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Biological and biochemical effects of organo-synthetic analogues of Trypsin Modulating Oostatic Factor (TMOF) on *Aedes aegypti*, *Heliothis virescens* and *Plutella xylostella*

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Abstract: The three-dimensional conformation of *Aea*-TMOF (*Aedes aegypti* Trypsin Modulating Oostatic Factor), a decapeptide (YDPAPPPPPP) isolated from mosquito ovaries that inhibits the translation of many trypsin-like serine proteases, e.g. in mosquitoes, flies and lepidopterans, was used as a model for the synthesis of 10 aromatic and aliphatic organic acid and ester analogues. The organic TMOF analogues were tested against herbivorous pest lepidopterans and larval *Ae. aegypti*. The compounds administered to microtiter plates or to leaf disks caused mortality to mosquito larvae and the diamondback moth, *Plutella xylostella,* larvae 3-6 days after treatment. The surviving diamondback moth larvae were sluggish, immobile and stopped feeding. A biochemical analysis showed that in larval *P. xylostella* the trypsin activity was low and correlated with the observed mortality. No activity was found against *Heliothis virescens* when four of the synthetic compounds that affected *P. xylostella* and *Ae. aegypti* were tested, although the decapeptide, TMOF, does affect trypsin biosynthesis in *H. virescens*.

Keywords: TMOF, trypsin biosynthesis, peptide mimics, mosquitoes, lepidopterans, insect growth inhibition, novel organo-insecticides

INTRODUCTION

The major digestive enzymes of many insect species are midgut serine proteases, including trypsin and chymotrypsin. Thus, controlling insects by interfering with their digestive enzymes by using trypsin inhibitors has been tried to control insects [1-4]. Anautogenous adult mosquitoes and their larvae, use trypsin and chymotrypsin as their major digestive enzymes to digest the blood meal in adult females, and proteinacious food found in the water for larval development [5, 6]. Similar to mosquito larvae, protein digestion in the tobacco budworm (*Heliothis virescens*) is mediated by endo- and exopeptidases secreted from the midgut epithelial cells into the luminal fluid of the midgut [7]. Although the factors that directly stimulate trypsin biosynthesis in mosquitoes and lepidopterans are not known, a decapeptide (YDPAPPPPPP) named trypsin modulating oostatic factor (TMOF) that terminates trypsin biosynthesis in mosquitoes and in one lepidopteran, *H. virescens* has been identified and characterized [8, 9].

TMOF has been originally isolated and characterized from the ovaries of female *Aedes aegypti*. The hormone is a decapeptide and was shown to be the physiological factor that stops trypsin biosynthesis in the mosquito gut by translational control of the trypsin message. Applying the hormone immediately after the blood meal to female mosquitoes or feeding the hormone to mosquito larvae causes cessation of trypsin biosynthesis in the midgut of adults and larvae. Mosquitoes need trypsin to digest the blood for egg development, but can survive on nectar in the field. On the other hand, larvae need the enzyme for survival. Thus, feeding mosquito larvae with TMOF can be used to arrest larval growth and starve the insects to death.

Since many agricultural pest insects, like lepidopterans (*H. virescens* and *Plutella xylostella*) also use trypsin as their major proteolytic enzyme to digest food, it was of interest to find out if trypsin biosynthesis in lepidoptera, is controlled by a TMOF molecule like in diptera. In an earlier study we showed that a TMOF-like molecule is circulating in the hemolymph of late 4th instar *Heliothis* larvae [8]. The physical property of this molecule was similar to the known mosquito decapeptide. Purification of the peptide by HPLC yielded a fraction that cross-reacted with mosquito TMOF antibodies. Injection of the *Heliothis* fraction into mosquitoes produced the same effect that one expects from mosquito TMOF, i.e., inhibition of trypsin biosynthesis, blood digestion and egg development [8].

Thus, it is now possible to develop chemical analogues that will resemble the 3D conformation of mosquito TMOF, feed them to mosquito larvae, assay

for the inhibition of trypsin biosynthesis and observe larval mortality. Because of the similarities in the TMOFs of mosquitoes, lepidopterans and nematodes (GenBank accession number CEY37D8A.21; YDPLPPPPPP for nematodes) one can use the same analogues to control agricultural insect pests that produce trypsin as their major digestive enzyme. If these analogues are effective, one expects to see larval starvation, and death. Because agricultural pest insect larvae feed on plant leaves it is relatively simple to follow larval mortality and leaf damage by feeding individual larva in well plates on leaf discs that can be treated with different amounts of TMOF analogues.

Because TMOF and its analogues target the larval gut by binding to a specific gut receptor that shuts down the translation of the trypsin message, one can also isolate and sequence TMOF gut receptor in different insects and express the receptor on the surface of cells. Using recombinant cells that express the TMOF-receptor one can easily find organic analogues that bind to the receptor that could be used as potential insecticides. These analogues will cause larval mortality by preventing normal food digestion that is essential for larval growth and development.

With these goals in mind, we investigated if several organo-synthetic mimics of TMOF can be effective in future control of insect pests. Our report describes the insecticidal activity of several TMOF chemical analogues that were synthesized using a 3D modelling approach [10].

MATERIALS AND METHODS

TMOF synthetic mimics

Ten putative mimics of Aea-TMOF (Figure 1) were synthesized by Professor Linderman at the department of chemistry in North Carolina State University as was described for IBI-152, 156, 219 and 220 [10]. The newly synthesized compounds were assayed by NMR, purified by chromatography (purity >95%) and were provided to us by IBI (Durham, North Carolina, USA). Stock solutions of 10 mg ml^{-1} of each compound were prepared in acetone for lepidopteran and in DMSO for larval mosquitoes for testing their insecticidal efficacy.

Insects

Second and 3rd instar larvae of the diamondback moth, *P. xylostella*, were grown on cabbage leaves. Third instar larvae of the tobacco budworm, *H.virescens*, were grown on artificial diet. All larvae were maintained in the laboratory at room temperature (22 °C), 50% relative humidity and an ambient

photoperiod. *Ae. aegypti* larvae were hatched from eggs at 27 °C in water containing Brewer's yeast and 24 hours later larvae were tested for larvicidal activity of the TMOF synthetic mimics.

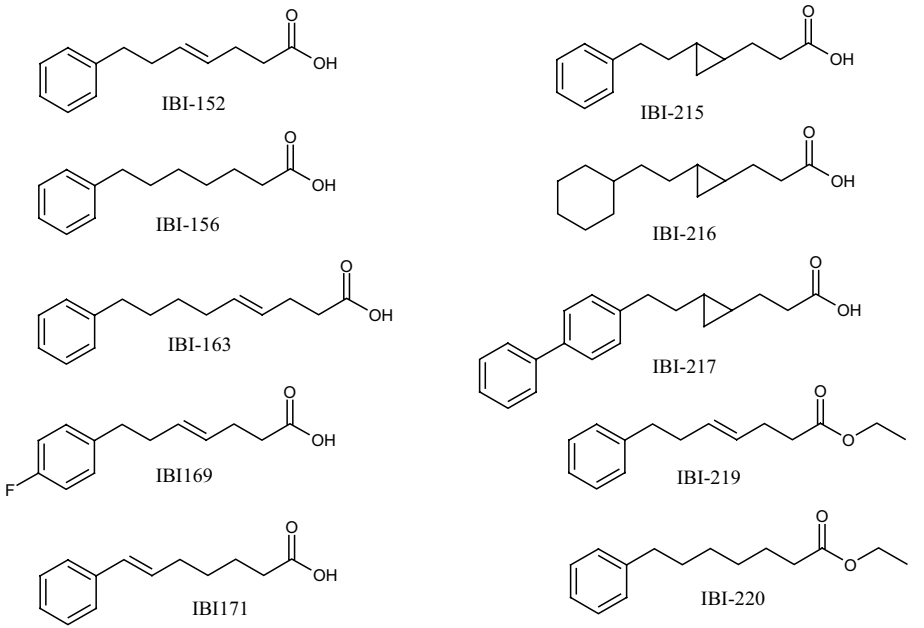

Figure 1. TMOF organo-synthetic mimics: IBI-152 = *E-7-phenylhept-4-enoic acid*; IBI-156 = *7-phenylheptanoic acid*; IBI-163 = *9-phenyl-non-4-enoic acid*; IBI-169 = *7-(4-fluoro-phenyl)-hept-4-enoic acid*; IBI-171 = *7-phenyl-hept-6-enoic acid*; IBI-215 = *3-(2-phenethyl-cyclopropyl)-propionic acid*; IBI-216 = *3-[2-(2-cyclohexyl-ethyl)-cyclopropyl]-propionic acid;* IBI-217 = *3-[2-(2-biphenyl-4-yl-ethyl)-cyclopropyl]-propionic acid*; IBI-219 = *E-7-phenyl-hept-4-enoic acid ethyl ester*; IBI-220 = *7-phenyl-heptanoic acid ethyl ester*.

Bioassays

Leaf-disk bioassay

Mature leaves of cabbage plants grown in the greenhouse were cut from the petioles, and circular disks (12 mm in diameter) were cut from the leaves using a cork borer. The leaf disks were then transferred with their lower side down to 24-well tissue culture plates containing 500 μl of agar (1% w/v) per well (see Figure 2).

Each leaf disk was then treated with 5 μl of acetone stock solution of each compound (representing 50 μg per disk). After a few seconds, the applied liquid evaporated and a dry deposit remained. Leaf disks which were treated with 5μl of only acetone served as controls. Twenty-four larvae of either *P. xylostella* (tested with all 10 compounds) or *H. virescens* (tested with IBI-152, 156, 163 and 169) were then transferred to the treated leaf disks and the tray was then covered with tissue paper and an appropriate plastic lid. Mortality and feeding was assessed after 3 days and live larvae (in groups of five) were collected for measuring trypsin activity.

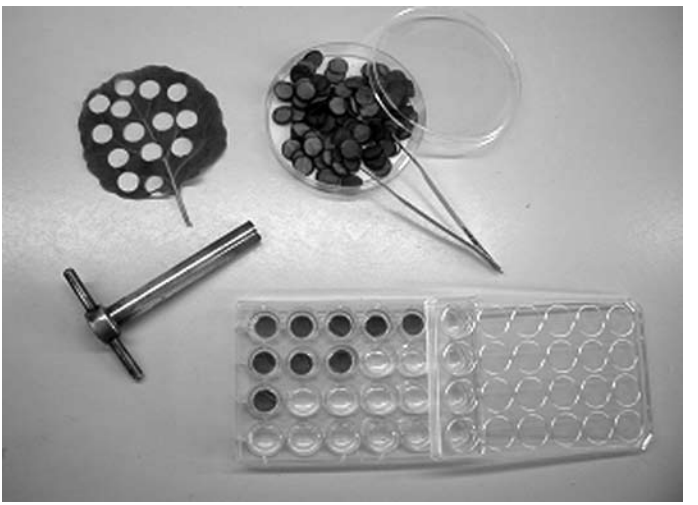

Figure 2. Leaf-disk bioassay in 24-well plates.

Leaf-dip bioassay

The leaf dip bioassay was performed to compare the efficacy of one of the most active compounds in a more common screening format. The stock solution of IBI-152 was diluted in 0.02% (v/v) aqueous Triton X-100 ten-fold, resulting in a dip concentration of 1000 ppm. Before the leaf disks were transferred into the 24-well plate they were dipped into the prepared dilution for 3 sec. After the disks dried, a single larva was transferred into each well. Mortality was scored after 3 days.

Larval mosquito bioassay

TMOF synthetic analogues were dissolved in dimethyl sulfoxide (DMSO) and aliquots of 0.25 to 10 μl were added to 96-well plates containing

188 μl sterile water, 200 μg Brewer's yeast and a 24 hours old first instar *Ae. aegypti* larva per well. Each experiment was repeated at least 3 times using 12 larvae per group with 5 to 6 different concentrations. Larval mortality was checked daily for 5 to 6 days and results are expressed as lethal concentrations at 50% mortality (LC_{50})$\pm$S.E.M. using EPA probit analyses as was described earlier (11).

Trypsin activity measurement

To measure trypsin activity, *P. xylostella* larvae (5 per group) were homogenized with a hand homogenizer in Eppendorf tubes containing 500 μl of 50 mM Tris-HCl, pH 8.2, 20 mM $CaCl_2$ buffer. The homogenates were centrifuged at 4 °C for 10 min at 10,000 g, the supernatants collected and assayed for trypsin activity. Assays were carried out in 96-well plates using 20 μl supernatant, 20 μl Tris-HCl buffer and 160 μl of a substrate solution of 1 mM N-α-benzoyl-DL-arginine-p-nitroanilide (BapNA, final concentration 0.8 mM). BApNA was prepared as a 50 mM stock solution in DMSO and then diluted 50-fold with buffer. The final DMSO concentration in the assay was <2%. To find out if *P. xylostella* synthesizes trypsin-like enzymes 20 μg of soybean trypsin inhibitor (SBTI) was added to the reaction mixture in 20 μl Tris-HCl buffer. The enzymatic reaction was continuously monitored at 405 nm over 20 min using the kinetic mode of a Molecular Devices microplate reader.

RESULTS AND DISCUSSION

In a first experiment, compounds IBI-152, 156, 163 and 169 were tested against both diamondback moth and tobacco budworm larvae. The diamondback moth larvae did not eat the leaf disks starting at day one and all starved to death. These compounds, were also shown to be very active in a different laboratory (results not shown) indicating that these TMOF mimics have an insecticidal potential against *P. xylosella*. No activity, however, was observed against 3rd instar *Heliothis* larvae, they fed the whole treated leaf disks without a toxic effect. Whether this is due to selectivity, pharmacokinetics or metabolism should be resolved at a later date. A few typical symptoms of starvation observed in *P. xylostella* larvae are shown in Figure 3 below. Larvae fed on leaf disks treated with IBI-156 did not cause leaf damage and did not grow as compared to controls that were fed on untreated leaf disks.

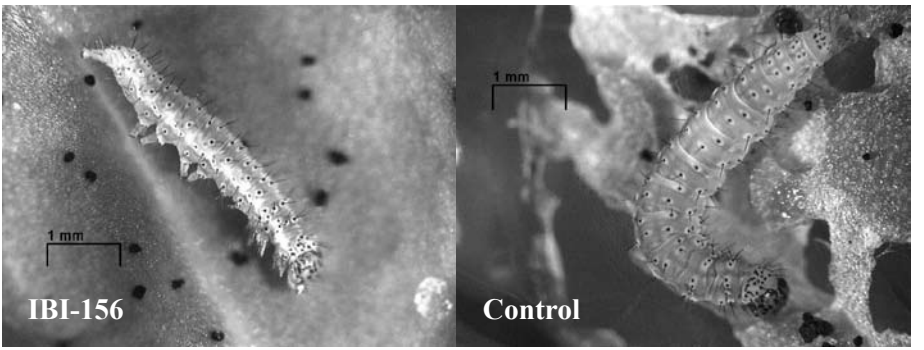

Figure 3. Symptoms of starvation observed in *P. xylostella* after feeding leaf treated with IBI-156 as compared with control larva feeding on an untreated leaf.

Table 1. Effect of IBI-compounds on *P. xylostella* larvae

Compound	Mortality, % 1d	**Mortality, % 3d**	Feeding, % 3d
IBI-171	4	**54**	59
IBI-215	8	**96**	19
IBI-216	17	**71**	36
IBI-217	4	**25**	71
IBI-219	0	**58**	50
IBI-220	4	**58**	48
Control	0	**0**	100

Two groups of larvae (8 per group) were fed on leaf disks treated and not treated with TMOF organic analogues. Mortality was followed at daily intervals and results are expressed as an average of 2 determinations with 5% to 10% difference between groups.

A second experiment was carried out with compounds IBI-171, 215, 216, 217, 219 and 220 (Figure 1). This time only the diamondback moth larvae were subjected to treated leaf disks. The most active compound was IBI-215 and the least active the biphenyl-derivative IBI-217 (Table 1). Mortality values show a strong reverse-correlation with the percentage of each leaf disk that was consumed by the larvae. We observed that more effective mimics caused higher larval mortality and protected the leaf disks from extensive larval damage (Table 1). The cyclopropane-containing compounds IBI-215 and 216 were most effective causing 96% and 71% mortalities, respectively. Whereas IBI-215 and 216 are novel most of the other compounds are known, or have been described in international patent disclosures, e.g., IBI-219 [EP (1988)-305189 and US

(1988)-5134128]. We suggest that more work is needed to study these novel TMOF mimics in order to elucidate their exact mode of action.

We also tested several of the TMOF organic mimics on *Ae. aegypti* larvae. Feeding the mimics with Brewer's yeast to mosquito larvae caused larval mortality within 5-6 days of the feeding (Table 2), in a similar fashion that was observed when TMOF peptide analogues were fed to first instar *Ae. aegypti* larvae (11). IBI-156, 163, 169, 171, 215 and 217 exhibited similar LC_{50} values even though IBI-217 is a biphenyl-derivative and IBI-215 has a cyclopropyl group instead of a double bond. When the double bond was placed in the middle of the molecule (IBI-152) and the benzene ring replaced by cyclohexane (IBI-216) the activities were greatly reduced (Table 2), due to different steric properties.

Table 2. Feeding of TMOF organic mimics to *Ae. aegypti* larvae

Compound	LC_{50} (mM±S.E.M.)
IBI-152	0.78±0.08
IBI-156	0.19±0.02
IBI-163	0.17±0.01
IBI-169	0.11±0.01
IBI-171	0.18±0.02
IBI-215	0.18±0.03
IBI-216	0.30±0.01
IBI-217	0.13±0.04

Three groups of first instar *Ae. aegypti* larvae (12 per group), were fed different concentration of TMOF organic analogues in microtiter plates in the presence of Brewer's yeast for 5-6 days. Larval mortality was followed daily and the results of 3 determinations were expressed as LC_{50} ± SEM using EPA probit analyses.

Biochemical studies

The kinetic plots for trypsin activity of the different TMOF mimics were followed for 15 min using two groups of homogenized larval *P. xylosella* (5 larvae per group), for each mimic. From the slopes of the Plots (mOD/min) the endpoint values at 15 min were determined (Table 3). Trypsin activity measured with BApNA in surviving groups of *Plutella* larvae is closely correlated with the observed mortality that these compounds induced by starving larval *Plutella* to death (Figure 4).

Table 3. Trypsin activity remaining in surviving *P. xylostella* larvae

Compound	Trypsin activity, OD 405 nm after 15 min
IBI-171	1.71
IBI-215	1.46
IBI-216	1.39
IBI-217	1.45
IBI-219	1.52
IBI-220	1.59
Control	1.92

Two groups (5 larvae each) of *P. xylostella* were homogenized and assayed for trypsin activity for 15 min and the kinetic plots of each reaction end point values (mOD/min) were determined. Each value is the average of 2 determinations with 1-5% deviation between groups.

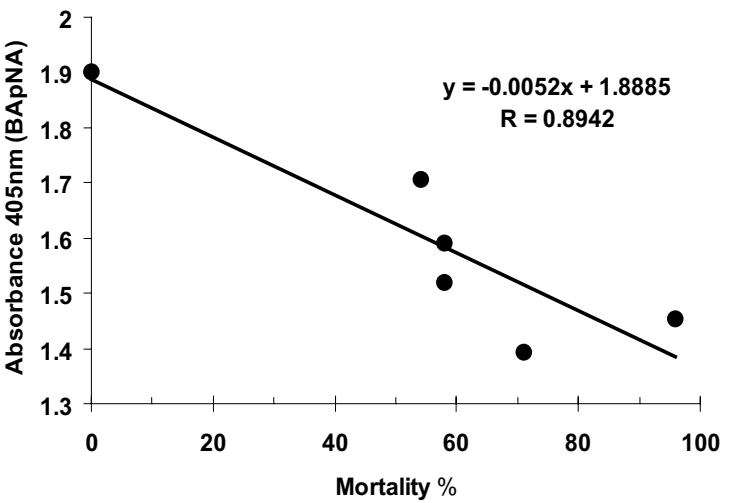

Figure 4. Correlation between trypsin activity and mortality in *P. xylostella*. Trypsin activity in the gut was followed by incubating two groups of 5 surviving larvae with BApNA. The results are expressed as averages of 2 determinations with differences of 5% to 8% between groups.

These results strongly suggest that larval mortality caused by the action of the synthetic compounds is correlated with the reduction of trypsin biosynthesis in the midguts of lepidopteran larvae. This report demonstrates that in principal synthetic mimics of TMOF eventually could be sprayed in the field and efficiently protect agricultural crops from larval feeding damage. Further investigations

are necessary in order to prove whether this approach is an appropriate tool in modern crop protection tactics.

Acknowledgment

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