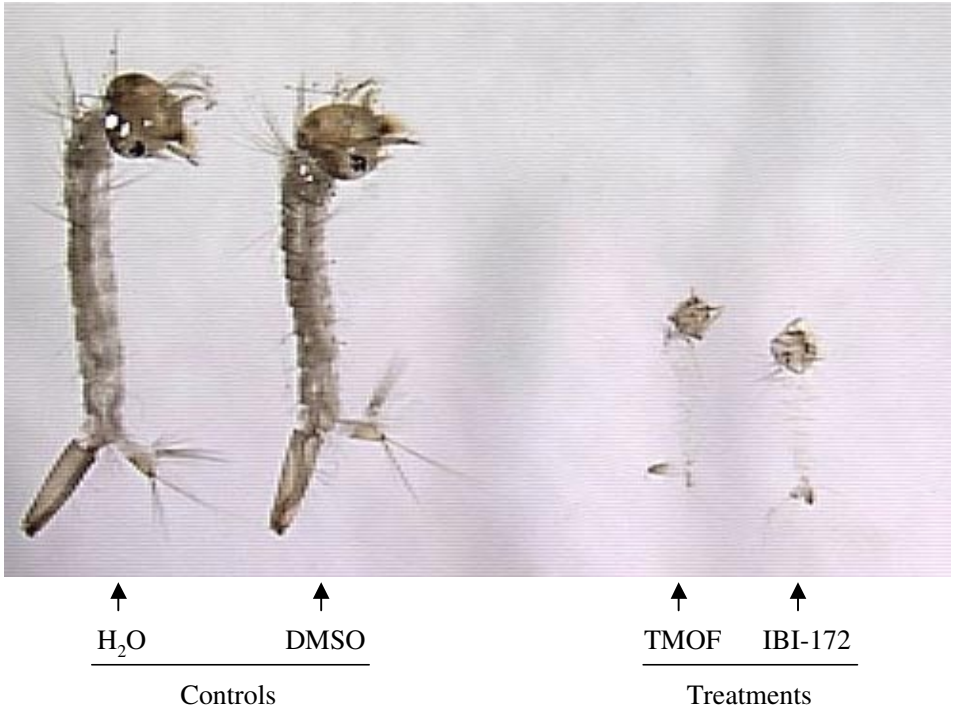

Figure 1. *Culex pipiens* larvae 5 days after exposure to H_2O (control for TMOF), 0.5% (v/v) DMSO in H_2O (control for TMOF analogs), TMOF or IBI-172. Larvae exposed to TMOF and IBI-172 are less developed than the controls.

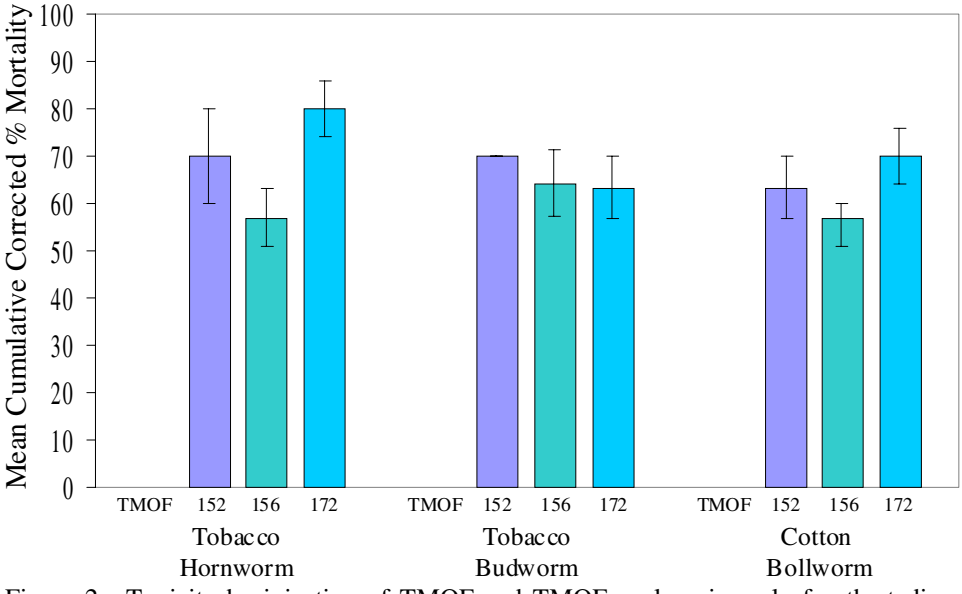

Figure 2. Toxicity by injection of TMOF and TMOF analogs in early fourth stadium lepidopteran species. Error bars are ±1 SEM. Absence of error bars indicates a SEM of zero for these data.

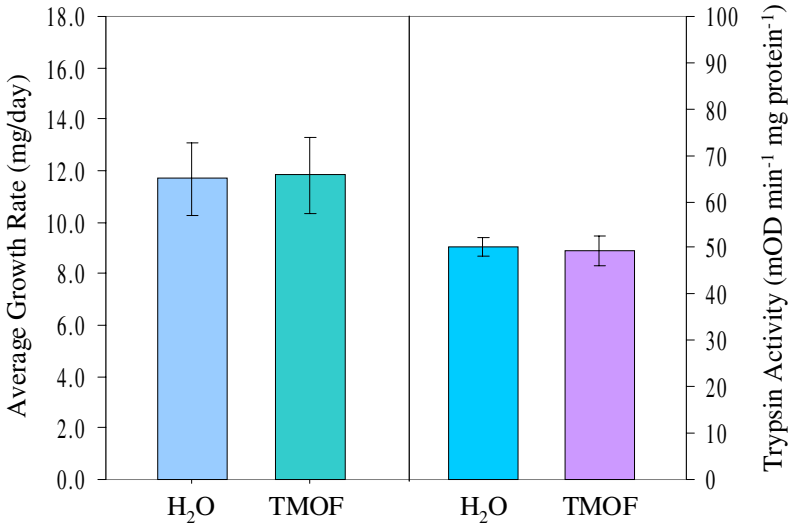

Figure 3. Average growth rate and trypsin activity for second stadium tobacco hornworms 24 hours after injection with TMOF or H_2O. Error bars are $\pm$1 SEM.

Figure 4. In a two-choice bioassay, early fifth stadium tobacco budworms were allowed to choose between diet pellets treated with IBI-152 or solvent control. A typical result observed in these experiments is presented. After 24 hours, the only thing remaining of the control pellet is frass. The control pellet was entirely consumed in 4 out of 5 trials.