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MENTHOFURANS FROM *CALAMINTHA ASHEI*: EFFECTS ON *SCHIZACHYRIUM SCOPARIUM* AND *LACTUCA SATIVA*

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IN MEMORY OF TONY SWAIN, 1922–1987

Key Word Index—*Calamintha ashei*; Labiatae; monoterpenes; menthofuran derivatives; germination; growth regulatory activity; *Schizachyrium scoparium*; *Lactuca sativa*.

Abstract—From the aerial parts of *Calamintha ashei* the new menthofuran, calaminthone, as well as the known terpenoids (+)-evodone, caryophyllene oxide and ursolic acid were isolated and identified by spectroscopic methods (NMR and MS). Menthofuran and 2,3-dihydroevodone were present in the volatiles as determined by GC-MS analyses. Evodone and calaminthone volatiles exhibited strong germination inhibitory activities towards *Schizachyrium scoparium* seeds. The aqueous solution of a fraction containing calaminthone, evodone and caryophyllene oxide totally inhibited germination of *S. scoparium* seeds but had no significant effects on *Lactuca sativa* seed germination. A saturated aqueous solution of pure evodone exhibited stimulatory effects on *S. scoparium* but inhibitory effects were observed when evodone was administered in a saturated aqueous solution of ursolic acid. The possible role of ursolic acid in the release and transport of allelopathic lipids from the source plant into the soil through rain leachates is discussed.

INTRODUCTION

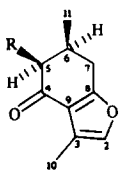
Previous chemical studies of the genus *Calamintha* have shown that the triterpene ursolic acid and its derivatives, [1, 2] as well as flavonoids and tannins [3] are common constituents within this genus. Volatiles of *C. nepeta* (L.) contain the known monoterpenes cineole, menthol, isomenthone and neoisomenthol as major constituents [4, 5]. A recent study of the volatiles of *C. nepeta* subsp. *glandulosa* provided piperitone oxide and piperitenone oxide as major components [6]. *Calamintha ashei*, a common plant in the Florida scrub community with documented inhibitory effects on the germination and growth of Florida sandhill grasses [7], had previously not been chemically investigated. In the course of our directed search for germination and growth inhibitors of Florida upland grasses by shrubs of the Florida scrub community, we found menthofurans to be the major monoterpenes in the essential oil of *C. ashei*. Extraction of *C. ashei* with nonpolar solvents also provided copious amounts of ursolic acid besides the mono- and sesquiterpenes. A nonpolar chromatographic fraction of this extract completely inhibited the germination of *Schizachyrium scoparium*, a native Florida sandhill grass. The results of our chemical studies and the allelopathic activities of these bioactive fractions and their pure constituents are discussed below.

RESULTS AND DISCUSSION

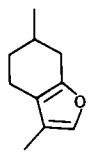
Chemical studies

Extraction of the aerial parts of *C. ashei* with petrol–CH_2Cl_2 (1:9) afforded a new monoterpene, calaminthone (**1**), and the known terpenes (+)-evodone (**2**), caryophyllene oxide and ursolic acid. Evodone (**2**) which is a major constituent in the petrol extracts, had a characteristic mass spectrum and ^{1}H NMR values in agreement with data reported for synthetic (+)-evodone [8–10]. Since the ^{13}C NMR spectral data of **2** had not been previously reported, they are included in Table 1. Caryophyllene oxide and ursolic acid were identified by comparison of their ^{1}H and ^{13}C NMR and mass spectral data with those of authentic samples.

Calaminthone (**1**), $C_{12}H_{14}O_4$, is an oil which in its mass spectrum exhibited a molecular ion peak at *m/z* 222, a base peak at *m/z* 43 $[Ac]^+$ and a prominent peak at *m/z* 162 $[M-AcOH]^+$. The latter two peaks supported the presence of an acetate moiety in the molecule. Facile loss of acetic acid from **1** was attributed to the



1 R = OAc
2 R = H



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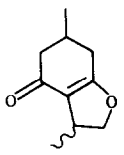

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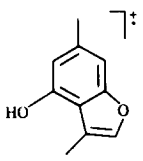

A *m/z* 162 (55)

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Table 1. ^{13}C NMR spectral data of compounds 1–3 (50.32 MHz, $CDCl_3$)

C	1	2	3
2	140.2 *d*	139.0 *d*	136.60 *d*
3	119.3 *s*	119.9 *s*	119.15 *s*
4	—	195.1 *s*	31.29 *t*
5	76.7 *t*	46.7 *t*	19.69 *t*
6	33.8 *d*	30.8 *d*	29.45 *d*
7	29.9 *d*	31.6 *t*	31.29 *t*
8	164.6 *s*	167.0 *s*	150.30 *s*
9	119.0 *s*	118.9 2	117.03 *s*
10	8.7 *q*	8.8 *q*	7.76 *q*
11	13.9 *q*	20.9 *q*	21.24 *q*
–C(=O)Me	170.1 s		
–C(=O)Me	20.8 q		

formation of a stable aromatic ion A(m/z 162). Further structural data were derived from detailed NMR experiments. ^1H NMR double irradiation of the broad singlet at δ7.11 (H-2) sharpened the three-proton signal at δ2.17 (Me-3) which suggested allylic coupling between these protons. Upon irradiation of the doublet at δ5.56 (H-5), the complex multiplet at δ2.66 (H-6) was affected. Irradiation of the latter multiplet caused changes in three additional proton signals. The three-proton doublet at δ1.09 (Me-6) collapsed to a singlet and the doublets of doublets centred at δ2.78 (H-7a) and 3.20 (H-7b) simplified into doublets with residual geminal couplings of 17 Hz. The results of the above double irradiation experiments were supported by the 2D-COSY ^1H NMR data. The methyl signal at δ2.17 (Me-3) in **1** exhibited a chemical shift at lower field than the equivalent methyl absorption of menthofuran (**3**) which absorbs at δ1.89. The deshielding of the C-3 methyl group suggested the presence of a carbonyl moiety at C-4 as in **2**, which exerts a through-space deshielding effect on the C-3 methyl group [11]. Correlation of the coupling constant between H-6 and H-5 ($J_{5,6}$=4 Hz) with dihedral angles of the two hydrogens derived from stereomodel inspections indicated a *cis* relationship between the protons at C-6 and C-5. ^{13}C NMR signals of **1** were assigned using chemical shift considerations and comparison with ^{13}C NMR data of the co-occuring evodone (**2**) and menthofuran (**3**) (Table 1).

GC-MS analysis of the volatiles obtained from a petrol extract (Experimental, Procedure A) indicated the presence of menthofuran (**3**). This was verified by MS-comparison with a standard. A closely related monoterpene which appeared as a shoulder on the GC trace of **2** showed a similar mass spectral pattern. As with **2**, the facile loss of 42 mass units from the parent ion (M^+, m/z 166) was interpreted as a retro-Diels–Alder cleavage. The loss of m/z 43 indicated saturation of the 2,3-double bond of the furan ring. Therefore, its structure was tentatively assigned as 2,3-dihydroevodone (**4**) with the chirality at C-3 to remain open.

Bioassays

A column chromatography fraction (see Experimental, Procedure B, fraction 6) composed of a mixture of calaminthone (**1**) as the major component and evodone (**2**) and caryophyllene oxide as minor compounds caused complete inhibition of *Schizachyrium scoparium* seed germination but had no significant effects on *Lactuca sativa* germination. In contrast evodone and caryophyllene oxide exhibited no significant inhibition when tested individually (Table 2). This difference in activity could be attributed to a very low solubility of the pure compounds in water in comparison to a considerably higher solubility (285 ppm) of the three-component mixture.

Since evodone was detected in the atmosphere around a living *C. ashei* plant, effects of volatiles of monoterpenes **1** and **2** on *S. scoparium* seeds were tested using a method similar to that of ref. [12]. Both, evodone and calaminthone volatiles exhibited highly significant inhibitory activity on seed germination. Evodone and caryophyllene oxide were also tested in an aqueous solution with ursolic acid which is present in copious amounts on the leaf surface of *C. ashei* (see Experimental). Sufficient quantities of calaminthone (**1**) were not available to be tested in solution with ursolic acid. A saturated aqueous solution of evodone (**2**) exhibited significant stimulatory

Table 2. Effects on germination (G) and radicle growth (R) of compounds from *C. ashei* as per cent of responses in distilled water control solutions

		Schizachyrium scoparium		*Lactuca sativa*	
Compound(s)	Conc. (ppm)	%G	%R	%G	%R
Mixture of **1**, **2** and caryophyllene oxide in aq. soln.	285	0*	—	97	61*
Calaminthone (**1**) (satd aq. soln)	250†	129	88	113	138*
Evodone (**2**) (satd aq. soln)	250†	185*	93	96	131*
Caryophyllene oxide (satd aq. soln)	625†	128	91	104	66*
Ursolic acid (UA) (satd aq. soln)	—	86	136	104	93
2 + satd aq. soln of UA	250*	57*	33*	97	81*
1 (volatiles)		36*	91		
2 (volatiles)		11*	77		

*Indicates significant difference from the control at $p<0.05$.

†This concentration represents the maximal concentration based on the total amounts weighed. In all cases only part of each sample dissolved.

effects on *S. scoparium* seed germination. In contrast, a solution of evodone (**2**) prepared by dissolving **2** in a saturated aqueous solution of ursolic acid exhibited a significant germination and radicle growth inhibitory activity on *S. scoparium* but had no significant effects on *Lactuca sativa* germination.

The above experiments clearly demonstrate that the presence of ursolic acid dramatically influences the activity of the allelopathic monoterpenes in the germination and radicle growth tests. These findings support our earlier proposal that a possible mechanism for the observed differences in bioactivity might be due to natural surfactants such as fatty acids and/or triterpene acids which are present on the leaf surface of an allelopathic source plant [13]. By this mechanism the water-solubility of relatively water-insoluble lipids could be increased due to micelle formation [14]. In our present study, the ursolic acid micelle matrix could possible act as a lipophilic host for lipids, thus increasing their leachate concentration and aid the transport of allelopathic agents from the leaf surface into the soil. Ursolic acid, which is present in copius amounts in the cutical wax of *C. ashei* leaves, would therefore allow the release and transport of *C. ashei* terpenes from the plant surface into the soil through natural rain leaching.

To investigate water leachates of *C. ashei* for possible micelle formation, a method using the fluorescence yield of acridine was applied [15]. This method is based on the fact that below the critical micelle concentration (CMC), the fluorescence of acridine is independent of surfactant concentrations while above the CMC, quantum yields decrease drastically with increasing surfactant concentrations. This is due to the behaviour of acridine which only fluoresces in protic solvents and is sufficiently hydrophobic to be mainly located within the hydrocarbon-like interior of the micelles. By using the acridine method, an aqueous leachate from leaves of *C. ashei* was tested for micellization. The concentration of the leachate solution was estimated by assuming a reasonable average M_r of 300. Figure 1 shows the dependence of relative fluorescence intensity of 1.2×10^{-5} M acridine solution versus concentration of *C. ashei* leachate. The dramatic decrease in fluorescence intensity near 10^{-4} and 10^{-3} molar concentrations clearly indicated micellization of the leachate solution [15]. This strongly suggests that ursolic acid, which is found in the leachate, represents the micellar host for bioactive compounds possibly increasing their concentration in water leachate and/or aid their transport to target plants. This effect is complementary to the 'volatility' mechanism of allelopathic terpenes, which was proposed by Muller [12]. The 'synergistic' effects of ursolic acid as exemplified by the presented bioassays are presently not known. Experiments are in progress which should allow us to distinguish between effects due to micelle-mediated concentration increases of allelopathic agents and/or transport mechanisms which aid possible destruction of and/or transport through membranes of target seeds by allelopathic agents.

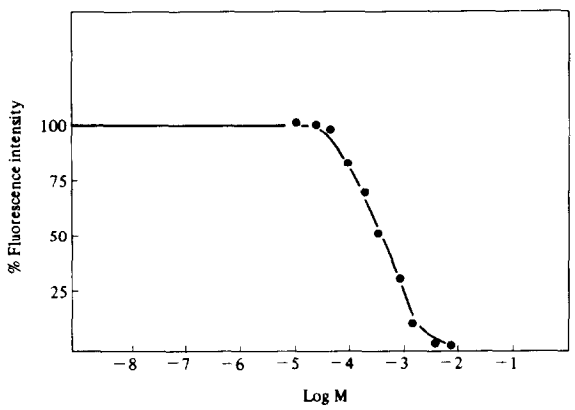

Fig. 1. Dependence of relative fluorescence intensity of 1.2×10^{-5} M acridine solution vs concentration of leachate from *Calamintha ashei*.

EXPERIMENTAL

Plant material. Calamintha ashei (Weatherby) Shinner was collected in June, 1984 in Polk County at Sun Ray, Florida (Donald R. Richardson and David W. Crewz, No. 724; voucher deposited at the University of South Florida Herbarium at Tampa; sheet No. 137245.) Further collections were carried out in Oct. 1984; Oct. 1985 and April 1986.

Isolation procedures. Procedure A: ground dry *C. ashei* leaves (125 g) collected in June 1984 were soaked successively in petrol, $CHCl_3$ and MeOH for 24 hr using 500 ml of each solvent. After filtration by suction the process was repeated with each solvent by resoaking the residue for 6 hr using 300 ml. The combined extracts yielded 2.2 g from petrol, 12.7 g from $CHCl_3$ and 6.2 g from MeOH.

The $CHCl_3$ extract gave 8.9 g of a ppt. upon slow evapn of the solvent to *ca* 100 ml. The solid was filtered off and repeatedly washed with CH_2Cl_2 and MeOH yielding 6.5 g of crude ursolic acid. Further purification of 25 mg by prep. TLC (silica gel) with Et_2O–EtOAc (5:1) provided pure ursolic acid for identification by NMR and MS comparison with authentic material.

Procedure B: dried ground *C. ashei* leaves (100 g), collected in Oct. 1984, were extracted with petrol–CH_2Cl_2 (1:9) providing 6.2 g crude extract. Part of this extract (3.4 g) was subjected to CC (silica gel, petrol–CH_2Cl_2, 3:7) providing eight 75 ml fractions. Fraction 5 contained caryophyllene oxide as the major component. It was purified by prep. TLC (silica gel, CH_2Cl_2) for identification by NMR and MS comparison with authentic material. Fraction 6 provided a mixture of evodone, calaminthone and caryophyllene oxide. Prep. TLC (silica gel, CH_2Cl_2–Me_2CO, 19:1) gave 112 mg calaminthone.

Calaminthone (**1**).* $C_{12}H_{14}O_4$; oil; ^{1}H NMR (200 MHz, $CDCl_3$, ppm): δ1.09(*d*, 3H, $J_{6,11}=7$ Hz,,H-11), 2.17(*d*, 3H, $J_{2,10}<1$ Hz, H-10), 2.21(*s*, 3H, COMe), 2.66(*m*, 1 H, H-6), 2.78(*dd*, 1H, $J_{7a,7b}=17$ Hz; $J_{7a,6}=3$ Hz, H-7α), 3.20(*dd*, 1H, $J_{7a,7b}=17$ Hz, $J_{7b,6}=5$ Hz, H-7b) 5.56(*d*, 1H, $J_{5,6}=4$ Hz, H-5), 7.11(*br s*, 1H, H-2). EIMS *m/z* (rel. int.): 222 $[M]^+$(5), 162 $[A]^+$ (55), 151 (14), 133 (10), 122 $(M-A-C_3H_4]^+$ (59), 94 $[M-A-C_3H_4-CO]^+$(29), 77 (10), 65 (21), 43 $[Ac]^+$ (100).

Procedure C: For the purpose of obtaining pure evodone (**2**), dry *C. ashei* leaves (150 g), collected in Oct. 1985 were repeatedly

*After submission of this paper the absolute configuration of the *p*-bromobenzoate derivative of desacetylcalaminthone (Macias, F. A., unpublished results) was determined by single crystal X-ray diffraction (Fronczek, F. R., to be published). This required revision of the attachment of the acetoxy group from C-7 to C-5 in the calaminthone structure [16]. The ^{1}H and ^{13}C NMR spectral assignments given in this paper represent corrected data in accord with the revised structure of calaminthone, the absolute configuration of which is shown (**1**).

extracted with petrol–CH_2Cl_2 (1:9). The extract (~6 g) was not taken to complete dryness to avoid possible loss of volatile compounds. Part of this crude extract (3 g) was separated by CC (silica gel) with petrol–CH_2Cl_2 (3:7) to yield eight 150 ml fractions. Fraction 5 mainly contained evodone (**2**) as shown by GC-MS analysis. Prep. TLC (silica gel; petrol–CH_2Cl_2–Me_2CO, 15:34:1) yielded pure **2** (28 mg) which was used for NMR and MS identification. Evodone decomposed upon prolonged exposure to air at ambient temp.

Menthofuran (**3**) *and* 2,3-*Dihydrocalaminthone* (**4**). A steam distillation was carried out by passing steam through 2 g of the petrol extract from *C. ashei* leaves collected in June, 1984 (Procedure A). The distillate (50 ml) was extracted with 10 ml of nanograde CH_2Cl_2. The volatiles were analysed by GC-MS. GC conditions: 30 m silica-bonded FSOT capillary column. Injection temp. 250°; oven 45° for 1 min then 5°/min for 15 min and finally 10°/min to 250°.

Methofuran (**3**). R_t 10.32 min.; EIMS *m/z* (rel. int.): 150 (40), 108 (100), 91 (8), 79 (15). 2,3-Dihydroevodone (**4**). M_r 166. R_t 12.82; EIMS *m/z* (rel. int.): 166 (35), 124 (100), 123 (45), 109 (5), 81 (15).

Measurement of relative acridine intensity. Acridine, which was purified on a silica gel column using CH_2Cl_2 as eluent, was dissolved in distilled H_2O with magnetic stirring for 4 hr, providing a soln of 6.0×10^{-4} M concentration. The initial test concentration was the one obtained by mixing the *C. ashei* leachate and acridine solutions in a 4:1 ratio. For dilution, aq. acridine soln (1.2×10^{-5} M) was added in a 1:1 ratio to the test soln after each measurement. The fluorescence yield obtained for various test solns was compared to pure acridine soln to determine the relative fluorescence intensity.

A SLM 4800 spectrofluorimeter with an excitation slitwidth of 8 nm and an emission slitwidth of 2 nm was used. Emission wavelength was chosen at 425 nm. While the excitation wavelength was 360 nm for compounds that did not absorb UV radiation at 360 nm, in cases where compounds did absorb at 360 nm, 395 nm was used as the excitation wavelength.

Bioassays. Germination and radicle growth assays were carried out on *Lactuca sativa* and *Schizachyrium scoparium* seeds. For each test species 1 or 2 petri dishes of 30 seeds each were used. Test soln (5 ml), prepared by dissolving test compound in distilled H_2O through sonication, was added to a petri dish lined with filter paper. Distilled H_2O was used instead of the test soln for the control. In the case where volatiles were bioassayed, an Al foil boat containing about 2 mg of the test compound was placed in the centre of the petri dish while the control contained an empty Al boat.

Solutions for bioassay of **2** and **3** in the presence of ursolic acid were prepared by adding the indicated maximum amounts of **1** and **2** to a satd aq. soln of ursolic acid with subsequent sonication and filtration.

The petri dishes were covered and kept in the dark for a period of 4 days for *L. sativa* and 15 days for *S. scoparium*.

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