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Antimicrobial phenolic abietane diterpene from *Lycopus europaeus* L. (Lamiaceae)

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ABSTRACT

A new acetylated highly oxygenated abietane-type diterpenoid named euroabienol was isolated in pure state from *Lycopus europaeus* L. (Lamiaceae) fruits and its structure elucidated through both extensive spectral and chemical means. The presence of a phenolic C ring with a rare substitution pattern in euroabienol and its high relative amount in the fruits (1%, based on the weight of the fruits) urged us to try to establish its possible biological role. Thus, it was screened for its in vitro antimicrobial activity against fifteen strains of bacteria and six fungal strains. Euroabienol showed a broad spectrum of activity and probably is a first line defense plant metabolite against pathogen attack. This is the first report on the occurrence of abietanes in the genus *Lycopus*.

Lycopus europaeus L. (Gypsywort in English, 'vučja noga' or 'gagalica' in Serbian) is a perennial plant native to Europe and Asia, and naturalized in the United States. It is reputed to have medicinal qualities, which are attributed to phenolic compounds,[1,2] and has been used by various peoples as an astringent, cosmetic, narcotic, and refrigerant.[3] Extracts from *Lycopi europaei herba* are traditionally used in patients with slight hyperthyroidism with vegetative-nervous disturbances as well as in tenseness and pain of the mammary gland.[4] A recent investigation showed a clear reduction of hyperthyroid symptoms in rats, particularly of cardiac symptoms and body temperature, following treatment with an extract of *L. europaeus*.[5] Previous phytochemical studies (dealing only with the aerial parts extracts) revealed this species to be a rich source of isopimarane-type diterpenoids[6–8] and alicyclic diterpenes.[9,10]

GC–MS analysis of a dichloromethane extract of the fruits of *L. europaeus* revealed the presence of a major volatile compound **1** showing the highest *m/z* value at 462 in its mass spectrum (Fig. 1). The MS fragmentation followed a characteristic pattern of consecutive losses of acetic acid and water molecules, giving raise to the second and third most intense peak in the MS—[M – 2AcOH] and [M – 2AcOH – H_2O], respectively, suggesting a diacetylated molecule. This was further strengthened by the existence of *m/z* 43 as the base peak. Subsequently, the compound was isolated in pure state by chromatography on silica gel. The yield of **1** calculated per weight of dry fruits was unexpectedly high ca. 1%. This permitted us to ascertain the structure of this metabolite through spectral and chemical means and test it in an antimicrobial assay to possibly determine its function, likely to be significant due to its abundant accumulation in the fruit. High resolution mass spectrometry of **1** indicated the molecular formula $C_{25}H_{34}O_8$ for this compound from now on termed euroabienol. Its IR spectrum was consistent with the presence of ester (1727, 1708 cm^{-1}), aromatic (3080, 1577 cm^{-1}) and hydroxyl (3389 cm^{-1}) groups. The silylation of compound **1** by a large excess of TMSCl/Et_3N resulted in a TIC chromatogram showing four peaks corresponding to the unreacted **1**, one disilylated and two monosilylated derivatives. The disilylated

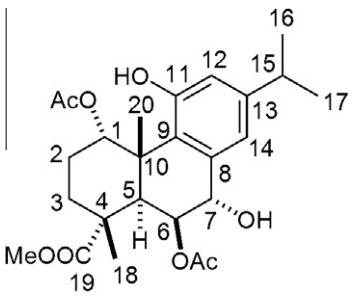

Figure 1. Structure of compound **1**.

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compound having an ion at *m/z* 606, [M(**1**) − 2H + 2Me$_3$Si]—compound **2**, possessed an analogous loss of two AcOH, giving significant fragment ions (base peak and the second most abundant). The remaining two monosilylated derivatives (**3** and **4**) formed in notably different relative amounts implied to the existence of two distinctive hydroxyl groups in the molecule. At least one of the two hydroxyl groups was found to be phenolic since the product of an attempted deacetylation procedure with sodium methoxide was soluble in water and was precipitated from the aqueous-methanol solution by the addition of hydrochloric acid. The GC–MS analysis of this precipitate demonstrated that it represented a single mono deacetylated derivative of **1** (compound **5**). UV absorption at λ_{max} 223 (log ε 4.00) and 284 (log ε 3.50) nm corroborated the presence in **1** of a hydroxyphenyl chromophore.[11] The ^{1}H and ^{13}C NMR spectra of **1** (Table 1) showed signals for an isopropyl group attached to an aromatic ring, two methyl groups attached to fully substituted sp^3 carbon atoms, two aromatic protons in the *meta*-position (J_m = 1.8 Hz; δ_H 6.77 d and 6.56 d; δ_C 121.8 and 114.6), a carbomethoxyl group (δ 3.76, 3H, s; δ 178.1 and 42.4) and two acetoxyl groups (δ_H 1.81, 3H, s, 2.03, 3H, s; δ_C 171.4 and 21.2, 170.4, and 21.4). The addition of a few drops of D_2O to the $CDCl_3$ solution of **1** resulted in the disappearance of two signals (a sharp singlet at 6.48 and a broad one at 2.68 ppm) from the ^{1}H NMR spectrum. These are consistent with the two distinct hydroxyl groups whose presence was hinted by the silylation results. In addition, euroabienol (**1**) possessed $(C)_2CH–CHOR–CHOH(C)_2$ (H-5, H-6, H-7) and $(C)CH_2–CH_2–CH(C)(OR)$ (H-2α and 2β, H-3α and 3β, H-4) structural moieties (closed spin systems δ_H 3.23, 5.06, 4.47; and δ_C 37.3, 75.2, 70.2; δ_H 1.49 and 2.15, 2.12 and 1.96, 6.32; and δ_C 31.3, 22.1, 74.6, respectively), evident from in the ^{1}H–^{1}H COSY. All these functionalities can be accommodated in a structure such as **1** for the new diterpenoid in agreement with NMR data reported for the analog leonubiastrin (methyl 6β-acetoxy-7α,11-dihydroxy-3-oxoabieta-1,8,11,13-tetraen-19-oate).[12]

In particular, the downfield resonance of the C-1 proton (δ 6.32), assumed to possess an α-acetoxy substituent, was only compatible with an abieta-8,11,13-triene structure possessing a phenol group at the C-11 position, which causes a strong deshielding effect on the C-1 proton due to its coplanarity with the aromatic ring C and its close proximity to the oxygen lone pairs of the C-11 hydroxyl group.[13] An analogous A ring to the one proposed for euroabienol, found in isopimarane diterpenes previously isolated from this species,[9,10] had the chemical shift value for H-1β in the range 4.84–4.97 ppm. Finally, the HMBC spectrum showed correlation between the H-6α proton (δ 5.06) and the carboxyl carbon of one of the acetates (δ 170.4 s), establishing that the second acetate group was at the 6 position. HMBC interactions that were important in the elucidation of the proposed structure are graphically represented in Figure 2. The C-15 methine proton (δ 2.79 sept) correlated with the C-16 and C-17 methyl carbons and with three aromatic carbons [δ 148.3 (C-13), 114.6 (C-12) and 121.8 (C-14)], whereas the H-7β (δ 4.47 d), H-12 (δ 6.56 d), H-14 (δ 6.77 d), Me-20 (δ 1.74 s) and the C-11 phenolic proton (δ 6.48 s) showed connectivities with C-9 carbon (δ 127.5).

These correlations confirmed the partial structure of rings B and C of **1**. Moreover, the methoxyl protons (δ 3.76, 3H, s), as well as the H-5 (δ 3.23 d) and the Me-18 protons (δ 1.46 s), showed connectivity with the carboxyl carbon at δ 178.1 (C-19), thus confirming the partial structure of ring A and establishing that the carbomethoxyl group of **1** must be attached to the C-4 position. Finally, the stereochemistry depicted in **1** was established from the NOESY spectrum. The axial H-5α proton showed cross-peaks of NOE with the H-6α and H-3α (δ 2.15), whereas the pseudoequatorial H-7β proton showed NOE with the H-14 and H-6 (pseudoequatorial as well) protons. The relative configurations of chiral centers on ring A were inferred from the NOESY interactions of Me-18 with Me-20 and H-1.

The previously mentioned compound (leonubiastrin,[12] from *Leonurus marrubiastrum*, Lamiaceae, subfamily Lamioideae) that resembles the most to the structure of euroabienol, is epimeric at carbon 4, and this is, from a biogenetic point of view, of interest to note, since a different set (or one) of enzymes seem to be operating in these species and puts further evidence in the placement of these genera in different subfamilies (Lamioideae and Nepetoideae). This is the first report on the occurrence of abietanes in the genus *Lycopus*. These metabolites (leonubiastrin and euroabienol) possess an unusual monohydroxylation in ring C, instead of the more frequent 11,12-dioxygenation pattern, adding interest to the present study. However, one should note the similarities that exist in the

Table 1
^{1}H NMR, ^{13}C NMR, and NOESY spectroscopic data for compound **1**

Position	δ_C[a] (ppm)	δ_H[b] (ppm)	NOESY
1	74.6	β: 6.32 (1H, br s)	20β-Me, H-2α, H-2β
2	22.1	α: 1.96 (1H, m)	H-3α, H-2β, H-1β
		β: 2.12 (1H, m)	H-3β, H-2α, H-1β
3	31.3	α: 2.15 (1H, m)	H-5α, H-2α, H-3δ
		β: 1.49 (1H, m)	H-3α, H-2β
4	47.6	/	/
5	37.3	α: 3.23 (1H, s)	H-6α, H-3α
6	75.2	α: 5.06 (1H, br s)	19α-COOMe, 18β-Me, H-7β, H-5α
7	70.2	β: 4.47 (1H, d, *J* = 1.5 Hz)	H-14, H-6α
8	136.2	/	/
9	127.5	/	/
10	42.5	/	/
11	153.3	/	/
12	114.6	6.56 (1H, d, *J* = 1.8 Hz)	H-15, H-16, H-17
13	148.3	/	/
14	121.8	6.77 (1H, d, *J* = 1.8 Hz)	H-17, H-16, H-15
15	33.1	2.79 (1H, septet, *J* = 7 Hz)	H-17, H-16, H-14, H-12
16	23.4	1.21 (3H, d, *J* = 7 Hz)	H-15
17	23.8	1.20 (3H, d, *J* = 7 Hz)	H-15
19	178.1	/	/
18 β-Me	18.2	1.46 (3H, s)	20β-Me, H-6α
20 β-Me	21.7	1.74 (3H, s)	18β-Me, H-1β
1α-OAc	171.4	/	/
	21.2	1.81 (3H, s)	/
6β-OAc	170.4	/	/
	21.4	2.03 (3H, s)	/
19α-COO*Me*	42.4	3.76 (3H, s)	H-6α
11-O*H*	/	6.48 (1H, s)	/
7α-O*H*	/	2.68 (1H, br s)	/

/- no interactions observed.
[a] Spectra were measured in $CDCl_3$ (125 MHz).
[b] Spectra were measured in $CDCl_3$ (500 MHz).

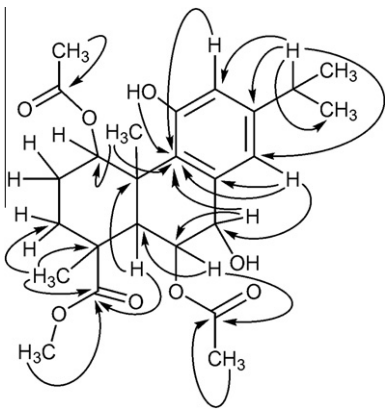

Figure 2. Important HMBC interactions of compound **1**.

Table 2
Antimicrobial activity of compound **1**—minimum inhibitory concentrations (MIC) and minimum bactericidal and fungicidal concentrations (MBC/MFC)

Bacterial strain		MIC (mg/mL)	MBC/MFC (mg/mL)	Tetracycline (μg/mL)	Nystatine (μg/mL)
Gram-negative					
E. coli	Clinical isolate	0.358	0.716	0.195	NT
E. coli	Food isolate	0.716	5.735	1.562	NT
E. coli	ATCC 25922	0.179	2.867	1.562	NT
K. pneumoniae	ATCC 10031	0.179	1.433	0.390	NT
K. pneumoniae	Clinical isolate	0.045	0.716	0.390	NT
P. aeruginosa	ATCC 9027	0.358	2.867	1.562	NT
P. vulgaris	ATCC 8427	0.179	5.735	0.390	NT
S. enterica	ATCC 13076	0.179	1.433	0.195	NT
Gram-positive					
B. subtilis	ATCC 6633	0.179	2.867	0.195	NT
B. cereus	Food isolate	0.089	0.716	0.195	NT
C. pyogenes	ATCC 19411	0.179	1.433	1.562	NT
S. aureus	ATCC 25923	0.179	5.735	0.098	NT
S. aureus	ATCC 6538	0.022	2.867	0.098	NT
S. aureus	Food isolate	0.022	0.716	0.098	NT
S. lutea	Food isolate	0.045	0.179	0.390	NT
Fungal strain					
C. albicans	ATCC 10231	0.358	1.433	NT	6.250
S. cerevisiae	ATCC 9763	0.089	0.089	NT	12.50
A. restrictus	Isolate	1.433	2.867	NT	0.781
A. chrysogenum	Isolate	>5.735	>5.735	NT	0.195
A. fumigatus	Isolate	0.089	0.358	NT	0.195
P. chrysogenum	Isolate	1.433	2.867	NT	0.390

NT—not tested.

oxygenation pattern of rings A and B of euroabienol and of isopimarane diterpenes already isolated from the aerial parts of *L. europaeus*.[9,10]

Through GC–MS analyses of CH_2Cl_2 extracts of the aerial parts free of any fruits we ascertained that euroabienol was present in other plant organs of *L. europaeus* and that it was either missed out in previous studies or that the population from Niš represents a new chemotype of this plant taxa. Although other variables that might result in the alteration of the secondary metabolites profile of *L. europaeus* such as harvest season, plant infection, insect attack etc., should not be dismissed.

Phenolic diterpenes are widely distributed natural products in the plant kingdom with various biological activities, for example, antioxidant,[14–17] antimicrobial,[18–22] cytotoxic,[23–25] anticancerogen,[26] anti-HIV[27] activities. Having this on mind and trying to decipher the possible biological role of euroabienol, compound **1** was screened for its in vitro antimicrobial activity against 15 strains of bacteria and six fungal strains using a microdilution assay. The obtained results are listed in Table 2. As it can be seen, compound **1** showed moderate antimicrobial activity compared to the standard antibiotics (Tetracycline and Nystatine) with no special selectivity towards any particular microorganism, but having a broad spectrum of activity. Against bacteria, this compound was active in the range from MIC = 0.022 mg/mL to 0.716 mg/mL, while against fungal strains, the active concentrations were in the range from 0.089 mg/mL to 1.433 mg/mL. The greatest reduction of bacterial growth was noted in the case of *Staphylococcus aureus* (both isolate and ATCC 6538 strains), but the lowest MBC value of 0.179 mg/mL was observed in the case of *Sarcina lutea*. On the other hand, the most resistant bacterial strain was *Escherichia coli* with the highest MIC and MBC values of 0.716 mg/mL and 5.735 mg/mL, respectively. Generally, the activity of compound **1** was slightly higher against Gram-positive compared to Gram-negative bacteria, whose greater resistance probably comes from the presence of certain cell-wall lipopolysaccharides.[28] Also, compound **1** manifested a wide range of antifungal activities—from being completely inactive against *Acremonium chrysogenum* to the activity that is comparable with the one of the standard used as the positive control. The most sensitive strains were the yeast *Saccharomyces cerevisiae* (MIC = MFC = 0.089 mg/mL), unexpectedly since it was the most resistant to Nystatine, and *Aspergillus fumigatus* (MIC = 0.089 mg/mL; MFC = 0.358 mg/mL).

Several previous studies that have dealt with antibacterial activity of similar abietane-type diterpenes but with the OH and isopropyl groups in *ortho* position reported MIC values against *S. aureus* in the range of 0.008–0.150 mg/mL (and higher) depending on the structure of the rest of the diterpene molecule.[19,21,22]

Urzúa et al. recently conducted a structure–activity study of various antimicrobial diterpenoids.[29] They concluded that diterpenes that possessed a substituted decalinic system, capable of insertion into a lipophilic region, and a hydrophilic fragment possessing one hydrogen-bond-donor (HBD) group, capable of interactions with hydrogen-bond-acceptor groups in the membrane were the most active ones. Compound **1** has these structural features and once again confirms their findings. Additionally, they proposed that the presence of two OH groups in the hydrophilic moiety of the molecule actually reduced its incorporation into the membrane (assuming such a mode of action for compound **1**). Acetylation of one of these groups had the result of increasing its incorporation. This might be a further direction of activity enhancement that could be perused in the case of compound **1** as well.

Ferruginol is a related phenolic abietane diterpene with a MIC value of 8 μg/mL and possessing the *ortho* orientation of the phenolic and isopropyl groups on ring C.[30] The difference in activity between these two might be sought in the intramolecular interactions between two hydrophilic groups (C1-OAc and C11-OH) observable in compound **1**, which might compete with intermolecular hydrogen-bonds between the HBD groups and the cell membrane.[29]

Further investigation is required on the specific mode of action of this compound in order to rationalize these observations.

The broad spectrum antimicrobial activity and high concentration of compound **1** in the fruits (ca. 1% w/w) make it an excellent first line defense plant metabolite candidate against pathogen attack (good examples of compounds having such a function are the fungal metabolites azaphilones[31] and 'green leaf' plant volatiles[32]).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.07.063.

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