Antibacterial Diterpenes from the Roots of *Salvia blepharochlaena*

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The roots of *Salvia blepharochlaena* have yielded two new diterpenoids, blephaein (**1**) and *O*-methylpisiferic acid methyl ester (**2**), together with eight known diterpenoids. The structures of the new compounds were established by spectroscopic analysis and by some chemical reactions. Potent antibacterial activity was exhibited by the known compounds horminone (**9**) and 7-acetylhorminone (**10**) against *Staphylococcus aureus* ATCC 6538 P, *Staphylococcus epidermidis* ATCC 12226, and *Bacillus subtilis* ATCC 6633. Horminone was also found to be active against *Enterococcus faecalis* ATCC 29212.

The antibacterial, antituberculous, and antiphlogistic activities of the constituents of *Salvia* species are well established.[1] In a recent study we have established antituberculous activity in some diterpenoids obtained from *Salvia multicaulis*.[2] Three of these diterpenoids were the same compounds (**5**−**7**) obtained in the present investigation from the roots *Salvia blephoraclaena* Hedge and Hub. Mor. (Lamiaceae). Diterpenoids were tested against *M. tuberculosis* and against standard bacterial strains such as *S. aureus* ATCC 6538 P, *S. epidermidis* ATCC 12226, *E. faecalis* ATCC 29212, *B. subtilis* ATCC 6633, *E. coli* ATCC 8739, *P. mirabilis* ATCC 14153, *Kl. pneumonia* ATCC 4352, *Ps. aeruginosa* ATCC 27853, and the yeast *C. albicans* ATCC 10231. Multicaulin (**5**), multiorthoquinone (**6**), and demethylmultiorthoquinone (**7**) showed strong antituberculous activity as established previously,[2] while horminone (**9**) and 7-acetylhorminone (**10**) were active against *S. aureus*, *S. epidermidis*, and *B. subtilis*. Compound **9** was also active against *E. faecalis*, *O*-methylpisiferic acid (**4**) was active only against *B. subtilis*, and ferruginol (**8**) had slight activity against the above given microorganisms except *E. faecalis* (Table 1).

Salvia blepharochlaena, a perennial herb, was collected from central Turkey. The dried roots of the plant material yielded 10 diterpenoids in the present investigation, with two of them being new compounds, namely, blephaein (**1**) and *O*-methylpisiferic acid methyl ester (**2**). The structures of the known compounds, pisiferic acid (**3**),[3,4] *O*-methylpisiferic acid (**4**),[5] multicaulin (**5**),[2] multiorthoquinone (**6**),[2] demethylmultiorthoquinone (**7**),[2] ferruginol (**8**),[6] horminone (**9**),[7] and 7-acetylhorminone (**10**),[7] were established by comparing their spectral data to those given in the literature and by TLC comparison with authentic samples. The ^{1}H NMR spectrum of blephaein (**1**) indicated the presence of four methyl signals at δ 0.80 (3H, s, Me-18), 1.05 (3H, s, Me-19), 1.17 (3H, d, J = 7 Hz), and 1.18 (3H, d, J = 7 Hz) (Me-17 and Me-16). The latter two signals, together with the signal at δ 3.25 (1H, septet, J = 7 Hz, H-15), showed the presence of an isopropyl group. The signal at δ 3.85 (3H, s) indicated a methoxy group on the aromatic ring, while the signal at δ 11.5 (1H, s) showed the presence of an acid hydroxyl proton, which was verified by the large shoulder at 2850 cm^{-1}, and the peak at 1695 cm^{-1} in its IR spectrum and at δ 182.2 in its ^{13}C NMR spectrum were consistent with an acid carbonyl. Other ^{1}H NMR signals of compound **1** were at δ 7.10 (1H, s, H-11) and 6.90 (1H, s, H-14), indicating aromatic protons, and a double doublet at δ 6.35 (1H, dd, J = 3, 8 Hz, H-6), which showed the presence of a double bond at C-5. Spin-decoupling experiments suggested a relationship between the H-6 (δ 6.35) and H-7 protons at δ 2.60 (m) and 1.80 (m). The HRMS of **1** indicated a molecular formula of $C_{21}H_{28}O_3$ (*m*/*z* 328.2058, calcd 328.2038), showing eight degrees of unsaturation as double bond equivalents, of which three were accounted for by a tricyclic ring system, four by double bonds, and the remaining one by the carbonyl group of the acid. The UV spectrum with a maximum at 240 nm indicated the presence of an unconjugated aromatic ring. The ^{13}C NMR (APT) signals revealed the presence of five methyls, four methylenes, four methines, and eight quaternary carbon signals, for 21 carbons in the molecule. The structure of blephaein **1** was thus deduced as 12-methoxyabieta-5,8,11,13-tetraen-20-oic acid and was supported by HETCOR and COLOC experiments (Table 2).

The HRMS of the second new compound, *O*-methylpisiferic acid methyl ester (**2**), indicated the molecular formula $C_{22}H_{32}O_3$ (*m*/*z* 344.2356, calcd 344.2352), showing seven degrees of unsaturation as double bond equivalents, of which three were accounted for by a tricyclic ring system, three by double bonds, and one by an the ester carbonyl group. The ^{1}H NMR spectrum of **2** showed aromatic proton signals at δ 6.88 (1H, s, H-11) and 6.76 (1H, s, H-14) and methoxy groups at δ 3.76 (3H, s, C-12) and 3.48 (3H, s, acid ester methyl group). The signal at δ 3.18 (1H, septet, J = 7 Hz, H-15) together with the signals at δ 1.14 (3H, d, J = 7 Hz) and 1.16 (3H, d, J = 7 Hz) (Me-16 and Me-17) suggested the presence of an isopropyl group. Two other methyl groups were observed at δ 0.95 (3H, s, Me-19) and 0.88 (3H, s, Me-18). The ^{13}C NMR spectrum showed the presence of six methyl groups, of which two were methoxy groups, five methylenes, four methines, and seven quaternary carbon signals for 22 carbon atoms. HETCOR and COLOC experiments supported the structural determination of **2** as 12,20-dimethoxyabieta-8,11,13-trien-20-oic acid methyl ester. The difference between compounds **2** and **4**

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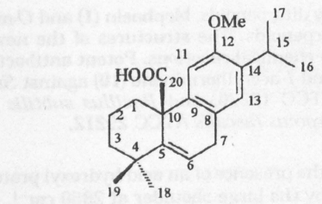
Table 1. Antibacterial Activity (MIC)[a] of Compounds **4** and **8**–**10**

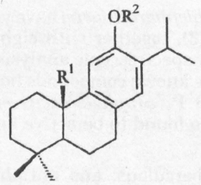
	organism[c]								
compd[b]	*S. aureus*	*S. epidermidis*	*E. faecalis*	*B. subtilis*	*E. coli*	*P. mirabilis*	*K. peneumonia*	*P. aeruginosa*	*C. albicans*
4	NA[d]	NA	NA	6.5	NA	NA	NA	NA	NA
8	>250	>250	NT[e]	>250	NT	NT	NT	NT	NT
9	6.5	1.5	14	1.5	NA	NA	NA	NA	NA
10	10	6	NA	3	NA	NA	NA	NA	NA

[a] Minimal inhibitory concentrations of the compounds are given in μg/mL. [b] The highest concentrations used were as follows: **4**, 10.7 μg/mL; **8**, 9.6 μg/mL; **9**, 10.5 μg/mL; **10**, 15.2 μg/mL. [c] Key to organisms: *Staphylococcus aureus* ATCC 6538, *Staphylococcus epidermidis* ATCC 12228, *Bacillus subtilis* ATCC 6633, *Enterococcus faecalis* ATCC 29212. [d] NA = not active (determined by the disk-diffusion method). [e] Not tested.

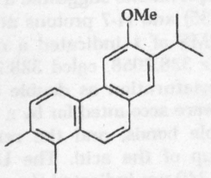
Chart 1



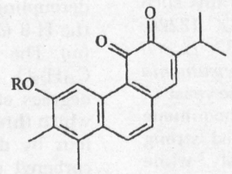
1



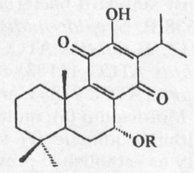
	R^1	R^2
2	COOMe	Me
3	COOH	H
4	COOH	Me
8	Me	H



5



6 R=Me
7 R=H



9 R=H
10 R=Ac

is that the former compound is a methyl ester of the latter. Compound **4** was therefore methylated, to yield the *O*-methylpisiferic acid methyl ester. The IR and ^{1}H NMR spectra of compound **2** and the methylated product of **4** were identical.

Experimental Section

General Experimental Procedures. Optical rotations were determined with an Optical Activity AA-5 LTD polarimeter. The UV spectra were recorded on a Shimadzu UV-1601 spectrophotometer. The IR spectra were measured on a Perkin-Elmer model 983 spectrometer; the NMR spectra, on Bruker AC-200 and 400 MHz instruments; and the HRMS and EIMS, on a VG ZabSpec instrument. Chromatographic separations were carried out on Si gel columns and ready-made TLC plates (1 mm thick, E. Merck).

Plant Material. The roots of *Salvia blepharochlaena* were collected from central Turkey (Nevşehir, Aktepe, at 1050 m altitude) in June 1999 by Dr. G. Kökdil and D. Kara and were identified by Prof. Dr. M. Vural (Ankara, Faculty of Sciences). A voucher specimen was deposited in the Herbarium of the University of Ankara, Faculty of Pharmacy (AEF 21128).

Extraction and Isolation. Dried and powdered roots of the plant material (1.8 kg) were extracted in a Soxhlet with acetone until exhaustion. The acetone extract was evaporated in vacuo to yield 54.2 g of a gummy residue. This residue was dissolved in the least possible amount of CH_2Cl_2 and mixed with Si gel, dried at room temperature, and added to the top of a Si gel column (5 × 80 cm). The column was eluted with hexane, and a gradient of CH_2Cl_2 was added up to 100%, followed by EtOH. Fractions showing similar spots were combined to yield five main fractions (A–E). Fraction A contained only fatty substances and fraction E steroidal compounds, which were not studied further. Fractions B–D were applied to smaller chromatographic columns (2 × 40 cm) and eluted with hexane and gradients of CH_2Cl_2 followed by EtOH. When necessary, compounds were finally purified on preparative TLC plates. Fraction B [eluted with hexane–CH_2Cl_2 (4:6)] yielded blephaein (**1**) (18 mg), multicaulin (**5**) (7 mg), multiorthoquinone (**6**) (5 mg), and demethylmultiorthoquinone (**7**) (10 mg). Fraction C [eluted with CH_2Cl_2] yielded pisiferic acid (**3**) (12 mg), *O*-methylpisiferic acid (**4**) (12 mg), and ferruginol (**8**) (15 mg), while fraction D [eluted with CH_2Cl_2–EtOH (99:1)] afforded horminone (**9**) (25 mg), 7-acetylhorminone (**10**) (19 mg), and *O*-methylpisiferic acid methyl ester (**2**) (20 mg).

Blaphaein (1): amorphous, colorless powder; $[\alpha]^{22}_D$ +14.3° (*c* 0.1, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 240 (3.9) nm; IR ($CHCl_3$) ν_{max} 2850 (br sh), 1695, 1605, 1500, 1480, 1360, 1240, 1160, 1060, 900, 840, 735 cm^{-1}; ^{1}H NMR and ^{13}C NMR (in $CDCl_3$), see Table 2; EIMS *m/z* 328 $[M]^+$ (3), 300 $[M - CO]^+$ (30), 284 $[M - COO-]^+$ (100), 269 $[284 - Me]^+$ (85), 253 (7), 241 (25), 228 (35), 213 (40), 185 (20), 165 (15), 126 (10), 91 (7), 82 (13), 69 (12); HRMS *m/z* 328.2058 calcd for $C_{21}H_{28}O_3$, 328.2038.

***O*-Methylpisiferic acid methyl ester (2):** amorphous, colorless powder; $[\alpha]^{22}_D$ +33° (*c* 0.2, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 270 (3.2), 240 (3.5); IR ($CHCl_3$) ν_{max} 2940, 2820, 1720, 1610, 1510, 1450, 1430, 1250, 1200, 1100, 1040, 950, 800 cm^{-1}; ^{1}H NMR and ^{13}C NMR ($CDCl_3$), see Table 2; EIMS *m/z* 344 $[M]^+$ (18), 328 $[M - Me - H]^+$ (52), 285 $[M - COOMe]^+$ (100), 269 $[285 - Me - H]^+$ (37), 242 (20), 212 (35), 203 (40), 191 (35), 179 (32), 83 (15), 69 (40); HRMS *m/z* 344.2356, calcd for $C_{22}H_{32}O_3$.

Table 2. NMR Data of Compounds **1** and **2**

	1			**2**		
	^{1}H	^{13}C	COLOC	^{1}H	^{13}C	COLOC
1α	2.75 m	29.8 t	H-6, H-11	1.3 m	29.3 t	H-11
1β	2.00 m			2.85 m		
2α	1.30 dd (5, 13)	18.0 t		1.50 dd (6, 12)	18.5 t	
2β	2.25 m			2.90 ddd (5,6,12)		
3α	1.45 dd (4, 12)	34.2 t		1.65 m	36.8 t	
3β	2.20 m			1.45 m		
4		37.0 s	H-6, H-18, H-19		34.9 s	
5		133.4 s	H-6, H-18, H-19	1.47 m	52.0 d	
6α	6.35 dd (3, 8)	101.1 d	H-14	2.42 m	20.3 t	
6β				1.50 m		
7α	2.60 m	38.9 t	H-11, H-14	2.70 m	41.7 t	
7β	1.80 m			1.60 m		
8		136.0 s	H-11, H-14		136.5 s	H-11, H-14
9		128.0 s	H-11, H-14		128.5 s	H-11, H-14
10		47.0 s			47.6 s	
11	7.10 s	125.8 d	H-15	6.88 s	127.0 d	H-14, H-15
12		156.3 s			156.0 s	
13		136.4 s			138.0 s	
14	6.90 s	106.9 d	H-11, H-15	6.76 s	107.4 d	H-11, H-15
15	3.25 sept (7)	33.1 d	H-11	3.18 sept (7)	33.1 d	H-11, H-14
16	1.18 d (7)	22.7 q	H-15	1.14 d (7)	22.4 q	H-15
17	1.17 d (7)	22.7 q	H-15	1.16 d (7)	22.7 q	H-15
18	0.80 s	23.1 q		0.88 s	20.2 q	
19	1.05 s	26.5 q		0.95 s	26.4 q	
20		182.2 s	H-1, H-6, H-11	-	180.0 s	H-1, H-11
21	3.85 s	55.1 q		3.76 s	55.4 q	
22				3.48 s	52.4 q	

Hydrogenation of Blephaein (1). Compound **1** (8 mg) was dissolved in 1 mL of MeOH, 50 mg of Pd/C (10%) were added, and hydrogen gas was passed through the solution for 2 h. The reaction mixture was filtered, evaporated to dryness, dissolved in CH_2Cl_2, and purified on a preparative TLC plate, with the reaction yield being 6 mg. The IR and ^{1}H NMR spectra of the hydrogenated product of **1** and *O*-methylpisiferic acid (**4**) were the same.

Methylation of *O*-Methylpisiferic Acid (4). Compound **4** (10 mg) was dissolved in MeOH and treated with excess CH_2N_2 in Et_2O at 5 °C. On evaporation of the solvent to dryness, a product was obtained and purified on a preparative TLC plate, with the reaction yield of **4** being 7.3 mg. The methyl ester of **4** and compound **2** were the same.

Microbiological Activity Tests. The broth microdilution method was used[8,9] for antituberculous activity. The details of the procedure were given in ref 2. The disk-diffusion method[10,11] was used to determine the inhibition zones of the diterpenoids against standard bacterial strains. Compounds **4** and **8**–**10**, with inhibition zones greater than 7 mm, were selected for a tube dilution test[12] to determine the antibacterial activity quantitatively as minimum inhibition concentrations (MIC).

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