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Letters

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Structure–Activity Relationship of a Broad-Spectrum Insect Odorant Receptor Agonist

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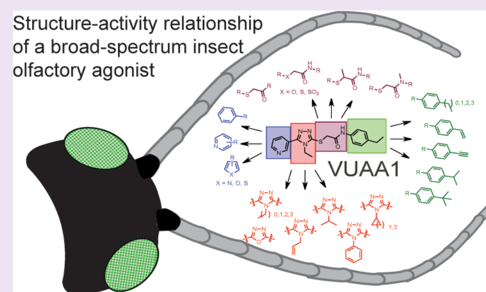
Supporting Information

ABSTRACT: Agonism of insect odorant receptor (OR) cation channels may represent a new strategy for the manipulation of destructive insect olfactory-driven behaviors. We have explored the chemical space around VUAA1, the first in class agonist of the obligate OR co-receptor ion channel (Orco), and describe novel compound analogues with increased potency across insect taxa. Functional analyses reveal several of these VUAA1 structural analogues display significantly greater potency as compared to the activity of the previously described active compounds in mobility-based behavioral assays on mosquito larvae.



Insects continually monitor their chemical environment and adjust their behavior in response to semiochemical cues. Many volatile odorants are detected and interpreted by surface receptors from the odorant receptor (OR) gene family, which are expressed on the dendrites of olfactory receptor neurons (ORNs) located at the base of hair-like cellular structures known as olfactory sensilla.[1] Insect ORs are 7-transmembrane domain proteins that form heteromeric cation channels composed of two ORs: the obligate OR co-receptor (Orco) and an odorant-specific "tuning" OR (ORx), which determines the range of odorant sensitivity.[2−5] Ligand binding to the ORx subunit leads to depolarization of ORN dendrites, which produce action potentials that propagate signals to downstream effector neurons.[4,6] In the absence of Orco, tuning ORs cannot form functional channels efficiently,[7,8] while Orco expressed alone can form functional cation channels but cannot be activated by classical odorants.[9,10] In general, tuning *Or* genes are highly divergent among and within insect species, whereas in contrast, *Orco* genes are highly conserved across all insect taxa,[11] making it a particularly attractive target for modulating a broad spectrum of chemosensory-driven insect behaviors.

High-throughput screening for small molecule activators of Orco/OR complexes expressed in HEK293 cells led to the discovery of VUAA1, the first compound to show allosteric agonism of OR complexes by specifically targeting Orco subunits.[9] The discovery of VUAA1 provided proof of concept that Orco channels can be directly modulated, thereby opening a new paradigm for insect control. Because VUAA1 class compounds activate OR ion channels in the absence of a natural ligand by interacting with the remarkably conserved Orco subunit, they have the potential to act broadly to reduce disease transmission and pest load from a variety of insects. Insect control strategies targeting a single ligand-sensitive tuning OR subunit may be limited by the number of ORNs that express that particular *Or* gene. In contrast, VUAA1 may activate all ORNs expressing Orco, theoretically eliciting a more powerful behavioral response. We now describe initial structure–activity relationship (SAR) studies around the VUAA1 scaffold, leading to the identification of more potent Orco agonists and the validation of ligand potency with a secondary larval behavioral assay.

VUAA1, a recently identified agonist of insect OR complexes, is thought to activate OR channels by binding to the obligate OR co-receptor (Orco), while volatile odorants activate the complex by binding to the classical ORx (Figure 1a). Following the discovery of VUAA1, we examined the Vanderbilt Institute for Chemical Biology (VICB) compound collection and assessed 480 structural analogues using high-throughput calcium mobilization imaging.[12−14] This preliminary screen revealed no agonist activity providing an extremely narrow SAR space; agonist activity was completely lost even with very subtle changes to the VUAA1 core structure (for representative structures see Supplementary Table 2). For example, a 4-methoxy aniline, a 4-methyl ketone substituted aniline, or a 3-ethylcarboxyaniline led to a loss of activity, as did a 2-,6-dimethyl aniline when paired with a methyltriazole variant of VUAA1. In light of these exceptionally narrow constraints, we elected to synthesize a small set of very focused analogues of

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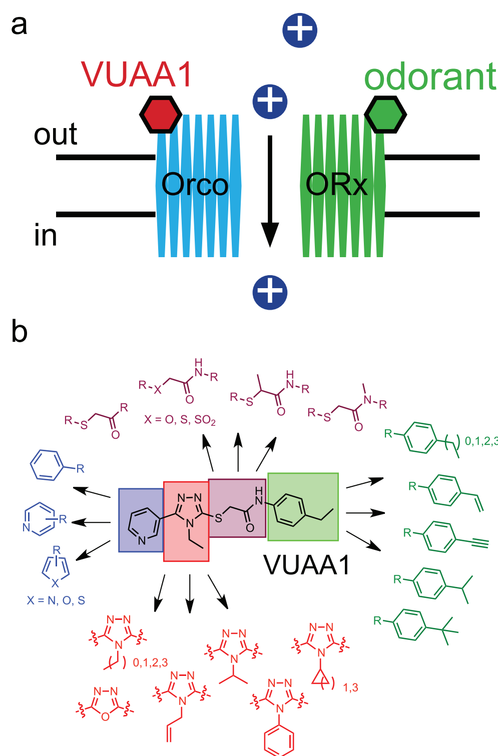

Figure 1. VUAA compounds agonize mosquito odorant receptors (ORs). (a) Insect OR channels are heteromeric cation channels made up of the obligate OR co-receptor (Orco) and a classical tuning OR (ORx). Volatile odorants are thought to activate the channel by binding to the odorant-specific ORx while VUAA1 allosterically activates the channel by interaction with the Orco subunit. (b) The structure of VUAA1 can be divided into regions based on chemical structure for systematic substitution at each position.

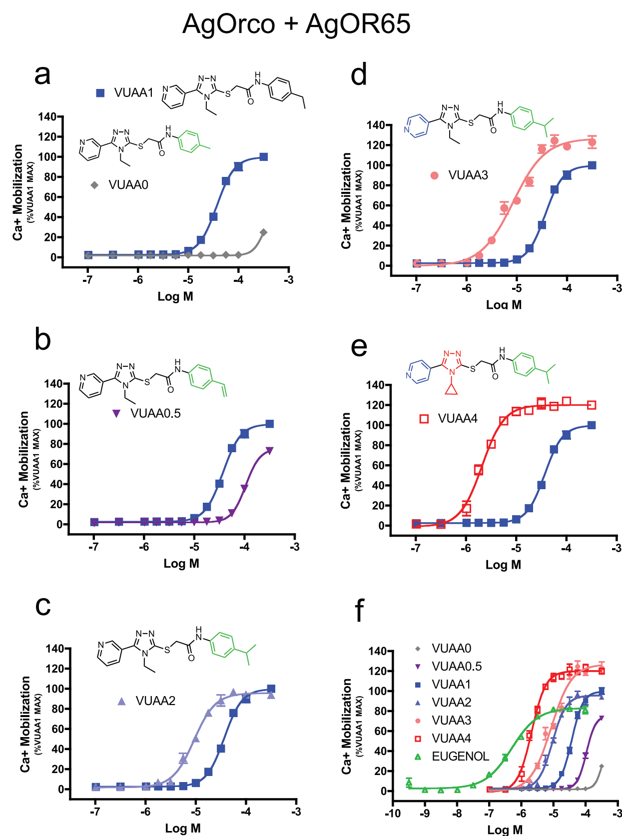

Figure 2. Very small changes to the VUAA1 structure greatly impact its ability to agonize AgORs in heterologous Ca^{2+} mobilization experiments. In HEK293 cells stably expressing AgOrco and AgOR65 and loaded with Ca^{++} indicator dye, Fluo-4 can be exposed to logarithmic dosage of potential VUAA-class agonists. VUAA0 (a) and VUAA0.5 (b) have reduced potency when compared to VUAA1, while VUAA2 (c), VUAA3 (d), and VUAA4 (e) display enhanced potency. The activity of improved compounds approaches the potency of the odorant eugenol (f). Error bars = SEM.

VUAA1 in hope of identifying more potent agonists. We divided the VUAA1 structure (Figure 1b) into four discrete regions for focused SAR exploration. Using an iterative medicinal chemistry approach, we synthesized a narrow range of compounds and found selected analogues that had improved potency relative to VUAA1.

As an example of the limited optimization space available around VUAA1, we found that the removal of even a single methyl from the *para* position of the aniline ring (VUAA0, Figure 2a) resulted in almost complete loss of potency when presented to heteromeric OR channels (AgOrco + AgOR65: VUAA1 $EC_{50} = 3.7 \times 10^{-5}$ M vs VUAA0 $EC_{50} > 3.4 \times 10^{-3}$ M). Introducing unsaturation at the same position (VUAA0.5, Figure 2b) also dramatically reduced potency ($EC_{50} = 1.1 \times 10^{-4}$ M). After evaluating a range of substituents at this position (Figure 1b), we discovered that the replacement of the ethyl group of VUAA1 with an isopropyl group (VUAA2, Figure 2c) improved potency relative to that of VUAA1 ($EC_{50} = 9.2 \times 10^{-6}$ M). All changes to the amide linker have thus far resulted in near total loss of agonist activity.

We next examined the effect of structural changes of the pyridine ring on Orco agonism. Again, most changes to this region of VUAA2 were not tolerated. However, shifting the nitrogen to the *para* position (VUAA3, Figure 2d) resulted in increased potency ($EC_{50} = 8.4 \times 10^{-6}$ M). Finally, changing the triazole *N*-ethyl group to a cyclopropyl group (VUAA4, Figure 2e) led to an additional increase in compound potency over previously tested compounds ($EC_{50} = 2.1 \times 10^{-6}$ M). Overall, VUAA4 represents a 10-fold improvement in agonist potency when compared to VUAA1. We have thus generated a series of modest substitutions that cover potency ranges from almost undetectable to nearly equivalent to a natural agonist, eugenol (Figure 2f). The extremely narrow SAR surrounding the VUAA-based family of Orco agonists suggests that putative binding relationship with Orco targets is complex and generally unforgiving.

We next compared the activity of VUAA analogues toward Orco orthologs derived from representative species of 3 different insect orders: diptera (*Anopheles gambiae*, AgOrco), lepidoptera (*Heliothis virescens*, HvOrco), and hymenoptera (*Harpegnathos saltator*, HsOrco). We found the relative potency of the VUAA series compounds (VUAA4 > 3 > 2 > 1) to be consistent regardless of the species origin of each Orco ion channel (Figure 3a). Odorant ligands are thought to activate the complex *via* interaction with the tuning ORx and thereby affect Orco/ORx channel properties.[15] However, the hierarchy of VUAA series potency was the same regardless of whether AgOrco was coexpressed with AgOR65 or AgOR48 tuning ORs (Figure 3b). In both cases, the potency of VUAA4 is within 1 order of magnitude of the respective cognate ligand (OR65: VUAA4 $EC_{50} = 2.1 \times 10^{-6}$ M, eugenol $EC_{50} = 5.0 \times 10^{-7}$ M; OR48: VUAA4 $EC_{50} = 1.7 \times 10^{-6}$ M, D-undecalactone $EC_{50} = 2.6 \times 10^{-7}$ M). In addition, we determined that VUAA1

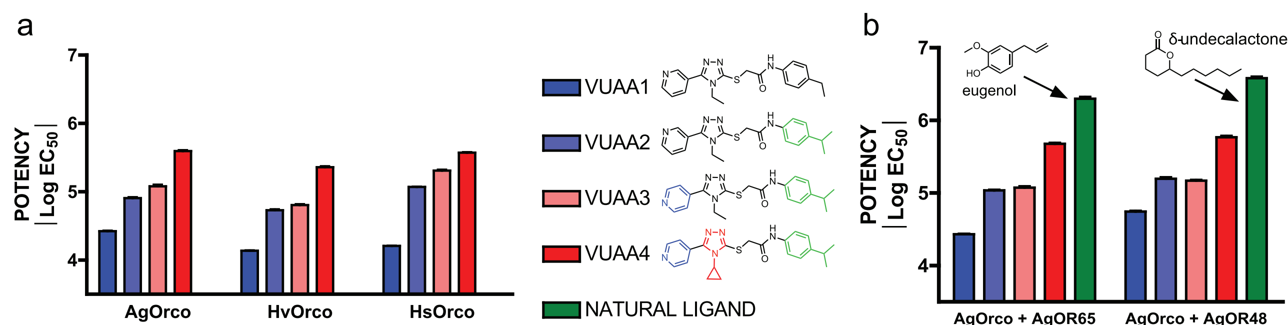

Figure 3. VUAA compounds agonize a diversity of insect odorant receptors (ORs). (a) VUAA analogues are able to agonize Orco proteins derived from *Anopheles gambiae* (AgOrco), *Heliothis virescens* (HvOrco), and *Harpegnathos saltator* (HsOrco). EC_{50} values (expressed as the absolute value of Log molarity) of each effective VUAA compound are relatively stable across evolutionary time. (b) VUAA compounds are effective regardless of the identity of the tuning ORx involved in the complex. Error bars = SEM.

and VUAA4 have very similar agonism potency when presented to heteromeric complexes containing orthologous Orco subunits derived from either *Anopheles gambiae* or *Drosophila melanogaster* (Supplementary Figure S1). These findings validate VUAA-class Orco modulators as potentially important compounds for the further development of broad-spectrum insect control strategies.

In order to further assess the agonist activity of the VUAA compound class, we have examined the behavioral effects of VUAA agonists against the *in vivo* spectrum of AgOrco-AgORx complexes on an organismal level and adapted a behavioral assay using larval-stage *An. gambiae* mosquitoes. Previously, we utilized similar behavioral assays to establish the involvement of AgORs in mediating larval ortho- and klinokinesis (movement and turning) in response to a series of semiochemicals.[16,17] Although such assays can narrowly define threshold concentrations of a behavioral effect, they do not measure attractive or repellent responses. Nevertheless, an added advantage is that because mosquito larvae are aquatic, compounds can be delivered to individual early fourth instar larvae and responses monitored regardless of volatility. In these assays, larval movements were automatically quantified over a 5-min period, and control larvae consistently moved the same number of times in the presence or absence of 0.1% DMSO ($p = 0.80$, $n = 31$) (Figure 4).

We next examined the effects of several compounds that are known to affect larval behavior[16] including 3-methylphenol (3MP), acetophenone (ACP), and the widely used synthetic insect repellent *N*,*N*-diethyl-*meta*-toluamide (DEET). In agreement with our previous findings,[16,16] exposure to 1×10^{-3} M 3MP (Figure 4a, green) significantly increases larval movements ($p = 0.0011$, $n = 23$), while 1×10^{-6} M ACPAcPH (Figure 4a, maroon) is sufficient to significantly decrease larval movements ($p = 0.02$, $n = 24$). DEET increased larval movements at and above a threshold concentration of 5×10^{-4} M (−3.3(Log M) $p = 0.013$, $n = 27$) (Figure 4a, yellow).

We next evaluated the effect of VUAA1 on larval behavior, and found a significant increase in the number of larval movements at and above a concentration of 5×10^{-9} M (−8.3(Log M) $p = 0.045$, $n = 35$) (Figure 4a, blue), a potency increase of several orders of magnitude over DEET, 3MP, and ACP. At higher concentrations of VUAA1 (5×10^{-5} M), larval movements decreased, a phenomenon that may reflect toxicity, off-target effects, or receptor effects such as desensitization. To determine the dependence of these larval responses on AgOrco agonism, we specifically silenced *AgOrco* mRNAs by injection of small interfering RNA (siRNA) oligonucleotides 48 h before evaluation of their behavior in response to VUAA1.[16] In these studies, VUAA1 responses persisted in larvae injected with buffer alone or with a nonspecific siRNA, while larvae treated with *AgOrco* siRNAs were no longer responsive to VUAA1 treatment ($p = 0.0025$, $n = 21$) (Figure 4a).

We next exposed larvae to VUAA0, VUAA0.5, VUAA2, VUAA3, and VUAA4 (Figure 4b). On the basis of our Ca^{++} mobilization experiments, we expected VUAA0 and 0.5 to have no effect on larvae and VUAA2, 3, and 4 to affect larvae in a similar manner to VUAA1 and DEET. We observed a dose-dependent increase in larval movements upon exposure to VUAA4 with a response-threshold at 10^{-9} M ($p = 0.047$, $n = 54$) with a decrease in movements at high concentrations similar to that observed for VUAA1. Exposure to VUAA0 caused a significant decrease in larval movements at several concentrations (1×10^{-8} M: $p = 0.0012$ $n = 54$; 1×10^{-6} M: $p = 0.0095$ $n = 42$; 1×10^{-5} M: $p = 0.008$ $n = 42$), which may be due to off-target effects or alternatively, a true receptor effect such as Orco channel antagonism. We observed no significant change in larval movements in response to VUAA0.5, 2, or 3.

These findings validate the VUAA4 structure as a potent new lead in the expansion of the VUAA structure for downstream applications. Furthermore, with respect to the larval responses to both VUAA2 and 3, these data highlight the necessity of additional validation of screening leads from heterologous systems in organism-level behavioral assays. Surprisingly, we observed a consistent lack of response to VUAA4 at 10^{-8} M, a currently inexplicable finding in light of the robust effects at both 10^{-9} M and 10^{-7} M. Overall, this assay demonstrates that some VUAA compounds elicit significant larval responses similar to those of known actives such as DEET, 3MP, and ACP, but at significantly lower threshold concentrations.

In conclusion, we have conducted studies to investigate and optimize the potency of VUAA-based Orco agonists. These studies reveal an extraordinarily narrow tolerance for changes around the VUAA1 parent structure, indicative of very tight constraints in the response relationships with Orco ion channel targets. This restriction may be due to a lack of a conventionally defined agonist-binding pocket on the Orco subunit, though at present the location and characteristics of the VUAA binding site are unknown. We have used iterative compound synthesis and testing to identify active VUAA analogues with as much as a 10-fold increase in potency relative to VUAA1.

This novel series of VUAA-family compounds are able to agonize Orco regardless of the identity of the tuning OR

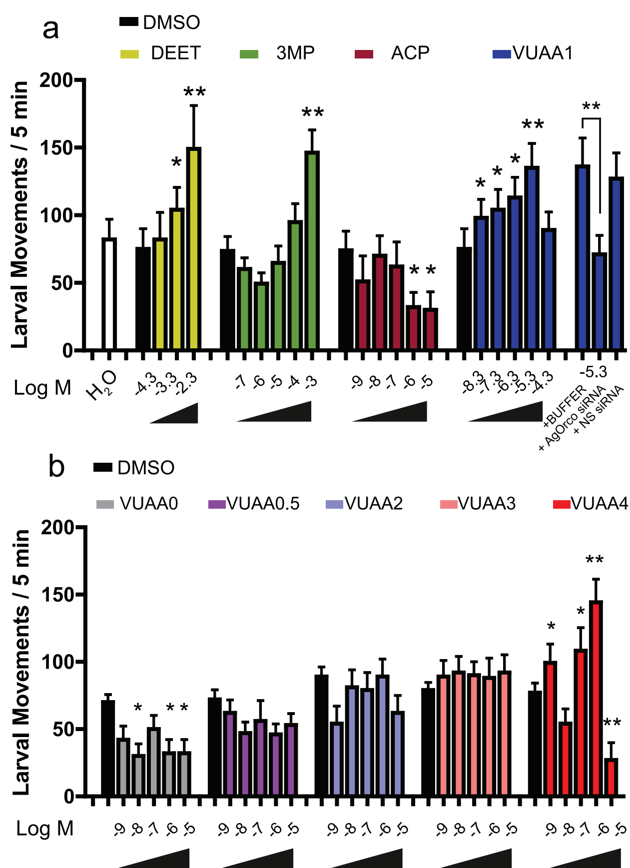

Figure 4. Larval mobility bioassays. Larval movement behavior can be monitored by automatic tracking over the course of 5 min in the presence of odorants and target compounds. This assay is carried out in 6-well plates using Daniovision (Noldus) instrumentation and analyzed using Ethovision (Noldus) software. (a) Stimulation by the commercial insect repellent DEET (yellow) increases the rate of larval movements in a dose-dependent manner. Larval behavior is also affected by high concentrations of 3-methyphenol (3MP) (green) and acetophenone (ACP) (maroon), which elicit increased and decreased rates of movement, respectively. VUAA1 (blue) treatment also increases larval movements in a dose-dependent manner, albeit at a reduced threshold concentration relative to DEET. The effect of VUAA1 is dependent on expression of Orco protein, as siRNA knockdown of Orco eliminates the effect, but injection of nonspecific (NS) siRNA has no effect. (b) VUAA0 (gray) reduces larval movements, possibly as the result of off-target effects or Orco antagonism. VUAA0.5 (purple) has no effect on larval movement behavior at any concentration. Neither VUAA2 (light blue) nor VUAA3 (light red) has any effect on larval movement behavior despite their ability to activate OR channels in heterologous expression systems (see Figure 1). VUAA4 (red) increases the number of larval movements over a 5-min period in a dose-dependent manner, with the inexplicable exception of the 1×10^8 concentration. Error bars = SEM (*$p < 0.05$, **$p < 0.005$).

subunit or, importantly, the species origin of the Orco ion channel subunit. Taken together, these characteristics enhance the utility of VUAA class agonists as emerging small molecule tools for laboratory studies of insect olfaction.

We have also attempted to validate the effectiveness of these compounds by determining the behavioral effect of compound exposure on the complex olfactory system of mosquito larvae. Recently, other groups have carried out similar efforts at improving Orco agonist activity through examination of VUAA1 analogues.[18,19] In agreement with our findings, Bohbot and Dickens have identified OrcoRAM2, in which a nitrogen at the *para* position utilizes a 4- instead of the 3-pyridine of the VUAA1 scaffold. This compound shows reduced activity relative to VUAA1 in *Aedes aegypti* . In addition, Chen and Luetje show that in *Culex quinquefasciatus* and *Drosophilia melanogaster* OLC3 (= OrcoRAM2) and OLC12 (= VUAA3) are both more potent insect Orco agonists than VUAA1. Chen and Luetje also agree with our finding that removal of a single methyl from the aniline ring (OLC5 = VUAA0) results in reduced activity.[19]

Our behavioral studies indicate that, despite their promising performance in heterologous expression systems, VUAA2 and 3 may not be effective for behavior modification on the organism level. In contrast, VUAA1 as well as our most potent compound, VUAA4, are able to affect larval mosquito behavior at significantly lower thresholds as compared to DEET or other compounds. These studies support the pursuit of VUAA-mediated Orco receptor agonism as the basis for the development of a comprehensive and broadly effective insect control strategy based on excito-repellency.

■ METHODS

Compound Synthesis. General Procedure. To a solution of substituted aniline (1.5 equiv) in CH_2Cl_2 were added triethyl amine (1.5 equiv) and chloroacetyl chloride (1.5 equiv). After 2 h, the solution was concentrated and redissolved in acetonitrile. To this solution were added substituted triazole (1 equiv) and cesium carbonate (2 equiv). After 16 h, the reaction mixture was concentrated, and the residue was purified by column chromatography with MeOH/CH_2Cl_2 (1:4) to afford 62−84% of the desired product. For synthesis details, refer to Supporting Information.

Calcium Fluorimetry. The creation and validation of the tetracycline-inducible AgOR-expressing HEK293 cell lines has been described previously.[12,14] Calcium mobilization fluorimetry was conducted at the high-throughput screening facility as part of the Vanderbilt Institute for Chemical Biology. A total of 20,000 cells were distributed to each well of black-walled, clear bottom 384-well assay plates (Greiner) coated with poly-D-lysine to aid cell adherence. After incubation at 37 °C for >5 h, OR expression was induced by addition of tetracycline at 0.3 μg/μL. One hour prior to fluorimetry, cells were loaded with 20 μL of 3 μM fluo-4/acetoxymethyl ester (Invitrogen) mixed at a 1:1 ratio with 10% (w/v) pluronic acid F-127 (Invitrogen) diluted in assay buffer (Hank's balanced salt solution, 20 mM HEPES, 2.5 mM probenecid). Fifteen minutes before fluorimetry, dye solution was replaced with assay buffer. Compounds to be assayed were prepared at a 100 mM stock solution in dimethyl sulphoxide (DMSO), and concentration series were generated in 15 mm 384-well polypropylene plates using an Echo 555 acoustic liquid handler (Labcyte) and diluted in assay buffer with a Multidrop Combi (Thermo Scientific).

Both liquid handling and fluorescence signal detection were carried out with a FDSS6000 plate reader (Hamamatsu). Fluo-4 fluorescence upon inward Ca^{++} flux was detected with an excitation of 470 ± 20 nm and an emission of 540 ± 30 nm. The fluorescent signal was recorded once per second over the course of a 3 min assay; 20 μL of 2X compound was added to each well after 20 s. OR channel activation was inferred by the ratio of max fluorescence following compound addition to minimum fluorescence before compound addition. Sigmoidal dose response curves and half-maximal effective concentrations (EC_{50}) were calculated with Prism4 software (Graphpad).

Mosquito Rearing and Larvae Selection. *An. gambiae sensu stricto*, originated from Suakoko, Liberia, was reared as described.[16] In order to propagate the colony, 5-day old females were blood-fed according to Vanderbilt Institutional Animal Care and Use Committee. For larval mobility assays, 12−15 early L4 stage larvae were manually selected, gently rinsed using deionized water, and kept in a clean plastic cup for starvation of 30 min under 27 °C, 75%

relative humidity. Immediately following starvation, larvae were maintained at RT (24 °C) for behavioral experiment.

Larval Mobility Assay. In order to avoid circadian oscillation that may potentially affect larval mobility, all assays in this study were carried out in the time window between 9 and 12 a.m. Nonsterile 6-well plates were used as the behavioral arena and discarded after every trial. A 4.0 mL portion of sterile, distilled water was pipetted to each well, and 4 μL of odorant dissolved in DMSO was then added. The plate was gently shaken to acquire homogeneous odor concentration. All odorant stocks used were of >99% pure or the highest grade commercially available. Immediately following plate preparation, 6 larvae were gently introduced into the 6-well plate, with each well containing only 1 larva. Larval locomotion was recorded using Daniovision (Noldus) with a frame rate of 12.5 f/s for a length of 5 min. Larval traces were automatically generated and analyzed with Ethovision (Noldus), and the tracking parameters in Ethovision were adjusted to guarantee a <0.01% error rate due to mis-recognition of the animal. Movements were calculated to assess the larval response to different compounds of varying concentrations, and the method is described as follows: if an individual larva moves more than 1.00 mm between consecutive recorded frames (3000 frames in total), it will be recognized as 1 movement, otherwise ignored. The threshold was established and strictly applied throughout the entire data analysis for the purpose of minimizing noise caused by larva vibrating while lacking any horizontal locomotion.

A minimum of 20 trials (20 independent larvae) were performed for each odorant concentration while wells containing only DMSO served as control. For statistical analysis, normality and equal variances of the data set was checked by Chi-square goodness of fit and Levene's test, respectively. The average of different experimental groups was compared relative to control using Dunnet's test following one-way Anova using JMP 9.0.2. Asterisks in the graph indicate a p value less than 0.05.

siRNA Preparation and Injection. For detailed methods, please refer to ref 16. Briefly, 27.6 nL of 100 nM siRNA targeting Orco (*An. gambiae*) or AT5G39360 (*Arabidopsis thaliana*) was injected into the dorsal side of the larval thorax (3rd instar) using a Nanoliter 2000 system (WPI, Sarasota, FL). Buffer-only injections were also undertaken to control for potential needle effects. Injected larvae were allowed to recover for 48 h in ∼100 mL 27 °C deionized water with 1 mL of larval food provided. Larval survival was checked at 24 h postinjection, and nonviable or severely damaged larvae were discarded. Before experiments, injected larvae were treated as described previously.

ASSOCIATED CONTENT

Supporting Information

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Notes

The authors declare the following competing financial interest(s): Appropriate patent applications are pending on the chemical novelty and the use of these compounds for insect control applications.

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