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Piperidinyl Amides with Insecticidal Activity from the Maritime Plant *Otanthus maritimus*

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Two new piperidine amides, *N*-[(2*E*,4*E*,8*Z*)-tetradecatrienoyl]piperidine (**1**) and *N*-[(2*E*,4*E*,8*Z*,11*Z*)-tetradecatetrenoyl]piperidine (**2**), along with the known metabolites *N*-[(2*E*,4*E*)-tetradecadienoyl]-piperidine (**3**), *N*-isobutyl-(2*E*,4*E*,)-tetradecadienamide (**4**), *N*-isobutyl-(2*E*,4*E*,8*Z*)-tetradecatrienamide (**5**), *N*-isobutyl-(2*E*,4*E*,8*Z*,11*Z*)-tetradecatetraenamide (**6**), sesamine (**7**), pinoresinol (**8**), and espeletone (**9**), were isolated from the dichloromethane/methanol extracts of the plant *Otanthus maritimus* Hoffman & Link collected from coastal areas in Greece. Pinoresinol (**8**) and espeletone (**9**) are reported for the first time as metabolites of *O. maritimus*. The structures of the new natural products were elucidated by interpretation of their NMR and high-resolution mass spectral measurements. The insecticidal properties of the crude extracts, essential oil, and isolated metabolites **1**−**9** were evaluated on *Crematogaster scutellaris* (Olivier) ants and *Reticulitermes balkanensis* (Clement) termites, showing significant levels of activity.

KEYWORDS: Insecticidal activity; *Otanthus maritimus*; *Crematogaster scutellaris*; *Reticulitermes balkanensis*; piperidinyl amides

INTRODUCTION

The genus *Otanthus*, found mainly in the Mediterranean region, belongs to Asteraceae and is represented by a single species (*1*, *2*). *Otanthus maritimus* has been reported in the past to exhibit a significant array of biological and pharmacological activities (*3*−*5*) including the treatment of dysentery and inflammation of the urinary bladder. Dry specimens of *O. maritimus* have been traditionally used as decoration and at the same time as a means of repelling flying insects from household areas.

A number of investigations on the chemical composition of *O. maritimus* have reported the isolation of amides, thiophenamides, polyacetylenes (*6*), and sesquiterpene hydrocarbons (*7*) from the roots of the plant. The aerial parts of the same plant have been found to contain terpenoids, aliphatic esters, flavonoids (*8*), sesquiterpene lactones, monoterpene diols (*9*), amides, and sesamine (*6*, *9*).

In continuation of our investigations toward the discovery of natural products with insecticidal activity or insect repellency from Greek endemic and Mediterranean plant species (*10*−*12*), we were able to collect and study the maritime plant *O. maritimus* growing wild on the sandy coasts of two Greek islands.

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MATERIALS AND METHODS

Plant Material. *O. maritimus* (L.) Hoffman & Link belongs to the family of Asteraceae and is a densely white-lanate and strongly aromatic perennial herb, growing wild in maritimal sands. Plant material was collected during anthesis (June−July) from the coastal plains of Halikounas in Corfu, Ionean Sea (collection A) and from Alykes area on Lemnos Island, Aegean Sea (collection B). Voucher specimens are kept at the Herbarium of the Pharmacognosy Laboratory, University of Athens (MT-123, MT-168).

Hydrodistillation, Extraction, and Isolation. Harvested aerial parts of *O. maritimus* were allowed to dry in a cool and dark chamber and were subsequently cut in small pieces and separately hydrodistilled for 2 h in a modified Clevenger apparatus with a water-cooled receiver, to reduce hydrodistillation overheating the artifacts. The essential oils were taken up in ether and dried over sodium sulfate. Quantities of the two plant collections (dry weight for collection A = 32 g, for collection B = 423 g) were separately exhaustively extracted at room temperature with mixtures of CH_2Cl_2/MeOH (2:1). The organic extracts after evaporation of the solvents afforded dark oily residues (1.43 g for A and 32.6 g for B). The crude solvent extract A was subjected to VCC on silica gel using cyclohexane with increasing amounts (in 10% increments) of EtOAc and finally MeOH. Fraction III (30% EtOAc in cyclohexane) (279 mg) was further purified by vacuum column chromatography (VCC) on silica gel using cyclohexane with increasing amounts (in 2% increments) of EtOAc. Fractions IV_a (20% EtOAc) (44 mg) and V_b (35% EtOAc) (41 mg) were subjected to normal phase HPLC chromatography, using as mobile phase cyclohexane/EtOAc (80:20 and 70/30), to yield pure compounds **7** (15.4 mg) and **8** (2.7 mg). The crude extract B was similarly subjected to VCC on silica gel using cyclohexane with increasing amounts (in 5% increments) of EtOAc.

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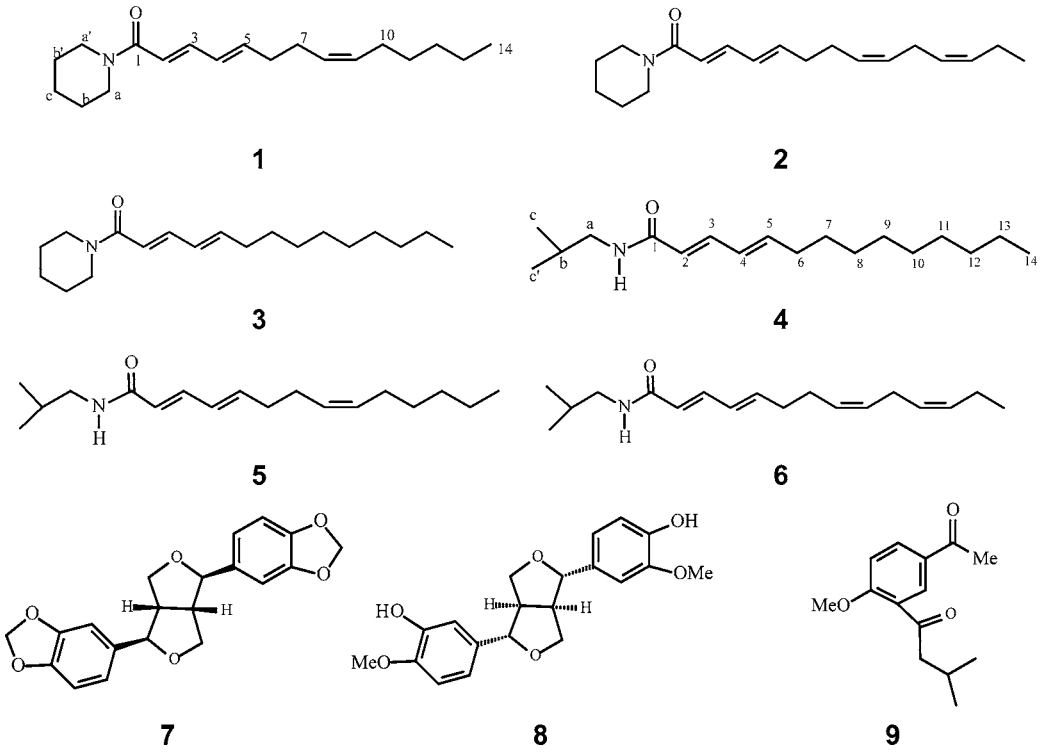

Figure 1. Isolated metabolites from *O. maritimus*.

The nonpolar fractions showed the presence of the previously isolated metabolites **7** and **8**. Fractions IV and V (35% and 40% EtOAc in cyclohexane) afforded 130 and 150 mg, respectively, and were further purified by VCC on silica gel. The fractions of interest were subjected to normal phase HPLC chromatography, using as mobile phase cyclohexane/EtOAc (80:20 and 75:25), to yield pure compounds **3** (7.1 mg), **9** (4.8 mg), **4** (6.3 mg), and **5** (5.8 mg) from fraction IV and **1** (2.4 mg), **2** (2.5 mg), and **6** (2.0 mg) from fraction V (**Figure 1**).

Chemical Analyses. Optical rotations were measured using a Perkin-Elmer model 341 polarimeter and a 10 cm cell. IR spectra were obtained using a Paragon 500 Perkin-Elmer spectrophotometer. NMR spectra were recorded using Bruker AC 200 and Bruker DRX 400 spectrometers. Chemical shifts are given on δ (parts per million) scale using TMS as internal standard (s, singlet; d, doublet; t, triplet; m, multiplet). The two-dimensional experiments (^{1}H$-^1$H COSY, HMQC, HMBC, NOESY) were performed using standard Bruker microprograms. High-resolution mass spectral data were provided by the University of Notre Dame, Department of Chemistry and Biochemistry, Notre Dame, IN. EIMS data were recorded on a Hewlett-Packard 5973 mass selective detector. VCC separations were performed on Kieselgel 60H (Merck), TLC analyses were performed with Kieselgel 60 F_{254} (Merck aluminum support plates), and spots were visualized upon spraying with 5% H_2SO_4 in MeOH and heating. HPLC separations were conducted using a Pharmacia LKB 2248 model and GBC LC-1240 refractive index detector, with a Spherisorb S10W (phase separation; column size, 10 $\times$ 250 mm) column.

Insect Collection and Bioassays. Assays were performed against the ant *Crematogaster scutellaris* (Hym., Formicidae) (*13*) and the termite *Reticulitermes balkanensis* (Iso., Rhinotermitidae) (*14*). The ants were collected from a colony in a stump of *Pinus halepensis* and the termites from a colony exploiting a fallen *Abies cephalonica* var. *graeca* tree. A sizable number of insects with the necessary dead wood substrate were transferred at the laboratory, where the queenless colonies were kept in Plexiglas rearing cages. These two colonies supplied the necessary insects for the bioassays. Each compound was dissolved in dichloromethane, and a volume of the solution containing 80 μg of the compound was micropipetted on the bottom of a glass flat Haggerton bottle (height = 10 cm and i.d. = 2.8 mm). Following evaporation of the solvent, five to seven insects were placed in each bottle, and the systems were sealed with a Whatman no. 4 filter paper.

The killed insects in each bottle were counted and recorded at specific intervals. For comparison purposes, as blank in every repetition set were used Haggerton bottles loaded only with solvent and treated under the same conditions.

The crude organic extract and the oil produced by hydrodistillation were tested on both insects, and the two recorded parameters were the times at which 50 and 100% of the insects were killed.

The killing efficiency of the isolated metabolites was measured by means of a dose−response model (*15*, *16*). The proportion of dead insects was considered to be the response. The duration between observations was considered as dose, and because the concentration of the killing agent is constant throughout the experiment, the measurements constitute serial time−mortality data (*17*) (**Figure 2**). This specific design was necessary due to the limited amounts of the isolated metabolites and the fact that speed of killing is of prime importance. The rationale behind this is that the tested compounds are continuously dissolved in the lipid layer lining the ectodermis of the insect cuticle and are transferred in the sensitive tracheal openings and the buccal cavity through the external mouthparts. Moreover, the proportion of dead insects is dependent on the proportion recorded at the previous measurement. For binary regression the algorithm proposed by Throne et al. was used for probit regression (*18*). Probit regression of the mean proportions of killed insects in all six replicates for each compound was used instead of logit or complementary log−log because of its simplicity in estimating LT_{50} and its smaller maximum likelihood D statistic (*15*, *17*).

Depending on the nature of the mortality curve the LT_{50} does not always reflect the killing efficiency of an agent. For this reason an additional value directly derived from experimental data was used. The potency of a compound as a killing agent was correlated with the area confined between the control and the tested compound curve. The parameter is expressed in time units and is directly comparable to the $1/LT_{50}$ calculated from the fitted probit model. The comparison of the killing efficiency in terms of the measured areas and those derived from fitting the probit model to the data was performed by means of the Spearman rank correlation coefficient (*19*).

RESULTS AND DISCUSSION

The chemical analyses of the essential oil constituents resulting from the two individual collections showed them to

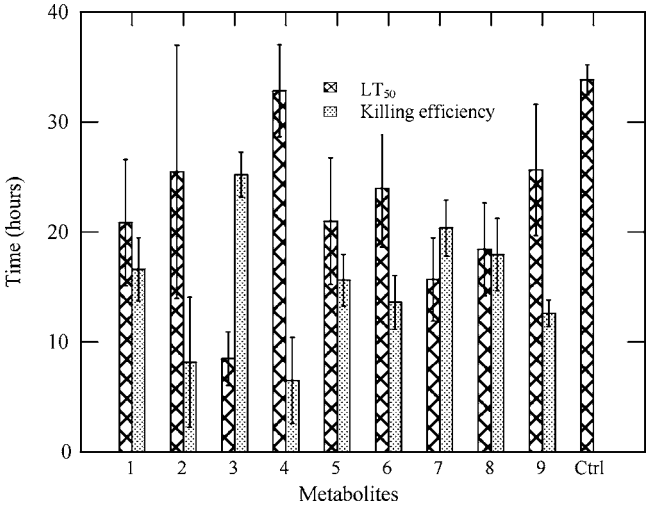

Figure 2. Killing efficiencies of metabolites **1**–**9** on *C. scutellaris* ants. Killing efficiency is represented by the area between curves corresponding to the mortality of the control and the tested metabolites as shown in **Figure 3**. LT_{50} is calculated after fitting the killing efficiency experimental data in the probit model. The control compound, "Ctrl", shows the time at which the experiment ended and does not reflect the lifetime of the ants. The error bars are in SE units.

have very similar chemical profiles with slight quantitative differences. The main metabolites of the essential oils, in order of contribution, were *cis*-chrysanthenyl acetate, 1,8-cineole, camphor, and artemisia alcohol. Extraction of the aerial parts of the two plant collections (studied separately) yielded, after a series of chromatographic separations by VCC and normal-phase high-pressure liquid chromatography, the new piperidine amides, *N*-[(2*E*,4*E*,8*Z*,)-tetradecatrienoyl]piperidine (**1**) and *N*-[(2*E*,4*E*,8*Z*,-11*Z*)-tetradecatetrenoyl]piperidine (**2**), along with the previously described metabolites *N*-[(2*E*,4*E*)-tetradecadienoyl]piperidine (**3**) (*6*, *20*), *N*-isobutyl-(2*E*,4*E*,)-tetradecadienamide (**4**) (*20*, *21*), *N*-isobutyl-(2*E*,4*E*,8*Z*)-tetradecatrienamide (**5**) (*22*, *23*), *N*-isobutyl-(2*E*,4*E*,8*Z*,11*Z*)-tetradecatetraenamide (**6**) (*23*, *24*), sesamine (**7**) (*25*), pinoresinol (**8**) (*26*), and espeletone (**9**) (*27*).

Compound **1** was obtained as colorless oil. Both ^{13}C NMR data and the HREIMS measurements (289.2409 obs, 289.2406 calcd) supported the molecular formula $C_{19}H_{31}NO$. The five degrees of unsaturation of this metabolite taking into account the presence of three double bonds and one carbonyl group, evident from the ^{13}C NMR spectrum, suggested a monocyclic structure. The intense stretch band at 1653 cm^{-1} in the IR spectrum was indicative of an amide moiety. The resonance at 3.54 (m, 4H) corresponding to two overlapping carbons at 45.0 ppm, along with the presence of an amide carbonyl signal at 163.5 ppm, suggested a symmetrical *N*-acylated nitrogen-containing ring. The characteristic mass fragment at *m*/*z* 84 and the coupling of the above-mentioned methylenes with a multiplet (1.56, 4H) that was sequentially coupled to a methylene (1.63, m, 2H) signal suggested the presence of a piperidine amide moiety. The presence of only one methyl (0.87, t), six aliphatic methylenes, and three disubstituted double bonds indicated a C14 acid residue. The chemical shifts of the two double bonds implied their conjugation with the amide, and their assignments were based in homo- and heteronuclear correlations. The *E* geometry of Δ_2 and Δ_4 double bonds is proposed on the basis of the coupling constants ($J = 14.7$ Hz for Δ_2, $J = 15.36$ for Δ_4) and comparison with those of the previously reported metabolite **3**. The position of the isolated double bond was assigned on the basis of the $^1H-^1H$ COSY correlations showing

Table 1. NMR Data for Metabolites **1** and **2**

	metabolite **1**		metabolite **2**	
no.	^1H NMR[a]	^{13}C NMR[b]	^1H NMR[a]	^{13}C NMR[b]
1		163.5		nd
2	6.24 (d, 14.7 Hz)[b]	129.0	6.21 (d, 14.7)	129.2
3	7.23 (dd, 14.7, 9.5 Hz)	141.8	7.21 (dd, 14.7, 10.6 Hz)	141.5
4	6.17 (dd, 15.4, 9.5 Hz)	128.7	6.17 (dd, 14.6, 10.6 Hz)	129.2
5	6.05 (dt, 15.3, 5.5 Hz)	142.8	6.04 (dt, 14.5, 5.5 Hz)	139.7
6	2.15 (m)	33.0	2.20 (m)	32.8
7	2.15 (m)	26.5	2.20 (m)	25.9
8	5.35 (m)	128.2	5.35 (m)	128.6[# c]
9	5.37 (m)	130.9	5.36 (m)	128.9[# c]
10	1.99 (m)	26.9	2.75 (m)	24.8
11	1.31 (m)	28.7	5.26 (m)	127.0[# c]
12	1.25 (m)	29.3	5.36 (m)	132.0[# c]
13	1.25 (m)	22.5	2.03 (m)	19.9
14	0.87 (t, 6.5 Hz)	14.1	0.95 (t, 7.2 Hz)	14.2
a–a′	3.54 (m)	45.0	3.55 (m)	46.8
b–b′	1.56 (m)	26.1	1.55 (m)	25.5
c	1.63 (m)	24.6	1.62 (m)	24.6

[a] 400 MHz, $CDCl_3$. [b] 50.3 MHz, $CDCl_3$. [c] Signals can be interchanged.

the conjugation of the C-14 terminal methyl with the C-13 methylene. The resonances of C-13 and C-12 methylene protons were overlapping, but the distinct carbon resonances allowed heteronuclear correlation with the C-11 methylene that through the $^1H-^1H$ COSY spectrum was found to be coupled with the vinylic C-10 methylene. The geometry of the isolated double bond was proposed on the basis of the C-7 and C-10 resonances, because the chemical shifts of vinylic carbons of linear olefins of *Z*-isomers resonate at 5 ppm higher fields ($\delta < 29$) than those of the *E*-isomers ($\delta > 31$) (*28*−*30*). Full assignment of the proton and carbon resonance is given in **Table 1**, and according to the above observations metabolite **1** was identified as *N*-[(2*E*,4*E*,8*Z*)-tetradecatrienoyl]piperidine.

Compound **2**, obtained as colorless oil, showed spectral characteristics very similar to those of metabolite **1**. The ^{13}C NNR along with the MS measurements supported the molecular formula $C_{19}H_{29}NO$ and showed the presence of a piperidine amide moiety and a lipid chain. The strong absorbance at 1658 cm^{-1} in the IR spectrum supported the presence of an amide carbonyl in the structure of metabolite **2**. The most significant difference was the occurrence of an additional double bond in the side chain. The presence of a doubly allylic methylene multiplet at 2.75 ppm (C-10) and the interpretation of the $^1H-^1H$ COSY correlations of the terminal methyl to C-13 methylene and C-12 olefinic proton allowed the assignment of the double-bond position in the side chain. The *Z* geometry of the two isolated double bonds was proposed on the basis of the chemical shifts of the adjacent allylic methylenes (25.9 for C-7, 24.8 for C-10, and 19.9 for C-13) (*28*−*30*). Full assignment of the proton and carbon resonances is given in **Table 1**, and according to the above spectral data metabolite **2** was identified as *N*-[(2*E*,4*E*,8*Z*,11*Z*)-tetradecatetrenoyl]piperidine.

The solvent extract *O. maritimus* showed significant killing efficiency on both *C. scutellaris* ants and *R. balkanensis* termites, whereas the essential oil exhibited moderate levels of activity (see **Table 2**). Due to the limited available amounts of the isolated metabolites and because *C. scutellaris* ants represent a more serious insect pest in Greece, it was decided to evaluate the activity of the individual constituents on this insect species. Earlier studies with a number of dienamides had indicated the insecticidal potential of this class of compounds on insects such as house flies, house mosquitoes, rice weevil, and pulse beetles (*20*), but this is, as far as we know, the first evaluation of their activity on ants.

Table 2. Killing Efficiency of *O. maritimus* Dichloromethane/Methanol Extract and Essential Oil

assayed material	insect	LT_{50} (h)
extract	*C. scutellaris*	8.02 (1.46)[a]
essential oil		31.57 (2.23)
control		35.63 (2.87)
extract	*R. balkanensis*	7.70 (1.53)
essential oil		29.20 (2.14)
control		33.82 (2.75)

[a] Numbers in parentheses represent the standard deviation. The amount of 80 μg of each assayed material was loaded once at the beginning of the experiment in the Haggerton bottles.

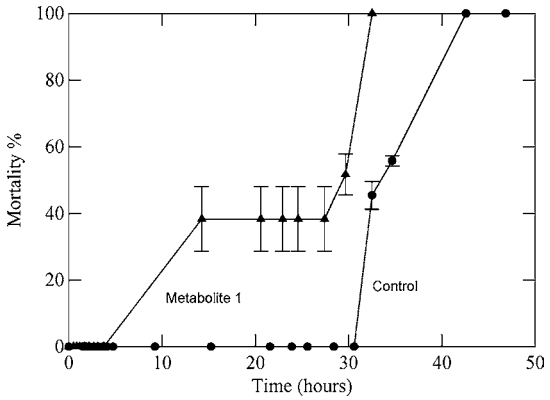

Figure 3. Mortality of *C. scutellaris* ants in contact toxicity bioassays with metabolite **1**. Killing efficiency is calculated by means of a dose–response model with the duration between observations considered as different doses. The error bars are in SE units.

Among all tested compounds, isolated in the present study, metabolite **3** exhibited the highest activity, whereas the new metabolite **1** showed the second best response among all tested enamides. The lowest response was observed for metabolite **4** (**Figure 3**). It is interesting to note that although metabolites **3** and **4** have the same lipid chain, they show the most dramatic activity difference, probably exerted from the nature of the nitrogen substituents. Comparison of the piperidine amides **1**−**3** revealed that the unsaturation of the lipid chain has a negative influence on the insecticidal activity of the compounds. Future studies with blends of the isolated metabolites, which exhibited the strongest activity, might reveal improved activities and lead to the development of a pest control system based partially or solely on this botanical formulation.

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