Mosquitocidal Compounds and a Triglyceride, 1,3-Dilinoleneoyl-2-palmitin, from *Ocimum sanctum*

Mark A. Kelm and Muraleedharan G. Nair*

Bioactive Natural Products Laboratory, Department of Horticulture and National Food Safety and Toxicology Center, Michigan State University, East Lansing, Michigan 48824-1325

The hexane extract of *Ocimum sanctum* was investigated using mosquito bioassay guided fractionation and yielded compounds **1** and **2**. The isolation of the triglyceride, 1, 3-dilinoleneoyl-2-palmitin (**3**) from *O. sanctum* leaves and stems is novel. The structures of these compounds were established using ^{1}H and ^{13}C NMR spectral data. Compounds eugenol (**1**) and (*E*)-6-hydroxy-4,6-dimethyl-3-heptene-2-one (**2**) demonstrated mosquitocidal activity at 200 and 6.25 μg mL^{-1} in 24 h, respectively, on fourth-instar *Aedes aegyptii* larvae.

Keywords: *Ocimum sanctum; Lamiaceae; sacred basil; 1,3-dilinoleneoyl-2-palmitin; eugenol; (E)-6-hydroxy-4,6-dimethyl-3-heptene-2-one; mosquitocidal*

INTRODUCTION

Ocimum sanctum L. (Lamiaceae) has been used for generations in Southeast Asian medicine and cuisine. *O. sanctum* or "sacred basil", the Ayurvedic herbal drug, has been used to treat a variety of human ailments (Thakur et al., 1989; Butani et al., 1982). Malarial fevers, ringworms, and other cutaneous afflictions have also been treated with this plant (Butani et al., 1982). According to African folk medicine, *O. sanctum* was reported to repel mosquito, and have subterfuge and poultice effects (Batta and Santhakumari, 1970). Moreover, the crude extracts from *O. sanctum* have also demonstrated biological activities against certain insects (Risvi, 1981). For example, crude alcoholic extracts had shown aphidcidal properties (Stein et al., 1988), antifeedant activities on Jute semilooper, *Anomis sabulifera* (Malik and Rafique, 1989), and mosquito repellent and toxic properties (Batta and Santhakumari, 1970; Deshmukh et al., 1982).

Previous phytochemical studies of *O. sanctum* have led to the isolation of flavones and flavone glycosides, which include the following: apigenin (Nair et al., 1982), apigenin 7-*O*-glucoside (Nair et al., 1982), cirsilineol (Nörr and Wagner, 1992), galuteolin (Nörr and Wagner, 1992), luteolin (Nair et al., 1982), luteolin 7-*O*-glucoside (Nair et al., 1982), molludistin (Nair et al., 1982), orientin (Nair et al., 1982), and vicenin 2 (Nörr and Wagner, 1992). Other compounds identified in *O. sanctum* were eugenol (Laakso et al., 1990), rosmarinic acid (Nörr and Wagner, 1992), β-sitosterol (Sukari et al., 1995), stigmasterol (Sukari et al., 1995), triacontanol ferulate (Sukari et al., 1995), and ursolic acid (Nair et al., 1982). More recently, the phenyl propane glycosides 4-allyl-1-*O*-β-D-glucopyranosyl-2-hydroxybenzene and 4-allyl-1-*O*-β-D-glucopyranosyl-2-methoxybenzene were isolated (Nörr and Wagner, 1992). GC/MS and GC/FTIR analyses of the essential oil of *O. sanctum* revealed the presence of 20 compounds (Laakso et al., 1990). From these studies eugenol, methylchavicol, and β-bisbolenes were found to be the major components in the essential oil.

Past research on *O. sanctum* has focused on the biological activity of crude extract. Typically, phytochemical investigations carried out on *O. sanctum* extracts have not led to the isolation and structural identification of biologically active compounds. The work in our laboratory, in part, involves the preliminary screening of many plant and microbial extracts to determine the presence of any biologically active compound. In this paper, we report, for the first time, the isolation and structure determination of a novel triglyceride (**3**) and two mosquitocidal compounds (**1** and **2**) from the leaf and stem hexane extract of *O. sanctum*.

MATERIALS AND METHODS

General Experimental Procedures. ^{1}H NMR, DQF-COSY, NOESY, and HMQC spectra were recorded at 300 and 500 MHz. ^{13}C NMR and DEPT spectra were recorded at 126 MHz. Chemical shifts were recorded in $CDCl_3$, and the values are in δ (ppm) based on residual of $CHCl_3$ at 7.24 and $CDCl_3$ at 77.0. Coupling constants, *J*, are in hertz. EIMS were recorded at 70 eV. Particle size of silica gel used in VLC and MPLC was 35−70 μm. All PLTC purifications were carried out on 250 and or 500 μm silica gel plates. Spots and bands were visualized under UV light (366 and 254 nm).

Gas chromatographic analyses were performed utilizing a flame ionization detector, which was set at 260 °C. The capillary column used for the analysis was a DB-5 (30 m × 0.25 mm i.d.). The temperature for the analysis was programmed from 150 (4 min) to 250 °C (5 min) at 4 °C min^{-1} with a helium carrier gas at a linear velocity of 34 cm s^{-1} and with split injections.

Plant Material. A voucher plant specimen (MSC 360851) was filed with the Beal−Darlington herbarium, Department of Botany and Plant Pathology, Michigan State University. Leaves and stems of *O. sanctum* were harvested from plants maintained in the Pesticide Research Center greenhouses at Michigan State University. Plant materials were then freeze-dried, milled, and stored at −20 °C until extraction.

Mosquitocidal Bioassay. Fourth-instar mosquito larvae, *Aedes aegyptii* L., were reared in our laboratory from eggs (courtesy of Drs. Alexander Raikal and Alan Hays, Department of Entomology, Michigan State University). Eggs were hatched

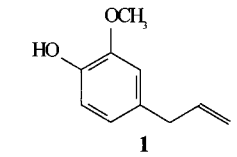
* Author to whom correspondence should be addressed.

S0021-8561(98)00304-5 CCC: $15.00
Published on Web 07/15/1998

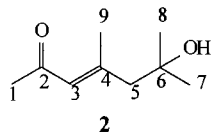
in 500 mL of distilled degassed water prepared by sonication (30 min). Approximately 5 mg of bovine liver powder was added to the water to provide a food source. After 4 days, the fourth-instar mosquito larvae were ready for bioassay. At least 10 larvae were placed in 980 μL of degassed distilled H_2O, and to this was added 20 μL of DMSO containing the appropriate concentration of test extract or purified compounds and left at room temperature. Extracts were tested at 250 ppm. Pure compounds were tested initially at 100−250 ppm and then serially diluted and subsequently bioassayed to determine LC_{100}. Four milliliter test tubes were used for the bioassay. There were three replicates per treatment. The numbers of dead larvae were recorded at 2, 4, and 24 h intervals. The control tube containing at least 10 larvae received 20 μL of DMSO alone, and mortality was recorded as in the case of test compounds. These bioassays were conducted according to previously published works (Roth et al., 1998; Nitao et al., 1991; Nair et al., 1989).

Saponification and Methylation of Compound 3. With stirring, 6.6 mg of **3** was treated with 5% NaOH in MeOH (1 mL) for 5 min. Methanolic 6 N HCl was then added to acidify this solution. This material was then dried under a stream of nitrogen. Diazomethane was prepared by reacting *N*-nitotroso-*N*-methylurea with concentrated KOH solution under ether. As the diazomethane product formed, it dissolved into the organic ether phase. This yellow ether solution containing the diazomethane product was then collected and used to methylate the free fatty acids obtained in the previous step. The methylated product was filtered to remove any solids prior to GC analyses.

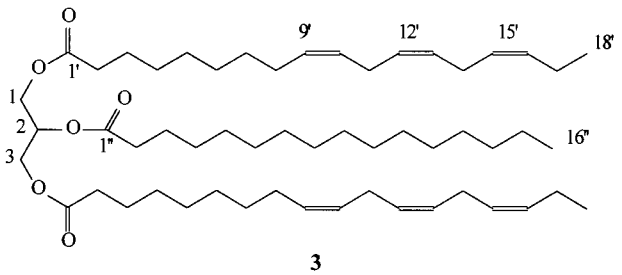
Extraction and Isolation of Compounds 1−3. The freeze-dried *O. sanctum* leaves and stems (440 g) were extracted sequentially with hexane, EtOAc, and MeOH; 750 mL of each solvent was used for the first 12 h. Thereafter, a



1



2



3

second 750 mL of fresh solvent was used to extract the plant material for an additional 12 h. The hexane extraction afforded 13.1 g of residue upon removal of solvent. A portion of this residue (10.4 g) was fractionated on a silica gel VLC (200 g) using: 4:1 pentane/Et_2O as the mobile phase. A 600 mL sintered funnel was used for the VLC. Dimensions of the MP column were 250 mm × 25 mm. Fraction collecting was based on the color of bands observed through the VLC sintered funnel as well as the MP column. Flow rates for MPLC experiments were approximately 1−2 mL min^{-1}. Fractions collected in the VLC experiment were 1 (375 mL), 2 (200 mL), and 3 (300 mL). Next, elution with 2:1 pentane/Et_2O yielded fractions 4 (650 mL) and 5 (250 mL). This was followed by elution with 1:1 pentane/Et_2O, which gave fractions 6 (250 mL), 7 (500 mL), and 8 (300 mL), and then elution with Et_2O gave fractions 9 (325 mL) and 10 (200 mL). Finally, elution with $CHCl_3$ and MeOH afforded fractions 11 (500 mL) and 12 (875 mL), respectively. All fractions were bioassayed, and only fraction 2 was found to be mosquitocidal. Fraction 2 was further purified by silica gel MPLC using hexane/Me_2CO solvent systems to yield seven fractions. The seven solvent systems used and the volume of each fraction collected were as follows: 1 (hexane, 110 mL); 2 (40:1 hexane/acetone, 90 mL); 3 (25:1 hexane/acetonee, 90 mL); 4 (10:1 hexane/acetone, 90 mL); 5 (4:1 hexane/acetone, 190 mL); 6 (acetone, 95 mL); and 7 (acetone, 250 mL). The seventh fraction was found to be mosquitocidal. The MeOH soluble portion of this fraction was then finally purified by repeated preparative TLC to give compounds **1** (31.9 mg), **2** (10.1 mg), and **3** (8.8 mg). Initially, compound **1** was purified using 100% EtOAc as the mobile phase. Hexanes/EtOAc (8:1) followed by hexane/acetone (10:1) mobile phase ultimately led to the isolation of compound **1**. Like compound **1**, compounds **2** and **3** were intially purified with 100% EtOAc as the mobile phase. Isolation of compounds **2** and **3** was accomplished using 2:1 and 1:1 EtOAc/hexane mobile phases, respectively.

Compound **1**, a pale brown oil: ^{1}H NMR ($CDCl_3$) δ 3.30 (d, 2H, J = 6.6 Hz, H-7), 3.86 (s, 3H, $-OCH_3$), 5.04 (dd, 1H, J = 9.0 Hz, J = 1.5 Hz, H-9 cis), 5.05 (dd, 1H, J = 18.0 Hz, J = 2.4 Hz, H-9 trans), 5.47 (s, 1H, −OH), 5.93 (m, 1H, H-8), 6.67 (d, 1H, J = 7.8 Hz, H-6), 6.67 (s, 1H, H-3), 6.83 (dd, 1H, J = 8.7 Hz, J = 1.5 Hz, H-5); ^{13}C NMR ($CDCl_3$) δ 39.9 (C-7), 55.8 ($-OCH_3$), 111.0 (C-9), 114.1 (C-8), 115.5 (C-6), 121.1 (C-5), 131.9 (C-3), 137.8 (C-4), 143.7 (C-2), 146.3 (C-1). The spectral data of this compound were identical to those of an authentic sample purchased from Aldrich.

Compound **2**, a colorless oil: ^{1}H NMR ($CDCl_3$) δ 1.23 (s, 6H, H-7, 9), 1.88 (s, 3H, H-8), 2.14 (s, 3H, H-1), 2.56 (s, 2H, H-5), 4.25 (s, 1H, −OH), 6.01 (s, 1H, H-3); ^{13}C NMR ($CDCl_3$) δ 21.0 (C-8), 27.8 (C-1), 29.3 (C-7, 9), 53.8 (C-5), 69.8 (C-6), 124.5 (C-3), 157.4 (C-4), 202.3 (C-2). Therefore, it is identified as (*E*)-6-hydroxy-4,6-dimethyl-3-heptene-2-one. ^{1}H NMR data were found to be in agreement with previously published data (Kimura et al., 1982).

Compound **3**, a pale yellow oil: EI-MS, *m*/*z* (rel intensity) 611 ($M^+ - C_{16}H_{31}O]^+$ (9), 262 [$C_{18}H_{29}O + H$, 85]$^+$ (85), 261 [$C_{18}H_{29}O]^+$ (42), 239 (15), 230 (15), 108 (69), 95 (100), 81 (96), 53 (93); ^{1}H NMR ($CDCl_3$) δ 0.86 (bt, 3H, H-16″), 0.95 (t, 6H, J = 7.5 Hz, H-18′), 1.25 [m, 40H, H-(4′-7′) × 2 and H-(4″-15″)], 1.58 [m, 6H, H-(3′ × 2), 3″], 2.01 [m, 8H, H-(8′, 17′) × 2], 2.29 (t, 4H, J = 7.5 Hz, H-2′ × 2), 2.28 (t, 2H, J = 7.5 Hz, H-2″), 2.7 (m, 8H, H-11′,14′ × 2), 4.12 (dd, 2H, J = 18.0, 6.0 Hz, H-1a,3a), 4.36 (dd, 2H, J = 16.2, 4.2 Hz, H-1b,3b), 5.24 (m, 1H, H-2), 5.34 (m, 12H, H-9′,10′,12′,13′,15′,16′ × 2); ^{13}C NMR ($CDCl_3$) δ 14.1 (C-16″), 14.3 (C-18′ × 2), 27.2 (C-8′,17′ × 2), 22.6, 22.7, 24.8, 24.9, 29.0−29.7 (C-4′-7′ × 2,4″-15″), 25.5, 25.6 (C-11′,14′ × 2), 31.5 (C-3′ × 2), 31.9 (C-3″), 34.0 (C-2′ × 2), 34.2 (C-2″), 62.1 (C-1,3), 68.9 (C-2), 127.1−131.9 (C-9′,10′,12′,13′,15′,16′ × 2), 172.3 (C-1′ × 2), 173.3 (C-1″). The spectral data confirmed that this compound is 1,3-dilinoleneoyl-2-palmitin.

RESULTS AND DISCUSSION

Three compounds, **1**−**3**, were isolated from the leaf and stem hexane extract of *O. sanctum* by successive silica gel VLC, MPLC, and preparative TLC. The ^{1}H NMR spectrum of compound **2** contained only five singlets. The peak furthest upfield at δ 1.23, integrated for six protons, indicated the presence of two deshielded magnetically equivalent methyl groups, C-7 and C-8. Deshielding of these methyl groups occurred as a result of a hydroxy group at C-6. Methyl protons at C-1 and C-9 at δ 2.14 and 1.88, respectively, were indicative of methyls attached to a carbonyl carbon and an olefinic carbon. From the ^{13}C NMR spectrum, it was concluded that compound **2** contained an α,β-unsaturated ketone moiety (C-3, C-4, and C-2, respectively) with an additional oxygenated carbon (C-6). This oxygenated carbon was determined to be a tertiary alcohol as indicated by the disappearance of a singlet at δ 2.56 following a D_2O shake in the ^{1}H NMR spectrum. The DEPT spectrum supported the ^{1}H NMR and ^{13}C NMR

data as well as indicating the presence of three nonprotonated carbons at 202.3, 157.4, and 69.8 ppm for one carbonyl, one olefinic, and one oxygenated carbon, respectively. Finally, the stereochemistry of **2** was determined to be *E* by NOESY because no correlations were observed for the olefinic proton on C-3 and the methyl protons on C-4. Therefore, compound **2** was confirmed to be (*E*)-6-hydroxy-4,6-dimethyl-3-heptene-2-one. The ^{1}H NMR spectrum of **2** was identical to previously published findings (Kimura et al., 1982). The structure of compound **3** was determined using ^{1}H and ^{13}C NMR, DEPT, and DQFCOSY spectral data. Also, two-dimensional HMQC proton−carbon correlations facilitated the assigning of saturated carbons in the fatty acid side chains and provided further evidence to 1D NMR experiments. The ^{1}H NMR signals at δ 4.12, 4.36, and 5.24 and the ^{13}C NMR signals at δ 62.09 and 68.87 along with MS fragments at *m*/*z* 611 $[M^+ - C_{16}H_{31}O]^+$, 262 $[C_{18}H_{29}O + H]^+$, and 261 $[C_{18}H_{29}O]^+$ confirmed that compound **3** is a triglyceride with C_{16} and C_{18} fatty acid esters. The overlapping multiplets at δ 5.34 for 12 protons correlated with unsaturated carbons at δ 127.09−131.94 in the HMQC spectrum indicated the presence of 6 double bonds in this molecule. Support for the chemical nature of the side chains came from the GC analyses of the methyl esters of fatty acids obtained from the hydrolyzed compound **3**. The GC profile confirmed the presence of the methyl esters of linolenic and palmitic acids with a ratio of 2:1, respectively. Also, retention times for both methyl esters were identical to those of authentic samples of linolenic and palmitic acid methyl esters analyzed under the same conditions.

The novel triglyceride, compound **3**, was not mosquitocidal. LD_{100} values were 200 and 6.25 μg mL^{-1} in 24 h for compounds **1** and **2**, respectively, when tested against fourth-instar *A. aegyptii* larvae. There was no mortality for control larvae. In a previous paper the phenylpropanoid eugenol was found to act as an attractant to the beetle *Maladera matrida* (Ben-Yakir et al., 1995). Eugenol, in previous studies, was found to comprise 30−70% of the essential oil in *O. sanctum* (Asthana et al., 1984). Compound **2** was originally isolated from green and red bell peppers, *Capsicum annum* L. (Kimura et al., 1982), and later identified in the culture broth $CHCl_3$ extract of *Streptomyces olivaceus* (Grote et al., 1990). The synthesis of **2** has been reported by Duperrier et al. (1975). To the best of our knowledge, compound **3** has not been reported as a natural product before.

The isolation and identification of mosquitocidal compounds in *O. sanctum* support earlier findings that had shown insecticidal activity in crude extracts (Batta and Santhakumari, 1970; Risvi, 1981; Stein et al., 1988; Malik and Rafique, 1989; Deshmukh et al., 1982). Additional insecticidal compounds should be found from subsequent extractions of *O. sanctum* leaves and stems using more polar solvents such as EtOAc and MeOH. We are in the process of investigating other biologically active compounds in EtOAc and MeOH extracts of *O. sanctum*.

ACKNOWLEDGMENT

This is a contribution from the Michigan State University Agricultural Experiment Station. *A. aegyptii* eggs were supplied by Drs. A. Raikhel and A. Hays, Department of Botany and Plant Pathology, Michigan State University.

LITERATURE CITED

Asthana, O. P.; Gupta, R. S. Effects of NP levels on physiological parameters at three growth stages in Sacred Basil (*Ocimum sanctum*). *Indian Perfum.* **1984**, *28*, 49−53.

Batta, S. K.; Santhakumari, G. The antifertility effect of *Ocimum sanctum* and *Hibiscus rosa sinensis*. *Indian J. Med. Res.* **1970**, *59*, 777−781.

Ben-Yakir, D.; Bazar, A.; Chen, M. Attraction of *Maladera matrida* (Coleoptera: Scarabaeidae) to eugenol and other lures. *J. Econ. Bot.* **1995**, *88*, 415−420.

Butani, D. K. Insect pests of *Tulsi* (*Ocimum sanctum* Linnaeus) and their controls. *Pesticides* **1982**, *16*, 11−12.

Deshmukh, P. B.; Chavan, S. R.; Renapurkar, D. M. A study of insecticidal activity of twenty indigenous plants. *Pesticides* **1982**, *12*, 7−10.

Duperrier, A.; Moreau, M.; Dreux, J. Action des organomagnésiens mixtes et de l'hydrure de lithium sur les dihydropyrones. II-Dihydro-3, 6-pyrones-2. *Bull. Soc. Chim. Fr.* **1975**, 2307.

Grote, R.; Zeeck, A.; Stümpfel, J.; Zähmer, H. Pyrrolizidinones Produced by *Streptomyces olivaceus*. *Liebigs Ann. Chem.* **1990**, *256*, 525−530.

Kimura, K.; Wada, T.; Endo, M.; Iwata, I. Lipids in Green and Red Bell Peppers. *J. Jpn. Soc. Food Nutr.* **1982**, *35*, 95−101.

Laakso, I.; Seppänen-Laakso; Hermann-Wolf, B.; Kühnel, N.; Knobloch, K. Constituents of the Essential Oil from Holy Basil or Tulsi Plant, *Ocimum sanctum*. *Planta Med.* **1990**, *56*, 527.

Malik, M. S.; Rafique, M. Effects of methanol extracts of *Ocimum sanctum* Linn. on jute semilooper. *Indian J. Entomol.* **1989**, *51*, 84−89.

Nair, A. G. R.; Gunasegaran, R.; Joshi, B. S. Chemical Investigation of Certain South Indian Plants. *Indian J. Chem.* **1982**, *21B*, 979−980.

Nair, M. G.; Putnam, A. R.; Mishra, S. K.; Mulks, M. H.; Taft, W. H.; Keller, B. E.; Miller, J. R.; Zhu, P.-P.; Meinhart, D.; Lynn, D. G. Faeriefungin: A new broad-spectrum antibiotic from *Streptomyces griseus* Var. *Autotrophicus*. *J. Nat. Prod.* **1989**, *52*, 797−809.

Nitao, J. K.; Nair, M. G.; Thorogood, D. L.; Johnson, K. S.; Scriber, M. J. Bioactive Neolignans from the Leaves of *Magnolia virginiana*. *Phytochemistry* **1991**, *30*, 2193−2195.

Nörr, H.; Wagner, H. New Constituents from *Ocimum sanctum*. *Planta Med.* **1992**, *58*, 574.

Risvi, S. J. H. Discovery of natural herbicides. *Pesticides* **1981**, *3*, 40−42.

Roth, G. N.; Chandra, A.; Nair, M. G. Novel Bioactivities of *Curcuma longa* Constituents. *J. Nat. Prod.* **1998**, *61*, 542−545.

Stein, U.; Sayaampol, B.; Klingauf, F.; Bestmann, H.-J.; Vostrowsky, O.; Classen, B. Aphidizide Wirkung ethanolisher Extracte aus dem Heiligen Basilikum, *Ocimum sanctum*. *Entomol. Gener.* **1988**, *13*, 229−237.

Sukari, M. A.; Rahmani, M.; Lee, G. B.; Takahashi, S. Constituents of Stem Barks of *Ocimum sanctum*. *Fitoterapia* **1995**, *66*, 552−553.

Thakur, R. S.; Puri, H. S.; Husain, A. *Major Medicinal Plants of India*; Central Institute of Medicinal and Aromatic Plants; Lucknow, India, 1989.

Received for review March 23, 1998. Accepted June 9, 1998. The NMR data were obtained on instrumentation that was purchased in part with the funds from NIH Grant 1-S10-RR04750, NSF Grant CHE-88-00770, and NSF Grant CHE-92-13241.

JF980304T