Linear Sesquiterpene Lactones from *Anthemis auriculata* and Their Antibacterial Activity

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Three linear sesquiterpene lactones, anthecotulide (**1**), hydroxyanthecotulide (**2**), and acetoxyanthecotulide (**3**), were isolated from the aerial parts of *Anthemis auriculata.* Five known flavonoids, taraxa-20(30)en-3β-ol (**4**), and methyl vanillate (**5**) were also isolated. The structures and the relative configuration of the new compounds **2** and **3** were deduced by spectroscopic methods. The in vitro activity of compounds **1**−**5** against 10 bacterial species and one fungus was tested using the microdilution method.

The genus *Anthemis* comprises about 130 species predominately distributed around the Mediterranean, but species are also found in Southwest Asia and South Africa, several of which are aromatic, herbal medicines, insecticides, and dyes.[1,2]

Anthemis auriculata Boiss. (Asteraceae), an annual herb, is an endemic plant of the South Balkan peninsula and Turkey.[1] As a continuation of our research on Greek *Anthemis* species,[3] we report here on the chemical profile and the antibacterial activity of secondary metabolites isolated from *A. auriculata.*

A lipophilic extract of the aerial parts of *A. auriculata* was chromatographed on a silica gel column using cyclohexane containing increasing amounts of EtOAc and acetone. Further chromatography of the main fractions afforded three linear sesquiterpene lactones, anthecotulide (**1**),[4−6] **2**, and **3**, as well as taraxast-20(30)-en-3β-ol (**4**)[4,5] and methyl vanillate (**5**).[7] From a polar extract of the same plant material five known flavonoids, namely, apigenin,[8] luteolin,[8] pectolinaringenin,[9] eriodictyol,[10] and luteolin 7-*O*-β-D-glucopyranoside,[11] were isolated. Compounds **2** and **3** are new natural products. The structures of the compounds were established by means of 1D and 2D NMR, MS, and UV spectroscopic analysis. The main constituents of *A. auriculata* were flavonoids, while sesquiterpene lactones were isolated in smaller amounts.

Compound **2** (Figure 1) showed in its mass spectrum a molecular ion $[M + H]^+$ at *m*/*z* 265.1440, compatible with the molecular formula $C_{15}H_{20}O_4$. The IR spectrum exhibited absorption bands typical of hydroxyl (3400 cm^{-1}) and carbonyl groups, 1770 (C═O, γ-lactone) and 1715 (C═O, ketone). The ^{13}C NMR spectrum of compound **2** displayed 15 carbons, which were assigned by HSQC, HMBC, and DEPT 135° experiments to the resonances of three tertiary methyls, one olefinic and two aliphatic methylenes, one oxygenated, one nonoxygenated, and two olefinic methines, three quaternary carbons, and two carbonyls. The presence of an α-methylene-γ-lactone moiety was confirmed by the ^{13}C NMR signals at δ 170.9 (OCO) and 134.3 and 124.6 ppm ($C═CH_2$). Detailed 2D NMR analysis showed that compound **2** is a linear type sesquiterpene lactone. The ^1H spin systems H-3/H-15a/H-15b and H-3/CH_2-14 were assigned by COSY. HMBC correlations between H-15a, H-15b/C-1, H-14a, H-14b/C-1, and H-3/C-1 suggested that these protons belong to a lactone ring. Further COSY correlations H-3/H-4 and H-4/H-5, as well as HMBC cross-peaks H-13/C-5, H-13/C-6, H-7/C-6, H-7/C-8, H-9/C-8, H-9/C-12, and H-9/C-11, revealed the structure as shown. The *trans*-configuration of the 5,6-double bond was confirmed by NOESY experiments.

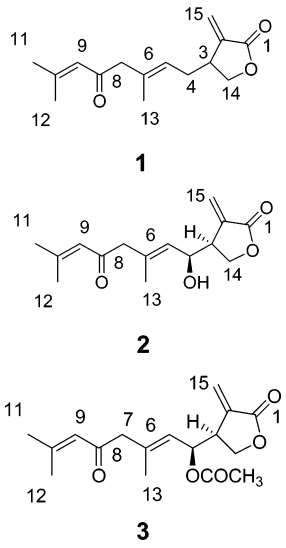

Figure 1. Structure of the isolated sesquiterpene lactones.

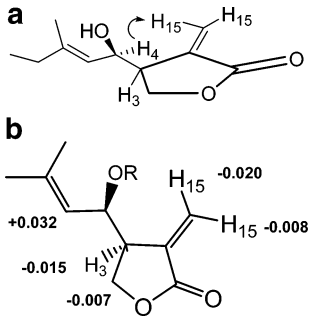

Figure 2. (a) Relative configuration of compound **2**. (b) Chemical shift differences, Δ_δ ($\delta_S - \delta_R$), for the (*S*)- and (*R*)-MTPA esters of compound **2**.

NOE interactions between H-3/H-15b and H-4/H-15b indicated that these protons have the same orientation (Figure 2a). From the above observations, compound **2** was assigned as (+)-4-[(*E*)-1-hydroxy-3,7-dimethyl-5-oxo-octa-2,6-dienyl]-3-methylenedihydrofuran-2-one [4-hydroxyanthecotulide].

The (3*S*,4*S*)-configuration of 4-hydroxyanthecotulide was confirmed by Mosher's ester methodology.[12] The (*S*)- and (*R*)-MTPA esters of compound **2** were prepared, and chemical shifts of the H-5, H-3, H-14a, H-15a, and H-15b protons were measured for both derivatives. Using $\Delta_\delta = \delta_S - \delta_R$ methodology for H-5, a positive value for Δ_δ was observed, and for protons H-3, H-14a,

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Table 1. ^{1}H NMR and ^{13}C NMR Data and HMBC Correlations for Compounds **2** and **3** (in $CDCl_3$)

	2[α]			**3**[α]		
position	δ_H	HMBC	δ_C	δ_H	HMBC	δ_C
1		H-3, H-14, H-15	170.9		H-14, H-15	170.9
2		H-3, H-14	134.3		H-3, H-14	135.0
3	3.13 m	H-4, H-14, H-15	44.2	3.39 m	H-4, H-14, H-15	42.2
4	4.41 dd (8.8, 7.8)	H-3, H-14	69.2	5.63 dd (9.3, 6.4)	H-14	71.9
5	5.23 brd (8.8)	H-3, H-4, H-13	128.4	5.19 brd (9.3)	H-13	128.1
6		H-4, H-7, H-13	135.5		H-7, H-13	138.1
7a	3.14 d (15.6)	H-5, H-13	54.4	3.09 brs	H-5, H-13	54.9
7b	3.07 d (15.6)					
8		H-7, H-9, H-11, H-12, H-13	198.2		H-7	198.0
9	6.04 s	H-11, H-12	122.9	6.03 brs	H-11, H-12	125.6
10		H-11, H-12	157.5		H-11, H-12	157.0
11	1.87 s	H-9, H-12	27.7	1.87 s	H-12	27.8
12	2.10 s	H-9, H-11	20.8	2.12 s	H-11	21.0
13	1.63 s	H-7	17.5	1.78 s	H-7	15.1
14a	4.37 dd	H-3, H-4, H-15	67.8	4.42 dd (9.3, 8.8)	H-4	67.8
14b	(6.9, 4.4)			4.21 dd (9.3, 4.9)		
15a	6.27 d (2.4)		124.6	6.37 d (2.4)		125.2
15b	5.77 d (2.4)			5.89 d (2.4)		
CH$_3$COO				2.15 s		20.9
CH$_3$*CO*O					CH$_3$	168.0

Table 2. Minimum Inhibitory Concentrations (MICs) of Compounds **1**−**5** (μM)

	Escherichia coli	*Proteus mirabilis*	*Agrobacterium tumefaciens*	*Pseudomonas aeruginosa*	*Pseudomonas tolaasii*	*Salmonella enteritidis*	*Staphylococcus aureus*	*Micrococcus luteus*	*Sarcina lutea*	*Bacillus cereus*	*Candida albicans*
1	101	202.0	101.0	202.0	202.0	202.0	101.0	101.0	51.0	51.0	101.0
2	189	189.0	94.7	189.0	189.0	189.0	47.0	94.7	47.0	94.7	189.0
3	81.7	163.4	81.7	163.4	163.4	163.4	81.7	81.7	40.8	81.7	81.7
4	117.4	117.4	29.3	117.4	117.4	117.4	29.3	58.7	14.7	58.7	117.4
5	274.7		274.7		274.7	274.7	137.4	137.4	137.4	137.4	274.7
streptomycin	68.6	137.2	68.6		137.2	137.2	34.3	34.3	34.3	34.3	
bifonazole											643.7

H-15a, and H-15b a negative value for Δ_δ was observed, respectively, indicating that these protons should be placed as shown in Figure 2b.

Compound **3** (Figure 1) showed in its mass spectrum a molecular ion $[M + H]^+$ at *m*/*z* 307.1474, compatible with the molecular formula $C_{17}H_{22}O_5$. The IR spectrum showed absorption bands typical of hydroxyl (3380 cm^{-1}) and carbonyl groups, 1770 (C═O, γ-lactone), 1718 (C═O, ketone), and 1735 (C═O, ester). The ^{13}C NMR spectrum of compound **3** displayed 17 carbons, which were assigned by HSQC, HMBC, and DEPT 135° experiments to the resonances of four tertiary methyls, one olefinic and two aliphatic methylenes, one oxygenated, one nonoxygenated, and two olefinic methines, three quaternary carbons, and three carbonyls. Interpretation of the ^{1}H NMR spectra showed structural similarities with compound **2** with the exception of the presence of an acetyl group, the position of which was determined at C-4 on the basis of the chemical shift of H-4 at δ_H 5.63 versus δ_H 4.41 in compound **2**. Moreover, the position of the acetyl group was assigned on the basis of the HMBC spectrum, due to the observed correlation between the carbonyl group and the H-4 proton. NOE interactions between H-3/H-15b and H-4/H-15b indicated that these protons have the same orientation. Consequently, compound **3** was assigned as (+)-4-[(*E*)-1-acetoxy-3,7-dimethyl-5-oxo-octa-2,6-dienyl]-3-methylenedihydrofuran-2-one [4-*O*-acetylanthecotulide].

This is only the second report of linear sesquiterpene lactones in the genus *Anthemis*. The first report was on *A. cotula* L.[4−6] belonging to sect. Maruta, while *A. auriculata* belongs to sect. Anthemis.

The isolated compounds were active against several bacteria and one fungus. Compound **5** was inactive against the clinical species of *Proteus mirabilis* and *Pseudomonas aeruginosa* (Table 2). Comparing these results with our previously published data[3] on the antimicrobial potential of sesquiterpene lactones of *A. altissima*, it is concluded that linear lactones are more active. In particular, they are almost twice as active against *Bacillus cereus* and *Staphylococcus aureus*, while they showed a moderate activity against *P. mirabilis* and *P. aeruginosa*, opposite of sesquiterpene lactones from *A. altissima*, which were inactive against these species. This differentiation in antimicrobial activity could be explained in terms of solubility, since the linear lactones are more lipophilic than the oxygenated eudesmanolides and germacranolides of *A. altissima*.

Experimental Section

General Experimental Procedures. Optical rotations were measured at 20 °C, in $CHCl_3$ (Uvasol), on a Perkin-Elmer 341 polarimeter. IR spectra were obtained on a Perkin-Elmer Paragon 500 FT-IR spectrophotometer. The ^{1}H NMR spectra (400 MHz) and ^{13}C NMR spectra (50.3 MHz) were recorded using Bruker DRX 400 and Bruker AC 200 spectrometers, respectively. Chemical shifts are reported in δ (ppm) values relative to TMS. COSY, HSQC, HMBC, and NOESY (mixing time 950 ms) were performed using standard Bruker microprograms. MS data were recorded at the University of Notre Dame, Department of Chemistry and Biochemistry, South Bend, IN. HR/EM FABMS data were recorded on a double-sector JEOL JMS-AX505HA mass spectrometer. Vacuum-liquid chromatography (VLC):[13] silica gel (Merck; 43−63 μm). Column chromatography: silica gel 60H SDS (40−63 μm), gradient elution with the solvent mixtures indicated in each case. Reversed-phase chromatography: CE 1100 liquid chromatography. HPLC support: Preparative HPLC was performed using a C_{18} 25 cm × 10 mm Kromasil column. Fractionations were monitored by TLC silica gel 60 F-254, Merck, art. 5554; Cellulose, Merck, art. 5716; RP 18 F-254, Merck, art. 5685 with visualization under UV (254 and 365 nm) with anisaldehyde−sulfuric acid reagent on silica gel and Neu's reagent on cellulose.[14]

Plant Material. The aerial parts of *A. auriculata* were collected at Gerania (Corinthia, Central Greece) in April 2001. The plant was authenticated by Dr. T. Constantinidis (Institute of Systematic Botany, Agricultural University of Athens), and a voucher specimen was deposited in the Herbarium (ACA-Constantinidis 9362).

Extraction and Isolation. The fresh aerial parts of *A. auriculata* (0.48 kg) were finely ground and extracted at room temperature with

cyclohexane−Et_2O−MeOH (1:1:1; extract A) and MeOH−H_2O (1:1; extract B), successively. Extract A was washed with brine, the aqueous layer re-extracted with EtOAc, and the organic layer dried with Na_2SO_4 and concentrated under reduced pressure. The residue (5.75 g) was fractionated by VLC on silica gel (10.0 × 5.0 cm), using cyclohexane−EtOAc−Me_2CO mixtures of increasing polarity as eluents to give 11 fractions of 500 mL each. Fraction B (cyclohexane−EtOAc, 75:25, 0.56 g) was subjected to further column chromatography on silica gel (3.0 × 16.0 cm; cyclohexane−EtOAc−MeOH, 10:0:0 to 0:0:10), which led to the isolation of **4** (62.9 mg). Fraction C (cyclohexane−EtOAc, 50:50, 0.78 g) was subjected to further column chromatography on silica gel (3.0 × 18.0 cm; cyclohexane−EtOAc−MeOH, 10:0:0 to 0:0:10), allowing the isolation of **1** (157.9 mg). Further purification by reversed-phase HPLC (MeOH−H_2O, 3:1, 2 mL/min) allowed the isolation of **3** (2.4 mg, t_R 19.2 min). Fraction D (cyclohexane−EtOAc, 25:75, 0.40 g) was subjected to further column chromatography on silica gel (3.0 × 14.0 cm; CH_2Cl_2−EtOAc−MeOH, 10:0:0 to 0:0:10), which led to the isolation of **3** (32.9 mg). Further purification by reversed-phase HPLC (MeOH−H_2O, 1:1, 2 mL/min) of fraction F (EtOAc−Me_2CO, 90:10, 89.5 mg) allowed the isolation of **2** (56.0 mg, t_R 24.3 min). VLC on silica gel (10.0 × 5.0 cm; cyclohexane−EtOAc−MeOH, 10:0:0 to 0:0:10) of fraction I (Me_2CO−MeOH, 50:50; 1.92 g) afforded 13 fractions. Fraction I_{11} (EtOAc−MeOH, 10:90, 0.98 g) of the latter VLC was subjected to further column chromatography on silica gel (3.0 × 11.0 cm; CH_2Cl_2−EtOAc−MeOH, 10:0:0 to 0:0:10) and yielded compound **5** (3.0 mg). VLC of extract B (89.6 g) followed by repeated column chromatography on silica gel and Sephadex LH-20 allowed the isolation of the flavonoids.

4-Hydroxyanthecotulide [(+)-(*E*)-4-(1-hydroxy-3,7-dimethyl-5-oxo-2,6-octadienyl)-dihydro-3-methylenetetrahydrofuran-2-one] (2): oil; $[\alpha]^{20}_D$ +53.9 (*c* 0.07, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 223.0 (5.11) nm; IR ($CHCl_3$) ν_{max} 3400, 1770, 1715 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; HR/EM FABMS *m*/*z* $[M + H]^+$ 265.1440, calcd for $C_{15}H_{20}O_4$ 264.1362.

4-*O*-Acetylanthecotulide [(+)-(*E*)-4-(1-acetoxy-3,7-dimethyl-5-oxo-2,6-octadienyl)-dihydro-3-methylenetetrahydrofuran-2-one] (3): oil; $[\alpha]^{20}_D$ +3.1 (*c* 0.07, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 218.0 (3.49) nm; IR ($CHCl_3$) ν_{max} 3380, 1769, 1735, 1718 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; HR/EM FABMS *m*/*z* $[M + H]^+$ 307.1474, calcd for $C_{17}H_{22}O_5$ 306.1468.

(*S*)- and (*R*)-MTPA Esters of Compound 2. To stirred solutions of two 4.0 mg aliquots of **2** in CH_2Cl_2 (0.5 mL) were added successively (*S*)-(+)- and (*R*)-(−)-MTPA chloride (5.0 μL each), DMAP (3.0 mg each), and Et_3N (4.0 μL each) at room temperature overnight. The esters were purified by preparative TLC (cyclohexane−EtOAc, 7:3) to give the (*S*)- and (*R*)-MTPA esters of compound **2**.

Bioassays. The compounds were dissolved at 10 mg/mL with DMSO and diluted with the nutrient medium to a concentration of 1000 μg/mL. Final concentrations ranging from 14.7 to 202.0 μM were used. The proportion of DMSO never exceeded 1% in the medium.[15]

The following Gram-negative bacteria were used: *Escherichia coli* (ATCC 35218), *Proteus mirabilis* (clinical isolates), *Agrobacterium tumefaciens* (A281), *Pseudomonas aeruginosa* (clinical isolates), *Pseudomonas tolaasii* (isolated from *Agaricus bisporus*), *Salmonella enteritidis* (ATCC 13076). The following Gram-positive bacteria were used: *Staphylococcus aureus* (ATCC 6538), *Micrococcus luteus* (clinical isolates), *Sarcina lutea* (ATCC 9341), *Bacillus cereus* (clinical isolates). One fungus was used: *Candida albicans* (clinical isolates).

To obtain quantitative data, a modified microdilution technique was used.[16,17] Determination of minimum inhibitory concentrations (MICs) was performed by a serial dilution technique using 96-well microtiter plates. Bacterial species were cultured overnight at 37 °C in TSB medium (Oxoid-CM129). Suspensions contained ~10^9 cells/mL. The spore suspension was adjusted with sterile saline to a concentration of approximately 1.0 × 10^5 in a final volume of 100 μL per well. *C. albicans* was maintained on Sabouraud agar (SDA), and the cultures were stored at +4 °C and subcultured once a month.[18] The inocula were stored at +4 °C for further use. Dilutions of the inocula were cultured on TSA (Oxoid-CM 131) for the bacteria and on LB for *C. albicans* to verify the absence of contamination and to check the validity of the inoculum. The plates containing the bacteria were incubated for 48 h at 37 °C, while that of *C. albicans* was incubated for 72 h at 28 °C. DMSO was used as a control, while streptomycin for bacteria and bifonazole for *C. albicans* were used as positive controls.

Clinical isolates were directly isolated from patients at the Centre for Preventive Medicine, Military Medicinal Academy, Department of Microbiology, Belgrade, Serbia & Montenegro.

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