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Control of *Aedes aegypti* larvae with *Aea*-TMOF mimics: aromatic derivatives of enoic acid

Dov BOROVSKY

University of Florida-IFAS, Florida Medical Entomology Laboratory,
200 9th St SE, Vero Beach, FL 32962, USA
e-mail: dobo@mail.ifas.ufl.edu

Abstract: Based on the 3D conformation of the N-terminus of TMOF in solution, six aromatic derivatives of enoic acid: 7-biphenyl-4-yl-hept-4-enoic acid (BPHE), 7-(4-butyl-phenyl)-hept-4-enoic acid (BuPHE), 7-cyclohexyl-hept-4-enoic acid (CyHE), 10-phenyl-dec-7-enoic acid (PDE), 7-p-tolyl-hept-2-enoic acid ethyl ester (THEEE) and 7-(4-methoxy-phenyl)-hept-2-enoic acid ethyl ester (MPEEE) were computer-modeled and synthesized. Treating first instar *Aedes aegypti* larvae with different concentrations of the TMOF mimics showed that addition of butyl to the benzyl ring, use of p-tolyl or converting the benzyl ring into cyclohexane increased the biological activity of the mimics by 5.2, 5.0 and 3.8-fold, respectively. Esterifying the carboxyl terminus into ethyl ester and addition of a methoxy group to the benzyl ring also increased, by 2-fold, the biological activity of the derivative. The position of the double bond in the aliphatic chain is important for enhanced biological activity. *Aea*-TMOF and CyHE fed to mosquito larvae equally inhibited trypsin biosynthesis in larvae for the first 24 h. The biological activity of CyHE, however, rapidly declined 2-3 days later, whereas TMOF activity stayed stable. These results indicate that TMOF organic mimics, although potent, need to be formulated in order to be more stable for future field applications.

Keywords: bio-organic insecticides, mosquitoes, trypsin biosynthesis, insect growth inhibition

INTRODUCTION

Yang and Davies [1] and Ho et al. [2] reported that trypsin and chymotrypsin are the main digestive enzymes during larval *Ae. aegypti* development. These authors reported that a decrease in trypsin and chymotrypsin biosynthesis occurred when the larvae pupated, indicating that trypsin biosynthesis can be terminated in larvae. Borovsky and co-workers have sequenced two trypsins and a chymotrypsin from the larval midgut of *Ae. aegypti* (GenBank AF487334, AF487426 and AY198134). The sequences of the two trypsins correspond to the early and late adult *Ae. aegypti* trypsins that were reported by Kalhok et al. [3] and Barrilas-Murry et al. [4], respectively. Borovsky and Meola [5] followed the increase and decrease of trypsin biosynthesis in the different stages of *Ae. aegypti* larval growth and development and found that the amount of trypsin in the first instar larval gut was 2.3-fold higher than chymotrypsin. The synthesis of trypsin and chymotrypsin enzymes in the gut of the second instar larval gut increased 7- and 3.4-fold, respectively. In third instar, the amount of trypsin increased by 3.4-fold whereas the amount of chymotrypsin stayed the same. The amount of trypsin in the gut of third instar larval gut was 3.8-fold higher than chymotrypsin. In the fourth instar and early pupal guts the amount of trypsin was at least 3-fold higher than chymotrypsin. These reports show that serine proteases play an important role in food digestion and growth of larval mosquito. Borovsky [6] and Borovsky et al. [7-10] reported that Trypsin Modulating Oostatic Factor (TMOF), which is synthesized in the ovaries of female mosquitoes after the blood meal, controls trypsin biosynthesis in the female mosquito's gut. Borovsky and Meola [5] reported that in larval *Ae. aegypti* TMOF is synthesized by neurosecretory cells found in the subesophageal, thoracic, abdominal ganglia of the central nervous system and the corpus cardiacum. These authors also showed that TMOF and its peptide analogues that were adsorbed onto yeast particles and were fed to *Ae. aegypti* and *Cx. quinquefasciatus* larvae caused a significant decrease in the level of trypsin biosynthesis in the larval gut, arrest of larval growth and development that caused anorexia and death. Based on this report a new study was initiated to find out if organic mimics of TMOF that show 3D conformational similarity to the folding of the N-terminus region of TMOF [11] where binding to the receptor is important [5] can be used as effective *Ae. aegypti* larvicides. Forty-four organic mimics were synthesized, six organic mimics exhibited high potency against mosquito larvae and their biological activity and effect on trypsin biosynthesis is described.

MATERIALS AND METHODS

Experimental animals

Larvae of *Ae. aegypti* were reared from eggs at 27 °C on a diet of Brewer's yeast, lactalbumin and lab chow (1:1:1) with 16:8 light:dark cycle. Adults were fed 5% sucrose solution or chicken blood.

Preparation and quantification of proteolytic enzymes

Whole larvae or dissected larval midguts were washed in saline, and homogenized with a Teflon homogenizer in 50 mM Tris-HCl buffer, pH 7.9. The homogenates were centrifuged at 14,000 *g*, and the supernatants were collected and incubated with [1,3-^{3}H] diisopropyl-fluorophosphate (DFP) (5 μCi, specific activity 35 Ci/mmol; Amersham, Arlington Heights, IL, USA) for 18 h at 4 °C. Following incubation, [1,3-^{3}H]diisopropyl-phosphoryl (DIP)-trypsin and (DIP)-chymotrypsin derivatives were spotted on paper squares, precipitated and washed with TCA, and assayed and quantified using liquid scintillation counter [12].

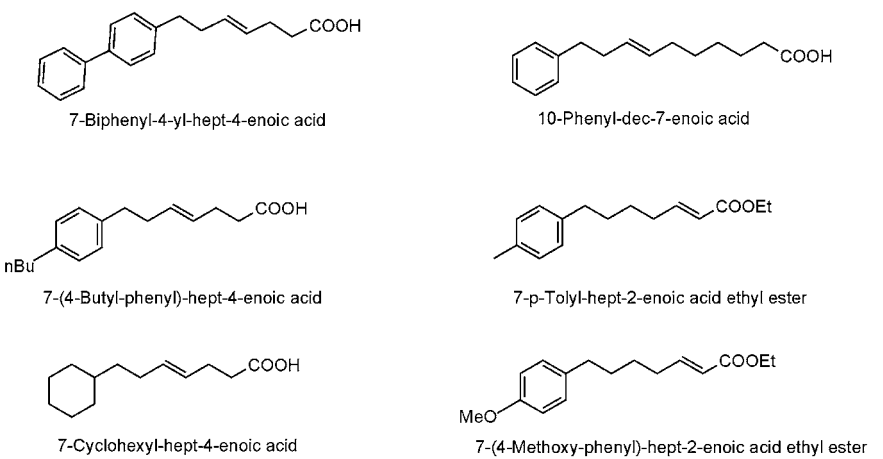

Figure 1. TMOF organo-synthetic mimics: 7-Biphenyl-4-yl-hept-4-enoic acid (BPHE); 7-(4-Butyl-phenyl)-hept-4-enoic acid (BuPHE); 7-Cyclohexyl-hept-4-enoic acid (CyHE); 10-Phenyl-dec-7-enoic acid (PDE); 7-p-Tolyl-hept-2-enoic acid ethyl ester (THEEE); 7-(4-Methoxy-phenyl)-hept-2-enoic acid ethyl ester (MPEEE).

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 [5]. Controls were fed Brewer's yeast in the presence of DMSO.

TMOF synthetic mimics

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 before for similar aromatic and aliphatic derivatives of TMOF [13]. The newly synthesized compounds were assayed by NMR, purified by chromatography (purity >95%) and were provided by IBI (Durham, North Carolina, USA). Stock solutions of 10 mg mL^{-1} of each compound were prepared in DMSO for larval testing.

RESULTS AND DISCUSSION

Aea-TMOF is an unblocked decapeptide (YDPAPPPPPP) hormone that stops trypsin biosynthesis in adult and larval mosquitoes, and is the physiological signal that terminates trypsin biosynthesis between larval molts, and after blood feeding and egg development in adult female mosquitoes, fleshflies and lepidoptera [14, 15, 18]. The hormone regulates the translation of the trypsin message and thus, it is more efficient than trypsin inhibitors that bind the enzyme's active site and stop trypsin activity, but not its biosynthesis. Because the hormone is a decapeptide it cannot penetrate the insect's cuticle, and for practical applications it has to be fed to larval mosquitoes by expressing it in yeast cells, or on the coat of tobacco mosaic virus [16, 17]. Aromatic and aliphatic mimics of TMOF that resemble the 3D stereo-specificity of the hormone can be used as possible effective mimics [13]. These organic compounds readily penetrate the insect's cuticle having aliphatic and aromatic lipophilic groups, and because they posses 3D stereo specificity that is similar to the N-terminus of TMOF [11] they can bind the TMOF's gut-receptor and stop trypsin biosynthesis like TMOF [15]. Applying TMOF organic mimics to larval mosquito in the marsh could effectively and economically control mosquito populations. To test this hypothesis, 6 leading compounds (BPHE, BuPHE, CyHE, PDE, THEEE and MPEEE; Figure 1) were synthesized. Three of the compounds (BPHE, CyHE and PDE) were tested recently on *Culex pipiens quinquefasciatus* [13]. We tested the organic mimics

on larval *Ae. aegypti* because TMOF was originally purified from this species [7], and the *Aedes* gut-receptor probably binds better these mimics because they closely resemble the larval native hormone.

Feeding first instar larvae with TMOF and yeast, or treating the larvae with the six organic mimics caused inhibition of larval development, apparent starvation, and death (Table 1). The LC_{50} of the six mimics was much lower than the LC_{50} of TMOF (0.2 mM) [5]. All the mimics exhibited enhanced activities that were 2.8 to 5.26-fold higher than the activity of TMOF (Table 1). The increase in the activity of the mimics can be attributed to the lipophilic nature of the compounds and to their ability to rapidly transverse through the cuticle into the larval hemolymph. On the other hand, TMOF has to bind to the yeast cells in the microtiter plates before they can be eaten by the larvae and transport the bound-hormone into the larval gut [5]. For TMOF to be effective, higher concentrations are needed because only a fraction, about 10% of the TMOF in the water, binds the yeast cells (Borovsky, unpublished observations). The LC_{50} observed with the six mimics are several fold lower than reported by Vanderherchen et al. [13] for *Culex quinquefasciatus*. These authors reported that the most effective mimic exhibited LC_{50} of 0.17 mM, whereas this report shows the most effective mimic THEEE exhibits a LC_{50} of 0.04 mM. PDE, the least active mimic, exhibits a LC_{50} of 0.089 mM against *Ae. aegypti* larvae as compared with a much higher value of 0.51 mM that was reported by Vanderherchen et al. against *Cx. quinquefasciatus* [13].

Table 1. Activity profile of TMOF and its organic mimics against mosquito larvae

TMOF mimics	N	LC_{50} (mM$\pm$ S.E.M.)	Activity (%)
TMOF	3	0.2 ± 0.015^{a}	100
BPHE	3	0.071 ± 0.006^{ab}	282
BuPHE	3	0.038 ± 0.003^{abc}	526
CyHE	3	0.052 ± 0.005^{abc}	384
PDE	3	0.089 ± 0.001^{ab}	225
THEEE	3	0.04 ± 0.0018^{abc}	500
MPEEE	3	0.046 ± 0.032^{abc}	434

Five to six different concentrations of TMOF and its organic mimics were incubated with 3 groups (12 larvae per group for each concentration) of late first instar larvae of *Ae. aegypti*. Larval mortality was checked daily and results are expressed as means of 3 probit analyses of lethal concentrations at 50% mortality (LC_{50}) $\pm$ S.E.M. Mortality of 1% to 5% was observed in controls that were fed Brewer's yeast. [a]Significantly different from TMOF ($p<0.05$), [b]Significantly different from BPHE and PDE ($p<0.05$), [c]Not significant different ($p>0.05$), by two-tailed Student's t test.

The results reported in Table 1 can partially be explained by the stereo specificity of the TMOF organic mimics that enabled them to bind to the TMOF's gut receptor. Toxicological and pharmacological effects of the aromatic and aliphatic mimics can also contribute to the enhanced mortality. Thus, it is important to find out whether larval growth-inhibition and death is due to anorexia prompted by down regulation of proteolysis in the gut similar to the effect of *Aea*-TMOF.

Table 2. The effect of TMOF and CyHE on serine proteases synthesis in larval *Ae. aegypti*

Treatment	N	Trypsin (ng/larva) ± S.E.M.		
		24 h	48 h	72 h
Control	9	5.15 ±0.28^{a}	12.62 ±0.42^{c}	13.56 ±0.9^{e}
TMOF	9	1.1 ±0.14^{ab}	1.17 ±0.14^{cd}	0.84 ±0.13^{ef}
CyHE	9	0.85 ±0.13^{ab}	7.73 ±0.62^{cd}	6.79 ±0.46^{ef}

Groups of first instar *Ae. aegypti* larvae (9 groups, 33 larvae per group) a day after larval eclosion were fed Brewer's yeast in the presence of TMOF (0.9 mM) or 7cyclohexyl-hept-4-enoic acid (CyHE) (0.12 mM). Both TMOF and CyHE at these concentrations cause 90% larval mortality 12 days and 6 days after the feeding, respectively. At 24 h intervals, 33 larvae were removed, homogenized and assayed for trypsin biosynthesis [12]. Results are expressed as means of 3 determinations ± S.E.M per time interval. ^{ace}Significantly different from the control ($p<0.05$), ^{df}Significant difference from TMOF ($p<0.05$), ^{b}No significant difference ($p>0.05$), by two-tailed Student's t test.

To find out if the TMOF organic mimics down regulate trypsin biosynthesis in the larval gut, CyHE which is 3.84-fold more effective than TMOF (Table 1) was added to microtiter wells (0.12 mM) in the presence of yeast cells and first instar *Ae. aegypti* larvae. At different intervals (24, 48 and 72 h), groups of larvae were removed and assayed for serine proteases. Although whole larvae were homogenized, over 90% of the trypsin and chymotrypsin in *Ae. aegypti* larva are synthesized by the gut [5]. Thus, the serine protease activity reported here reflects mainly the trypsin and chymotrypsin activity in the larval gut. The amount of serine proteases in the gut of larval mosquitoes fed TMOF and its mimic after 24 h was about 4.7 and 6-fold lower, respectively than in controls that were fed only yeast cells (Table 2). No significant difference was found in the amount of serine protease in guts of larvae that were fed TMOF or its mimic. However, at 48 h and 72 h CyHE was less effective. The amount of serine proteases in the guts of larvae that were fed the mimic was 1.6 and 2-fold lower, respectively than the controls. TMOF, on the other hand, reduced the level of serine protease at 48 h and 72 h by 10.8 and 16-fold, respectively (Table 2). Significant lower

levels of serine proteases in the larval gut at 48 h and 72 h of 6.6 and 8-fold, respectively were found when the levels of serine proteases after TMOF and CyHE treatments were compared (Table 2).

These results indicate that *Aea*-TMOF may be more stable than CyHE; the mimic is probably broken down in the water or detoxified inside the larva. It is possible that *Culex* larvae detoxify the TMOF mimics faster than *Aedes* and this is the reason for the differences in the larval LC_{50} between *Aedes* and *Culex* [13]. Future mimics will have to be constructed and formulated so they are stable in the marsh and are not readily detoxified by mosquito larvae.

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