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Mosquito Repellents Based on a Natural Chromene Analogue with Longer Duration of Action than *N,N*-Diethyl-*meta*-toluamide (DEET)

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ABSTRACT: Mosquito repellents play a major role in reducing bites and therefore mitigating transmission of mosquito-borne diseases. There is concern by some about the reported neurotoxic effects of the popular repellent DEET. Also, a product with longer effective activity after application is needed. This paper describes the synthesis and repellent activity of (2,2 dimethyl-2*H*-chromen-5-yl)methanol, a derivative of chromene amide that is a compound from the plant *Amyris texana*. This compound is more potent and provides longer duration of protection than DEET against *Aedes aegypti* (L.), the primary vector that transmits pathogens causing yellow and dengue fevers in humans.

KEYWORDS: *Aedes aegypti, DEET, mosquitoe, Amyris texana, chromene, repellent*

INTRODUCTION

Mosquitoes are responsible for transmission of pathogens of diseases such as malaria, arboviral encephalitis, dengue fever, Rift Valley fever, and yellow fever that cause severe mortality and morbidity in humans and livestock througout the world.[1] Pathogens are transmitted by female mosquitoes during a blood meal by injection of saliva into hosts. Female mosquitoes need protein from a blood meal to develop eggs.

Application of insecticides is one of the major control methods for these medically important insect pests. However, few new insecticides have been developed for mosquito control, and mosquitoes are evolving resistance to currently used products.[2] Recently, the most popular repellent, *N,N*-diethyl-*meta*-toluamide (DEET), was reported as a neurotoxin through inhibition of cholinesterase,[3] although it is an extremely weak inhibitor of this enzyme from humans.[4] Nevertheless, new mosquito repellents without this potential side effect are desired. Addtionally, more potent mosquito repellents that will not have to be applied as often as DEET would be a major advance in the use of repellents to avoid mosquito-bourne diseases.

The U.S. military is very interested in preventing disease transmission of mosquito-borne diseases as these diseases impact deployed military personnel in some parts of the world.[5] As part of an effort in search for mosquito repellents under the DWFP (Deployed War Fighter Protection) program of the U.S. Armed Forces Pest Management Board, we have synthesized some natural-product-derived chromene analogues that have higher duration of action and potency against *Aedes aegypti* female mosquitotes than does DEET in laboratory bioassays.

MATERIALS AND METHODS

General Experimental Procedures. Extracts were analyzed on silica gel TLC plates GF with a fluorescent indicator (250 μm, Analtech, Newark, DE, USA). Iodine vapor, UV light (at 254 and 365 nm), and Dragendorff and anisaldehyde spray reagents were used for the detection of compounds. Column chromatography was carried out with kieselgel 60 (particle size 0.063−0.2 mm, Merck) with mixtures of hexane and ethy acetate in varying amounts. Flash column chromatography was performed on Biotage Isolera Four (Biotage, Charlotte, NC, USA) using FLASH + silica gel cartridges with ultraviolet detection at 254 nm. All solvents were reagent grade and used without further purification. ^{1}H and ^{13}C NMR spectra were recorded on a Varian Mercury AS400 spectrometer operating at 400 MHz for ^{1}H NMR and at 100 MHz for ^{13}C NMR. The HR-ESIMS was measured using a Jeol ACCU TOF JMS-T1000 mass spectrometer. GC-MS analysis was carried out on an HP5790 MSD spectrometer (Hewlett-Packard, USA) equipped with GC 5890 using a DB-1 column (20m × 0.2 mm, 0.18 μm film thickness). The oven was temperature programmed from 60 °C (5 min) to 280 °C (20 min) at 5 °C/min with helium as the carrier gas.

Plant Material. Leaves of *Amyris texana* were collected in Cameron County in South Texas, in June, 2002. A voucher specimen (BUR 190204 a) is deposited at the University of Mississippi herbarium. The leaves were air-dried, ground, and stored at room temperature until they were extracted.

Extraction and Fractionation. Extraction and isolation of *N*-[2-(2, 2-dimethyl-2*H*-chromen-6-yl)-ethyl]-3,*N*-dimethyl-butyramide, chromene amide (**1**) was done according to previously published methods.[6]

Syntheses of Analogues. The general synthetic procedure involved reaction of the appropriate phenol with 3-chloro-3-methyl-1-butyne followed by pyran ring formation by heating with *N,N* diethylaniline (Figure 1). Compounds **2**−**19** (Figure 2)

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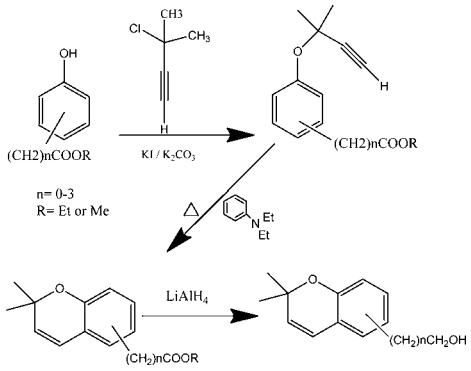
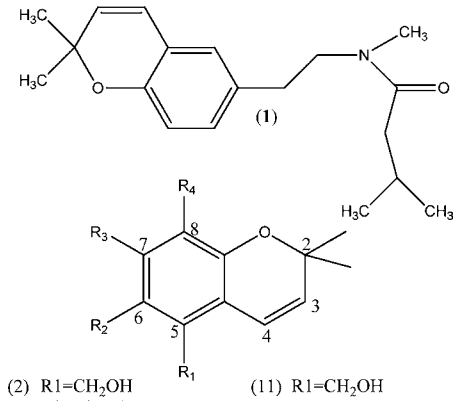


Figure 1. General synthetic procedure of chromene analogues.



(2) R1=CH_2OH
R2, R3, R4= H

(3) R_1=OH
R_2= $COCH_3$

(4) R1=OMe
R2, R3= H
R4=CH_2OH

(5) R1,R3, R4= H
R2=$C(O)OCH_3$

(6) R1=$C(O)OCH_3$
R2, R3, R4= H

(7) R2= CH_2OH
R1, R3, R4=H

(8) R3= $C(O)CH_3$
R1,R2, R4=H

(9) R2=CH_2CH_2OH
R1, R3, R4= H

(10) R2= $CH_2C(O)OCH_3$
R1, R3, R4=H

(11) R1=CH_2OH
R2, R3= H
R4= Cl

(12) R3=Cl
R1, R2, R4= H

(13) R1=COOH
R2, R3= H
R4=Cl

(14) R1=$COOCH_3$
R2, R3= H
R4=Cl

(15) R4=Cl
R1,R2, R3= H

(16) R1=$COOCH_3$
R3, R4= H
R2=Cl

(17) R2=Cl
R1, R3, R4= H

(18) R1=CH_2OCH_3
R2, R3, R4= H

(19) R2=$CH_2CH_2OCH_3$
R1,R3,R4= H

Figure 2. Chromene analogues synthesized and tested.

were prepared and identified using spectroscopic data according to published methods.[6−10]

*5-(Methoxymethyl)-2,2-dimethyl-2H-chromene (**18**).* To **2** (1.9 g, 0.01 mols) under N_2 in dry THF (50 mL) was added methyl iodide (5.6 g, 0.04 mols), followed by NaH (60 g, 1.5 mols, 60% dispersion in mineral oil). The reaction mixture was stirred for 12 h at 80 °C. The solvent was evaporated, the residue was partioned between water (100 mL), and diethyl ether (100 mL) and the ether layer were dried over anhydrous Na_2SO_4. The solvent was evaporated to afford a pale yellow oil, which was purified by silica gel flash column chromatography using 5% EtOAc in hexane to afford (**18**) (yield, 1.7 g, 0.0083 mol, 83%). ^{1}H NMR ($CDCl_3$) δ: 1.41 (6H, s), 3.34 (3H, s), 4.44 (2H, s), 5.64 (^{1}H, d, J = 12 Hz), 6.58 (^{1}H, d, J = 8 Hz), 6.75 (^{1}H, d, J = 8 Hz), 6.81 (^{1}H, d, J = 8 Hz), 7.05 (^{1}H, t, J = 8 Hz). ^{13}C NMR ($CDCl_3$) δ: 27.82, 57.87, 72.25, 116.61, 118.95, 119.90, 127.71, 128.41, 131.03, 133.37, 153.15. HRMS (ESI-TOF) m/z: $[M - H]^-$ calcd for $C_{13}H_{15}O_2$, 203.10720; found 203.09436.

*6-(2-Methoxyethyl)-2,2-dimethyl-2H-chromene (**19**).* To (**9**) (2.04 g, 0.01 mols) under N_2 in dry THF (50 mL) was added methyl iodide (5.6 g, 0.04 mols) followed by NaH (60 g, 1.5 mols, 60% dispersion in mineral oil). The reaction mixture was stirred for 12 h at 80 °C. The solvent was evaporated to afford a pale yellow oil which was purified by silica gel flash column chromatography using 5% EtOAc in hexane to afford (**19**) (yield, 1.9 g, 0.0088 mol, 87%). ^{1}H NMR ($CDCl_3$) δ: 1.41 (6H, s), 2.77 (2H, t, J = 8 Hz), 3.34 (3H, s), 3.55 (2H, t, J = 8 Hz), 5.28 (^{1}H, s), 5.59 (^{1}H, d, J = 8 Hz), 6.29 (^{1}H, d, J = 8 Hz), 6.69 (^{1}H, d, J = 8 Hz), 6.82 (^{1}H, d, J = 2 Hz), 6.94 (^{1}H, dd, J = 8 Hz, 2 Hz). ^{13}C NMR ($CDCl_3$) δ: 27.96, 35.35, 53.43,58.63, 73.85, 76.01, 116.12, 121.08, 122.32, 129.28, 130.77, 130.98, 151.30. HRMS (ESI-TOF) m/z: $[M - H]^-$ calcd for $C_{13}H_{15}O_2$, 203.10720; found, 203.09732.

Bioassay for Mosquito Repellent Activity. The repellent efficacy was compared to that of the standard repellent, DEET. Experimental compounds were assessed in one of two ways: (1) a range of concentrations was evaluated to determine the minimum effective dosage (MED), which was the concentration threshold at which the repellent began to fail and allowed bites, or (2) a predetermined concentration of each repellent was applied to cloth, and the activity was evaluated over several days (or in some cases months).[11,12] When conducting assays for repellency, a failure point was also predetermined for these measurements. Since experiments described herein used about 500 female *Ae. aegypti* or *Anopheles albimanus* mosquitoes per test, the failure point was set at 1% (equal to five bites received) during a test interval. From three to five human volunteers participated in the screening of each set of chemicals.

Minimum Effective Dosage (MED) Test. Experimental compounds were prepared in solution by combining 0.15 g of experimental chemical with 2 mL of acetone in a 7.1 mL screwtop glass vial. This solution was then serially diluted to obtain nine concentrations: 1.5, 0.75, 0.375, 0.187, 0.094, 0.047, 0.023, 0.011, and 0.006 mg/cm^2. The standard *N,N*-diethyl-*meta*-toluamide (DEET) was also included at the same concentration levels to serve as a positive control and a comparison for relative repellency. A 50 cm^2 (5 cm × 10 cm) patch of muslin cloth was rolled lengthwise and placed into each of the glass vials and sealed with a screwtop so it could soak up the solution. Just prior to the experiment, the pieces of treated cloth were removed from the vials, affixed to card stock tabs (5 cm × 3 cm) with staples, and hung with masking tape on a rack to dry and allow acetone to evaporate. Volunteers wore a latex glove over their hands, a nylon stocking over their arm and then wrapped a plastic Velcro-sealed sleeve over their entire forearm with a 32 cm^2 (4 cm × 8 cm) window cut-out to allow mosquitoes to bite through. The nylon stocking acted as a barrier between the dried cloth and the skin, an additional precaution with experimental compounds. Dried cloth patches were stretched across the window in the plastic sleeve and held in place with masking tape. Volunteers placed their sleeve-covered arms into a screened cage for a 1 min period with approximately 500 female *Ae. aegypti* mosquitoes that have been preselected for host-seeking behavior using a draw box.[13] A cloth patch receiving a 1% bite through level, or five mosquito bites within 1 min, resulted in a failure of the compound to repel, whereas a cloth patch receiving 0−4 bites

Table 1. *Ae. aegypti* Minimum Effective Dosage (mg/cm^2)[a]

	M4	M5	M8	M9	M10	av	SD
3	>1.500	0.187	>1.500	>1.500	>1.500	N/A	
4	>1.500	0.375	0.375	0.750	>1.500	N/A	
5	>1.500	0.375	0.187	0.375	>1.500	N/A	
2	0.011	0.011	0.011	0.094	0.047	0.035	0.037
DEET	0.011	0.023	0.023	0.023	0.023	0.021	0.005
acetone control	NR	NR	NR	NR	NR	N/A	

[a]Human subject raw data by coded identifier (M4, M5, M8, M9, M10). NR = Not repellent, N/A = not applicable due to no repellency at highest concentration for some or all volunteers.

within a minute was a passing result. The MED recorded was the lowest passing concentration level tested for the experimental compound. These results from all the volunteers were averaged for each experimental compound and reported as the mean MED. All human volunteers in the study provided informed consent to participate of a protocol approved by the University of Florida Human Use Institutional Review Board (IRB # 636-2005).

Complete Protection Time Test. Complete protection time (CPT) on treated cloth is a method of screening that is used to determine the repellent duration for experimental chemicals that have not been examined for safe use on humans. The standard repellent DEET was used as a positive control as a benchmark by which to compare the repellents. Experimental compounds were tested as in MED tests.

Table 2. *Ae. aegypti* Minimum Effective Dosage (mg/cm^2)[a]

	M1	F1	M2	av	SD
10	0.750	0.375	0.375	0.500	0.217
9	0.375	0.375	0.375	0.375	0.000
16	>1.500	>1.500	>1.500	N/A	N/A
14	>1.500	>1.500	>1.500	N/A	N/A
11	0.011	0.011	0.011	0.011	0.000
13	>1.500	>1.500	>1.500	N/A	N/A
12	0.750	0.750	0.750	0.750	0.000
15	0.094	0.094	0.187	0.125	0.054
2	0.023	0.023	0.023	0.023	0.000

[a]Human subjects raw data by coded identifier (M1, F1, M2). N/A = not applicable due to no repellency at highest concentration for some or all volunteers.

Table 3. Duration of Protecton Against *Ae. aegypti* (in Days)

	dosage (mg/cm^2)	M-3 days protection	proportion to DEET
9	0.75	1	0.50
11	0.75	2	1.00
13	0.75	1	0.50
17	0.75	1	0.50
2	0.75	6	3.00
DEET	0.75	2	1.00

RESULTS AND DISCUSSION

Chromene amide (**1**) (Figure 2) was isolated from the ethyl acetate extract of the leaves of *A. texana*. A series of chromene derivatives (Figure 2) were synthesized as shown in Figure 1 and tested for mosquito repellent activity (Tables 1−5). In the preliminary assay for the MED (Table 1), the chromene analogue **2** (MED 0.035 mg/cm^2) showed a comparable level of activity to that of DEET (MED 0.021 mg/cm^2). The control acetone was not repellent. Compound **3** with an OH group at C-5 and a $COCH_3$ group at C-6 was the least repellent because four of five volunteers found it not repellent at the highest concentration tested (1.500 mg/cm^2). Compound **4** with an OMe group at C-5 and a CH_2OH group at C-8 and **5** with an acetate group at C-6 had mixed results, where the highest concentration was not repellent for some of the volunteers. Based on these initial data, more analogues were synthesized and tested (Table 2). Analogue **6**, the acetate analogue of **2**, was ineffective at the highest dosage tested. Similarly, **7** and **8**, the regioisomers of **2** and **5**, respectively, were ineffective, suggesting that the position of CH_2OH in the chromene molecule is critical for the activity. Compounds **9** and **10**, analogues of **7** and **8** with an additional CH_2 group, were less active but still exhibited some repellency (MED 0.375 mg/cm^2 and 0.500 mg/cm^2, respectively. Compound **11**, an analogue of **2** with a Cl attached to C-8, was highly active with the average MED value of 0.011 mg/cm^2). The other chlorinated analogues (**12**−**17**) were less active. When the active compounds were tested for duration of protection, **2** performed best with 6 days of protection, whereas **11** and DEET had only 2 days of protection against *Ae. egypti* mosquitoes (Table 3). These data suggest that **2** has the best activity and the longest duration of protection. When these compounds were tested against the mosquito species *Anopheles albimanus*, they were less active than DEET as repellents (Table 4). Analogue **18**, the methylated analogue of **2**, showed diminished repellent activity, further suggesting the importance of the CH_2OH group of **2** for the activity. Similarly, **19**, the methylated analogue of **9**, did not show any improvement of activity (Table 5).

We hypothesized that if **2** has a different molecular target than DEET, it might be synergistic with DEET. Compound **2**

Table 4. *An. albimanus* Minimum Effective Dosage (mg/cm^2)[a]

	M4	M5	M8	M9	M10	av	SD
3	0.094	>1.500	>1.500	0.375	>1.500	N/A	
4	>1.500	>1.500	>1.500	0.750	>1.500	N/A	
5	0.187	0.375	>1.500	0.187	0.750	N/A	
2	0.047	0.375	0.187	0.375	0.187	0.234	0.141
DEET	0.011	0.047	0.023	0.023	0.023	0.025	0.013
acetone control	NR	NR	NR	NR	NR	N/A	

[a]NR=not repellent, N/A = Not applicable.

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Table 5. *Ae. aegypti* Minimum Effective Dosage (mg/cm²)[a]

	M4	M5	M8	av	SD	highest dose (mg/cm²)
10	>0.375	>0.375	>0.375	N/A		0.375
7	>0.187	>0.187	>0.187	N/A		0.187
6	>0.375	>0.375	>0.375	N/A		0.375
8	>0.375	>0.375	>0.375	N/A		0.375
2	0.011	0.011	0.006	0.009	0.003	0.375
18	0.047	0.047	0.187	0.094	0.081	0.375
19	>0.375	>0.375	>0.375	N/A		0.375
DEET	0.011	0.011	0.023	0.015	0.007	0.375

[a]N/A = compound ineffective at highest available dose.

Table 6. MED Values of Varying Molar Ratios of 2 and DEET Against *Ae. Aegypti*

molar ratio of **2**/ DEET	M4	M5	M8	av	SD	highest dose (mg/cm²)
1:0	0.0156	0.0313	0.0313	0.0260	0.009	4.0
1:1	0.0156	0.0156	0.0313	0.0208	0.009	4.0
1:2	0.0313	0.0313	0.0313	0.0313	0.000	4.0
1:3	0.0156	0.0313	0.0156	0.0208	0.009	4.0
1:4	0.0313	0.0313	0.0156	0.0260	0.009	4.0
1:5	0.0313	0.0313	0.0156	0.0260	0.009	4.0
1:6	0.0156	0.0156	0.0156	0.0156	0.000	4.0
1:7	0.0313	0.0156	0.0156	0.0208	0.009	4.0
1:8	0.0156	0.0156	0.0156	0.0156	0.000	4.0
2:1	0.0313	0.0313	0.0156	0.0260	0.009	4.0
3:1	0.0156	0.0313	0.0156	0.0208	0.009	4.0
4:1	0.0078	0.0313	0.0156	0.0182	0.012	4.0
5:1	0.0156	0.0313	0.0156	0.0208	0.009	4.0
6:1	0.0313	0.0313	0.0313	0.0313	0.000	4.0
7:1	0.0156	0.0313	0.0313	0.0260	0.009	4.0
8:1	0.0156	0.0313	0.0313	0.0260	0.009	4.0
0:1	0.0313	0.0313	0.0313	0.0313	0.000	4.0

was evaluated with DEET for synergistic activity. Test samples were prepared at varying molar ratios of **2**:DEET and the MED values were evaluated (Table 6, Figure 3). MED data of the mixtures of **2** and DEET showed at molar ratios of **2**/DEET of 1:8, 1:6, and 4:1 that there is some enhanced effect. It is not clear if this effect is due to synergism. These results indicate that the compound mixture could be a useful repellent preparation against mosquitoes with significantly reduced amounts of DEET.

We have previously shown that chromene analogues possess termiticide activity and algecide activity.[8,10] There is no correlation of the structural requirements of the chromene analogues that showed termiticide activity with those that showed mosquito repellent activity, even though **2** showed termiticide activity.[10] There are various reports in the literature of chromene analogues as insecticides.[14−16] This is the first report of chromene analogues as mosquito repellents.

Some chromene derivatives possess juvenile hormone antagonistic activities.[17,18] We have found that that the mosquito repellent chromenes are not insecticidal on topical application to mosquitoes and termites (data not shown). However, **2** has moderate but slow insecticide activity on termites when ingested.[10]

In summary, **2** is highly effective as a repellent against mosquitoes, lasting 3 times (6 days) longer than DEET (2 days) in our bioassay. Similar formulations of **2** or **11** to those of DEET can be achieved, as **2** and **11** are also colorless, viscous oily compounds like DEET. Furthermore, **2** formulated with DEET has the potential to reduce the overall molar concentration of repellents needed for effective repellency. Thus, chromene analogues, based on the natural chromene amide, particularly **2** and **11**, have potential for further development as mosquito repellents.

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Notes

The authors declare no competing financial interest.

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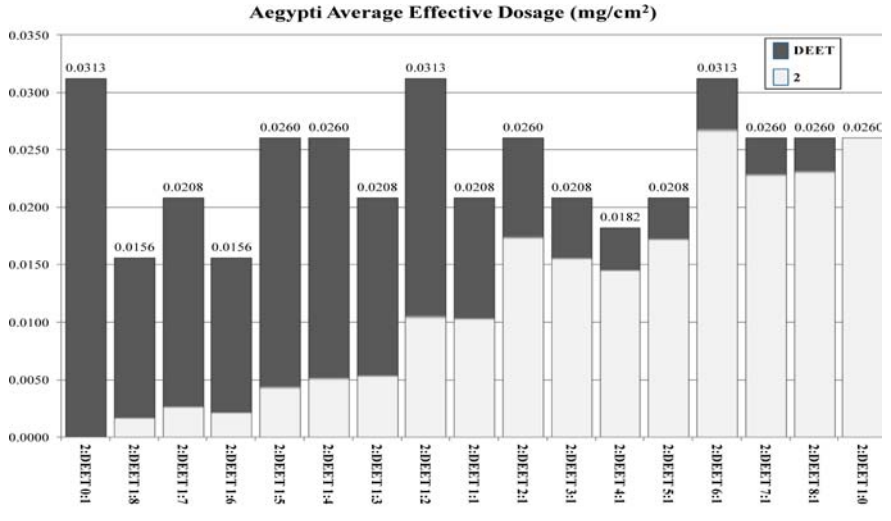

Figure 3. Graphical representation of MED values of varying molar ratios of 2 and DEET with *Ae. aegypti*.

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