Isolongifolenone: A Novel Sesquiterpene Repellent of Ticks and Mosquitoes

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ABSTRACT A naturally occurring sesquiterpene, isolongifolenone, derivatives of which have been used extensively as ingredients in the cosmetics industry, was discovered to effectively repel blood-feeding arthropods that are important disease vectors. We show that (−)-isolongifolenone deters the biting of the mosquitoes, Aedes aegypti (L.) and Anopheles stephensi Liston, more effectively than the widely used synthetic chemical repellent, N,N-diethyl-3-methyl benzamide (DEET), in laboratory bioassays. The compound also repelled blacklegged ticks, Ixodes scapularis Say, and lone star ticks, Amblyomma americanum (L.), as effectively as DEET. Isolongifolenone is easily synthesized from inexpensive turpentine oil feedstock. We are therefore confident that the compound has significant potential as an inexpensive and safe repellent for protection of large human populations against blood-feeding arthropods.

KEY WORDS Aedes aegypti, Anopheles stephensi, Ixodes scapularis, Amblyomma americanum, N,N-diethyl-3-methyl benzamide

Human diseases vectored by blood-feeding arthropods represent a serious threat to public health worldwide. More than 700 million cases of mosquito-transmitted disease have been reported annually (Shell 1997). Over three billion people live under the threat of malaria, which kills more than 1 million each year (UNICEF 2005). In the United States, mosquito-transmitted West Nile virus infected >8,000 people from 1999 to 2005, resulting in >780 deaths (DeBiasi and Tyler 2006). Clearly, there are strong reasons to pursue discovery of effective chemical tools that might help alleviate this suffering. The worldwide market for personal insect repellents has been estimated at more than $2 billion annually (Gilbert and Firestein 2002), with N,N-diethyl-3-methyl benzamide (DEET) being the most widely used compound (Elston 1998, Fradin and Day 2002). However, it is a synthetic compound that dissolves certain plastics and safety and environmental concerns have arisen regarding its use (Robbins and Cherniack 1986, Clem et al. 1993, Ross et al. 2004).

Isolongifolenone is a natural product that was found in Humiria balsamifera St. (Aubl.) Hill (Humiriaceae), a plant commonly found in South America (Da Silva et al. 2004). Isolongifolenone is odorless and some of its derivatives have a characteristic woody smell (Hall 1973, Pickenhagen and Schatkowski 2002, De Bruyn et al. 2003). Therefore, derivatives of isolongifolenone have been widely used as fragrances in cosmetics, perfumes, space sprays, detergents, deodorants, fabrics, fibers, and paper products (Curtis et al. 1971, 1972, 1978, Pickenhagen and Schatkowski 2004). In addition, (−)-isolongifolenone inhibits tyrosinase that is a multifunctional copper-containing enzyme for melanin biosynthesis in plants and animals (Choudhary et al. 2003). Also, hydrogenated (−)-isolongifolenone has been used as a bridged core to prepare a chiral ligand for the estrogen receptor that could be useful in regulating fertility, preventing, and treating breast cancer, and for menopausal hormone replacement (Muthyala et al. 2003).

gifolene, which is commercially available as a feedstock in kilogram to ton quantities.

Given the widespread need for naturally occurring and environmentally benign repellents, our laboratories have sought to discover new natural chemicals to fulfill this need. This report shows the effectiveness of (−)-isolongifolenone in deterring feeding of two species of mosquitoes and repelling two species of ticks of medical importance.

Materials and Methods

Chemicals. (−)-Isolongifolenone was prepared as the major product from (−)-isolongifolene (Sigma, St. Louis, MO). The preparation, using tert-buty1 hydroperoxide as an oxidant, chromium hexacarbonyl as the catalyst, and acetonitrile and benzene as the solvent, yielded ≥90% product with high purity (≥99%) in a reaction time of ≈2 h (Wang and Zhang 2008, Zhang et al. 2008). DEET (>98% purity; Morflex, Greensboro, NC), which is considered to be the best mosquito repellent ever developed (Elston 1998), served as our standard for comparing the efficacy of (−)-isolongifolenone.

Mosquito Bioassay. Aedes aegypti (L.) (red eye Liverpool strain) and Anopheles stephensi Liston (Delhi strain) used in the bioassays came from colonies maintained at the Walter Reed Army Institute of Research, Division of Entomology, Silver Spring, MD. The mosquitoes were reared using the procedure of Gerberg et al. (1994). Larvae were fed ground tropical fish flakes (Tetramin Tropical Fish Flakes; Tetra Sales, Blacksburg, VA). Colonies were maintained in a photoperiod of 12:12 h (L:D with lights on at 0600 hours) at 27°C and 80% RH. Adult mosquitoes were maintained with cotton pads moistened with 10% aqueous sucrose solution. Twenty-four hours before testing the sugar pads were taken from the mosquitoes and replaced with water-moistened pads. Mated nulliparous female mosquitoes were 5–15 d old when they were used in bioassays.

The mosquito-feeding deterrent efficacies of (−)-isolongifolenone and DEET were compared using the in vitro K&D bioassay system. The in vitro K&D bioassay system is a proven method for discovery of new candidate repellent chemicals for human use. Examples of discovery include this work with isolongifolene and previous work involving isolation of bioactive compounds from Callicarpa spp. plants that are effective against mosquitoes and ticks (Cantrell et al. 2005, Carroll et al. 2007).

The K&D assay system consisted of three components: (1) a Plexiglas K&D module composed of six adjacent 39 by 47 by 48-mm cells each designed to hold mosquitoes and each having a rectangular 30 by 40-mm floor that opened and closed by a sliding door, (2) a Plexiglas water-bath warmed reservoir with six 30 by 40 by 7-mm-deep wells designed to match the sliding-door openings of the K&D module. The wells contained warmed (38°C) human red blood cells (6 ml), and they were covered with an Edicol collagen membrane (Devro, Sandy Run, SC). The blood-membrane unit simulated a human host for mosquito feeding, and (3) a 297 by 71 by 4-mm Teflon separator having six 30 by 40-mm rectangular openings identical to the K&D module cell floors. In the study, test compounds in 95% ethanol solution were applied to 30 by 40-mm rectangular cloth areas marked with ink pen on a 297 by 71-mm piece of organdy cloth. We routinely applied a 110 μl ethanol solution to the rectangular areas and 5 cm outside of the ink-marked areas to ensure that mosquitoes were subsequently exposed only to treated cloth. The chemically treated cloth was placed over the membrane-covered blood wells. The Teflon separator was placed over the treated cloth. The function of the separator was to prevent direct contact of the K&D module with the chemically treated cloth. In this way, chemical contamination of the module was avoided. The K & D module, holding five mosquitoes in each of the adjacent module cells, was positioned over the Teflon separator, and the mosquitoes were exposed to the cloth treatments for three minutes by opening the module’s sliding doors. The number of mosquitoes biting (proboscis inserted through the cloth and/or observed blood-engorged) within each cell in the 3-min exposure was recorded, mosquitoes were prodded from the cloth back up into the cell and module doors were closed. Mosquitoes were used once in a test and frozen.

The quantitative feeding-deterrent effects of compounds observed using the in vitro K&D bioassay system approximate the effects seen when compounds are applied topically (in vivo) to the skin of human volunteers (Klun et al. 2005). However, the relative effectiveness of a compound in the in vitro and in vivo K&D assay modes can differ moderately. As an example, in earlier in vitro tests with DEET, nepetalactones, and SS220, the SS220 ranked as the most effective, whereas nepetalactones did not differ significantly from each other or from DEET. However, with in vivo tests the nepetalactones did not deter mosquito biting as effectively as DEET or SS220. In all tests, in vitro or in vivo, all compounds differed significantly from the control (Chauhan et al. 2005).

In a fixed dose study, compounds in 95% ethanol solution were randomly applied to rectangular areas of organdy cloth. A bioassay replicate consisted of three treatments: 25 nmol (−)-isolongifolenone/cm² cloth, 25 nmol DEET/cm² cloth, and 95% ethanol treated cloth as control. The 25 nmol compound/cm² cloth dose was used, because it was known from previous dose–response bioassays (Klun et al. 2003, 2005) with DEET and other effective–bioactive compounds that the dose suppressed mosquito biting by ≈80%. We conducted replicated tests with treated cloth covering the six wells of a reservoir. In these tests, treatments were each evaluated against 200 Ae. aegypti and 200 An. stephensi females (40 replicates). In a dose–response assay, Ae. aegypti was tested against DEET and (−)-isolongifolenone at 4, 9, 16, 25, and 36 nmol compound/cm² cloth and a blank control (95% ethanol). Two hundred mosquitoes were tested (40 replicates) against each dose of each compound. As a mosquito
quality control procedure in both the fixed dose and the dose–response assays, we used data sets in which three or more females bit (≥0.4 proportion not biting) in the control cell. This approach assured that chemicals were evaluated against aggressively feeding mosquitoes.

**Tick Bioassay.** Blacklegged tick, *Ixodes scapularis* Say, nymphs were reared from larvae obtained from the laboratory colony at Oklahoma State University, Stillwater, OK, and fed on rats (Beltsville Area Animal Care and Use Committee Protocol 05-022) at the USDA–ARS, Beltsville Agricultural Research Center, Beltsville, MD. Lone star tick, *Amblyomma americanum* (L.), nymphs were obtained from a colony at the USDA–ARS, Knipling-Bushland U.S. Livestock Insects Research Laboratory, Kerrville, TX. Both species of ticks were maintained in a photoperiod of 16:8 h (L:D with lights on at 0600 hours) at 24°C and 100% RH until testing. Nymphs of *I. scapularis* were tested 2–3 mo and *A. americanum* 2–3 mo after they had fed as larvae.

Tick responses were evaluated using a fingertip bioassay modified from Schreck et al. (1995) and described in detail by Carroll et al. (2005, 2007). Test solutions were applied to the outer layer of a strip of cloth (7 by 7 mesh organdy), allowed to dry for 10–12 min and the cloth doubly wrapped around the middle phalax of the left index finger of a volunteer (depicted in Carroll et al. 2005). The cloth was shaped like an ice hockey stick. It was 9-cm long section, 4.5-cm short section, and 4–4.5 cm wide. The cloth extended 5–6 mm proximally beyond the deepest crease of the middle joints of the finger and overlapped 1–3 mm. The boundaries of the area to receive the chemical treatment area (between the deepest creases of the distal and middle joints) were marked on the cloth with a lead pencil. Calculations used in preparing test solutions used to generate nmol doses/cm² cloth were based on the dimensions of the middle phalax of the left index finger of a volunteer (the area marked on the cloth). When on the finger, the cloth was kept from unraveling by three small dabs of beeswax between the layers. As with Schreck et al. (1995), it was necessary to screen *I. scapularis* nymphs for active individuals. After the treated cloth dried, *I. scapularis* nymphs were transferred by forceps from a holding vial to a finger. Only nymphs that crawled ≥5 mm were used in the bioassay. Using forceps, the 10 selected ticks were placed on the tip of the horizontally held finger between the nail and the edge of the cloth. When the 10th tick was placed on the finger, the finger was slowly tipped vertically with the tip downward. Host-seeking *A. americanum* are notably more active than *I. scapularis*, so *A. americanum* nymphs were allowed to crawl directly from an open vial onto the fingertip. For both species, the finger was held over moated petri dishes while at least one tick remained on the finger. The locations of ticks remaining on the finger at 15 min after their release on the finger were recorded as were the number of ticks that fell from the finger or crawled completely across the cloth. As soon as a tick crawled across the cloth, it was removed from the finger. During the bioassays, temperatures ranged from 23 to 26°C and 10–56% RH.

In a dose–response test, three doses (19.5, 39, and 78 nmol compound/cm² cloth) of (−)-isolongifolenone, DEET, and a blank control (95% ethanol) were tested against four groups of 10 *I. scapularis* nymphs (four replicates). We tested *A. americanum* nymphs against six doses (78, 155, 310, 465, 620, and 775 nmol compound/cm² cloth) of (−)-isolongifolenone and DEET and an ethanol control. Against (−)-isolongifolenone, three groups of 10 nymphs each were tested at the 155-, 465-, and 620-nmol doses and eight groups of 10 nymphs each were tested at the 78-, 310-, and 775-nmol doses (unequal replication). Three groups of 10 nymphs were tested against all six doses of DEET (equal replication). Ticks were considered repelled if they had dropped from the finger or were on the untreated tip at 15 min after they were placed on the finger.

**Statistical Analyses.** The mosquito deterrence and tick repellent data were converted to proportions of not biting or proportions repelled, respectively, and transformed by the standard variance stabilizing transformation for proportions (arcsine √p, where p is the original proportion) to fit the assumption of homogeneity of variances for analysis of variance (ANOVA). Mean proportions of mosquitoes not biting or ticks repelled were compared by one-way ANOVA and by Ryan-Einot-Gabriel-Welsch range test for significance at α = 0.05 level, and the dose–response data were analyzed using regression analysis (SPSS 10.0 for Windows; George and Mallery 2002).

**Results**

**Mosquito Bioassay.** Bioassay data showed that (−)-isolongifolenone effectively deters the biting of *Ae. aegypti* and *An. stephensi* at 25 nmol compound/cm² cloth (Fig. 1). The proportions of mosquitoes not biting were significantly larger for (−)-isolongifolenone and DEET than for the control, and (−)-isolongifolenone deterred the biting of *Ae. aegypti* and *An. stephensi* more effectively than DEET (n = 40; F = 67.03; df = 5,234; P < 0.0001). The data also showed that on the average more than three of five *Ae. aegypti* and *An. stephensi* bit in controls. This indicates that the two species of mosquitoes bit above our arbitrary biting-threshold limit. The data also showed that *Ae. aegypti* was more susceptible to DEET and (−)-isolongifolenone than was *An. stephensi*.

The biting deterrent activity of (−)-isolongifolenone and DEET against *Ae. aegypti* increased with increasing concentrations. Regression analysis of the data for the proportion not biting showed the dose–response relationship was linear (R = 0.9753 and 0.9917, respectively; Fig. 2). The proportions not biting at the 25 nmol DEET/cm² dose approximated 0.8, and this confirms the previous dose–response relationship observed earlier by Klun et al. (2003, 2005) and the fixed-dose assay result shown in Fig. 1. The proportions of mosquitoes deterred by (−)-isolongi-
folenone were significantly greater than the proportions deterred by DEET at doses of 25 nmol compound/cm² cloth \((n = 40; F = 107.47; \text{df} = 2,117; P < 0.0001)\) and 36 nmol compound/cm² cloth \((n = 40; F = 329.38, \text{df} = 2,117; P < 0.0001)\), respectively.

**Tick Bioassay.** \((-\)-isolongifolenone also effectively repelled ticks. All *I. scapularis* nymphs were repelled by \((-\)-isolongifolenone and by DEET at 78 nmol compound/cm², yet all the nymphs remained on the untreated fingertip. In contrast, *A. americanum* were prone to dropping off the finger, when exposed to a repellent. For example, when the cloth was treated with 155 nmol compound/cm², 19 of the 20 *A. americanum* nymphs repelled \([\text{totals of all replicates the same for both \((-\)-isolongifolenone and DEET}] fell from the finger.

The repellent activity of \((-\)-isolongifolenone and DEET against *I. scapularis* increased with the concentration reaching 1.0 at 78 nmol compound/cm² cloth for both compounds and regression analysis of the data for the proportions repelled was linear \((R = 0.9894\) and 0.9878, respectively; Fig. 3). The proportions of *I. scapularis* repelled by \((-\)-isolongifolenone did not differ significantly from the proportions repelled by DEET in the dose range tested \((all P > 0.05)\). Both DEET and \((-\)-isolongifolenone significantly repelled the *A. americanum* nymphs at 25 nmol compound/cm² compared with ethanol controls \((P < 0.004)\). Higher concentrations of DEET and \((-\)-isolongifolenone were needed to repel *A. americanum* than *I. scapularis*. Over the range of concentrations tested, \((-\)-isolongifolenone and DEET repelled a maximum of 0.80 proportion of *A. americanum* nymphs. Even at the highest concentration tested, which was \(10\) times greater than the dose needed to completely repel *I. scapularis*, neither compound repelled all *A. americanum*. Although overall proportions of *A. americanum* repelled by \((-\)-isolongifolenone were in the same range as those of DEET, the response were highly variable with no clear dose–response relationship exhibited cross the

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**Fig. 1.** Mean proportions of female *Ae. aegypti* and *An. stephensi* not biting exposed to \((-\)-isolongifolenone, DEET, and blank control in the in vitro K&D bioassay. Error bars denote SEM. Means followed by different letters are significantly different at \(\alpha = 0.05\).

**Fig. 2.** Dose–responses of female *Ae. aegypti* not biting in the in vitro K&D bioassay. Error bars denote SEM.
range of the doses tested ($R = 0.6852$ and $0.5097$, respectively; Fig. 4).

Discussion

Dethier et al. (1960) defined a deterrent as “a chemical that inhibits feeding when present in a place where insects would, in its absence, normally feed.” Thus, the in vitro bioassays using the K&D module system specifically quantified the biting (feeding) deterrent properties of $(-)-$isolongifolenone. Our in vitro bioassay data indicated that $(-)-$isolongifolenone and DEET had biting deterrent properties against the two species of mosquitoes. Both compounds were more effective against *Ae. aegypti* than *An. stephensi*. At 25 nmol $(-)-$isolongifolenone/cm$^2$ cloth, the proportions of mosquitoes not biting were 0.92 for *Ae. aegypti* and 0.75 for *An. stephensi*. However, at the same concentration of DEET, the proportion not biting was 0.81 for *Ae. aegypti* and 0.65 for *An. stephensi* (Fig. 1). Differential sensitivity to repellents from one species to another is not uncommon among mosquitoes (Rutledge et al. 1978, Robert et al. 1991, Klun et al. 2004). Despite the differences between these two species of mosquitoes in the percentages deterred from biting, $(-)-$isolongifolenone was generally more effective than DEET as a biting deterrent.

Between the species of ticks, we observed a pronounced difference in sensitivity to the repellent effects of $(-)-$isolongifolenone and DEET. The tick-bioassay data showed that *I. scapularis* nymphs were significantly more sensitive to $(-)-$isolongifolenone and DEET than were *A. americanum* nymphs. At 78 nmol/cm$^2$ cloth, $(-)-$isolongifolenone and DEET repelled all of the *I. scapularis* nymphs (Fig. 3), but only 0.45 and 0.24 proportions of the *A. americanum* nymphs were repelled (Fig. 4). These results confirm the earlier finding by Carroll et al. (2007) that *A. americanum* was clearly more tolerant to DEET and three other repellents than *I. scapularis*.

Many natural product chemicals and terpenoids such as cadalenol, $\alpha$-cadinol, and nepetalactone, iso-
lated from plants and essential oils have proven to have repellent effects against arthropods (He et al. 1997a, b, Grace 2002, Omolo et al. 2004, Panella et al. 2005, Trongtokit et al. 2005, Schultz et al. 2006). Most often, such compounds never attain commercial development, and their use is limited or impractical because they are expensive and/or not available in pure and large quantities. In contrast, pure (-)-isolongifolone can be made inexpensively from turpentine oil feedstock in large quantities for large-scale commercial applications, making it unique among known natural product repellent chemicals.

Our findings showed the effectiveness of (-)-isolongifolone against four important disease vectors. A patent application has been granted for preparation and use of the (-)-isolongifolone for repellent and feeding deterrent purposes (Wang and Zhang 2008, Zhang et al. 2008). Derivatives of this compound have been used as ingredients in the cosmetics industry. Therefore, we surmise that (-)-isolongifolone has considerable potential as a natural repellent that can be made available sustainably in large quantities to safely protect human populations from blood-feeding arthropods.

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References Cited


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