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Review

Trypsin-modulating oostatic factor: a potential new larvicide for mosquito control

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Summary

Trypsin-modulating oostatic factor (TMOF), a mosquito decapeptide, terminates trypsin biosynthesis in the mosquito gut. The hormone is secreted from the ovary, starting 18 h after the blood meal, circulates in the hemolymph, binds to a gut receptor and stops trypsin biosynthesis by exerting a translational control on trypsin mRNA. Because of the unique primary amino acid sequence of the hormone (YDPAPPPPPP) and its stable three-dimensional conformation, TMOF is not degraded by gut proteolytic enzymes and can traverse the gut epithelial cells into the hemolymph of adults and larvae. Using this unique property, hormone fed to different species of mosquito larvae stops food digestion and causes larval mortality. To determine the shortest amino acid sequence that can bind to the gut receptor and still cause high larval mortality, 25 analogues of TMOF were synthesized and tested. The tetrapeptide (YDPA) was as effective as the decapeptide, indicating that the binding to the gut receptor is at the N-terminus of the molecule. Cloning and expressing the hormone on the coat protein of tobacco mosaic virus (TMV) in *Chlorella* sp. and *Saccharomyces cerevisiae* cells and feeding the recombinant cells to mosquito larvae caused larval mortality. These results indicate that TMOF can be used as a new biorational insecticide against mosquito larvae.

Key words: TMOF, trypsin-modulating oostatic factor, mosquito, *Aedes aegypti*, larvicide, trypsin biosynthesis, TMOF receptor, 3-dimensional modeling.

Introduction

Mosquitoes transmit many diseases, such as malaria, dengue and yellow fever, that have a social and economical impact in tropical countries. Rapid increases in population, limited funds and know-how, together with environmental change and an increase in the resistance of vectors and pathogens to insecticides and drugs and a shift in vector-control operations from long-term preventive measures to on-the-spot responses have led to an increase in vector-transmitted diseases (Gubler, 1998). Malaria causes a 1.3% loss in economic growth in Africa per year, and the long-term impact over a 15-year period is estimated at a 20% loss in the gross national product (Zaim and Guillet, 2002). Thus, vector control is an important strategy in controlling and preventing vector-borne diseases such as malaria.

Chemical insecticides are the most important components of integrated vector control. However, safe and cost-effective insecticides are rapidly disappearing because of the development of resistance, abandonment of many compounds for reasons of environmental safety, and new registration requirements that are more stringent. A global economy that has suffered setbacks in the past 10 years worldwide and limited investments into research and development of new compounds that control vectors of public health importance have slowed the development of new insecticides against mosquitoes. Because it takes 7–10 years and more than $50 million to develop and register a new insecticide (Rose, 2001), industry is very reluctant to take on new ventures that are mainly aimed at third world countries and, thus, deemed non-profitable.

In Africa, pyrethroids are used as the main insecticides for treating mosquito nets. However, the use of pyrethroids, DDT, organophosphates and carbamate has led to resistance in major malaria vectors worldwide (Zaim and Guillet, 2002). Since 1970, there has been a steady decrease in the development of alternative insecticides for use in public health (Tomlin, 2000). The pyrethroids, introduced in 1980 for indoor residual spraying and for treatment of mosquito nets, induced resistance to this entire class of compounds, as well as cross resistance to other compounds, limiting the number of effective alternatives suitable for vector control. Industrial thrust of developing more-selective compounds for agricultural use, either acting by ingestion or cloned and expressed in transgenic crops, could

be used as models to develop new public health insecticides that are environmentally friendly and can be used as effective larvicides.

Currently, there are two effective mosquito larvicides on the market; methoprene, a juvenile hormone analogue that interferes with pupal-to-adult development, and *Bacillus thuringiensis* subsp. *israelensis* (Bti), a bacterium toxin that binds to the gut epithelial cells, forming non-specific pores that lead to gut swelling and larval death (Henrick et al., 1973; Schnepf et al., 1998). The present review describes the physiological and biochemical roles of *Aedes aegypti* trypsin-modulating oostatic factor (TMOF) in adults and larvae and its potential as a future biorational larvicide.

The discovery of TMOF

Antigonadotrophins, or factors that inhibit egg development (oostatic hormones and TMOF), have been demonstrated in the cockroach *Blattella germanica* (Iwanov and Mescherskaya, 1935), decapod crustaceans (Carlisle and Knowles, 1959) and the housefly *Musca domestica* (Adams et al., 1968; Kelly et al., 1984). In mosquitoes, Meola and Lea (1972) and Else and Judson (1972) similarly demonstrated an ovary-produced humoral factor secreted during oogenesis that inhibited yolk deposition in less-developed follicles. In *Rhodnius prolixus*, oostatic hormone produced by the abdominal neurosecretory organs is a small peptide of M_r 1411 that inhibits the action of juvenile hormone on vitellogenic follicle cells and prevents the ovary from accumulating vitellogenin from the hemolymph (Liu and Davey, 1974; Davey, 1978; Davey and Kunster, 1981). In *M. domestica*, oostatic hormone seems to inhibit the release or synthesis of egg developmental neurosecretory hormone (EDNH; Adams, 1981), but in mosquito it was proposed that the hormone acts directly on the ovary (Meola and Lea, 1972). Kelly et al. (1984) injected a crude extract of oostatic hormone from *M. domestica* into the autogenous mosquito *Aedes atropalpus* and demonstrated inhibition of both egg development and ecdysteroid biosynthesis.

Borovsky (1985) reported that the mosquito ovary is a rich source of ‘oostatic hormone’. Injections of the hormone into female mosquitoes inhibited yolk deposition and vitellogenin biosynthesis (Borovsky, 1985). The hormone did not block the release of EDNH from mosquito brain and, thus, it was assumed that the hormone acted directly on the ovary, either by preventing pinocytosis or by inhibiting ecdysteroid biosynthesis. When partially purified oostatic hormone was injected into female *Aedes aegypti*, both egg development and blood digestion were inhibited (Borovsky, 1988). Injections of the hormone into decapitated and ovariectomized females (these females do not synthesize ecdysteroids and do not develop eggs but synthesize protease in their gut) inhibited trypsin-like enzyme biosynthesis and blood digestion in their midgut. These results suggested that oostatic hormone inhibits trypsin biosynthesis in cells of the midgut and not in the ovary or in the endocrine system as was earlier suggested (Borovsky, 1988). The hormone is not species specific, as injection of the hormone caused inhibition of egg development and trypsin biosynthesis in the mosquitoes *Culex quinquefasciatus*, *Culex nigripalpus* and *Anopheles albimanus* (Borovsky, 1988). Even though the target tissue of the hormone is the mosquito midgut and not the ovary or the brain, the hormone was named trypsin-modulating oostatic factor (TMOF). Borovsky and co-workers purified, sequenced and, using mass spectrometry, characterized the hormone as an unblocked decapeptide (NH_2-YDPAPPPPPP-COOH; Borovsky et al., 1990). Several peptide analogues were synthesized and shown to possess TMOF activity (Borovsky et al., 1990, 1991, 1993). The solution structure of the hormone was determined by NMR studies (Curto et al., 1993), which confirmed earlier suggestions (based upon computer modeling) that the polyproline portion of TMOF is a left-handed α helix in solution (Borovsky et al., 1990, 1993; Fig. 1).

Biological activity and mode of action of TMOF

When injected into the hemolymph of intact mosquitoes after the blood meal, TMOF is metabolized in the thorax (half-life of 1.6 h), probably by proteases that are anchored to the cell membranes (Borovsky et al., 1993). Thus, these authors followed inhibition of trypsin biosynthesis in the midgut in ligated abdomens that synthesize trypsin but do not metabolize TMOF. At concentrations of 3×10^{-9} mol l^{-1} and 6.8×10^{-6} mol l^{-1}, TMOF inhibited 50% and 90% of trypsin-like enzyme biosynthesis in the midgut of *Aedes aegypti*, respectively (Borovsky et al., 1993). The amount of TMOF present in the hemolymph of control, untreated mosquitoes at 30 h and 38 h after the blood meal was determined by enzyme-linked immunosorbent assay (ELISA; Borovsky et al., 1992)

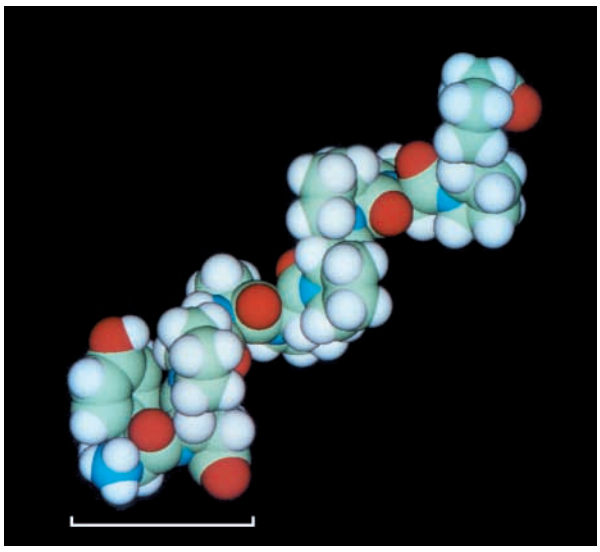

Fig. 1. A three-dimensional NMR model of *Aedes aegypti* trypsin-modulating oostatic factor (*Aea*-TMOF). A left-handed helix of six prolines can be observed at the C-terminus. The N-terminus with the first four amino acid sequence (YDPA) that is important in binding to the TMOF gut receptor is underlined.

to be between 33 ng and 37 ng, which is at least 30-fold higher than the amount that was found to cause 90% inhibition in the TMOF-treated mosquitoes (Borovsky et al., 1993). Similar results were recently obtained with hemolymph of female *C. quinquefasciatus* (D. Borovsky, unpublished observations). TMOF does not act as a classical trypsin inhibitor [e.g. TLCK (*p*-tosyl-L-lysine chloromethyl ketone hydrochloride), TPCK (*p*-tosyl-L-phenylalanine chloromethyl ketone) and soybean trypsin inhibitor] that binds to the active site of serine proteases and prevents protein hydrolysis. TMOF binds to a specific gut epithelial cell receptor and then stops trypsin biosynthesis (Fig. 2; Borovsky et al., 1990, 1994a).

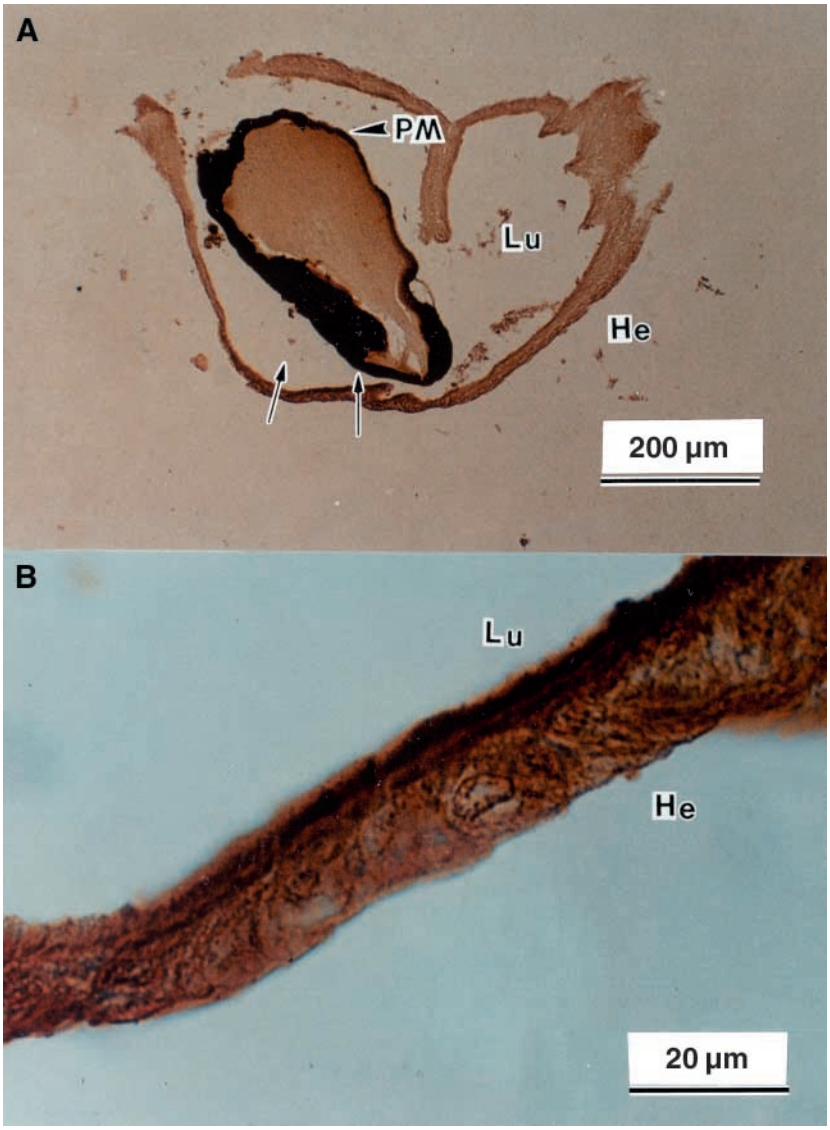

Fig. 2. Immunolocalization of trypsin-modulating oostatic factor (TMOF) binding to mosquito midgut receptor 48 h after the blood meal. (A) A transverse section (7 µm) was incubated with TMOF and polyclonal antibodies. Distinct binding of TMOF to its receptor was observed on a small area of the transverse section (dark area between arrows). (B). Fourfold magnification of the binding region. PM, peritrophic membrane; Lu, gut lumen; He, hemolymph side of the gut.

Inhibition of trypsin biosynthesis by TMOF in other insects

Mosquito TMOF or its analogues inhibited trypsin biosynthesis in the cat flea *Ctenocephalides felis*, in the stable fly *Stomoxys calcitrans*, in *M. domestica* and in the midge *Culicoides variipennis* (Borovsky et al., 1990, 1993). TMOF from the grey flesh fly *Neobellieria bullata* has been sequenced and characterized. The hormone is an unblocked hexapeptide (NH_2-NPTNLH-COOH), that, like *Aedes* TMOF (*Aea*-TMOF), stops trypsin biosynthesis and egg development in the flesh fly (Bylemans et al., 1994). The mosquito hormone did not affect trypsin biosynthesis in the flesh fly, and the flesh fly's hormone did not affect trypsin biosynthesis in the mosquito. Both hormones specifically terminate trypsin biosynthesis in the gut of the mosquito or flesh fly, respectively, after the protein meal has been digested (Borovsky, et al., 1990, 1992, 1993; DeLoof et al., 1995; Bylemans et al., 1995).

Synthesis and secretion of TMOF

TMOF is synthesized by the mosquito ovary, starting 18 h after the blood meal, and is rapidly secreted into the hemolymph. The synthesis yields a maximum concentration at 33 h and rapidly declines thereafter to a minimum at 48 h after the blood meal (Borovsky et al., 1994b). Approximately 33% of the TMOF synthesized in the ovary is secreted and circulating in the hemolymph at the peak of TMOF synthesis (33 h; Borovsky et al., 1994b). Cytoimmunochemical studies indicate that the site of synthesis of TMOF in the mosquito and the flesh fly is the follicular epithelium of the ovary. TMOF was not initially detected in the mosquito brain, indicating that the hormone is not a neuropeptide (Borovsky et al., 1994b; Bylemans et al., 1996). Recent studies of *Aea*-TMOF, however, have identified an immunoreactive material (IR) in all the ganglia of the central nervous system in larvae as well as in male and female *A. aegypti* adults. The subesophageal, thoracic and abdominal ganglia contained TMOF-IR cells located in the same regions in both larvae and adults. However, the localization and presence of TMOF-IR material differed in the brain and corpus cardiacum between larvae and adults. In larvae, TMOF-reactive cells were found in the prothoracic and abdominal ganglia, indicating that TMOF is also a neurosecretory hormone that controls trypsin biosynthesis in adult female mosquitoes through the ovaries

and in larvae by the neurosecretory cells (Borovsky and Meola, in press).

Characterization and localization of TMOF receptors

Specific TMOF-binding sites on the midgut epithelial cells increased after the blood meal and were visualized by cytoimmunochemical staining. The binding of TMOF to mosquito gut membranes was characterized at 24 h and 72 h after the blood meal. Two classes of binding sites were found on the midgut membrane: high affinity (K_{d1}=4.6×10^{-7}± 0.7×10^{-7} mol l^{-1}; K_{assoc}=2.2×10^{6} mol l^{-1}; B_{max}=0.1 pmol gut^{-1}) and low affinity (K_{d2}=4.43×10^{-6}±1×10^{-6} mol l^{-1}; K_{assoc}= 2.3×10^{5} mol l^{-1}; B_{max}=0.2 pmol gut^{-1}). The total binding sites for high and low affinity classes of TMOF per gut were estimated as 6.3×10^{10} and 1.1×10^{11} sites, respectively (Borovsky et. al, 1994a). Thus, at 24 h, when trypsin biosynthesis is at its highest level, the follicular epithelium of the ovary begins to synthesize and release TMOF into the hemolymph. TMOF binding to the gut epithelial cells signals them to cease trypsin biosynthesis, which rapidly declines and stops at 48 h (Borovsky, 1985; Borovsky et al. 1990, 1993, 1994a,b).

Genetic characterization and expression of TMOF

The effect of TMOF on the trypsin gene was first studied in *Neobellieria.* After injecting TMOF into these flies, the biosynthesis of trypsin mRNA was followed using northern analysis (Borovsky et al., 1996). Feeding these flies a liver meal caused degradation of the endogenous trypsin early mRNA and synthesis of a new mRNA that corresponded with late trypsin biosynthesis associated with post-meal digestion. In flesh flies that were injected with *Neobellieria* TMOF (10^{-9} mol l^{-1}), the early mRNA did not disappear and the late mRNA that was synthesized was not translated. These results indicate that TMOF controls the translation of the late trypsin mRNA, as would be expected for a hormone that is released after trypsin mRNA has already been transcribed (Borovsky et al., 1996). Injecting TMOF into female *A. aegypti* and *C. quinquefasciatus* and following the late trypsin mRNA by RT-PCR and northern blot analysis confirmed the observations that were reported for *Neobellieria* (D. Borovsky, unpublished observations); TMOF did not affect trypsin mRNA transcription but did affect its translation, i.e. inhibition of trypsin biosynthesis, as was shown for *Neobellieria* (Borovsky et al., 1996).

The effect of TMOF and its analogues on mosquito larvae

Feeding of [^{3}H]TMOF mixed with the blood meal to female *A. aegypti* stopped trypsin biosynthesis and inhibited egg development in the ovaries (Borovsky and Mahmood, 1995). The [^{3}H]TMOF was also found circulating in the hemolymph, indicating that TMOF traversed the mosquito gut into the hemolymph and bound a gut receptor on the hemolymph side

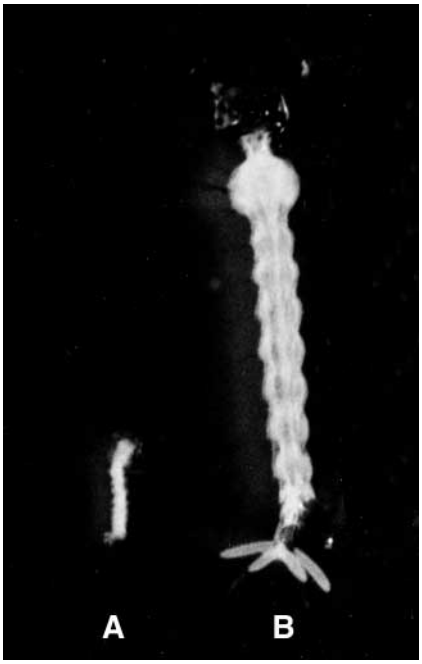

Fig. 3. Effect of feeding *Aedes aegypti* trypsin-modulating oostatic factor (*Aea*-TMOF) on larval growth and development. Mosquito larvae were fed Brewer's yeast (20 μg) with either (A) 188 μg TMOF or (B) no TMOF. Larval growth and development were compared after 6 days.

of the gut (Borovsky et al., 1994a; Fig. 2). When *A. aegypti* and *C. quinquefasciatus* larvae were fed TMOF that was adsorbed onto yeast cells (188 μg TMOF per 20 μg yeast cells), the larvae stopped synthesizing trypsin (88% and 91.7% inhibition, respectively; Borovsky and Meola, in press) and stopped growing (Fig. 3). These results indicate that shutting off trypsin biosynthesis with TMOF can be used as a new approach to control larval growth and development, possibly leading to new biorational insecticides, which are desperately needed (Zaim and Guillet, 2002). Nauen et al. (2001) have reported that tobacco budworm (*Heliothis virescens*; Lepidoptera) larvae control their trypsin biosynthesis with a hormone that is similar to *Aea*-TMOF. Either injecting or feeding *Aea*-TMOF to 4th instar *H. virescens* larvae caused inhibition of trypsin biosynthesis and larval growth. Tortiglione et al. (2002) cloned and expressed six *Aea*-TMOF genes in transgenic plants and reported an increase in mortality of 20–33% in *H. virescens* larvae that were fed on the transgenic plants. These observations prompted us to feed the hormone to different species of mosquitoes to determine the concentrations of the hormone that cause 50% mortality (LC_{50}). *A. aegypti* larvae were the most sensitive to *Aea*-TMOF (LC_{50} of 0.2±0.015 mmol l^{-1}; Table 1). The lower sensitivities of other mosquito species to TMOF indicate that they synthesize a TMOF that is either slightly different from *Aea*-TMOF or that its transport through the gut of different mosquito species is different. Because mosquito larvae are filter feeders (Clements, 1992) that internalize only the TMOF

Table 1. *Effect of* Aea-*TMOF on different mosquito species*

Mosquito species	LC_{50} (mmol l^{-1})
Anopheles quadrimaculatus	0.383±0.005[a]
Culex quinquefasciatus	0.458±0.02[a]
Culex nigripalpus	1.056±0.097[a]
Aedes aegypti	0.2±0.015[a]
Aedes taeniorhynchus	0.483±0.049[a]

Three groups of larvae (12 larvae per group) of first instar larvae were individually grown in microtiter plates containing 188 μl sterile distilled water, yeast (20 μg) and different concentrations of TMOF (1.46–376 μg). Larval mortality was followed for 5–7 days. Mortality in control wells lacking TMOF was ≤5%. Larval mortality at 50% lethal concentrations (LC_{50}) was determined using Probit and two-tailed Student's *t*-test of matched pairs analyses and are expressed as a mean of three determinations ± S.E.M.

[a]Significant difference from *A. aegypti* (LC_{50}) at $P<0.0148$.

that is adsorbed onto the yeast particles, the LC_{50} values that are presented in Table 1 are much higher than the true LC_{50} values. When recombinant yeast cells that produced TMOF were fed to mosquito larvae, and the absolute amounts of TMOF in the cells were determined using ELISA, nanogram amounts of TMOF were sufficient to kill *A. aegypti* larvae (Nauwelaers and Borovsky, 2002). Thus, the LC_{50} values in the mmol l^{-1} concentrations presented in Table 1 are, in practice, more likely to be in the μmol l^{-1} range.

To determine the shortest sequence of TMOF that binds its receptor, 25 analogues were synthesized and evaluated (Table 2). First, the maximum size of a TMOF analogue that will not traverse the gut into the hemolymph, and thus would not bind to the TMOF receptor, was established. TMOF to which an arginine was added at the C-terminus (Arg11; YDPAPPPPPPR) was not active (Table 2). When the same peptide was injected into female *A. aegypti* that were fed a blood meal and immediately ligated (Borovsky et al., 1993), trypsin biosynthesis in the midgut was 70% and 80% inhibited with 100 ng and 250 ng of TMOF, respectively (D. Borovsky, unpublished observations). These results indicate that the addition of Arg to TMOF inhibited the transport of the hormone through the gut into the hemolymph rather than its ability to bind to a TMOF gut receptor (Borovsky et al., 1994a). Several longer analogues of 20 mer [$(H)_6$IEGRYDPAPPPPPP and $(YDPAR)_4$] and 16 mer [$(DPAR)_4$] to which trypsin cleavage sites were added (IEGR, R and R, respectively) were cleaved in the midgut to smaller peptides of 10, five and four amino acids each. These short peptides traversed the gut into the hemolymph and inhibited trypsin biosynthesis. The multiple peptides showed enhanced activities of 2-fold and 4-fold for $(YDPAR)_4$ and $(DPAR)_4$, respectively (Table 2). Although the six prolines at the N-terminus have a very low biological activity (18%; Table 2), removal of three prolines from the C-terminus of TMOF lowered the activity to 45%, and removal of five prolines lowered the activity to 31%. Alternatively, removal of the six

Table 2. *Activity profile of TMOF and its analogues on mosquito larvae*

TMOF analogues	LC_{50} (mmol l^{-1})	Activity (%)
YDPAPPPPPP	0.2±0.015[a,b]	100
YDPAPPPPPPR	>71.6	0
$(H)_6$IEGRYDPAPPPPPP	0.34±0.032[b]	59
YDPAPPPP	0.44±0.05[b]	45
YDPAPP	0.64±0.032[b]	31
YDPAP	0.64±0.028[b]	31
YDPAPR	0.24±0.01[a]	80
YDPAPK	⩾2.9	0
YDPA	0.21±0.01[a]	95
YDPAR	0.12±0.017[a]	166
$(YDPAR)_4$	0.095±0.007[b]	210
YDP	2.3±0.36[b]	9
YDPR	0.24±0.02[a]	80
YD	1.24±0.05[b]	16
DPA	0.4±0.03[b]	50
DPAR	0.46±0.011[b]	43
$(DPAR)_4$	0.048±0.002[b]	417
DPAP	0.98±0.017[b]	20
DPAPPPPPP	0.44±0.015[b]	45
PAPPPPPP	0.58±0.029[b]	34
APPPPPP	1.18±0.065[b]	17
PPPPPP	1.1±0.025[b]	18
PPPP	1.5±0.085[b]	13
PP	1.83±0.07[b]	11
PAP	6.4±0.23[b]	3

TMOF and its analogues were fed to first instar mosquito larvae (three groups of 12 larvae per group) as described in Table 1. Larval mortality was followed for 6 days. Lethal concentrations at 50% mortality (LC_{50}) were obtained by Probit analyses and are expressed as means of three determinations ± S.E.M. Statistical analyses were done using two-tailed Student's *t*-test of paired samples. Mortality in control wells containing Brewer's yeast and without TMOF and its analogues was 0–5%.

[a]No significant difference between analogues and TMOF (LC_{50}) ($P>0.05$).

[b]Significant difference between analogues and TMOF (LC_{50}) at $0.001<P<0.05$.

prolines at the C-terminus increased the activity to 95%, and the biological activity was not significantly different from *Aea*-TMOF activity (Table 2). Thus, it seems that the smallest size of TMOF that binds a TMOF gut receptor and maintains biological activity of the original decapeptide is the tetrapeptide YDPA (Fig. 1). The six prolines at the C-terminus form a left-handed helix (Borovsky et al., 1990, 1993; Curto et al., 1993; Fig. 1). When three prolines or more are removed, the truncated molecule cannot form a stable left helix at the C-terminus, and thus the binding of TMOF to its receptor and the biological activity of the truncated molecule are reduced (Table 2). When all the prolines are removed, the tetrapeptide (YDPA) assumes a more stable conformation, and the binding to the receptor is enhanced, bringing it to the same level

exhibited by the decapeptide (Table 2). Similar results were obtained when eightmer and fivemer TMOF analogues (YDPAPPPP and YDPAP, respectively) were injected into female *A. aegypti* (Borovsky et al., 1993).

Cloning and expression of *Aea*-TMOF in *Saccharomyces cerevisiae*

Feeding of TMOF and its analogues to mosquito larvae indicates that TMOF has potential as an effective larvicide. Although TMOF by itself is useful for establishing activity against mosquito larvae, the cost of chemical synthesis and its water solubility (mosquito larvae are filter feeders) limits its usefulness as a commercial product. Consequently, several biological methods for producing TMOF were tried. TMOF was fused to the coat protein of tobacco mosaic virus (TMV), and the recombinant protein was fed to mosquito larvae, causing inhibition of trypsin biosynthesis and larval mortality (Borovsky et al., 1998). TMOF was also expressed in *Chlorella* sp., and the recombinant cells caused larval mortality within 72 h of feeding (Borovsky et al., 1998). Although these results are encouraging, plant leaves are not natural food for mosquito larvae, and *Chlorella* sp. grows slowly. We then selected a haploid strain of *S. cerevisiae* and cloned TMOF and GFP–TMOF (TMOF fused with green fluorescent protein) using homologous recombination and free plasmid expression (Nauwelaers and Borovsky, 2002). Synthesis of TMOF and GFP–TMOF was followed by ELISA (Borovsky et al., 1992) and fluorescence microscopy. Mosquito larvae that were fed recombinant yeast cells that synthesized TMOF or GFP–TMOF did not digest the yeast cells, stopped growing and 38–83% of the larvae died. By contrast, only 4–8% of larvae that were fed cells that were transformed with plasmids that were not carrying TMOF, or larvae that were fed Brewer's yeast, died (Nauwelaers and Borovsky, 2002). These results are encouraging; however, to increase the amount of TMOF that is produced in yeast cells, new promoters and a combination of multigene insertions of TMOF and its analogues will have to be developed.

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