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Insect-Repellent and Mosquitocidal Effects of Noreremophilane- and Nardoaristolone-Based Compounds

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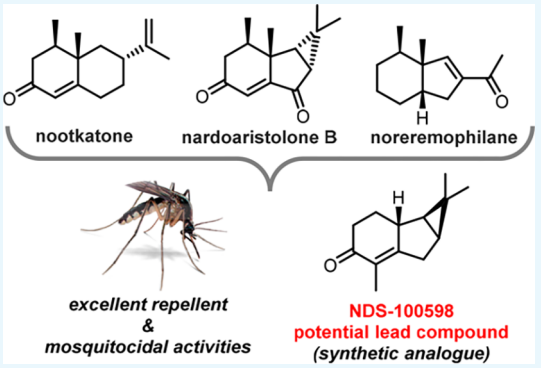
Supporting Information

ABSTRACT: Here, we disclose novel mosquito-repellent synthetic hydrindanes based on noreremophilanes and nardoaristolone B which show increased activity against adult females of *Aedes aegypti*. The noreremophilanes and nardoaristolone B with hydrindane skeleton are structurally related to nootkatone with decalin skeleton, a well-studied natural product extracted from a grape fruit. Out of our library of compounds synthesized based on the noreremophilanes and nardoaristolone B scaffolds, NDS-100598 (compound **20**) exhibits higher repellent and knock-down effects at a very low concentration (0.25 mg/cm^2), while a few analogues showed considerably enhanced activity compared to racemic nootkatone. This is the first report documenting insect-repellent and knock-down activity of the noreremophilanes class of compounds.



INTRODUCTION

Mosquito bites can potentially result in the transmission of several infectious diseases and remain as one of the main causes of worldwide human health concern. The most effective way of tackling the mosquito menace is through personal protective measures, including the use of mosquito repellents.[1] Pyrethroids, well-known insecticides, are the mainstay of vector-borne disease eradication programs.[2] However, growing resistance to the commonly used pesticides, particularly pyrethrins,[3−7] is resulting in the need to increase concentrations of the active ingredients to improve efficacy, thereby surging the pesticide load in the environment. Earlier efforts led to the identification of *N*-*N*-dialkylamide and diol class of compounds as two promising leads toward the development of mosquito repellents.[8] Advancement in repellent chemistries in subsequent years brought about some novel structures including piperidine and terpene classifications.[9−14] Garson and Winnike[15] suggested that compounds containing amides, imides, phenols, alcohols, hydroxy esters, glycols, and hydroxyl esters were active as repellents in comparison with the parent hydrocarbons. Several compounds with repellent activity, including DEET, picaridin, IR3535 (structurally similar to β-alanine), and oil of eucalyptus (*p*-menthane-3,8-diol) are now commercially available (Figure 1).[16] Recently, metofluthrin, a spatial repellent, and a catnip formulation have been registered by the EPA.[17,18] Hence, there is a need to develop new, safer insect repellents with novel modes of action. (+)-Nootkatone is a naturally occurring sesquiterpene, known to possess very impressive insect repellent and/or insecticidal activity against various ticks, mosquitoes, termites, bed bugs, fleas, and so forth.[19−26] It has been isolated from the essential oil of the heartwood of Alaska yellow cedar, *Chamaecyparis nootkatensis*.[27] Nootkatone is reported to be as effective as DEET against ticks (*Ixodes scapularis*).[11] Recently, two derivatives of (+)-nootkatone, tetrahydronootkatone, and 1,10-dihydronootkatone were shown to act as insecticide and repellent against several arthropods including termites, ants, cockroaches, and ticks.[28] Because of their impressive insecticidal activity, we explored structurally resembling compounds like noreremophilane and nardoaristolone for their insecticidal properties.[29,30] In this article, for the first time, we are reporting the repellent activity and mosquitocidal effects of *cis*-hydrindane derivatives synthesized based on noreremophilane and nardoaristolone B against adult females of *Aedes aegypti*, a vector of yellow fever, dengue fever, dengue hemorrhagic fever, and Zika virus. Our studies show that these compounds exhibit better activity than some of the commercially available mosquito repellents/mosquitocides.

RESULTS AND DISCUSSION

Synthesis. The preparation of (±)-nootkatone, (±)-noreremophilane, and analogues was achieved by applying key Diels−Alder/Aldol methodology developed by our group followed by several functional group interconversions (Figure

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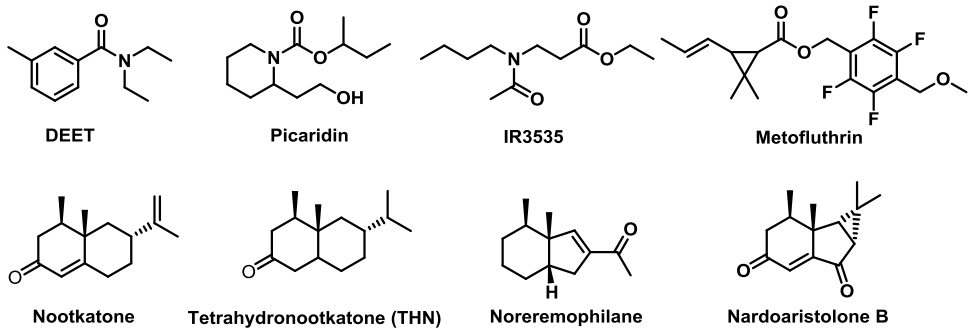

Figure 1. Selected insect repellents and natural products.

2).[29−31] Similarly, synthesis of (±)-nardoaristolone B and its analogues with cyclopropyl ring fusion were prepared.[30]

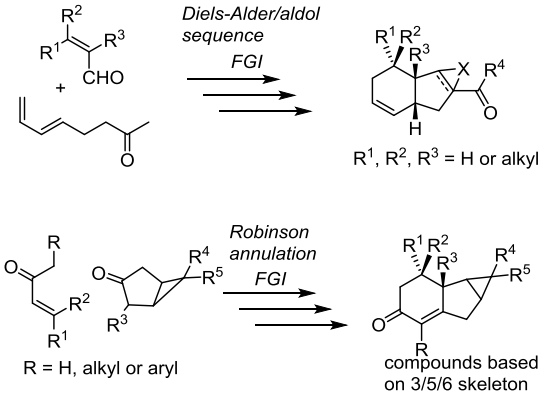

Figure 2. Synthetic strategies for the preparation of the compounds used in the present study.

Synthesis of compounds **12**−**17** were described in a recently published paper.[32] All the compounds were fully characterized by IR, ^{1}H NMR, ^{13}C NMR, and HRMS. Compounds **8**, **19**, **21**, and **23** contain ∼10% diastereomer which was difficult to separate and they were screened as such; the remaining compounds were >95% pure as single diastereomers (judged from NMR). The detailed experimental procedures and supporting spectral data are available for the abovementioned compounds in the cited papers from our group. The commercial sample of DEET was obtained from Allyl Amines Chemicals Ltd., Daund, Maharashtra, India, which served as the positive control.

Mosquito Repellence Bioassays. Mosquito-repellent activity was assessed on the basis of the protection period (minute) offered by various analogues of nootkatone and nardoaristolone B against adult females of *A. aegypti*. The protection period was measured on the basis of the concept "time until the first bite".[33] Repellence tests were carried out between 09.00 and 17.00 h against 3−5 days old, disease-free, blood-starved but sucrose-fed (0.5 M solution) nulliparous female mosquitoes of *A. aegypti* drawn from an established laboratory colony maintained at 27 ± 1 °C, 70 ± 5% RH, and a 12:12 h light/day cycle. The light intensity was regulated at 300−500 lux. Volunteers with no history of allergic reactions to arthropod bites, stings, or repellents were selected for the study. A human volunteer's hand washed with unscented soap and subsequently rinsed with 70% ethanol was air-dried. The treated arm was covered with a polythene glove which had a muslin screen stuck over a small window (2 × 2 cm) on which the required concentrations of the test compound(s) were applied. Various analogues of nootkatone and/or DEET were loaded on the muslin cloth screen instead of direct skin application so as to avoid the potential risk involved in the evaluation of natural products of unknown mammalian toxicity. Stock solution (1%) of each compound was prepared using analar grade acetone. This stock solution (100 and 200 μL) was spread evenly on the muslin cloth screen (thereby yielding a dose of 0.25 and 0.50 mg/cm^2, respectively), and the solvent was allowed to evaporate. A similar polyethylene glove with a muslin cloth screen was treated with solvent alone, which served as a control. Before the start of the experiment, an untreated arm of a volunteer (control) was placed in the mosquito cage (30 × 30 × 30 cm) containing about 200 blood starved mosquitoes for 15 s to estimate the readiness of mosquitoes to take a bite. Subsequently, the mosquitoes were blown from the hand before any blood was taken. After introduction of the hand covered with the polythene glove with the treated muslin screen into the mosquito cage, the number of mosquito bites received in the subsequent 5 min was counted. In the event of no bites during the initial 5 min exposure, the test hand was exposed repeatedly after every consecutive 1/2 h for 5 min test till the time a confirmed bite was received. The time period between the application of the compound and the first two bites or two bites in successive observation was recorded as the protection time. In control, the frequency of mosquito bite was 10−12 bites/min with the first bite occurring within 10 s. The above test was repeated with human volunteers (male—3 and female—2) using a new batch of mosquitoes for each test.

Contact Toxicity Bioassays. Studies on contact toxicity of compound **20** were pursued following the protocol described by Brogdon and McAllister[34] and Brogdon and Chan[35] with slight modifications. Briefly, the interior of Erlenmeyer flasks (250 mL) was coated by pipetting 1 mL of the following concentrations of the test compound dissolved in isopropyl alcohol—0.0125, 0.025, 0.05, and 0.1%. The flasks were swirled so that all surfaces get exposed to the solution. The process was continued until all the solvent had evaporated. An aspirator was used to introduce 20 blood starved adult females of *A. aegypti* aged 3−5 days. They were blown gently to expel the mosquitoes into the flasks. The mouth of the flasks was covered with muslin cloth. Observations on mortality were taken at 1 min intervals until 5 min and then at 5, 10, 20, 30, 40, 50, and 60 min and also after 24 h. Five replicates were conducted for each concentration.

Statistical Analysis. A two-way ANOVA followed by Tukey's test (JASP) was used to separately compare the mean time to first bite for the 25 synthetic analogues. Data on

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Table 1. Mean ± SE[a] to First Bite by Adult Females of *A. aegypti* after Exposure to Different Synthetic Analogues of Nootkatone and Nardoaristolone B (n = 5)

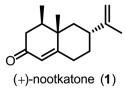
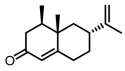
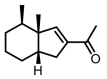
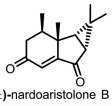
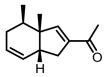
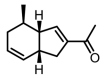
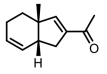
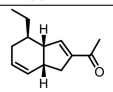
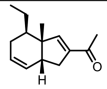
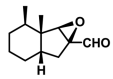
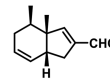
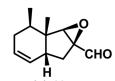
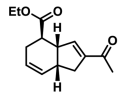
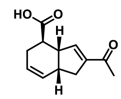
Compounds	Protection period in minutes @ concentration			
	0.25 mg/cm^2		0.5 mg/cm^2	
	Mean	Range	Mean	Range
 (+)-nootkatone (**1**)	324 ± 2.85[a]	315 – 330	433.2 ± 5.17[a]	420 – 450
 (±)-nootkatone (**1**)	187.2 ± 3.50[b]	180 – 198	300 ± 9.88[b]	264 – 318
 (±)-noreremophilane (**2**)	138.6 ± 2.40[c,i]	132 – 144	247.2 ± 2.04[c,h]	240 – 249
 (±)-nardoaristolone B (**3**)	217.2 ± 9.58[d,m,n]	198 – 240	368.4 ± 4.07[e]	360 – 378
 (±)-**4**	185.4 ± 2.40[b]	180 – 192	324 ± 3.29[b,d]	312 – 330
 (±)-**5**	146.4 ± 8.42[c,e]	138 – 180	154.8 ± 10.31[f]	138 – 180
 (±)-**6**	81.6 ± 4.5[f,k]	66 – 90	258.4 ± 5.07[b,c]	246 – 273
 (±)-**7**	128.4 ± 4.08[g,i]	120 – 138	144.60 ± 1.99[f]	138 – 150
 (±)-**8**	21.6 ± 2.4[h,j]	18 – 30	96 ± 9.83[g]	78 –120
 (±)-**9**	117.6 ± 7.26[c,o,p]	90 – 132	196.2 ± 3.37[f]	186 – 207
 (±)-**10**	28.8 ± 1.20[h,i,j]	27 – 33	67.2 ± 3.37[g]	60 – 75
 (±)-**11**	77.4 ± 1.12[f,k,o]	75 – 81	124.2 ± 2.94[f,g]	120 – 135
 (±)-**12**	250.8 ± 4.42[l]	240 – 258	283.2 ± 10.31[b,c]	258 – 300
 (±)-**13**	63.6 ± 3.6[f,k]	60 – 78	190.8 ± 4.42[f]	180 – 198

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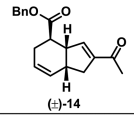
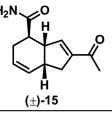
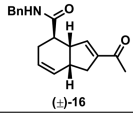
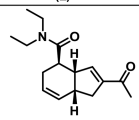
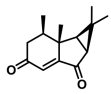
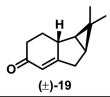
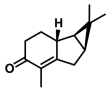
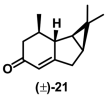
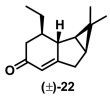
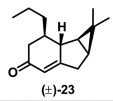
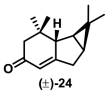
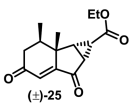
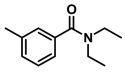
Table 1. continued

Compounds	Protection period in minutes @ concentration			
	0.25 mg/cm^2		0.5 mg/cm^2	
	Mean	Range	Mean	Range
 (±)-14	0^{h}	–	130.8 ± 4.42^{f,g}	120 – 138
 (±)-15	0^{h}	–	71.40 ± 4.69^{g}	60 – 81
 (±)-16	0^{h}	–	0^{i}	–
 (±)-17	194.4 ± 3.6^{b,m}	180 – 198	268.8 ± 8.16^{b,h}	258 – 300
 (±)-18	247.8 ± 3.75^{b,l}	240 – 258	321 ± 5.21^{b,c,j}	306 – 333
 (±)-19	201.6 ± 4.79^{b,d,n}	189 – 213	314.4 ± 5.33^{b,k}	300 – 330
 (±)-20	326.4 ± 11.19^{a}	300 – 366	372.6 ± 3.85^{e}	360 – 384
 (±)-21	313.2 ± 4.10^{a}	300 – 324	382.8 ± 2.25^{d}	378 – 390
 (±)-22	91.8 ± 7.34^{f,i,k,o}	78 – 120	352.8 ± 28.43^{d,e,j,k}	240 – 390
 (±)-23	108.60 ± 8.94^{f,g,i,p}	84 – 126	119.4 ± 6.81^{f,g}	93 – 129
 (±)-24	131.4 ± 4.29^{g,e}	120 – 138	136.2 ± 2.62^{f,g}	129 – 144
 (±)-25	71.4 ± 1.99^{f,k}	66 – 78	82.8 ± 2.25^{g}	78 – 90
 DEET	510 ± 8.33^{q}	493 – 526	630 ± 10.2^{q}	609 – 650

^{a}Means in each column followed by the same letter are not significantly different (p > 0.001) by Tukey's test. Overall SE of the mean is 7.167.

experiments on contact toxicity was analyzed by the log probit method of Finney[36] using Probit software. Times to 50% knock-down and 95% knock-down (KDt_{50} and KDt_{95}) were estimated by the probit software.

Studies on Repellency. The repellent activity of nootkatone, noreremophilane, and nardoaristolone B and its analogues against adult females of *A. aegypti* was measured in terms of protection time (min), and the results are compiled in

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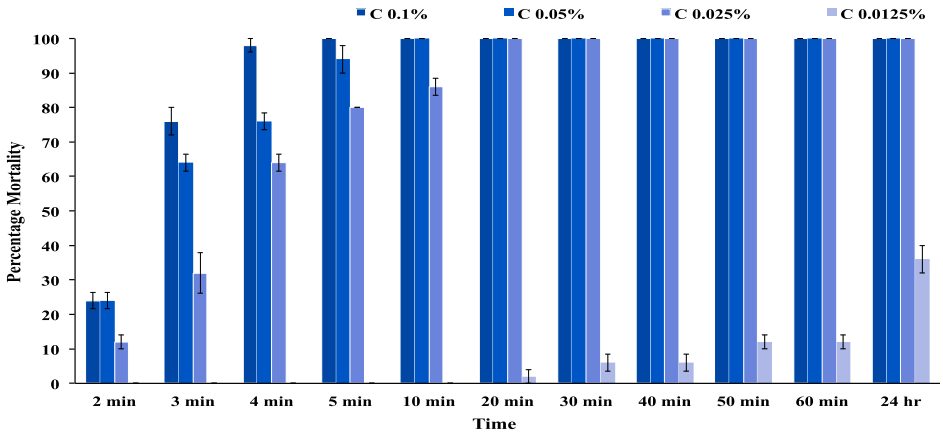

Figure 3. Contact toxicity effects of compound **20** against adult females of *A. aegypti* in Erlenmeyer flasks (n = 20).

Table 1. The two-way ANOVA indicates significant differences between the activities of different analogues (F = 641.36, df 26, p < 0.001) and their concentration (F = 2079.86; df 1, p < 0.001), which clearly reflects in the interaction between compound/concentration (F = 44.91; df 26, p < 0.001) (see Supporting Information for details). The analysis of contrasts by Tukey's method indicated differential activity of the individual analogues, DEET and their concentration (Table 1). At a concentration of 0.25 mg/cm^2, the mean protection time for (+)-nootkatone **1** was 325 min, which was comparable with the compounds **20** and **21** but was significantly lower for the remaining 23 analogues. However, at a concentration of 0.5 mg/cm^2, the mean of protection time (433 min) was significantly higher for (+)-nootkatone **1** than the remaining compounds. However, at both concentrations, the mean protection time for DEET was significantly higher than that of nootkatone, noreremophilane, and nardoaristolone B and its analogues. Because we had synthesized all the *cis*-hydrindane compounds in the racemic form, we performed comparative evaluation of racemic nootkatone **1**. Under the same assay conditions, *rac*-nootkatone provided 187 and 300 min protection from mosquitoes at 0.25 and 0.5 mg/cm^2 concentrations, respectively. On the basis of initial screening results, we also synthesized natural products noreremophilane **2** and nardoaristolone B **3** in their racemic form and screened for their activity. Both of these compounds showed significant repellent activity with good dose response; in fact, compound **3** showed higher activity when compared with *rac*-nootkatone **1**. Following up on these initial leads, other analogues of the natural products noreremophilane and nardoaristolone were synthesized and evaluated for their repellent activity. These experiments show that introduction of an additional double bond in the six-membered ring of hydrindane significantly improved the potency (compare compounds **4** with **2**). In fact, compound **4** showed more than 185 and 324 min protection at 0.25 and 0.5 mg/cm^2, respectively. Furthermore, removal of one of the methyl groups in compound **4** showed a negative effect, thereby illustrating the role of both the methyl groups in noreremophilane skeleton (**5** and **6**) in improving the activity. Interestingly, extension of the carbon chain (**7** and **8**) as well as manipulation of five-membered ring (**9**, **10**, and **11**) was found to be detrimental for the repellent activity. Similarly, carboxylic acid or its derivatives at the six-membered ring (**12** to **16**) reduced the activity. However, the corresponding diethyl amide derivative (**17**) showed modest improvement in the activity. Perhaps, the relative improvement in activity on the addition of this moiety is similar to what has been observed in the case of DEET, a well-known insect repellent. Additionally, we observed very interesting results in the case of compounds belonging to the nardoaristolone skeleton. Of the eight compounds belonging to this skeleton, half of them showed more potency than the racemic nootkatone. Two of these compounds, **20** and **21**, were observed to have about 360 min (6 h) or more protection from mosquitoes, at 0.5 mg/cm^2 concentration. Unlike in the case of noreremophilane skeleton, removal of methyl groups did not affect the potency of the molecules. However, increased chain length, placing of gem-dimethyl group on the six membered ring, or introduction of carboxylic ester moiety on the cyclopropyl ring has drastically reduced the activity.

Studies on Contact Toxicity. Bioassays on knock-down and mortality were evaluated for the most promising compound **20** on adult females of *A. aegypti*. Studies on contact toxicity in Erlenmeyer flasks revealed that compound **20** showed a dose-dependent effect for KDt_{50}, KDt_{95}, and mortality. At a concentration of 0.1%, compound **20** exhibited an average mortality of 98% within 4 min reaching 100% at 5 min (Table S1, Figure 3). In contrast, 100% mortality was achieved at 10 min at 0.05% and 20 min at 0.025%. At a lower concentration of 0.0125%, maximum mortality of 36% was achieved at the end of 24 h. The surface area of a 250 mL Erlenmeyer flask was calculated using the expression

$$S = \pi(r_1 + r_2)\sqrt{(r_1 - r_2)^2 + h^2}$$

where S = surface area, r_1 = radius at the base of the Erlenmeyer flask, r_2 = radius at the open end of the Erlenmeyer flask, and h = height of the Erlenmeyer flask.

Thus, the inner surface area of a 250 mL Erlenmeyer flask works out to be approximately 285 cm^2, and correspondingly, the adult females of *A. aegypti* were exposed to an amount of 3.5, 1.75, 0.88, and 0.44 μg/cm^2 of the compound corresponding to concentrations of 0.1, 0.05, 0.025, and 0.0125%, respectively.

Ever since the recognition of pyrethrins as repellents by WHO, they have been extensively used for vector control.[37] Other than this class of compounds, other molecules from the plant source have been explored as insect repellents,[38−42] although these are not essentially insecticidal but mimic the repellant action by masking the human skin to impede detection by insects and arthropods. As mentioned previously, most of the prevalent repellents suffer from serious limitations.

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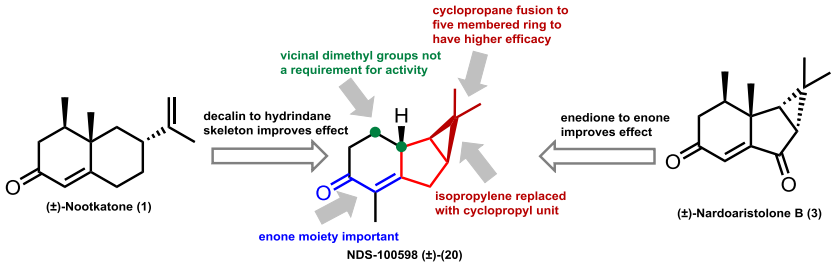

Figure 4. Summary of SARs of NDS-100598 and key changes from nootkatone (**1**) and nardoaristolone B (**3**).

To address this lacuna, we have explored novel class of compounds from the nootkatone family. In the present study, two of the compounds synthesized, **20** and **21**, offer a protection period of more than 360 min when applied at 0.5 mg/cm^2. It is interesting to note that, unlike the well-studied nootkatone, which possesses a decalin ring system, the present scaffolds based on noreremophilane and nardoaristolone belong to the hydrindane skeleton. To our knowledge, this is the first mosquito-repellent study on this scaffold.

Although it is difficult to compare results obtained in various studies because of variations in the protocols and/or the concentrations used, results from the present study clearly indicate that the new compounds reported here are promising insect repellents, in particular for *A. aegypti*. Initially, three natural products (noreremophilane, nootkatone, and nardoaristolone B), synthesized in the racemic form, were subjected to mosquito repellence activity and found to be active and amongst them, nardoaristolone B was observed to be having higher efficacy. Perhaps, the higher efficacy of nardoaristolone B could be due to its close structural similarity with nootkatone. Based on these initial results, a focused library of compounds was synthesized. SAR analysis of 25 hydrindane-based compounds suggests a few basic structural requirements for significant repellent activity. Repellent assays with them showed a clear trend in structure−activity relationships (SARs). Some of the findings are: (i) replacement of decalin scaffold with hydrindane improves the effect; (ii) enone moiety in the six-membered ring of hydrindane seems to improve the potency (Table 1, see compounds **3**, **18**−**25**); (iii) cyclopropane fusion to the five-membered ring is a requirement with an exception, compound **25**; (iv) vicinal dimethyl groups are not a requirement for activity; and (v) replacement of enedione to enone improves the protection time (Figure 4). Overall, compounds **18**, **19**, **20**, and **21** based on hydrindane scaffold are the best amongst the lot having equipotent or better activity, compared to that of *rac*-nootkatone. In addition, all three compounds showed good dose response. By considering activity and its synthetic feasibility to make them in sufficient quantities, compound **20** (NDS-100598) was selected for further studies on possible knock-down effects. There have been very few reports of repellents having quick knock-down effects. Licciardi et al.[43] compared the toxic effects of DEET, IR 3535, and KBR 3023 and observed DEET at 7% (i.e., 0.25 mg/cm^2) to cause a significant amount of knock-down. Comparing these results with the present study, we found that compound **20** is effective at very low concentrations of 0.035 and 0.017 mg/cm^2, resulting in 100% mortality in adult females of *A. aegypti*, within 5 and 10 min, respectively.

Despite DEET being a very efficacious molecule, there are concerns over health risks to infants.[44] Foul odor, oily greasy feel, and plasticizing nature of DEET are the other drawbacks, which limit its use. In addition, several species of insects have developed resistance to pyrethrins, which have been the mainstay for control of insect vectors. Current research and development in the area of vector control has focused on finding suitable alternatives that are botanical in origin and preferably have alternative mode(s) of action.

CONCLUSIONS

The present study identifies compounds based on noreremophilane and nardoaristolone, in particular compound **20**, with good repellent activity and also exceptional mosquitocidal effects at very low concentrations. These parameters are comparable to those for racemic nootkatone. Thus, the compounds reported here have the potential to be developed as an alternative product to control mosquitoes. Although the mode of action for nootkatone has not yet been well established, it was suggested to act on octopamine receptors[45] Thus, the present study paves ways for the development of novel candidate(s) for vector control against insects that have developed resistance toward currently used pesticides.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.8b03652.

Studies on contact toxicity and summary of two-way ANOVA and test for equality of variances (PDF)

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Notes

The authors declare no competing financial interest.

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