ABSTRACT

Vanderherchen, Matthew Barton: Trypsin Modulating Oostatic Factor (TMOF) and Non-peptidic Analogs as Novel Insecticides and Arthropod Repellents. (Under the direction of Dr. R. M. Roe, Chair of Advisory Committee)

Eight nonpeptidic chemical analogs 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 (LC_{50}) for these analogs, TMOF and FDPAP, a peptidic TMOF analog, was estimated for larvae of the northern house mosquito, *Culex pipiens*, using a static 5-day bioassay. Four of these compounds demonstrated the same larvicidal activity as TMOF, while three of these compounds were 1.2 - 2.5-fold more active than TMOF. There was a relationship found between the lipophilicity of this series of analogs and toxicity to mosquito larvae. All eight TMOF analogs were toxic by injection at some level in fourth instars of the tobacco hornworm (*Manduca sexta*) while TMOF and FDPAP were non-toxic. Injection of TMOF and FDPAP into fourth stadium and TMOF into second stadium *M. sexta* had no effect on trypsin activity, larval growth or mortality. Apparently the mosquito hormone is inactive in this species and at the developmental stages examined. Three TMOF analogs (CHEA, PHEA, PHA), demonstrating the highest activity by injection in *M. sexta*, were also found to be toxic by injection in fourth instars of the tobacco budworm (*Heliothis virescens*) and the cotton bollworm (*Helicoverpa zea*) as well as adult male German cockroaches (*Blattela germanica*). Finally, results of a two-choice feeding bioassay with *H. virescens* indicated that one of the TMOF analogs, PHEA, has anti-feedant properties.

Novel aromatic and aliphatic organic acids, esters and ketone mimics of the mosquito trypsin modulating oostatic factor (TMOF) were synthesized and assayed as repellents for ticks and as an anti-feeding agent for mosquitoes. *E*-7-(cyclohexyl)hept-4-enoic acid (CHEA), *E*-7-phenylhept-4-enoic acid (PHEA), ethyl *E*-7-(cyclohexyl)hept-4-enoate (CHEN) and ethyl *E*-7-phenylhept-4-enoate (PHEN) had repellent activity against the soft tick, *Ornithodorous parkeri* (Acari: Argasidae) in a two-choice bioassay. PHEN, an aromatic organic ester, was the most active. Only 15% of the ticks tested selected the surface treated with 5 $\mu g/cm^2$ of PHEN as compared to the untreated surface. Commercially available 2-undecanone, a natural product found in the trichomes of wild tomatoes, was found to also mimic the structure of TMOF and was active as a repellent at 50 $\mu g/cm^2$. Since this compound is a natural botanical product with low mammalian toxicity, further tests were conducted to determine its anti-feeding activity against mosquitoes. The median effective dose to prevent 50% of adult yellow fever mosquitoes, *Aedes aegypti*, from obtaining a blood meal (ED_{50}) was estimated as 91.5 $\mu g/cm^2$ of human skin as compared to 1.6 $\mu g/cm^2$ for deet. 2-Undecanone applied as a 30 and 40% solution (vol/vol) in isopropyl alcohol to human skin was 100% active for 15 min in preventing mosquito feeding and 40% provided 93% protection 30 min after application. Persistence is limited by the high volatility of 2-undecanone although it appears that once mosquitoes come into contact with the repellent, host seeking is inhibited more than 2 h.

TRYPSIN MODULATING OOSTATIC FACTOR (TMOF) AND NON-PEPTIDIC ANALOGS AS NOVEL INSECTICIDES AND ARTHROPOD REPELLENTS

by

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DEDICATION

First and foremost, I would like to thank God for strength, patience and His many provisions. I would like to thank all of my family; my parents, Bart and Lynn, my sister Kelly and my brother-in-law, Jimmy, my niece, Kaycie, and my nephew, Christian, and my wife's family, Roger, Sally, Mark and Matt, for all of their support and encouragement.

This thesis is dedicated to my wife, Heather. She has been an inspiration and a blessing and I could not have done it without her.

BIOGRAPHY

Matthew Barton Vanderherchen was born on October 27, 1974 in Beloit Wisconsin to Fredric Barton and Carol Lynn Vanderherchen. He spent his early life in several places including Forth Worth, Texas, Gywnn's Island, Virginia, Williamsburg, Virginia, and eventually settled in Chesterfield County, Virginia. After graduating from Lloyd C. Bird high school in Chesterfield County, Matthew graduated Magna Cum Laude with a Bachelor of Science degree in 1999 from Virginia Commonwealth University located in Richmond, Virginia. Matthew married Heather Suzanne Nisley in December of 1999 and immediately moved to Raleigh, North Carolina to attend graduate school in the Department of Entomology at North Carolina State University.

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Introduction

Chapter I: The Regulation of Insect Digestion

The digestive system is the primary interface between insects and transgenic insecticidal crops which are expected to play an ever-larger role in integrated pest management (IPM) strategies in the future. A more complete understanding of the digestive physiology of insects will be critical in order to develop new strategies to prevent crop failure in the inevitable appearance of resistance of pest species to cultivars expressing insecticidal proteins derived from the bacterium *Bacillus thuringiensis* (Bt). The number of possible cultivars expressing Bt toxins is finite and the development time lengthy, necessitating exploration of other modes of action (MOA).

The morphological and biochemical organization of digestion in insects has been shown to correlate well with the phylogenic position and nutritional requirements of insects (Terra, 1990). There are many lines of evidence including biochemical, molecular and histological data that support this conclusion. The inherent similarities and differences in the digestive physiology of insects are amendable to exploitation and should be seriously considered in the search for new MOAs. Inhibition of protease biosynthesis in the insect midgut is one MOA that has potential applications in the control of many important agricultural pests. The primary function of proteases or peptide hydrolases is to breakdown dietary protein into amino acids necessary for insect growth and development (Terra and Ferreira, 1994). Proteases are divided into two main classes on the basis of preferred cleavage sites. Endopeptidases or proteinases preferentially cleave peptide bonds within the protein. Exopeptidases preferentially remove the amino- or carboxy-terminal amino acids of proteins. The relative importance of endo- and exopeptidases is determined by the phase of

digestion. During the initial phase of digestion, endopeptidases such as trypsin and chymotrypsin are more important and therefore are present at higher levels in the gut lumen. Endopeptidases cleave proteins into smaller oligopeptides, which are subsequently attacked by exopeptidases such as aminopeptidase and carboxypeptidase during the intermediate phase of digestion. During the final phase of protein digestion, dimers are reduced to monomers by dipeptidases and absorption through the midgut epithelium and distribution throughout the insect usually follow. Proteases can be further classified based on substrate specificities, required cofactors, amino acid sequence of the enzyme and the length of amino acid chains preferentially cleaved.

According to Woods and Kingsolver (1999), who developed a mathematical "chemical reactor" model describing protein digestion in lepidopteran larvae, the transformation of dietary protein into biomass occurs in four main serial steps: consumption of dietary protein, digestion of protein into amino acids, absorption of amino acids and construction of tissues and biological molecules from these amino acids. The hypothesis generated by the model proposed by these authors is that either absorption or post-absorption processes are the rate-limiting step(s) in nutrition acquisition. Observations of insect feeding behavior and biochemical analysis of insect digestive processes have provided support for this hypothesis. Further investigation of these processes especially those involving receptor mediated transfer across the gut epithelium may provide target sites for new insecticides.

Alternatively, if digestion could be made functionally limiting by decreasing the abundance of the major gut endopeptidase trypsin, then absorption and biomass accumulation would be secondarily compromised. This has been accomplished with limited success by plants expressing proteinase inhibitors such as Soybean Trypsin Inhibitor (STI). These

inhibitors bind and competitively inhibit trypsin, which is the major gut endopeptidase in some insect digestive systems. Unfortunately, it has been demonstrated that trypsin inhibitors (TIs), while effective against specific isozymes, induce isozymes resistant to or not affected by TIs in the midgut of lepidopteran larvae (Jongsma et al., 1995; Mazumdar-Leighton and Broadway, 2001). The mechanism(s) by which this apparent induction occurs is unknown although it has been hypothesized that either or both of the processes of digestive enzyme synthesis or secretion are regulated by factors produced in the gut or other tissues composed of endocrine cells. These factors may modulate trypsin levels in response to TIs (Broadway, 1997; Gatehouse et al.,1997).

There are several lines of evidence suggesting that insect digestion, specifically enzyme synthesis or secretion, is regulated by hormonal factors. The similarity in the number and types of digestive enzymes and the spatial organization of digestive processes between mammals and insects has stimulated speculation that comparable regulatory mechanisms exist (Huang et al., 1998). Dipterans and lepidopterans in general possess a complement of mammalian-like proteinases and these enzymes and associated metabolic functions are compartmentally organized as in mammals to maximize digestive efficiency (Terra et al., 1996).

In mammals, the level of trypsin in the small intestine is determined by the complex interplay of at least two peptidergic hormones, monitor peptide (MP) and cholecystokinin (CCK). MP, so named for its trypsin modulating activity, is a 61 amino acid monomeric protein originally isolated from rat pancreatic juice (Iwai et al., 1987) and subsequently found to have two distinct but related functions. MP is synthesized in the pancreatic acinar cells and stored in zymogen granules (Tsuzuki et al., 1991). MP is trypsin sensitive and

similar in amino acid sequence to pancreatic secretory trypsin inhibitors (PSTI), which are classified as kazal type inhibitors. One function of MP is to serve as a substrate for trypsin in the small intestine in the absence of dietary protein, preventing degradation of the lining of the intestinal mucosa cells. After ingestion of a meal, dietary protein competes with MP for the active site of trypsin, allowing progressively more MP to serve a second function, the stimulation of CCK release into the blood stream from I-cells of the proximal small intestine (Sharara et al., 1993).

CCK is an enteric hormone with multiple physiological functions including induction of satiety, regulation of gastric emptying, stimulation of gall bladder contraction and the stimulation of pancreatic secretion of digestive enzymes including trypsin (Liddle, 1995). To summarize, MP regulates the level of trypsin activity in the small intestine of surveyed mammals. In the absence of dietary protein, MP inhibits by competitive interaction with the enzyme, the release of trypsin into the intestinal lumen. In the presence of dietary protein, unbound MP triggers CCK release, which in turn stimulates the secretion of trypsin into the lumen.

It is possible that the complexity of trypsin regulation in the mammalian digestive system is mirrored in insects. While MP-like peptides have never been reported in insects, other mammalian-like gut proteins such as pancreatic polypeptide-like and CCK-like peptides have been detected in insects by immunohistochemical and biochemical techniques (Garcera and Tamarelle, 1995; Sehnal and Zitnan, 1996; An et al., 1998; Huang et al., 1998). These factors have not yet been completely characterized or their function elucidated. It is my hypothesis that endogenous factor(s) regulate trypsin biosynthesis and secretion in larval

Lepidoptera and that these factors may have both stimulatory and inhibitory activity towards trypsin biosynthesis and secretion.

The regulation of trypsin biosynthesis has been studied extensively in Culicidae. In mosquitoes, distinct roles for at least two trypsin families have been assigned and are related to the physiological phases of digestion. Early trypsin is regulated at the transcriptional level by juvenile hormone (JH) and at the translational level by dietary protein and predominates in the midgut of *Aedes aegypti* (Diptera: Culicidae) females during the first few hours following a blood meal (Noriega et al., 1997). Late trypsin is the predominant form of this enzyme detected in the second phase of digestion. Late trypsin is regulated at the transcriptional level indirectly by early trypsin. The mechanism by which this regulation occurs is currently unknown.

A hormone has been has isolated, purified and characterized from the ovaries of *A. aegypti* that also regulates trypsin biosynthesis in this species (Borovsky et al., 1993). This decapeptide, trypsin modulating oostatic factor (TMOF), inhibits trypsin biosynthesis following digestion of the bloodmeal in the adult female. 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). Nauen et al. (2001) found that synthetic TMOF causes a reduction in the levels of trypsin in the midgut lumen of larvae of the tobacco budworm, *Heliothis virescens* (Lepidoptera: Noctuidae). These workers also found a factor in the hemolymph of this species that cross reacted with an antibody to TMOF. This factor was subsequently shown to reduce luminal trypsin levels in *H. virescens*. It is unknown if TMOF or TMOF-like factors regulate trypsin biosynthesis in other lepidopteran species.

The primary intent of the studies described in this thesis was to investigate the regulation of digestion in larval Diptera (Culicidae) and Lepidoptera (Sphingidae). Nonpeptidic chemical mimics of TMOF (referred to hereafter also as TMOF analogs) were synthesized based on the structure of the native peptide. Chapter I of this thesis describes the use of these mimics as chemical probes to explore the regulation of digestion in the northern house mosquito, *Culex pipiens* (Diptera: Culicidae) and the tobacco hornworm, *Manduca sexta* (Lepidoptera: Sphingidae).

Chapter II: Application of Novel Potential Arthropod Repellents

In the course of the studies undertaken in Chapter I, it was discovered that the nonpeptidic chemical analogs of TMOF possessed desirable insect control properties unrelated to the inhibition of trypsin biosynthesis, namely anti-feedant and deterrent/repellant properties. In chapter II of this thesis, we wanted to investigate the effects of these novel chemistries on the behavior of biting arthropods. As mammalian toxicology data for these chemistries do not currently exist, testing was restricted to two-choice bioassays with the soft tick, *Ornithodoros parkeri* (Acari: Argasidae). To our knowledge, this is the first report of the use of representatives of this novel chemical class for the protection of persons against arthropods of medical and veterinary importance.

The naturally derived 2-undecanone (methyl nonyl ketone) is similar in structure to the TMOF analogs and may also have potential as an arthropod repellent. The 11-carbon methyl ketone, 2-undecanone, is an oily liquid at room temperature and has a strong odor (Budavari, 1996). This compound can be isolated from a variety of natural sources. 2-Undecanone has been identified as one of the volatile compounds emitted from the

pathogenic bacterium, *Esherichia coli* (Yu et al., 2000) and is found among other volatile compounds in the aroma of Camembert cheese (Kubickova and Grosch, 1997). Szauman-Szumski et al. (1998) identified 2-undecanone in whole body extracts of the olive bark beetle, *Phloeotribus scarabaeoides* (Coleoptera: Scolytidae) and in the wood of olive trees (*Olea europea*) but only after invasion of this wood-boring pest.

2-Undecanone is also found in a variety of plant species in the family Rutaceae (Guenther, 1949). Rue oil derived from *Ruta graveolens* L. ("garden rue") and *R. montana* L. ("summer rue") have been found to contain 2-undecanone as the major constituent. This compound is also found to a lesser extent in the rue oils distilled from *R. bracteoas* L. ("winter rue"). The rue oils isolated from these plants have been used at various times in flavorings, perfumery and soaps. Oils containing a high percentage of 2-undecanone were once important as starting material for the preparation of methyl nonyl acetaldehyde. 2-Undecanone has also been isolated from the oil of jaborandi leaves (*Pilocarpus spp*., Rutaceae) where this compound is the main constituent. However, this oil is not currently of commercial importance. 2-Undecanone has been found to be the major constituent of the essential oils derived from other species in the family Rutaceae including *R. chalepensis* L. (Baser et al., 1996), *Zanthoxylum pinnatum* L. (Brophy et al., 2000), *Glycosmis pentaphylla* (Cor.) (Ahmed et al., 2000) and the Japanese pepper ("sansho"), *Zanthoxylum piperium* DC. (Kasahara and Osawa, 1998).

Other plant sources of 2-undecanone include *Humulus lupulus* L. (Moraceae) from which the oil of hops used in beer and malt beverage production is derived (Guenther, 1952a), the fatty oil of coconuts, *Cocus nucifera* L. (Palmae) (Guenther, 1952b) and from the lichen ("oak moss"), *Euernia prunastri* L. (Usneaceae) (Guenther, 1952c). 2-Undecanone

has also been found to be a constituent of the essential oil of wild tomato, *Lycopersicon hirsutum f. glabratum* (Solanaceae) (Soost et al., 1968; Lundgren et al., 1985) where this compound has been shown to play a role in resistance to herbivorous arthropods.

In plant accession PI 1344717 of the wild tomato, 2-undecanone was found in the type VI glandular trichomes at a concentration of 0.66 % fresh weight (Farrar et al., 1987). At a similar concentration, these authors found that synthetic 2-undecanone incorporated into artificial diet caused pupal deformity and mortality in the tomato fruitworm, *Heliothis zea* (Lepidoptera: Noctuidae), when ingested by the larvae. Lin et al. (1987) found that topical application of exudates and extracts of type VI trichomes containing 2-undecanone as well as synthetic 2-undecanone were toxic to neonates of the tomato pinworm, *Kiefera lycopersicella* (Lepidoptera: Gelechiidae) and the beet armyworm, *Spodoptera exigua* (Lepidoptera: Noctuidae). These authors proposed that 2-undecanone, a volatile liquid, keeps 2-tridecanone, a solid 13-carbon methyl ketone also found in type VI trichomes, dissolved while 2-tridecanone prevents the evaporation of 2-undecanone from the trichome glands. Resistance to some arthropods from these chemicals may be in part physical: As the arthropod ruptures the gland, 2-undecanone rapidly evaporates, leaving the arthropod attached to the viscous residue that remains.

Synthetic 2-undecanone has also been shown to be toxic to larvae (planidia) of a tachnid parasitoid of *H. zea*, *Archytas marmoratus* (Diptera: Tachinidae), by direct contact with filter paper treated with this compound (Farrar et al., 1992). Toxicity of synthetic 2-undecanone to the two-spotted spider mite, *Tetranychus urticae* (Acari: Tetranychidae), has also been reported (Chatzivasileiadis and Sabelis, 1997). Apparently the methyl-ketone based resistance of the wild tomato also adversely affects these species.

For our studies, the non-toxic effects of 2-undecanone on arthropods were of greater interest. Kashyap et al. (1991) noticed that some factor(s) associated with the type VI glandular trichome of wild tomato reduced the searching efficiency and landing rates of *Telenomus sphingis* (Hymenopetera: Scelionidae), a parasitic wasp attacking the eggs of the tobacco hornworm, *Manduca sexta* (Lepidoptera: Sphingidae). The same or possibly different factor(s) was also observed to stimulate prolonged grooming behavior in this species. These authors could not conclude for certain that the repellency observed for *T. sphingis* was due to 2-undecanone, 2-tridecanone or some other volatile component of the trichomes.

Barbour et al. (1993) found that egg consumption was lower and mortality was higher for *Coleomegilla macula* (Coleoptera: Coccinellidae) and *Geocorris punctipes* (Hemiptera: Lygaeidae), two insect predators of *H. zea* eggs, when these insects were maintained on the foliage of resistant tomatoes and also when provided eggs on filter paper treated with 2-undecanone and 2-tridecanone. These authors suggested that volatile compounds associated with the type VI trichomes of the resistance varieties of tomatoes may have repellent or deterrent properties against these two predator species. Chatzivasileiadis and Sabelis (1997) found that 2-undecanone was repellent or deterrent to the two spotted spider mite as the mites avoided areas of leaf discs treated with this compound and oviposited predominantly on the untreated areas. Conversely, Szauman-Szumski et al. (1998) found that 2-undecanone was attractive to male and female olive bark beetles. Apparently, 2-undecanone elicits markedly different responses depending on the arthropod species tested.

More recently, 2-undecanone has been found to be repellent or deterrent to the German cockroach, *Blattela germanica* (Blattaria: Blattellidae), green peach aphids, *Myzus*

persicae (Sulzer) (Homoptera: Aphididae) and western flower thrips, *Frankiniella occidentalis* (Pergande) (Thysanoptera: Thripidae) (R.M. Roe, unpublished). The purpose of this study was to investigate the effects of 2-undecanone on the host seeking behavior of the yellow fever mosquito, *A. aegypti* (Diptera: Culicidae) and the soft tick, *O. parkeri.* 2-Undecanone is currently registered by the EPA for use as an animal repellent (EPA, 1995). Therefore the mammalian toxicology data for this compound is already available. The toxicology profile of 2-undecanone shows that it is comparable and in some aspects favorable to deet, the current standard among insect repellents. The studies described in Chapter II of this thesis were undertaken in part to determine if 2-undecanone has the potential to be a reasonable alternative to the currently limited arsenal of arthropod repellents available for personal protection. To our knowledge, this is the first report of the use of this compound for the protection of persons against biting arthropods of medical and veterinary importance.

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Chapter I

Toxicity of novel aromatic and aliphatic organic acid and ester analogs of trypsin modulating oostatic factor (TMOF) to larvae of the northern house mosquito, *Culex pipiens*, and the tobacco hornworm, *Manduca sexta*.

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ABSTRACT

Eight nonpeptidic chemical analogs 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 (LC_{50}) for these analogs, TMOF and FDPAP, a peptidic TMOF analog, was estimated for larvae of the northern house mosquito, *Culex pipiens*, using a static 5-day bioassay. Four of these compounds demonstrated the same larvicidal activity as TMOF, while three of these compounds were 1.2 - 2.5-fold more active than TMOF. All eight TMOF analogs were toxic by injection at some level in fourth instars of the tobacco hornworm, *Manduca sexta,* while TMOF and FDPAP were non-toxic when injected at 50 μg per insect. Injection of TMOF and FDPAP into fourth stadium and TMOF into second stadium *M. sexta* had no effect on trypsin activity, growth or mortality. Apparently the mosquito hormone is inactive in this species and at the developmental stages examined. Three TMOF analogs (CHEA, PHEA, PHA) demonstrating the highest activity by injection in *M. sexta*, were also found to be toxic by injection in fourth instars of the tobacco budworm (*Heliothis virescens*) and the cotton bollworm (*Helicoverpa zea*) as well as adult male German cockroaches (*Blattela germanica*). Finally, results of a two-choice feeding bioassay with *H. virescens* indicated that one of the TMOF analogs, PHEA, has anti-feedant properties.

1. 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 [1]. This decapeptide, Tyr-Asp-Pro-Ala-Pro-Pro-Pro-Pro-Pro-Pro [2], actuates a specific receptor on the mosquito gut signaling the termination of biosynthesis of the major digestive enzyme trypsin [3]. 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 use as a mosquito larvacide.

TMOF or peptidic analogs of TMOF 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) [4]. 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) [5] and the tobacco budworm, *Heliothis virescens* (Lepidoptera: Noctuidae) [6]. 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 expand the utility of this new class of insecticides, eight nonpeptidic chemical analogs of TMOF (referred to hereafter also as TMOF analogs) were synthesized based on the structure of the native peptide. In this work, we evaluated TMOF, FDPAP and our novel chemistry in the northern house mosquito, *Culex pipiens* (Diptera: Culicidae) with the intent of characterizing the essential structural characteristics necessary for toxicity to mosquito

larvae. In order to extend the TMOF technology into the agricultural and urban pest management arenas, TMOF analogs were also tested against the tobacco hornworm, *Manduca sexta* (Lepidoptera: Sphingidae), tobacco budworm, cotton bollworm, *Helicoverpa zea* (Lepidoptera: Noctuidae) and the German cockroach, *Blattella germanica* (Blattaria: Blattellidae). The effects of TMOF and FDPAP on the trypsin activity and growth rate of *M. sexta* were also examined. Finally, feeding experiments were conducted with *H. virescens* in an effort to elucidate the mode of action of the TMOF analogs.

2. Materials and methods

2.1. Insects

Culex pipiens egg rafts were purchased from Carolina Biological Supply Co. (Burlington, NC). Mosquito larvae were allowed to eclose in covered beakers containing sterile distilled water. Unfed first stadium day 1 larvae were used in mosquito bioassays. *M. sexta* larvae (the original Yamamto strain [7]) were maintained at the NCSU Insectary on artificial diet according to Bell and Joachim [8]. Experiments with fourth instar *M. sexta* were iniatiated with larvae that were physiologically synchronized at head-capsule slippage from third to fourth instar. Following treatment as fourth stadium day zero lavae, *M. sexta* were maintained in 1-oz plastic cups until the end of the fourth stadium and then transferred to 16-oz plastic cups for the remainder of the observation period. Experiments with second instar *M. sexta* were iniatiated with larvae that were physiologically synchronized at head-capsule slippage from first to second instar. Following treatment as second stadium day zero larvae, *M. sexta* were maintained in 1-oz plastic cups. *H. virescens* and *H. zea* larvae were obtained from colonies maintained at the NCSU Insectary on artificial heliothine diet [9] in 1-oz plastic cups. The *H. virescens* colony was propagated from individuals collected on

tobacco in 1997 in Clayton, NC. The *H. zea* colony was propagated from individuals collected on cotton in 1997 in Plymouth, NC. *C. pipiens*, *M. sexta*, *H. virescens* and *H. zea* were maintained at 27±2°C, 50±10% RH, and a 14:10 (L:D) photoregime following treatment.

Blatella germanica were provided by Coby Schal, NCSU, and were from a colony that originated from an American Cyanmid Co. (Princeton, NJ) stock. Following treatment, cockroaches were transferred to 16-oz plastic cups (10 insects per cup) and maintained on Purina No. 5012 Rat Chow (Purina Mills, St. Louis, MO) in the dark at ambient laboratory conditions. Water was provided in microcentrifuge tubes with cotton stoppers.

2.2. Synthesis of TMOF analogs

Preparation of Ethyl E-7-Phenylhept-4-enoate (PHEN). E-7-Phenylhept-4-enoic acid was prepared from commercially available 3-phenylpropanal (dihydrocinnamaldehyde) in four steps as follows. Dihydrocinnamaldehyde was added to an excess of vinyl magnesium bromide in tetrahydrofuran (THF) at 0°C and allowed to warm to room temperature and remain there for 2 hr. The reaction was quenched with saturated ammonium chloride solution and extracted twice with ether. The combined ether extracts were dried over anhydrous sodium sulfate and after evaporation of the ether *in vacuo*, the residue was purified by column chromatography on silica gel to yield 5-phenyl-3-hydroxypent-1-ene. The allylic alcohol was then dissolved in triethyl orthoacetate with a catalytic amount of propionic acid and heated at 140°C for 6 hr (following distillation of the ethanol). After cooling to room temperature, the excess triethyl orthoacetate was removed under reduced

pressure, and the resulting residue purified by flash chromatography to give E-7-phenylhept-4-enoate in >95% purity.

Preparation of E-7-Phenylhept-4-enoic acid (PHEA). Ethyl E-7-Phenylhept-4-enoate was saponified in methanolic sodium hydroxide at room temperature. The resulting acid, E-7-phenylhept-4-enoic acid, was obtained in >95% purity after chromatography.

Preparation of E-7-Phenylheptanoic Acid (PHA). E-7-Phenylheptanoic acid was prepared from E-7-phenylhept-4-enoic acid by hydrogenation at 40 psi using 5% palladium on carbon as the catalyst. Quantitative conversion of the alkene to the alkane was observed by thin layer chromatographic analysis. The product acid was obtained in >95% purity after chromatography on silica gel.

Preparation of E-7-(Cyclohexyl)hept-4-enoate (CHEN). Ethyl E-7-(cyclohexyl)hept-4-enoate was prepared in two steps from the known compound 3-cyclohexylpropanal. A THF solution of 3-cyclohexylpropanal was treated with vinyl magnesium bromide (as a solution in THF) at 0^{o}C. The resulting allyl alcohol derivative, 5-cyclohexyl-3-hydroxypent-1-ene, was purified by chromatography. The allylic alcohol was then dissolved in triethyl orthoacetate and heated to 140^{o}C to effect a Claisen rearrangement. The product, ethyl E-7-(cyclohexyl)hept-4-enoate, was obtained in >95% purity after chromatography.

Preparation of Ethyl E-7-Phenylheptanoate (PHN). Ethyl E-7-Phenylheptanoate was prepared from ethyl E-7-phenylhept-4-enoate by hydrogenation at 40 psi using 5% palladium on carbon as the catalyst. Quantitative conversion of the alkene to the alkane was observed by thin layer chromatographic analysis. The product ester was obtained in >95% purity after chromatography on silica gel.

Preparation of E-7-(Cyclohexyl)hept-4-enoic Acid (CHEA). Ethyl E-7-(Cyclohexyl)hept-4-enoate was saponified in methanolic sodium hydroxide at room temperature. The resulting acid, E-7-(cyclohexyl)hept-4-enoic acid, was obtained in >95% purity after chromatography.

Preparation of Ethyl E-7-((4-phenyl)phenyl)hept-4-enoate (PPHEN). Ethyl E-7-((4-phenyl)phenyl)hept-4-enoate was prepared in two steps from the known compound 3-((4-phenyl)phenyl)-propanal. A THF solution of 3-((4-phenyl-phenyl)propanal was treated with vinyl magnesium bromide (as a solution in THF) at 0^{o}C. The resulting allyl alcohol derivative, 5-((4-phenyl)phenyl)-3-hydroxpent-1-ene, was purified by chromatography. The allylic alcohol was then dissolved in triethyl orthoacetate and heated to 140^{o}C to effect a Claisen rearrangement. The product, ethyl E-7-((4-phenyl)phenyl)hept-4-enoate, was obtained in >95% purity after chromatography

Preparation of E-7-((4-phenyl)phenyl)hept-4-enoic Acid (PPHEA). Ethyl E-7-((4-phenyl)phenyl)hept-4-enoate was saponified in methanolic sodium hydroxide at room temperature. The resulting acid, E-7-((4-phenyl)phenyl)hept-4-enoic acid, was obtained in >95% purity after chromatography

The structures of the final products were verified by the appropriate spectral analysis (infrared and nuclear magnetic spectroscopy) and combustion analysis.

2.3. Mosquito bioassays

The median lethal concentration (LC_{50}) for TMOF (SynPep Corp., Dublin, CA), FDPAP (SynPep Corp., Dublin, CA) 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, personal communication). Day 1 first stadium *C. pipiens* were transferred singly to individual wells of a 96-well microtiter plate in approximately 20 µL of sterile distilled water. All water was removed and 150 µL of a 0.14% yeast (Brewers-debittered, inactive; ICN Biochemicals, Cleveland, OH) solution was immediately added to each well. Stock solutions of TMOF and FDPAP were prepared in sterile distilled water. Stock solutions of nonpeptidic chemical analogs were prepared in technical grade DMSO. Dilutions from all stock solutions were then made into sterile distilled water, so when 50 µL was transferred to each well of the microtiterplate, the desired final concentration of the test compounds were obtained in a total volume of 200 µL per well. Controls for TMOF and FDPAP were treated with 50 µL of water and controls for TMOF analogs were treated with 50 µL of 2% (v:v) DMSO in water. Following treatment, plates were covered with plastic transparent lids and placed in sealed plastic containers. The floor of these containers were covered with water before sealing, creating an environment of 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). In all LC_{50} estimations, treatments (5 doses) and solvent controls were replicated at least three times with 12 insects per replicate. Abbot's correction was applied to all data from dose response experiments [10]. LC_{50}s were determined by plotting log dose versus probit plus 5 mortality [11-13].

2.4. Injections

Initial testing of TMOF, FDPAP and TMOF analogs was conducted using *M. sexta*. Insects were selected at head capsule slippage to the fourth stadium. Fourth stadium day zero

larvae (L4D0) were injected in separate experiments at a high dose, 1.5 mg per insect, and a low dose, 50 µg per insect**.** Larvae were anesthetized on ice for one minute prior to treatment. All injections were made in the perivisceral hemocoel through an abdominal proleg using 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. Ten insects were assayed per treatment and solvent control in each experiment. Experiments were replicated three times. Mortality (failure to respond to a blunt probe in 10 seconds) was assessed after eight days, corrected according to Abbot (10) and is expressed as mean cumulative corrected percent mortality ± SEM.

For the injections at 1.5 mg per insect, TMOF analogs were injected undiluted in a volume of 1.4-1.6 µL, depending on the density of the compound. The TMOF analogs (except for PPHEA) are oils at room temperature with a viscosity similar to olive oil. For this reason, controls for injections at 1.5 mg per insect were injected with 1.5 mg (≈1.4 µL) of olive oil. PPHEA is a solid at room temperature, is not soluble in common injection solvents at this concentration and was therefore not injected at 1.5 mg per insect.

From the results of the previous injections at the high dose, it was determined that the carboxylic acid analogs, CHEA, PHEA and PHA, were more toxic by injection to *M. sexta* than the ethyl ester analogs. The former were therefore tested simultaneously at 50 µg per insect injected in 0.5 µL of acetone. Controls were injected with 0.5 µL of acetone alone. Cumulative corrected percent mortality was assessed after eight days. Mortality data were normalized by transforming to arsine √ (%/100) prior to analysis using linear ANOVA in PROC GLM [14]. Means were separated by the Tukey multiple comparison procedure (α =

0.05) [14]. The remaining acid analog, PPHEA was not soluble at 50 μg in 0.5 of μL acetone and was therefore injected as 50 μg in 1.0 μL of acetone in a separate experiment. Controls were injected with 1.0 μL of acetone alone. L4D0 larvae were also injected with 50 μg CHEN and PHEN in 0.5 μL of acetone in a separate experiment. Controls were injected with 0.5 μL of acetone alone. TMOF and FDPAP were injected in 50 μL of sterile distilled water in a separate experiment. Controls for TMOF and FDPAP were injected with 0.5 μL of water alone.

Preliminary data suggested that the ester analogs, CHEN and PHEN, and the peptides, TMOF and FDPAP, were less toxic by injection than the acid analogs of TMOF at 50 μg per insect. Therefore in addition to mortality, insects injected with these compounds at 50 μg per insect were also weighed daily for eight days following treatment. For these same larvae, frass was removed and weighed 24 and 48 hours following treatment. The number of days required to molt to the fifth stadium, the number of days to pupation and the weights of day 1 pupae were also recorded during these experiments. These developmental data were used to investigate any sub-lethal effects suggestive of a mode of action similar to TMOF in mosquitoes. These growth parameters were assessed only for those larvae alive after the eight-day observation period. Days to molt and days to pupation were log transformed prior to analysis. Daily weights, frass weight, days to molt (log transformed), days to pupation (log transformed) and day 1 pupae were analyzed using linear ANOVA in PROC GLM. Means were separated by the Tukey multiple comparison procedure ($\alpha = 0.05$) [14].

2.5. Effect of TMOF and FDPAP on trypsin activity

The effects of TMOF and FDPAP on the growth rate and trypsin activity of fourth stadium larvae and the effects of TMOF on these same parameters for second stadium larvae were examined. L4D0 *M. sexta* were first weighed and then injected with 0.5 μL of sterile distilled water containing 50 μg TMOF or FDPAP. Controls were injected with 0.5 μL of water alone. After feeding for 24 hours, larvae were reweighed. For tissue preparation larvae were dissected in 0.15 M NaCl. Midguts were removed, separated from fatbody and malpighian tubules by gently teasing these tissues from the gut wall, and rinsed in 0.15 M NaCl to remove traces of hemolymph. Midguts were pooled into microcentrifuge tubes (3 midguts per tube, 3 tubes per treatment) and frozen at -80^{o}C for enzyme assays.

Nauen et al. [6] found that injection of second instar *H. virescens* with TMOF resulted in reduced total trypsin activity when larvae were assayed 24 hours after treatment. Therefore, in a separate experiment, day zero second stadium *M. sexta* were first weighed and then injected with 0.5 μL of sterile distilled water containing 20 μg of TMOF. Controls were injected with 0.5 μL of water alone. After feeding for 24 hours, larvae were reweighed, pooled into microcentrifuge tubes (3 insects per tube, 2 tubes per treatment) and frozen at -80^{o}C for enzyme assays.

Midgut (fourth stadium larvae) and total body (second stadium larvae) homogenates were prepared in buffer (0.1M Tris-HCl, 20 mM $CaCl_2$, 0.01% PTU, pH 9.0) followed by centrifugation at 10,000g at 4^{o}C for 10 minutes. The supernatant was centrifuged again at 10,000g at 4^{o}C for 10 minutes and the resulting clear supernatant was placed on ice for enzymes assays.

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) [15] using a protocol modified from Lam et al. [16]. Reactions were initiated by adding 150 μL of substrate solution (0.1 M 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 (0.1M Tris-HCl, 20 mM $CaCl_2$, 0.01% PTU). 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 briefly and incubated at 30°C in a Thermomax® microtiterplate 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 minutes. Protein determinations were made according to Bradford [17] using bovine serum albumin (Fisher Scientific, Fair Lawn, NJ) as a standard. Trypsin activitiy was expressed as mOD/min/mg protein and growth rate was expressed as the change in wet weight per day (mg/day).

Data are the mean (± SEM) of four and three replicates, for the experiments with fourth and second stadium larvae, respectively. For the experiments with fourth stadium larvae, data were analyzed using linear ANOVA in PROC GLM. Means were separated by the Tukey multiple comparison procedure ($\alpha = 0.05$) [14]. For the experiments with second stadium larvae, data were analyzed using Student's t-tests ($\alpha = 0.05$).

2.6. Topical applications

The compounds demonstrating the highest activity in *M. sexta* by injection (CHEA, PHEA and PHA) were chosen for topical applications to compare the insecticidal activity

produced by TMOF analogs by this route of administration with the activity produced by injection. L4D0 *M. sexta* were anesthetized on ice for one minute prior to treatment. Compounds were applied to the mesothorax as 0.5 mg in 0.5-µL acetone with a 10-µL Hamilton syringe (Hamilton Company, Reno, NV). Controls were treated with 0.5 µL of acetone alone. Insects were observed until all of the solvent had evaporated before being placed on diet. Five insects were assayed per treatment and solvent control. This experiment was replicated three times. Cumulative corrected percent mortality was assessed after eight days. Mortality data was normalized by transforming to arcsine $\sqrt{}$ (%/100) prior to analysis using linear ANOVA in PROC GLM. Means were separated by the Tukey multiple comparison procedure ($\alpha = 0.05$) [14].

2.10. Species range of TMOF analogs

In separate experiments, adult male *B. germanica* (14 days after eclosion) and early fourth stadium *H. virescens* and *H. zea* were chosen to investigate the specificity of the compounds determined to be the most active by injection in *M. sexta* (CHEA, PHEA and PHA). TMOF analogs were injected at 306 mg/kg, a dose equivalent to the low dose (50 µg per insect) tested against *M. sexta* larvae. Injections with the TMOF analogs were made in 0.5 µL acetone. Controls were injected with 0.5 µL of acetone alone. *B. germanica* were anesthetized with CO_2 gas for 30 seconds and then injected between two ventral abdominal plates. *H. virescens* and *H. zea* were anesthetized on ice for one minute prior to treatment. Analogs were injected in the perivisceral hemocoel through an abdominal proleg.

Ten insects were assayed per treatment and solvent control. These experiments were replicated three times. Cumulative corrected percent mortality was assessed eight days after

treatment. Mortality data were normalized by transforming to arcsine √ (%/100) prior to analysis using linear ANOVA in PROC GLM. Means were separated by Tukey's procedure ($\alpha = 0.05$).

2.11. Two-choice feeding bioassay

Microtiter plates (96-well, flat bottom; Becton Dickinson, Fair Lakes, NJ) containing artificial heliothine diet were frozen at -80°C and lyophilized (Bench Top 6, Virtis, Gardiner, NY; cold trap = -70°C, ≈200 mTorr, ambient temperature ≈23°C) for 24-48 hours to form hydratable meal pellets [18]. Dehydrated meal pellets were saturated with PHEA diluted in acetone. Control pellets were saturated with acetone alone. Following evaporation of the acetone in a fume hood, pellets were rehydrated with sterile distilled water. The final concentration of PHEA 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 instar *H. virescens* 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.

3. Results and discussion

3.1. Toxicity of TMOF, FDPAP and TMOF analogs in C. pipiens

TMOF, FDPAP and the nonpeptidic chemical analogs of TMOF were tested against larvae of the northern house mosquito, *C. pipiens*, in an effort to determine the essential structural characteristics required for biological activity in mosquitoes. The structures of TMOF and FDPAP are shown in Figure 1. The results of the mosquito bioassay as well as the structures of the nonpeptidic chemical analogs of TMOF are shown in Table 1. LC_{50}s for

compounds were considered significantly different based on failure of respective 95% confidence intervals to overlap. The LC_{50} for FDPAP (0.29 mM) was 1.5-fold lower than the LC_{50} for TMOF (0.43 mM) (Table 1). Apparently when five prolines are removed from the C-terminus of TMOF and the amino acid at the N-terminus is switched from tyrosine to phenylalanine, larvicidal activity against *C. pipiens* is increased. Borovsky et al. [2] concluded that both the C- and N-termini of TMOF contribute to the biological activity in mosquitoes. The relative importance of these modifications with respect to the toxicity of TMOF and FDPAP in *C. pipiens* is not clear at this time and requires testing of additional peptidic analogs of TMOF in this species.

The LC_{50} for CHEA was 1.5-fold lower and the LC_{50} of PHA was 1.2-fold lower than the LC_{50} for TMOF (Table 1). PPHEN was the most active compound tested with an LC_{50} of 0.17 mM; 2.5-fold lower than the LC_{50} for TMOF. There were no significant differences found between the toxicities of the other TMOF analogs and TMOF except for CHEN which was not toxic at 1.0 mM; the highest dose tested. The lack of toxicity of CHEN cannot be explained by the rest of the data and therefore will not be considered in subsequent analysis. There were no observable physical differences between larvae that had survived a five-day exposure to TMOF or any of the compounds tested. Larvae surviving exposure to TMOF, FDPAP and the TMOF analogs were smaller in size compared with respective controls at the end of the 5-day observation period. 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.

The LC_{50}s for the ethyl ester analogs, PHEN and PPHEN, were significantly lower than the LC_{50}s for the corresponding acids, PHEA and PPHEA (Table 1). The biphenyls, PPHEA

and PPHEN, also have significantly lower LC_{50}s than the corresponding phenyls, PHEA and PHEN (Table 1). The substitution of the phenyl ring (PHEA) for the cyclohexane ring (CHEA) resulted in a 1.8-fold increase in toxicity to mosquito larvae (Table 1). One possible explanation for these trends may be that the increased lipophilicity of those analogs possessing an ester, biphenyl or phenyl substituent compared to the corresponding analogs possessing an acid, phenyl or cyclohexane substituent, resulted in increased binding of the former to food particles leading to higher levels of consumption. Once consumed, increased lipophilicity would also be predicted to enhance sequestration and reduce elimination of these compounds [19]. Alternatively or in conjunction with the former hypotheses, if these analogs are acting as TMOF mimics, these structural characteristics may enhance binding to the TMOF receptor, requiring lower levels of these compounds to achieve effective levels of trypsin inhibition. Testing of these hypotheses necessitates further experimentation.

To examine the possibility of a relationship between lipophilicity and toxicity in this series of TMOF analogs, a linear regression of biological potency, defined as the logarithm of the inverse of the LC_{50} estimate ($\log_{10}(1/LC_{50})$), on the logarithm of the octanol-water partition coefficient (P) was conducted according to Hansch [20] and McFarland [21] using linear ANOVA in PROC GLM [14]. This model is based on the assumption that the probability of success for a compound in traversing the multiple aqueous and lipid phases in a biological system from the site of administration to the site of action is dependant on the hydrophobic bonding capacity (reflected by P) of the molecule. The octanol-water partition coefficients for nonpeptidic analogs of TMOF were calculated using ChemDraw [22]. The relationship between biological potency and Log P was significant ($F = 22.58$, $P = 0.0177$, treatment df =1, error df = 4) for this analysis when PPHEA, CHEN and PHN were omitted.

As previously mentioned, CHEN was not toxic at the highest dose tested (1.0 mM) and was therefore not included in this analysis. When PPHEA and PHN were included, the relationship between biological potency and Log P was not significant ($F = 4.52$, $P = 0.0869$, treatment df =1, error df = 6). Other factors including steric and electronic effects determine toxicity [19] and may explain why CHEN was not toxic and the toxicities of PPHEA and PHN were lower than would have been predicted by this model. The relative importance of these factors as well as hydrogen bonding capacity will be explored in future work following synthesis and testing of additional analogs of TMOF.

3.3. Toxicity by injection of TMOF, FDPAP and TMOF analogs

To determine the effects of the nonpeptidic chemical analogs on lepidopeteran larvae, L4D0 *M. sexta* were injected in series of experiments with a high dose (1.5 mg) and a low dose (50 µg) of TMOF analogs. Mean cumulative corrected percent mortality is shown in Table 2. The acids, CHEA, PHEA, and PHA, produced 100% mortality at 1.5 mg per insect. Larvae treated with these analogs at this dose either did not feed or feeding was minimal, producing few if any fecal pellets. The injections were made into the perivisceral cavity through a last abdominal proleg. Before death, larvae 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. The average time to death for these treatments was 2 days (data not shown). The esters, CHEN, PHEN and PHN, were 2-fold less toxic than the acids at 1.5 mg per insect (Table 2) while PPHEN was only slightly toxic at this dose (Table 2). The average time to death for CHEN and PHEN was 4 days and the average time to death for PHN was 3 days (data not shown). The effects of these ethyl

ester analogs of TMOF on susceptible larvae were different than those described previously for the acids. In general, larvae injected with the ester analogs of TMOF demonstrated reduced growth compared to controls. There was a slight but significant delay in molting for surviving larvae treated with CHEN and for larvae surviving treatment with PHN compared to the controls (data not shown).

L4DO *M. sexta* were also injected with 50 µg of the nonpeptidic chemical analogs except for PHN and PPHEN which were unavailable for testing at this dose. TMOF and FDPAP were also tested at 50 µg per insect. Mean cumulative corrected percent mortality is shown in Table 2. No significant differences were found in the toxicity of CHEA, PHEA and PHA ($F = 2.37$, $P = 0.1739$, treatment df = 2, error df = 6). All three of these acid analogs produced greater than 50% mortality eight days after injection in *M. sexta*. In addition to the effects described previously for these compounds at 1.5 mg per insect, complications during molting were also observed and many of the treated insects, including survivors, were unable to completely shed the exuviae. These effects were different from those observed in mosquitoes that were fed TMOF, suggesting a possible different mode(s)-of-action of TMOF analogs in lepidopteran larvae. In a separate experiment, PPHEA was found to be 2.1-3.0 less toxic than the CHEA, PHEA and PHA when injected at 50 µg per insect (Table 2). The effects observed for larvae treated with PPHEA were similar to those observed for larvae treated with the other acids.

In preliminary experiments, the esters CHEN and PHEN were found to be non-toxic to larvae of *M. sexta*, therefore these compounds were tested at 50 µg per insect in a separate experiment. In addition to mortality, daily weights, frass production, time to molting, time to pupation and day 1 pupae weights were also assessed. CHEN and PHEN were only slightly

toxic to *M. sexta* in this experiment (Table 2), producing less than 10% mortality. There was a significant effect of treatment on the daily weights of larvae treated with CHEN and PHEN. The daily weights for larvae treated with PHEN were significantly different from controls (Tukey's, $\alpha = 0.05$) (not shown) from day 1 to day 8 of the eight-day observation period (Fig. 2). The daily weights for larvae treated with CHEN were not significantly differently from the controls (Fig. 2a). There was also a significant effect of treatment on the frass production measured 24 hours following injection ($F = 8.19$, $P = 0.0193$, treatment df = 2, error df = 6) and 48 hours following injection ($F = 5.62$, $P = 0.0422$, treatment df = 2, error df = 6) (Table 3). Day 1 frass production was 2.2-fold lower for insects injected with PHEN compared to controls. For these same insects, day 2 frass production was 1.9-fold lower than controls. Day 1 and day 2 frass production for larvae injected with CHEN were not significantly different from the controls (Table 3). In this experiment, there was also a significant effect of treatment on the time to molt to the fifth stadium ($F = 10.5$, $P = 0.011$, treatment df = 2, error df = 6). Insects injected with IBI-219 molted 2 days later than the controls (Table 3). The number of days required to molt to the fifth stadium for insects injected with 50 µg CHEN was not significantly different from the controls. Apparently, *M. sexta* larvae differ with respect to its response to these two ethyl ester analogs. No significant effects on the time to pupation ($F = 1.75$, $P = 0.2519$, treatment df = 2, error df = 6) or day 1 pupae wt ($F = 1.4$, $P = 0.3164$, treatment df = 2, error df = 6) were detected among insects treated with CHEN, PHEN and control (Table 3).

In subsequent experiments designed to investigate the reason for the delay in growth and development of larvae injected with PHEN, it was discovered that these larvae do not begin feeding for 2-4 hours following injection. These same larvae are responsive to prodding and

generally recover within 4 hours following injection. Additionally, larvae injected with PHEN at 50 μg per insect do not show any signs similar to those insects injected with the acids at this dose. Based on these observations, it is unlikely that the delay in growth and development of *M. sexta* caused by PHEN is related to the inhibition of trypsin biosynthesis and suggests an alternative mode(s)-of-action for this compound.

TMOF and FDPAP were non-toxic to *M. sexta* at 50 μg per insect (Table 2). At this dose, these peptides also did not have any effects on growth (Fig. 2b). There were also no significant differences in frass production, time to molt, time to pupation or the day 1 pupae weights for insects injected with these peptides compared to controls (Table 4).

3.4. Effect of TMOF and FDPAP on trypsin activity

To determine if TMOF or FDPAP affect trypsin levels in the gut of fourth stadium *M. sexta*, fourth stadium day zero larvae were injected with TMOF or FDPAP 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 ($F = 0.9$, $P = 0.4401$, treatment df = 2, error df = 9) or trypsin activity ($F = 0.12$, $P = 0.8894$, treatment df = 2, error df = 9) between insects treated with 50 μg TMOF, FDPAP and the control. To determine if TMOF is active on an earlier instar of *M. sexta*, second stadium day zero larvae were injected with TMOF, and 24 hours later the average growth rate and trypsin activity were compared to controls. There were no significant differences in the average growth rate (Student's t-test, $P = 0.95$) between insects injected with 20 μg TMOF (11.7 mg/day) and control (11.8 mg/day). For these same insects, there were also no significant differences in trypsin activity (Student's t-test, $P = 0.84$) between insects injected

with 20 µg TMOF (50.1 mOD min^{-1} mg $protein^{-1}$) and control (49.3 mOD min^{-1} mg $protein^{-1}$). Similar results were obtained when insects were treated with 50 and 100 µg of TMOF (data not shown). Nauen et al. [6] found that injection of second instar *H. virescens* with TMOF resulted in 50% inhibition of trypsin biosynthesis after 24 hours at a dose of 0.2 ng. Apparently, *M. sexta* differs from *H. virescens* relative to its response to TMOF injections and its effect on trypsin activity.

3.5. Topical toxicity of TMOF analogs

There was a significant effect of treatment on the mortality produced by topical application of 0.5 mg of CHEA, PHEA and PHA ($F = 7.6$, $P = 0.0266$, treatment df = 2, error df = 6) for *M. sexta* larvae. PHEA did not produce any mortality at 0.5 mg per insect (Table 2). The mean cumulative corrected percent mortality produced by PHA and CHEA (Table 2) were not significantly different based on Tukey's comparison ($\alpha = 0.05$) (not shown). Susceptible insects did not feed or feeding was minimum, producing few if any fecal pellets. These insects were responsive to prodding until death. In general, CHEA, PHEA and PHA were less active in *M. sexta* by topical administration than by injection.

3.6. Species range of TMOF analogs

To investigate the species range of TMOF analogs, early fourth stadium *H. virescens* and *H. zea* and adult male *B. germanica* were injected with those analogs demonstrating the highest activity by injection in *M. sexta* (CHEA, PHEA and PHA). TMOF analogs were injected at a dose of 300 mg of active ingredient/kg. There were no significant differences among treatments with TMOF analogs for *H. virescens* ($F = 0.38$, $P = 0.6968$, treatment df =

2, error df = 6) or *H. zea* (F = 1.45, P = 0.3062, treatment df = 2, error df = 6). All three analogs produced greater than 50% mortality eight days after injection in these species (Fig. 4). In general, the effects of these analogs in *H. virescens* and *H. zea* were similar to those described previously for *M. sexta* and suggest a possible different mode(s)-of-action of TMOF analogs in lepidopteran larvae compared to mosquitoes. There was a significant difference among treatments in mortality for *B. germanica* (F = 6.98, P = 0.0272, treatment df = 2, error df = 6). Mortality was 2.2-fold higher in insects treated with PHEA then in insects treated with CHEA (Fig. 4). There were no significant differences in the toxicity of PHEA and PHA or in the toxicity of PHA and CHEA in these insects. Before death, cockroaches injected with these compounds showed signs of reduced activity but where otherwise normal in appearance.

TMOF was non-toxic to early fourth stadium *H. virescens* and *H. zea* and adult male *B. germanica* when injected at 306 mg/kg (data not shown). Apparently, the mosquito hormone and TMOF analogs evoke markedly different responses from *H. virescens*, *H. zea* and *B. germanica*.

3.7. Anti-feedant activity of TMOF analogs

In preliminary feeding experiments with TMOF analogs incorporated into artificial diet, second instars of *H. virescens* demonstrated an obvious preference for control diet pellets over those treated with PHEA (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 *H. virescens*, diet pellets treated with solvent alone were chosen over pellets treated with PHEA 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 PHEA. 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 PHEA has anti-feedant activity against *H. virescens* 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.

In summary, seven of the eight nonpeptidic chemical analogs of TMOF were found to be toxic to larvae of the northern house mosquito, *C. pipiens*. There was a relationship found between the lipophilicity (as indicated by the octanol-water partition coefficient) of this series of analogs and toxicity to mosquito larvae. The mode(s) of action of these analogs are currently unknown, however, larvae surviving treatment with these chemistries were similar in appearance to larvae treated with TMOF, a peptide known to inhibit trypsin biosynthesis in mosquito larvae resulting in death by starvation. Additional studies must be conducted to elucidate the mode(s) of action of TMOF analogs in mosquito larvae. Representatives of this novel chemical class were also found to be toxic to larvae of *M. sexta*, *H. virescens*, and *H. zea* as well as adult male *B. germanica*. Also, results of a two-choice bioassay with fifth instars of *H. virescens* suggest that some of these analogs may have anti-feedant properties. These findings suggest that the TMOF technology may be applicable to the control of important agricultural and urban pests. This study represents the first attempt to develop a stable non-peptidic chemical analog of TMOF. Future synthesis and testing projects will focus on the development of some analogs with higher activity against mosquito larvae and others that demonstrate broad-range control of important pest species. Finally, it was

interesting to note that TMOF had no effects on the development or trypsin activity of *M. sexta* in these studies. The regulation of digestion in larval Lepidoptera is currently not well understood and additional studies in this area of research are warranted. If a peptidic hormone could be isolated from lepidopteran larvae that down-regulates digestive enzymes, similar to TMOF in mosquitoes, transgenic plants expressing this hormone may be a viable alternative to the currently available control strategies for these pests.

Acknowledgements

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Fig. 1. Structures of TMOF and FDPAP.
(A) TMOF (NH_2-Tyr-Asp-Pro-Ala-Pro-Pro-Pro-Pro-Pro-Pro-COOH). Box indicates region of the peptide from which nonpeptidic chemical analogs were designed.
(B) FDPAP (NH_2-Phe-Asp-Pro-Ala-Pro-COOH)

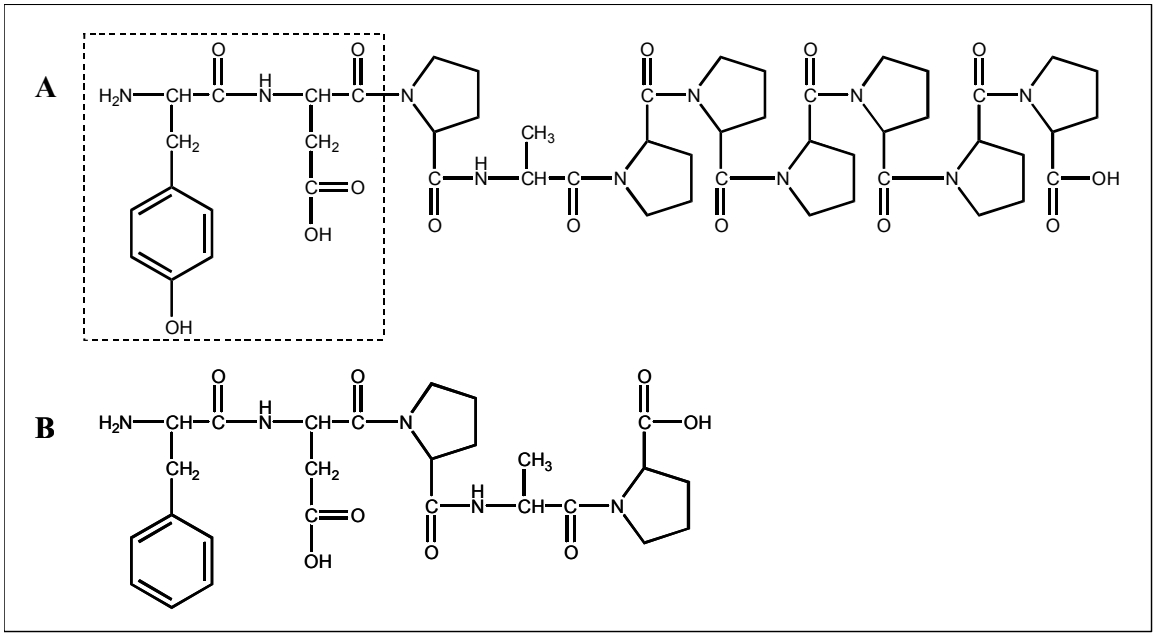

A
B

Fig. 2. Effects of CHEN, PHEN, TMOF and FDPAP on the growth of *M. sexta* larvae. Days are counted beginning on the day of treatment (Day 0). Each data point is the mean of three replicates (10 insects per replicate). Stages for control are indicated. (A) Wet weight (± SEM) of *M. sexta* injected as fourth stadium day zero larvae with 50 μg CHEN or PHEN. Controls were injected with acetone. (B) Wet weight (± SEM) of *M. sexta* injected as fourth stadium day zero larvae with 50 μg TMOF or FDPAP. Controls were injected with water.

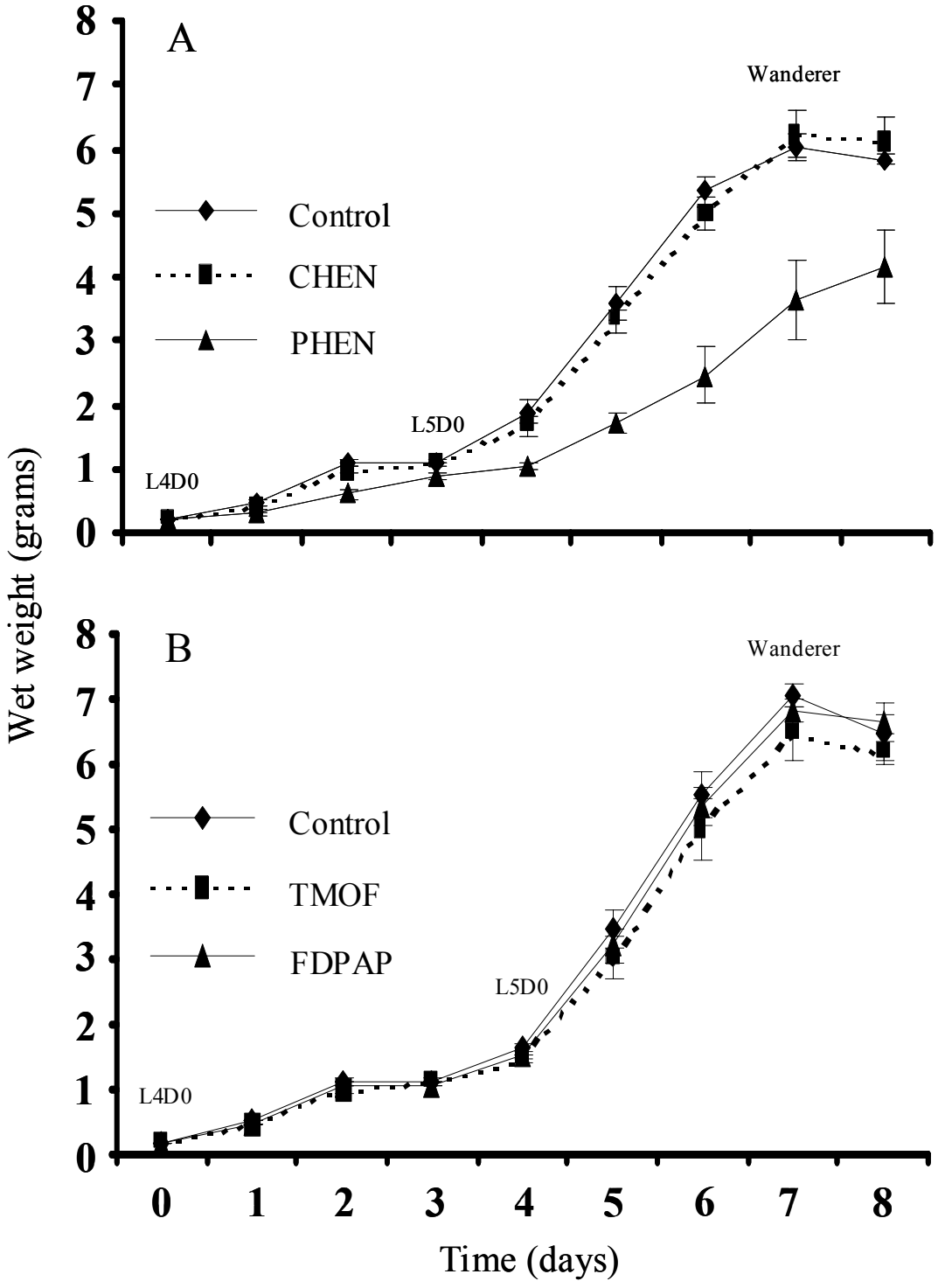

A
Wanderer
Control
CHEN
PHEN
L5D0
L4D0
B
Wanderer
Control
TMOF
FDPAP
L5D0
L4D0
Wet weight (grams)
Time (days)
0
1
2
3
4
5
6
7
8

Fig. 3. Average growth rate and trypsin activity for fourth stadium *M. sexta* 24 hours after injection with TMOF or FDPAP. Controls were injected with water. Error bars are $\pm$ SEM. Means separated by Tukey's comparison ($\alpha = 0.05$). Means indicated by the same letter are not significantly different.

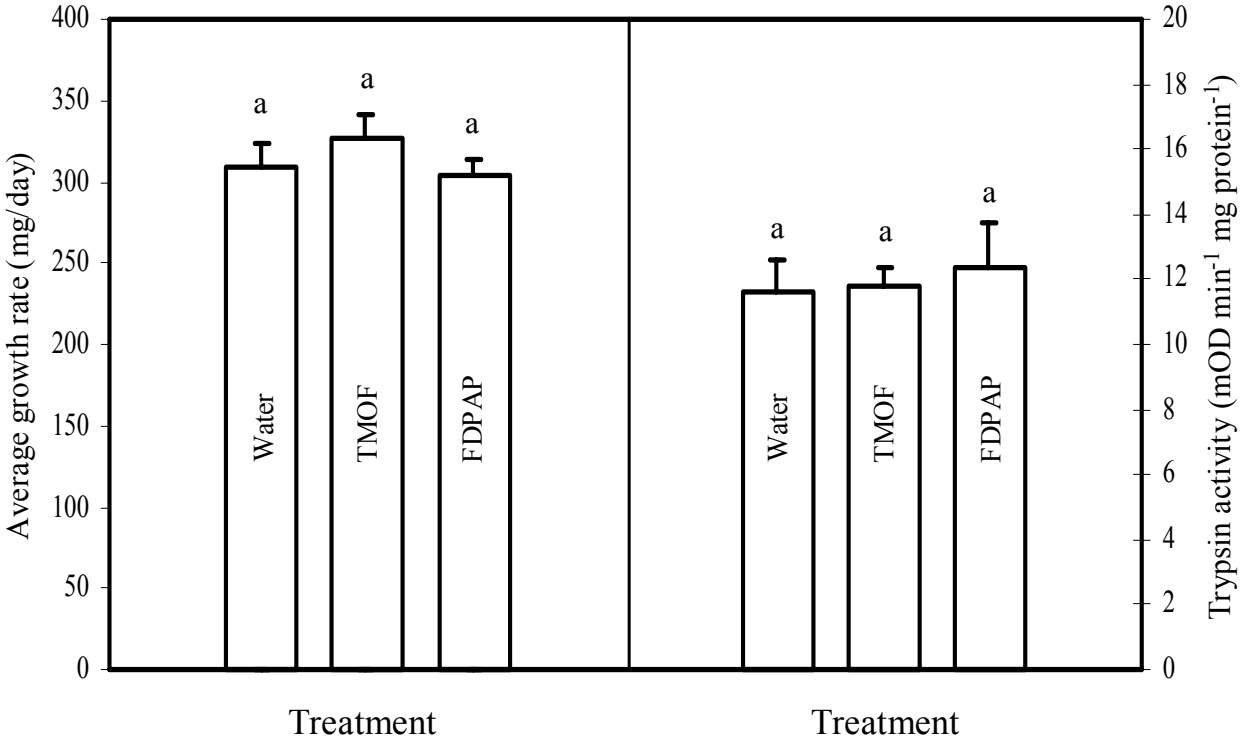

Average growth rate (mg/day)
Trypsin activity (mOD min-1 mg protein-1)
400
350
300
250
200
150
100
50
0
20
18
16
14
12
10
8
6
4
2
0
a
a
a
a
a
a
Water
TMOF
FDPAP
Water
TMOF
FDPAP
Treatment
Treatment

Fig. 4. Toxicity by injection of nonpeptidic chemical analogs of TMOF in early fourth stadium *H. virescens* (A) and *H. zea* (B) and adult male *B. germanica* (C). Error bars are $\pm$ SEM. Absence of error bars indicates a SEM of zero for these data. Means separated by Tukey's comparison ($\alpha = 0.05$). Means indicated by the same letter are not significantly different.

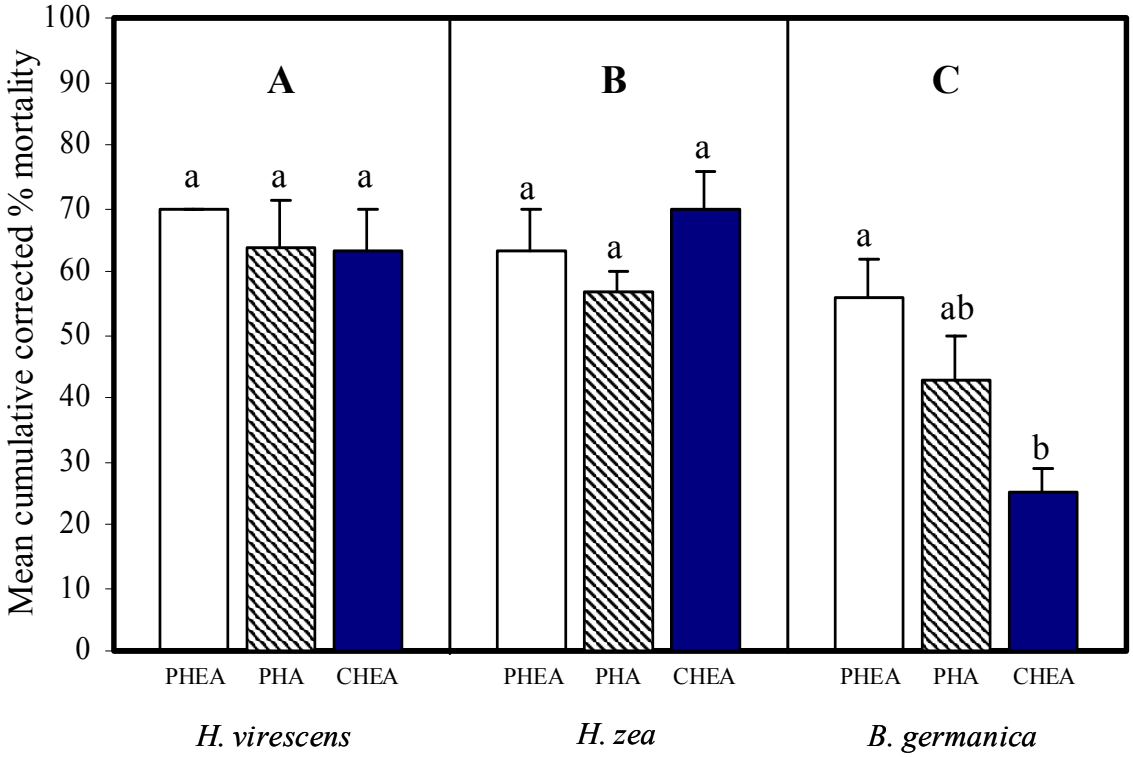
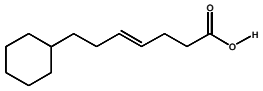
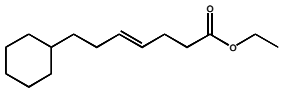
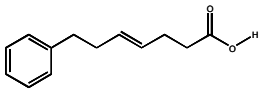
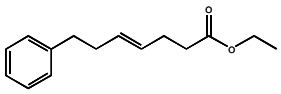
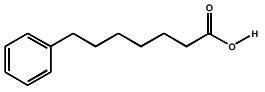
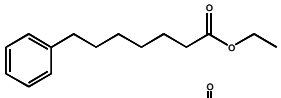
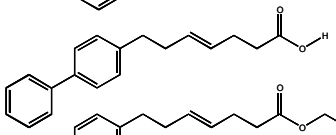
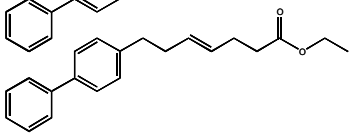

Mean cumulative corrected % mortality
100
90
80
70
60
50
40
30
20
10
0
A
B
C
a
a
a
a
a
a
a
ab
b
PHEA
PHA
CHEA
PHEA
PHA
CHEA
PHEA
PHA
CHEA
H. virescens
H. zea
B. germanica

Table 1
Comparison of LC_{50}s 5 days after exposure to TMOF, FDPAP or nonpeptidic chemical analogs of first stadium day 1 *C. pipiens*. Data are presented in mM. Structures of nonpeptidic chemical analogs of TMOF are also shown.

Structure	Compound	LC_{50}	Regression curve	R^2	95% CI	N
	TMOF	0.43	y = 8.46x + 3.08	0.99	0.38-0.49	240
	FDPAP	0.29*	y = 7.06x + 3.84	0.99	0.25-0.33	180
	CHEA	0.28*	y = 10.8x + 5.91	0.99	0.28-0.29	240
	CHEN	> 1.00				
	PHEA	0.51	y = 8.24x + 2.39	0.99	0.47-0.56	192
	PHEN	0.39	y = 3.81x + 1.56	0.99	0.36-0.42	228
	PHA	0.36*	y = 12.16x + 5.46	0.99	0.34-0.37	204
	PHN	0.39	y = 3.98x + 1.63	0.99	0.33-0.46	120
	PPHEA	0.42	y = 3.93x + 1.49	0.99	0.37-0.47	156
	PPHEN	0.17*	y = 1.79x + 1.39	0.99	0.14-0.20	144

* Significantly different from TMOF based on non-overlapping 95% confidence intervals.
CI, confidence interval; N, number of individuals tested.

Table 2
Comparison of the toxicities of TMOF, FDPAP and nonpeptidic chemical analogs of TMOF in *M. sexta*. Mortality assessed eight days after treatment.

		Mean cumulative corrected percent mortality[a]		
		Injected[b]		Topical[c]
	Compound	1.5 mg	50 µg	0.5 mg
Analogs	CHEA	100	80 ± 6	33 ± 13
	PHEA	100	70 ± 10	0
	PHA	100	57 ± 7	13 ± 11
	PPHEA	nt	27 ± 3	nt
	CHEN	48 ± 12	7 ± 4	nt
	PHEN	34 ± 14	8 ± 8	nt
	PHN	43 ± 3	nt	nt
	PPHEN	3 ± 3	nt	nt
Peptides	TMOF	nt	0	nt
	FDPAP	nt	0	nt

[a] Data are the mean (± SEM) of three replicates.
[b] N = 30 individuals tested.
[c] N = 15 individuals tested.
nt, not tested

Table 3
Effects of injection of 50 μg of CHEN or PHEN on the frass production and development of *M. sexta* treated as fourth stadium day zero larvae. Controls were injected with acetone. Data are the mean (± SEM) of three replicates (10 insects per replicate).

	Treatment[a]		
	Control	CHEN	PHEN
L4D1 frass weight (mg)	74.8 ± 8.6 A	60.0 ± 6.1 AB	34.3 ± 6.5 B
L4D2 frass weight (mg)	233.6 ± 24.0 A	207.4 ± 25.2 AB	123.0 ± 23.9 B
Days to molt	3 ± 0 A	4 ± 0 AB	5 ± 0 AB
Days to pupation	12 ± 0 A	13 ± 0 A	13 ± 1 A
Day 1 pupae wt (g)	3.9 ± 0.1 A	3.7 ± 0.1 A	3.6 ± 0.2 A

[a] Horizontal means separated by Tukey's comparison ($\alpha = 0.5$). Means followed by the same letter are not significantly different. Days counted beginning on the day of treatment (Day 0).

Table 4
Effects of injection of 50 µg of TMOF or FDPAP on the frass production and development of *M. sexta* treated as fourth stadium day zero larvae. Controls were injected with water. Data are the mean (± SEM) of three replicates (10 insects per replicate).

	Treatment[a]		
	Control	TMOF	FDPAP
L4D1 frass weight (mg)	81.9 ± 2.3 A	75.3 ± 7.1 A	76.7 ± 4.5 A
L4D2 frass weight (mg)	221.8 ± 6.9 A	203.0 ± 6.4 A	210.4 ± 11.2 A
Days to molt	4 ± 0 A	4 ± 0 A	4 ± 0 A
Days to pupation	12 ± 0 A	12 ± 0 A	12 ± 0 A
Day 1 pupae wt (g)	4.0 ± 0.1 A	3.9 ± 0.2 A	4.1 ± 0.1 A

[a] Horizontal means separated by Tukey's comparison ($\alpha = 0.5$). Means followed by the same letter are not significantly different. Days counted beginning on the day of treatment (Day 0).

Chapter II

Synthesis and assay of novel aromatic and aliphatic organic acids, esters and ketone mimics of Trypsin Modulating Oostatic Factor (TMOF) for arthropod repellent and anti-feeding activity

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ABSTRACT

Novel aromatic and aliphatic organic acids, esters and ketone mimics of the mosquito trypsin modulating oostatic factor (TMOF) were synthesized and assayed as repellents for ticks and as an anti-feeding agent for mosquitoes. *E*-7-(cyclohexyl)hept-4-enoic acid (CHEA), *E*-7-phenylhept-4-enoic acid (PHEA), ethyl *E*-7-(cyclohexyl)hept-4-enoate (CHEN) and ethyl *E*-7-phenylhept-4-enoate (PHEN) had repellent activity against the soft tick, *Ornithodorous parkeri* (Acari: Argasidae) in a two-choice bioassay. PHEN, an aromatic organic ester, was the most active. Only 15% of the ticks tested selected the surface treated with 5 $\mu g/cm^2$ of PHEN as compared to the untreated surface. Commercially available 2-undecanone, a natural product found in the trichomes of wild tomato plants, was found to also mimic the structure of TMOF and was active as a repellent at 50 $\mu g/cm^2$. Since this compound is a natural botanical product with low mammalian toxicity, further tests were conducted to determine its anti-feeding activity against mosquitoes. The median effective dose to prevent 50% of adult yellow fever mosquitoes, *Aedes aegypti*, from biting (ED_{50}) was estimated as 91.5 $\mu g/cm^2$ of human skin as compared to 1.6 $\mu g/cm^2$ for deet (N, N-diethyl-m-toluamide). 2-Undecanone applied as a 30 and 40% solution (vol/vol) in isopropyl alcohol to human skin was 100% active for 15 min in preventing mosquitoes from biting and 40% provided 93% protection 30 min after application. Persistence is limited by the high volatility of 2-undecanone although it appears that once mosquitoes come into contact with the repellent, host seeking is inhibited more than 2 h.

1. Introduction

The active ingredient most commonly found in repellent formulations for personal protection against biting arthropods is deet (N, N-diethyl-m-toluamide) [1]. Deet is a broad spectrum repellent with activity against arthropods of medical and veterinary importance including mosquitoes, biting flies, fleas and ticks [2]. While Osimitz and Grothaus [3] concluded that the risks associated with the normal use of deet are low, recent concerns about the safety of this repellent [2, 4-5] along with a decrease acceptance of deet by the general public have prompted the search for new active ingredients that are safer than deet, while retaining the same or similar activity.

Trypsin modulating oostatic factor (TMOF) is a peptide hormone produced by the adult ovary of the mosquito and is transported by insect hemolymph to the gut wall, where it reduces trypsin biosynthesis [6]. There has been significant interest in the chemistry of TMOF because of its mosquito larvicidal activity. When larvae ingest TMOF, protease activity in the gut declines. The larvae become satiated, stop feeding and they eventually die from starvation. Recently, Vanderherchen et al. (unpublished) found that chemical mimics of TMOF originally designed as mosquito larvicides with the same mode of action as TMOF, had anti-feedant activity against lepidopteran larvae and repellent activity against the soft tick *Ornithodorous parkeri* (Acari: Argasidae).

Interestingly, naturally derived 2-undecanone (methyl nonyl ketone) is similar in structure to the chemical mimics of TMOF studied by Vanderherchen et al. (unpublished). Undecanone is the major volatile constituent of the essential oils derived from several plant species in the family Rutaceae [7-11] and can be found in oil derived from hops, *Humulus lupulu* (Moracae) [12], coconut oil, *Cocus nucifera* L. Palmer [13] and in wild tomato plants,

Lycopersicon hirsutum f. glabratum (Solanaceae) [14-15]. In the latter species, 2-undecanone has been shown to play a major role in plant resistance to herbivorous arthropods [16-18]. 2-Undecanone is currently registered by the EPA for use as an animal repellent [19], and the extensive mammalian toxicology data are comparable and in some aspects more favorable than deet. 2-Undecanone has been shown to affect the behavior of arthropods exposed to this compound *in planta* or when exposed to synthetic material. 2-Undecanone has been found to be repellent to German cockroaches, *Blattela germanica* (Blattaria: Blatellidae), green peach aphids, *Myzus persicae* (Sulzer) (Homoptera: Aphididae) and western flower thrips, *Frankiniella occidentalis* (Pergande) (Thysanoptera: Thripidae) (Roe and Long, personal communication). The effects observed differ markedly depending on the species tested [18, 20-22]. The goal of the current study is to synthesize novel chemical mimics of TMOF, and examine these mimics including 2-undecanone for their repellent activity to *O. parkeri* and the yellow fever mosquito, *Aedes aegypti*.

2. Materials and methods

2.1. Test arthropods

Ornithodoros parkeri Cooley and *Aedes aegypti* (L.) were maintained at 25±2° C (85±2% relative humidity) and 16:8 h (light:dark) cycle with 0.5 h of crepuscular light before and after the photophase. The photophase begins at 0600 h. *O. parkeri* were originally obtained from J.H. Oliver, Georgia Southern University, Statesboro, GA and maintained at NC State University. All stages are fed on white mice (*Mus musculus)*. Ticks used were from a colony that had not been bloodfed for at least six months. Both males and females of the nymphal and adult stages of *O. parkeri* were used in the experiments that follow. *A.*

aegypti larvae were originally collected from Raleigh, NC in 1986. Larvae were fed a 2:1 mixture (wt:wt) of liver powder (ICN Biochemicals, Cleveland, OH) and brewers yeast (ICN Biochemicals), respectively, on a standardized schedule [23]. Adults were maintained according to Benzon and Apperson [24] in 30 x 30 x 30 cm clear acrylic plastic cages with a front cotton access sleeve and provided 20% sucrose *ad libitum*. Mosquitoes were starved (provided only water) 24 h prior to testing.

2.2. Test compounds

Technical grade 2-undecanone (99.7%) was obtained from Mclaughlin Gormley King Co. (Minneapolis, MN). Technical-grade deet (N, N-diethyl-m-toluamide, 97%) was obtained from Sigma Chemical Co. (St. Louis, MO).

2.3. Synthesis of nonpeptidic chemical analogs of TMOF

Preparation of Ethyl *E*-7-Phenylhept-4-enoate (PHEN). *E*-7-Phenylhept-4-enoic acid was prepared from commercially available 3-phenylpropanal (dihydrocinnamaldehyde) in four steps as follows. Dihydrocinnamaldehyde was added to an excess of vinyl magnesium bromide in tetrahydrofuran (THF) at 0^{o} C and allowed to warm to room temperature and remain there for 2 hr. The reaction was quenched with saturated ammonium chloride solution and extracted twice with ether. The combined ether extracts were dried over anhydrous sodium sulfate and after evaporation of the ether *in vacuo*, the residue was purified by column chromatography on silica gel to yield 5-phenyl-3-hydroxypent-1-ene. The allylic alcohol was then dissolved in triethyl orthoacetate with a catalytic amount of propionic acid and heated at 140^{o} C for 6 hr (following distillation of the ethanol). After

cooling to room temperature, the excess triethyl orthoacetate was removed under reduced pressure, and the resulting residue purified by flash chromatography to give *E*-7-phenylhept-4-enoate in >95% purity.

Preparation of *E*-7-Phenylhept-4-enoic acid (PHEA). Ethyl *E*-7-Phenylhept-4-enoate was saponified in methanolic sodium hydroxide at room temperature. The resulting acid, *E*-7-phenylhept-4-enoic acid, was obtained in >95% purity after chromatography.

Preparation of *E*-7-(Cyclohexyl)hept-4-enoate (CHEN). Ethyl *E*-7-(cyclohexyl)hept-4-enoate was prepared in two steps from the known compound 3-cyclohexylpropanal. A THF solution of 3-cyclohexylpropanal was treated with vinyl magnesium bromide (as a solution in THF) at 0^{o} C. The resulting allyl alcohol derivative, 5-cyclohexyl-3-hydroxypent-1-ene, was purified by chromatography. The allylic alcohol was then dissolved in triethyl orthoacetate and heated to 140^{o} C to effect a Claisen rearrangement. The product, ethyl *E*-7-(cyclohexyl)hept-4-enoate, was obtained in >95% purity after chromatography.

Preparation of *E*-7-(Cyclohexyl)hept-4-enoic Acid (CHEA). Ethyl *E*-7-(Cyclohexyl)hept-4-enoate was saponified in methanolic sodium hydroxide at room temperature. The resulting acid, *E*-7-(cyclohexyl)hept-4-enoic acid, was obtained in >95% purity after chromatography.

The structures of the final products were verified by the appropriate spectral analysis (infrared and nuclear magnetic spectroscopy) and combustion analysis.

2.4. Tick repellent assays

To determine if 2-undecanone, CHEA, PHEA, CHEN and PHEN have repellent activity against soft ticks, a two-choice bioassay was conducted using unfed *O. parkeri* nymphs and

adults. Half-circle test papers (radius = 24 mm) were prepared from Whatman filter paper (#2 Qualitative, Whatman Inc., Clifton, New Jersey). Half-circle test papers were saturated with different doses of 2-undecanone, CHEA, PHEA, CHEN or PHEN (5-100 μg/cm^2) diluted in 100 μL acetone. A test paper saturated with 100 μL of acetone served as the control. The solvent was allowed to evaporate at room temperature in the fume hood for 1 h prior to testing.

The arenas for the two-choice test were prepared by placing one half-circle of paper treated with the test compound (in acetone) and one half-circle of paper treated with acetone only into a small glass petri dish (diameter = 60 mm, height = 15 mm). The two half-circle test papers completely covered the bottom of the petri dish. Ten ticks were transferred into the center of each arena and then covered with the lid of the glass petri dish. For each arena, the number of ticks in contact with the treatment and the acetone control were recorded 2 h after the introduction of ticks into the arena. Experiments with each dose of 2-undecanone, CHEA, PHEA, CHEN, PHEN and the no-choice control were replicated four times.

2.5. Mosquito anti-feedant assays

Anti-feedant tests were conducted at room temperature (25^o C and 60% RH) from 0700-1000 and 1800-2100 h each day. All mosquito tests were performed using a testing system similar to the one used by Canyon et al. [25]. The test consisted of a 50 mL clear polypropylene conical tube (Fisher Scientific, Hampton, NH) covered at one end with cotton stockinette (Alba Health, Rockwood, TN). On the day of testing, nulliparous females 7-12 days old, which had been provided only water for 24 h, were separated from the adult colony based on readiness to feed on a human arm placed in contact with the netted side of the

rearing cage. Females attempting to probe and bite through the netting were transferred into the test modules and allowed to acclimate for 1h prior to testing. Each module contained ten host-seeking females. Separate modules and experimentally naive mosquitoes were used for each treatment and replicate.

Tests were conducted using Protocol 138-02-6 approved by the Institutional Review Board at NC State University modified from Klun and Debboun [26]. A human subject wearing short pants was seated normally. Test compounds were applied in 55 μL of isopropyl alcohol to previously marked areas of the subject's upper and lower legs. The application areas were 20 cm^2 rectangles previously marked with non-toxic waterproof ink. Control areas were treated with isopropyl alcohol alone. Two minutes were allowed for solvent evaporation before testing began. The end of the module covered with cotton stockinette was pressed tightly against the treated area of skin. Mosquitoes were then allowed to probe and bite through the stockinette for 5 min. The number of mosquitoes taking a blood meal during this time was determined by the presence of a red distended abdomen and confirmed by rupturing individual insects in a transparent plastic bag. Percent protection was calculated from the total number of bites received for each treatment and control as % Protection = [(control-treatment) / control] x 100%.

Dose response tests were conducted on human skin using the test module with 2-undecanone and deet applied as various concentrations (0.4-200 $\mu g/cm^2$) diluted in isopropyl alcohol. During these experiments, the human subject experienced one chemical treatment (5 doses and solvent control) per day. The experiment was replicated five times for each chemical on consecutive days. The human subject was instructed to wash treated areas of skin thoroughly with soap and water between replicates.

From the dose response experiments, it was determined that 2-undecanone provided 100% protection against bites at a concentration of approximately 20% vol:vol (approximately 440 μg/cm^2) (data not shown). The persistence of 2-undecanone at 20, 30 and 40% was then measured by applying the treatments (3 doses and solvent control) to 20 cm^2 areas of skin as previously described. Tests were conducted as follows. Two treatments were conducted simultaneously, one on either side of the upper and lower areas of the left leg. The remaining two treatments were conducted in the same manner on the right leg. The treatments conducted on the left and right legs were staggered with respect to time, allowing a total of 20 independent observations to be taken in 75 min. The number of mosquitoes taking a blood meal in a 5 min period was determined every 15 min until 2-undecanone failed to provide protection. The entire experiment was replicated three times on consecutive days. The human subject was instructed to wash treated areas of skin thoroughly with soap and water between replicates.

To determine if 2-undecanone is toxic under our conditions, ten mosquitoes were exposed in the test module for 5 min to skin treated with 40% 2-undecanone, the highest concentration tested. Controls were exposed to skin treated with isopropyl alcohol alone. The solvent was allowed to evaporate from the skin for two minutes prior to the mosquito exposure. Following exposure, insects were transferred to 23 x 23 x 23 cm clear acrylic plastic cages with a front cotton access sleeve and provided 20% sucrose. Insects were monitored every 24 h for two days for mortality (failure to respond to a blunt probe in 10 seconds). This experiment was replicated three times.

2.6. Statistical analysis

Percent protection for each dose of deet and 2-undecanone was transformed into the probit scale and dosages were transformed into the logarithmic scale. For each compound, the median effective dose to prevent 50% of the mosquitoes from biting (ED_{50}) was calculated by the method of Sokal and Rohlf [27] and Finney [28] in Microsoft Excel [29]. Significant differences were determined by comparing the 95% confidence intervals between effective doses. Significance tests were conducted using Student's t-tests in SAS [30].

3. Results and discussion

3.1. Repellent activity of TMOF mimics for ticks

To evaluate TMOF mimics for repellent activity against the soft tick, *O. parkeri*, a two-choice bioassay was conducted. The compounds tested were considered to have repellent activity if, in the same arena, the number of ticks observed on the half-circle of filter paper treated with the test compound was significantly different (paired t-tests, $P<0.05$) from those found on the half-circle solvent control. Prior to the addition of ticks to the test arenas, the filter paper from the treatment and the solvent control were allowed to stand at room temperature in the fume hood for 1 h to allow evaporation of the acetone carrier. It was obvious from time course studies that at 1 h, no detectable residual solvent was found on the filter paper as determined by the dry appearance of the paper and lack of any obvious solvent odor. To confirm that the distribution of ticks between the two sides of the arena was random, no-choice experiments were also conducted. No-choice experiments consisted of two half-circles of filter paper, both treated with solvent alone. The results of these experiments as well as the structures of the TMOF mimics synthesized are shown in Table 1.

CHEA, PHEA, CHEN and PHEN were designed to mimic the amino-terminal region of the TMOF decapeptide (Tyr-Asp-Pro-Ala-Pro-Pro-Pro-Pro-Pro-Pro; [31]). Specifically, these compounds were designed as stable chemical analogs of the dipeptide Tyr-Asp, predicted to have an important role in binding to the TMOF receptor. To determine if the Asp carboxylate group is required for receptor binding, two carboxylic acid analogs (CHEA and PHEA) and corresponding ethyl esters (CHEN and PHEN) were synthesized. The replacement of the carboxylic acid with an ethyl ester was also predicted to enhance analog stability in aqueous media and in insect hemolymph. The volatility of the ethyl esters would also be expected to be greater than for carboxylic acids, enhancing repellent activity against arthropods. Interestingly, the non-aromatic backbone of these mimics is similiar to that of other natural products such as citronellal, rhodinal and geraniol, all of which have been documented to influence arthropod behavior.

It was apparent from the no-choice experiments (Table 1), that the distribution in the arena after 2 h was random, i.e., the same number of ticks were found on both half-circles of filter paper (paired t-test, alpha = 0.05). Therefore, any differences from a 50:50 distribution must be the result of repellency of the test compound. There was no mortality in any of the tests conducted. The 2 h exposure period after ticks were introduced into the arena was chosen because at earlier times, the distribution was not always random, and the ticks were still actively seeking to escape.

The average number of ticks on the side with 2-undecanone, CHEA or PHEA at 100 and 50 $\mu g/cm^2$, ranged from 0 to 1, while the solvent control averaged 9.2 to 10 (Table 1). These results indicated that 2-undecanone, CHEA and PHEA have repellent activity against *O. parkeri* at these concentrations (Table 1). These compounds were not active, however, at 10

$\mu g/cm^2$. At the latter dose, the distribution of ticks between the treated and control side of the arena was apparently 50:50 as was the observed distribution of ticks in the no-choice control (Table 1).

CHEN and PHEN were active as repellants at 10 $\mu g/cm^2$. The average number of ticks on filter paper treated with CHEN and PHEN ranged from 0 to 2.2, compared to the 7.8 to 10 ticks for the solvent control. In addition, PHEN was also active at 5 $\mu g/cm^2$, however, this compound failed at 1.0 $\mu g/cm^2$ (data not shown).

The ethyl ester chemical mimics of TMOF, CHEN and PHEN, demonstrated higher repellent activity against *O. parkeri* than the corresponding acids, CHEA and PHEA. This was not surprising as compounds containing esters would be expected to have a lower boiling point and a higher vapor pressure than carboxylic acids of similar molecular weight and composition, causing more volatilization of the compound [32]. The strong intermolecular forces afforded to carboxylic acids by hydrogen bonding may explain this trend. At a concentration of 5 $\mu g/cm^2$, PHEN was active as a tick repellent while CHEN was not, suggesting that the substitution of the phenyl- for the cyclohexyl ring may have resulted in an increase in repellent activity against *O. parkeri*. PHEN appeared to be at least an order of magnitude more active than the other compounds tested suggesting that further structure activity studies are warranted to examine the potential for optimization of this repellent activity.

Undecanone in our tests was not the most active repellent, especially as compared to PHEN. The boiling point for undecanone is 233-234^{o} C, and it could be that the 1 h incubation in the hood at room temperature might result in a significant lost of active ingredient prior to the beginning of the bioassay. As our limited structure activity studies

indicate, volatility is likely a component of repellant activity, and further consideration of this will be needed to fully understand the usefulness of our novel TMOF mimics as arthropod repellants. However, undecanone is of special interest because of its tick repellant activity and the fact that it is a well known botanical secondary plant compound (7-15) that plays an important role in plant resistance to herbivorous arthropods (16-18). Further tests were conducted with 2-undecanone to better understand its repellant activity on human skin against mosquitoes. Similar tests were not conducted with CHEA, PHEA, CHEN and PHEN because these are novel compounds, and their mammalian toxicology is unknown. In addition, CHEA, PHEA, CHEN and PHEN are not commercially available.

3.2. *Anti-feedant activity of 2-undecanone*

2-Undecanone demonstrated anti-feeding activity when applied to human skin. The ED_{50}, the concentration to reduce biting of adult *A. aegypti* by 50%, was 91.5 μg undecanone/cm^2 (Table 2). The female mosquitoes used in these experiments were 7-12 days old from emergence, were reared with males until the day of testing and therefore were assumed to be mated, were not sugar fed for 24 h, and had demonstrated host seeking behavior prior to being separated from the main mosquito population. It is clear from these studies, that 2-undecanone has mosquito anti-feeding activity in our bioassay system. The ED_{50} for deet under the same assay conditions as that used for undecanone was 1.6 μg/cm^2. It is apparent that deet was active at a lower concentration.

The mode of action of deet in mosquitoes is apparently related to the inhibition of attraction to L-lactic acid, a major component of sweat [33]. The behavioral mode of action of 2-undecanone in mosquitoes is currently unknown. When 40% 2-undecanone was applied

to skin, the solvent was allowed to evaporate for 2 min, and then *A. aegypti* exposed to this treated skin for 5 min. It appears that undecanone is not insecticidal in our bioassays; no mosquito mortality was observed even 48 h after treatment. However, treated mosquitoes do not host seek for at least 2 h even when presented untreated human skin (data not shown). In cage studies where a human hand is treated with 2-undecanone and then inserted into a cage of *A. aegypti*, >95% of the host seeking behavior including blood feeding is inhibited at 0 time and even at 3 and 6 h after treatment (Dr. A.E. Brandt, Insect Biotechnology, Durham, NC, personal communication).

Various other methods have been applied to determine the ED_{50} for deet against *A. aegypti*, and the reported values of these estimates range from 1.6-350 $\mu g/cm^2$ [reviewed in 34]. Lower estimates (1.6, 1.8 and 3.4 $\mu g/cm^2$) have resulted from tests conducted on human skin while considerably higher estimates (260 and 350 $\mu g/cm^2$) were obtained with treated membrane feeding devices. The difference in the level of attractiveness between human skin and feeding devices containing human or animal blood may explain the markedly different responses observed using these approaches. The failure of membrane feeding devices to adequately reproduce the complex stimuli used by host-seeking mosquitoes apparently results in a lower biting rate for the controls. Consequently, ED_{50}s determined using these devices are higher and comparisons between treatments less sensitive.

Using our test module, the mean (± 1 SEM) number of mosquitoes biting and taking a bloodmeal from skin treated with isopropyl alcohol alone was 8.9 ± 0.2. The maximum number of mosquitoes that could potentially bite was ten in each test, indicating that our test module may be a better alternative to membrane feeding devices when the toxicity of the test compounds is known and human subject testing is possible. Also, the simple construction of

these modules combined with the small number of mosquitoes required for each test may facilitate rapid screening of potential repellent compounds. This approach could also be readily adapted for animal testing. Because the mosquitoes are restricted in their movement in the 50 ml conical tubes and to a relative close distance to the skin, it is not clear whether we are measuring repellent activity in a similar manner to that of a cage test. Therefore, we defined our results as anti-feeding activity. However, the ED_{50} that we obtained for deet in our bioassay was similar to values reported in the literature for deet using cage studies, and argues that our assay is also a measure of repellent activity.

3.3. Persistence of 2-undecanone

The results of persistence tests with 2-undecanone are presented in Figure 1. In these studies and at each time point after the application of undecanone, naive mosquitoes are used to measure anti-feeding activity. 2-Undecanone at the concentrations of 20, 30 and 40% (v/v in isopropyl alcohol) provided >95% protection against bites after 15 min, and biting was inhibited >93% after 30 min at 40%. However, there was no statistically significant difference (Student's t-test, $P > 0.05$) from zero after 45 min. Feeding inhibition at this time was 11% for 20% undecnaone, 11% for 30% undecanone and 18% for 40% undecanone. 2-Undecanone did not provide any protection against mosquito bites after 1 h at any of the doses tested (data not shown).

The short time to failure for 2-undecanone in our studies was not surprising considering that this compound is volatile (vapor pressure ≈ 0.05 mm Hg) [19]. It is apparent after 30 min, that the amount of undecanone on the skin is greatly reduced. If 2-undecanone is to be

used directly on skin to inhibit mosquito feeding, a formulation that would reduce volatility may increase the length of time for its anti-feeding activity for naive mosquitoes.

3.4 Mechanism of action and potential uses of 2-undecanone

There are a number of characteristics about 2-undecanone that would argue that it may be useful as a novel insect repellent to protect human and animals from arthropod feeding. For example, 2-undecanone is naturally found in plants as an insect anti-feeding agent, and our current studies clearly demonstrated that this same chemistry can be used to inhibit mosquito feeding on human skin. Because of the past and current commercial uses for 2-undecanone, a significant amount of toxicology information is already available. 2-Undecanone is listed by EPA as a Category IV chemistry [19], the safest classification for pesticides. As a comparison, deet is in Category III. 2-Undecanone is on the EPA GRAS (Generally Regarded As Safe) list, is currently registered for commercial use as an animal repellent, is found in nature in a number of plants including food plants and is a component of food supplements used in some countries [7,9,12,13]. 2-Undecanone was successfully patented as a method of repelling insects by Roe (2002).

Deet has become the gold standard for insect repellents and has been proven to be safe and effective. In our studies, the ED_{50} for 2-undecanone was 57-times greater than that for technical deet, indicating that the latter is a better anti-feeding agent under the specific conditions of our assay. We also found that although 2-undecanone demonstrated almost 100% anti-feeding activity against mosquitoes for 30 min, it failed at 45 min. It was not surprising that the persistence of 2-undecanone was less than 45 min. It is well known that 2-undecanone is more volatile than other repellents like deet, and formulation studies are

needed to determine if the persistence of undecanone and its long-term efficacy can be increased by reducing its volatility.

It is clear that the anti-feeding activity based on a measure of the concentration needed to inhibit 50% biting was significantly greater for deet than that for undecanone. However, when mosquitoes were exposed to 2-undecanone at time zero in our tests, they failed to host seek again for more than 2 h, even for untreated skin. In other studies, where deet or 2-undecanone are applied to a human hand and then the treated hands inserted into a cage of adult mosquitoes 0-6 h after treatment, both chemistries reduced host seeking and biting even 6 h after application (Alan E. Brandt, CEO, IBI, personal communication). These studies were conducted at each time point after 0 time exposure without replacing the mosquitoes. It is apparent that the mechanism of anti-feeding activity for deet and 2-undecanone are different, and that the latter inhibits host seeking behavior in mosquitoes for a considerable amount of time. In the future, additional studies will be needed to better understand the mechanism of action of 2-undecanone and how this mechanism might be used as an anti-feedant and repellant. One obvious use other than as an application on human skin, is in devices to produce a chemical barrier.

In summary, the concentration of 2-undecanone to inhibit 50% mosquito feeding was greater than that for deet. However 2-undecanone is known to be safe (classified by EPA as a category IV pesticide as compared to deet, category III), is a natural product, has anti-feeding activity, produces a sustained reduction in host seeking behavior, and has repellent activity against the soft tick, *O. parkeri*. Further studies on the use of this compound as an arthropod repellent are warranted. The repellent activity of other chemical mimics of TMOF, CHEA, PHEA, CHEN and PHEN, against soft ticks combined with our previous findings

that these chemistries have anti-feedant properties against lepidopteran larvae, suggest that this chemistry class in general might be used for a variety of applications in the control of arthropods of medical, veterinary and agricultural importance.

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Fig. 1. Persistence of 2-undecanone (2-U) on human skin at different concentrations (vol:vol in isopropyl alcohol) assessed for feeding deterrence to *A. aegypti*. Data are the mean (± 1 SEM) of three replicates.

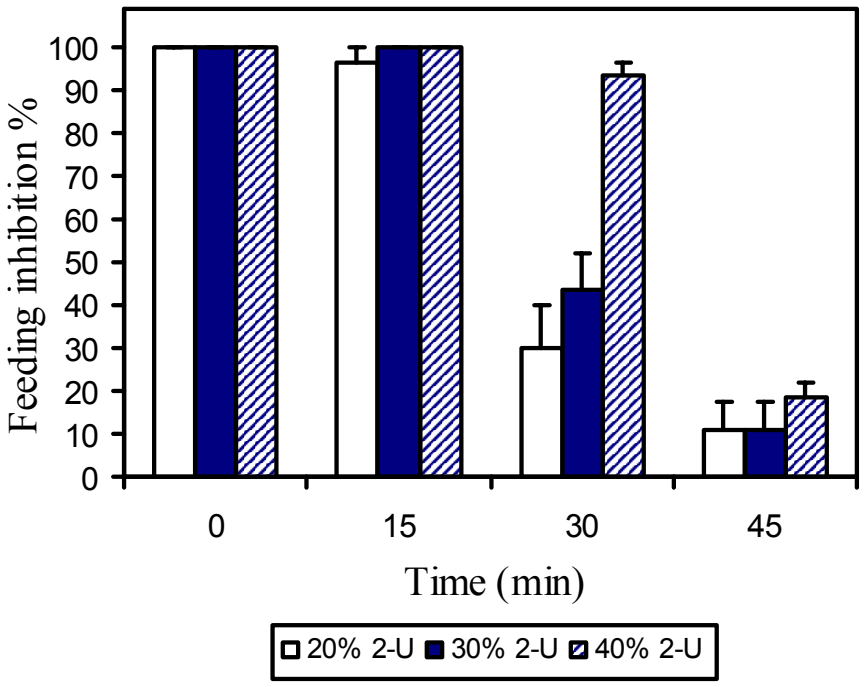
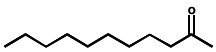
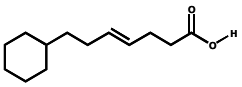
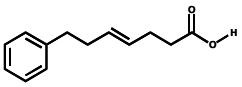
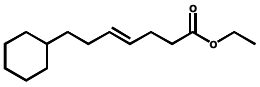
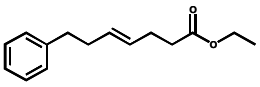

Feeding inhibition %
100
90
80
70
60
50
40
30
20
10
0
0
15
30
45
Time (min)
20% 2-U
30% 2-U
40% 2-U

Table 1
Results of repellency assays with *O. parkeri*. Structures of TMOF mimics are also shown.

Compound			µg/cm^2 100	50	10	5
1.	 (2-Undecanone)	c =	9.0 ± 0.4 a[1]	9.5 ± 0.3 a	5.5 ± 0.3 a	–
		t =	1.0 ± 0.4 b[1]	0.5 ± 0.3 b	4.5 ± 0.3 a	–
2.	 (CHEA)	c =	10.0 ± 0.0 a	10.0 ± 0.0 a	4.5 ± 0.3 a	–
		t =	0.0 ± 0.0 b	0.0 ± 0.0 b	5.5 ± 0.3 a	–
3.	 (PHEA)	c =	10.0 ± 0.0 a	9.2 ± 0.3 a	4.8 ± 0.3 a	–
		t =	0.0 ± 0.0 b	0.8 ± 0.3 b	5.2 ± 0.3 a	–
4.	 (CHEN)	c =	–	9.8 ± 0.2 a	7.8 ± 0.6 a	6.0 ± 1.0 a
		t =	–	0.2 ± 0.2 b	2.2 ± 0.6 b	4.0 ± 1.0 a
5.	 (PHEN)	c =	–	10.0 ± 0.0 a	10.0 ± 0.0 a	8.5 ± 0.6 a
		t =	–	0.0 ± 0.0 b	0.0 ± 0.0 b	1.5 ± 0.6 b
	no-choice	c =	4.8 ± 0.5 a			
		t =	5.3 ± 0.5 a			

[1]Mean number of ticks on the untreated (c) versus the treated surface (t) ± 1 SEM. Means followed by different letters were significantly different as determined by paired t-test (α=0.05).

Table 2
Median effective dose (ED_{50}) for bite protection against *A. aegypti*. Results are expressed as $\mu g/cm^2$.

Repellent	ED_{50}	Regression curve	R^2	95% CI	N
deet	1.6	y = 2.9x - 0.6	0.98	1.0 - 2.8	300
2-undecanone	91.5	y = 2.3x - 4.5	0.98	67.2 - 123.9	300

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