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Sesquiterpene Lactones from *Anthemis altissima* and Their Anti-*Helicobacter pylori* Activity

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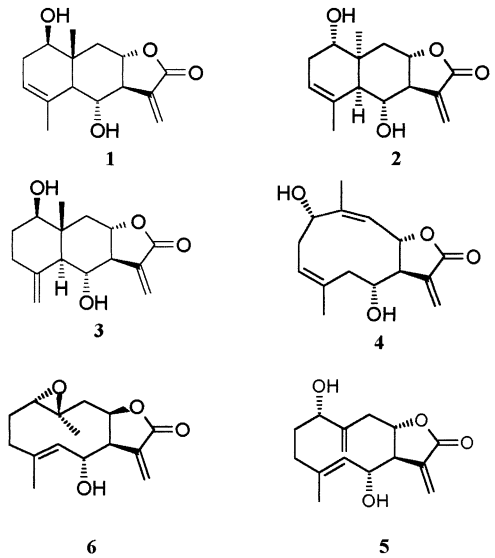
Seven sesquiterpene lactones, (−) sivasinolide (**1**), a new naturally occurring eudesmanolide (altissin, **2**), desacetyl-β-cyclopyrethrosin (**3**), tatridin-A (**4**), 1-*epi*-tatridin B (**5**), 1α,10β-epoxy-6-hydroxy-1,10*H*-inunolide (**6**), and spiciformin (**7**), were isolated from *Anthemis altissima*. Also isolated were 10 known flavonoids, namely, apigenin (**8**), kaempferol 4′-methyl ether (**9**), quercetin (**10**), quercetin 3-methyl ether (**11**), isorhamnetin (**12**), rhamnetin (**13**), 6-hydroxyquercetin 3,6,4′-trimethyl ether (**14**), isoquercetrin (**15**), taxifolin (**16**), and eriodictyol (**17**), and one phenolic acid, chlorogenic acid (**18**). The structure and the stereochemistry of compound **2** were deduced by spectroscopic methods. The in vitro activity of the sesquiterpene lactones (**1**−**5**) against *Helicobacter pylori*, as well as against three Gram-positive and three Gram-negative bacteria growing aerobically, was tested using the microdilution method. Compounds **8**−**18** have also been tested against *H. pylori*.

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

A lipophilic extract of the aerial parts of *Anthemis altissima* L. (Asteraceae) was chromatographed on a Si gel column using CH_2Cl_2 containing increasing amounts of MeOH. Further chromatography of the main fractions on silica column chromatography and on reversed-phase HPLC afforded (−)-sivasinolide (**1**),[3] altissin (**2**), a new naturally occurring eudesmanolide with a rare *cis*-decalin skeleton, desacetyl-β-cyclopyrethrosin (**3**),[4] tatridin-A (**4**),[5] 1-*epi*-tatridin B (**5**),[6] 1α,10β-epoxy-6α-hydroxy-1,10*H*-inunolide (**6**),[7] and spiciformin (**7**),[8] as well as several phenolic compounds, mainly flavonoids.



Compound **2** showed in its mass spectrum a molecular ion $[M]^+$ at *m*/*z* 264.1358, compatible with the molecular formula $C_{15}H_{20}O_4$, and the IR spectrum afforded absorption bands typical of hydroxyl (3448 cm^{-1}) and lactone carbonyl (1752 cm^{-1}) groups. The ^1H and ^{13}C NMR spectra (Table 1) of compound **2** showed typical signals that suggested a eudesmane framework.[9] The ^{13}C NMR spectrum displayed 15 carbons, which were assigned by HSQC, HMBC, and DEPT 135° experiments to the resonances of four quaternary, six methine, three methylene, and two methyl carbon atoms. The presence of a α-methylene-γ-lactone moiety was confirmed by the ^{13}C NMR signals at δ 170.4 (OCO), 134.0, and 120.0 ppm ($C{=}CH_2$). COSY NMR experiments enabled H-7 to be placed at δ 2.57 according to its allylic couplings to H-13a (d, J = 3.2 Hz) and H-13b (d, J = 3.2 Hz). Moreover, the coupling constants between H-7 (tt, J = 11.0/2.9 Hz) and H-8 (dt, J = 3.8/12.1 Hz) indicated a *trans* attachment of the α-methylene-γ-lactone to the decalin ring system.[10] The relative stereochemistry was proved by using J couplings and NOEs derived from NOESY spectra (Figure 1).

Therefore, it was revealed that **2** has a eudesmanolide nucleus with functionalization and stereochemistry similar to (−)-sivasinolide (**1**), except for C-1 and C-10. Further analysis of ^1H and ^{13}C NMR spectra, as well as a NOESY experiment, revealed the presence of an unusual *cis*-decalin ring: H-5 was shifted upfield at δ 1.85 (vs δ 2.07 in compound **1**), while protons H-1, H-6, and H-14 were shifted downfield by 0.1−0.2 ppm, compared to **1**. As a result of the *cis*-fusion of rings A and B, the methyl group on C-10 is equatorially oriented, giving NOE cross-peaks with H-5, H-9a, and H-9b, while it did not interact with H-1. C-1 was placed axially at C-10, and therefore H-1 was spatially close to protons H-6 and H-8, giving rise to NOE cross-peaks. In the ^{13}C NMR spectrum, particularly notable was the relatively low-field shift of the methyl group (δ 20.2) at the ring junction, which in the case of the *trans* isomer, sivasinolide (**1**), appeared at δ 14.1. The downfield shift of C-6 from δ 76.6 (compound **1**) to δ 75.1 could be attributed to the magnetic anisotropy effect of the Δ^3 double bond, while the high-field shift of C-1 (δ 66.4 vs 75.7 in compound **1**) could be rationalized in terms of the 1,3-

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Table 1. ^{1}H NMR Data and NOESY Correlations of Compounds **2** and **6** (400 MHz, $CDCl_3$)

	2			**6**		
position	δ (m)	NOESY	*J* (Hz)	δ (m)	NOESY	*J* (Hz)
1	3.78 (dd)	2a, 6, 8	8.9/6.4	2.70 (dd)	2a	10.6/3.1
2a	2.44 (m)	1, 2b		2.20 (m)	1, 2b	
2b	1.95 (m)	2a, 14		1.60 (m)	2a	
3a	5.40 (brs)	2a, 15		2.40 (dd)	5	8.2/7.9
3b				2.18 (m)		
5	1.85 (d)	7, 14	9.6	5.24 (brd)	3a	9.2
6	3.88 (t)	1, 8	9.8	4.27 (t)	15	9.6
7	2.57 (tt)	5, 9b	11.0/2.9	2.98 (m)	5, 8, 9, 13b, 14	
8	4.03 (dt)	1, 6, 9a	3.8/12.1	4.09 (ddd)	5, 7	9.9/5.1/4.8
9a	2.76 (dd)	8, 9b, 14	12.4/3.8			
9b	1.37 (t)	7, 9a, 14	12.4	2.04 (m)	7, 14	
13a	6.13 (d)	13b	3.2	6.37 (dd)	13b	2.7/1.0
13b	5.91 (d)	13a	3.2	6.15 (dd)	7, 13a	2.4/1.0
14	0.98 (s)	2b, 9a, 9b		1.42 (s)	5, 7, 9	
15	1.91 (brs)	3		1.82 (s)	6	

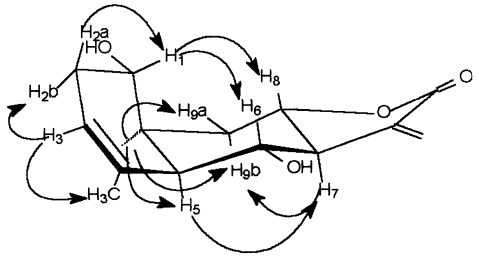

Figure 1. Relative stereochemistry of compound **2**.

diaxial interaction involving C-1 and the axial protons of C-6 and C-8.[11] Thus, compound **2** was established as 4,6-dihydroxy-5a,9-dimethyl-3-methylene-3a,4,5,5a,6,7,9a,9b-octahydro-3*H*-naphtho[1,2-*b*]furan-2-one and has been accorded the trivial name altissin.

The conformation and relative stereochemistry of compound **6** were established by using *J* values and NOE data derived from ^{1}H NMR and NOESY spectra. The occurrence of a NOE between H-14 and protons H-7 and H-5 suggested that these protons are oriented on the same side of the middle ring. The lack of any NOEs between H-14 and H-1 suggested that they have an opposite orientation. Therefore, compound **6**, although having identical ^{1}H NMR data described by Bohlmann et al.[7] for 1β,10α-epoxy-6α-hydroxy-1,10*H*-inunolide, should, based on our results, be corrected to 1α,10β-epoxy-6α-hydroxy-1,10*H*-inunolide.

We also report here the ^{13}C NMR data of compounds **3** and **6** (Table 2), as they have not been previously reported in the literature. The ^{1}H and ^{13}C NMR data in $CDCl_3$ of compound **5** (Table 2) are also reported here, as the previous data are different, since the spectra were measured in $CDCl_3$ plus CD_3OD at 57 °C.[5]

The structures of the known compounds were established by means of 1D and 2D NMR, MS, and UV for the flavonoid[12] spectral analyses. The major constituents of *A. altissima* were flavonoids, while sesquiterpene lactones were isolated in smaller amounts.

Concerning the antibacterial potential of **1**−**7**, it was possible to test only compounds **1**−**5**, as the epoxides **6** and **7** rapidly decomposed. *Helicobacter pylori* is a Gram-negative curved rod bacterium, which colonizes the gastric epithelial surface and withstands the stomach's hostile ambience by microaerophilic growth capacity.[13] Among the bacterial chronic infections of the gastrointestinal system, it is a causitive agent of chronic gastritis, peptic ulceration, and gastric cancer in humans.[14,15] Sivasinolide (**1**) and tatridin-A (**4**) proved active against *H. pylori* (Table 3). To our knowledge, there is no previous report concerning the potential anti-*H. pylori* activity of sesquiterpene lactones. As to the inhibitory effects of flavonoids (**8**−**17**), most of the aglycons proved more active than the one glycoside tested (**17**). Apigenin (**8**) and 6-hydroxyquercetin 3,6,4′-*O*-trimethyl ether (**12**) were totally inactive. The compounds with potent inhibitory activity have an *ortho*-hydroxy group system. The presence of one methoxy group did not reduce dramatically the inhibitory activity, as already mentioned in the literature.[16] Chlorogenic acid (**18**), bearing also an *ortho*-hydroxy-group system at the phenolic ring, was also active. Sivasinolide (**1**), altissin (**2**), quercetin (**9**), and isorhamnetin (**10**) showed moderate activity against *Bacillus cereus*. All compounds were inactive against *Pseudomonas aeruginosa* and *Proteus mirabilis*.

Experimental Section

General Experimental Procedures. Optical rotation values 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 instrument. The ^{1}H NMR spectra (400 MHz) and ^{13}C NMR spectra (50.3 and 100.6 MHz) were recorded using Bruker DRX 400 and Bruker AC 200 spectrometers. Chemical shifts are reported in δ (ppm) values relative to TMS. COSY, HMQC, HSQC, HMBC, and NOESY (mixing time 950 ms) were performed using standard Bruker microprograms. High-resolution EI mass spectral data were recorded on a JEOL GCmate mass selective detector and were provided by the University of Notre Dame, Department of Chemistry and Biochemistry, Notre Dame, IN. Low-resolution EI mass spectral data were recorded on a HP 1100 MSD API-electrospray (using Na as reagent). Vacuum-liquid chromatography (VLC): silica gel (Merck; 43−63 μm). Column chromatography: silica gel (SDS; 40−63 μm), gradient elution with the solvent mixtures indicated in each case. HPLC: CE 1100 liquid chromatography pump; Techsil 10 C_{18} (250 × 10 mm) column. Absorbents for TLC: Merck RP 18 F_{254s}, Art. 5685; Merck silica gel 60 F_{254s}, Art. 5554; Merck cellulose, Art. 5716. Detection on TLC plates: UV light, anisaldehyde−H_2SO_4 spray reagent on silica gel; Neu spray reagent on cellulose.[17]

Plant Material. The aerial parts of *A. altissima* L. were collected at Erythres (Viotia, Central Greece; altitude 350 m, latitude 38°13′ N, longitude 23°18′ E) in June 1999. The plant material was authenticated by Dr. T. Constantinidis (Institute of Systematic Botany, Agricultural University of Athens), and a voucher specimen is deposited in the Herbarium of the Institute of Systematic Botany, University of Patras, under the number Constantinidis 8550.

Extraction and Isolation. The fresh aerial parts of *A. altissima* (0.7 kg) were finely ground and extracted at room temperature with cyclohexane−Et_2O−MeOH (1:1:1). The extract was washed with brine, the aqueous layer re-extracted

Table 2. ^{1}H NMR Data of **5** and ^{13}C NMR Data of Compounds **2**, **3**, and **5** (400 and 50.3/100.6 MHz, respectively, $CDCl_3$)

H	5	2		3		5	
1	3.83 (dd,4.4, 10.2)	66.4	CH	78.3	CH	70.4	CH
2a	2.07 (dd,8.6, 11.7)	33.3	CH_2	31.5	CH_2	30.9	CH_2
2b	2.03 (dd, 5.7, 11.0)						
3a	2.23 (m)	121.5	CH	34.5	CH_2	34.3	CH_2
3b	2.19 (m)						
4		137.5	C	144.2	C	136.5	C
5	5.02 (brd, 10.2)	58.6	CH	57.2	CH	130.8	CH
6	4.25 (t, 9.8)	70.6	CH	67.3	CH	70.7	CH
7	2.80 (dddd, 2.9, 3.2, 6.7, 9.2)	54.9	CH	54.0	CH	51.6	CH
8	3.93 (m)	75.1	CH	76.4	CH	78.6	CH
9a	2.94 (brd, 14.6)	36.9	CH_2	40.3	CH_2	41.7	CH_2
9b	2.35 (dd,10.9, 14.0)						
10		40.6	C	42.8	C	146.7	C
11		134.0	C	137.3	C	136.5	C
12		170.4	C=O	170.2	C=O	169.8	C=O
13a	6.34 (d, 2.8)	120.0	CH_2	120.3	CH_2	125.6	CH_2
13b	6.18 (d, 1.6)						
14a	5.14 (brs)	20.2	CH_3	13.8	CH_3	114.7	CH_2
14b	5.09 (brs)						
15	1.69 (d, 0.9)	26.2	CH_3	108.7	CH_2	17.4	CH_3

Table 3. Minimum Inhibitory Concentrations (MICs, μg/mL) of Compounds **1**−**5** and **8**−**18** and Plant Extracts A and B

compound	*H. pylori*	*E. coli*	*P. mirabilis*	*P. aeruginosa*	*S. aureus*	*M. flavus*	*B. cereus*
1	12.5				50		25
2	50				50		12.5
3	50	50			50	50	50
4	12.5				50		50
5	50	50			50	50	50
8							
9	6.25					50	25
10	50						50
11	12.5	50					
12							
13	50						
14							
15	25						
16	25						
17	50						
18	6.25						
plant extract A	312.5−1250				1250		
plant extract B							
S[a]		200	400	500	100	100	100
A[b]	HP-ATCC: 1 clin. isolates: 2						

[a] S: streptomycin. [b] A: ampicillin.

with EtOAc, and the organic layer dried with Na_2SO_4 and concentrated under reduced pressure. The residue (5.98 g) was fractionated by VLC on silica gel (8.5 × 6.0 cm) using cyclohexane−EtOAc−Me_2CO mixtures of increasing polarity as eluents to give nine fractions of 500 mL each. Fractions C (cyclohexane−EtOAc, 1:1, 1.00 g), D (cyclohexane−EtOAc, 25:75, 0.29 g), and E (EtOAc, 0.27 g) were subjected to further chromatographic separation. Column chromatography on silica gel (3.0 × 17.0 cm; CH_2Cl_2−MeOH, 10:0 to 4:6) of fractions C and D followed by reversed-phase HPLC (MeOH−H_2O, 1:1, 2 mL/min) allowed the isolation of **1** (1.0 mg), **2** (2.9 mg), **3** (2.0 mg), **4** (3.3 mg), **5** (2.5 mg), **6** (16.8 mg), and **7** (8.0 mg), with t_R values 17.0, 19.5, 13.1, 11.3, 10.4, 36.2, and 29.0 min, respectively. Further column chromatography *o*f D on silica gel and Sephadex LH-20, as well as preparative TLC on cellulose plates (CH_3COOH−H_2O, 3:7), yielded the following flavonoids: apigenin (0.4 mg), kaempferol 4′-*O*-methyl ether (3.7 mg), quercetin (3.9 mg), quercetin 4′-*O*-methyl ether (7.0 mg), quercetin 3′-*O*-methyl ether (3.4 mg), quercetin 3-*O*-methyl ether (12.7 mg), 6-hydroxyquercetin 3,6,4′-*O*-trimethyl ether (4.8 mg), isoquercetrin (3.5 mg), isorhamnetin (51.0 mg), dihydroquercetin (1.2 mg), eriodictyol (3.7 mg), and the phenolic acid, chlorogenic acid (18.5 mg).

4,6-Dihydroxy-5a,9-dimethyl-3-methylene-3a,4,5,5a,6,7,-9a,9b-octahydro-3*H*-naphtho[1,2-*b*]furan-2-one (altissin, 2): oil; $[\alpha]^{20}_D$ −10.5° (*c* 0.13; $CHCl_3$); IR (KBr) ν_{max} 3450−3300, 1752 cm^{-1}; ^{1}H NMR data, see Table 1; ^{13}C NMR data, see Table 2; HREIMS *m*/*z* $[M]^+$ 264.1358, calcd for $C_{15}H_{20}O_4$ 264.1362.

Desacetyl-*β*-cycloperethrosin (3): oil; $[\alpha]^{20}_D$ +3.0° (*c* 0.07; $CHCl_3$); ^{13}C NMR data, see Table 2.

1-*Epi*-tatridin B (5): oil; $[\alpha]^{20}_D$ −8.16° (*c* 0.21; $CHCl_3$); ^{1}H and ^{13}C NMR data, see Table 2.

1α,10*β*-Epoxy-6-hydroxy-1,10*H*-inunolide (6): oil; $[\alpha]^{20}_D$ −8.8° (*c* 0.17; $CHCl_3$); IR (KBr) ν_{max} 3450−3300, 1762, 1720 cm^{-1}; ^{1}H NMR data, see Table 1; ^{13}C NMR data, see Table 2.

Bioassays. Plant extracts A and B were dissolved at 1000 mg/mL with DMSO and diluted with the nutrient medium to a concentration of 100 mg/mL. Final concentrations of 5000, 2500, 1250, 625, 312.5, 156.25, 78.12, and 39.06 μg/mL were tested. 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 of 50.0, 25.0, 12.5, and 6.25 μg/mL were used. The proportion of DMSO did not exceed 1% in the medium.[18]

Thirteen randomly selected clinical strains of *H. pylori* from antral biopsies and one reference strain (ATCC 43504) were used for this study. Following primary selective isolation, *H. pylori* bacterial cells were identified by the usual diagnostic procedures, i.e., according to colony morphology, Gram-staining, microaerophilic growth (at 37 °C), and biochemical tests (APICAMPY, Bio Mérieux, 24800). Growth of *H. pylori* was

maintained under a humid, microaerobic atmosphere (Genbox microaer, Bio Mérieux, 96125) in an anaerobic jar (Bio Mérieux, 96127) at 37 °C for 3 days. Then, the *H. pylori* isolates were used immediately in the tests. Inocula of approximately 5×10^5 CFU were inserted into brain-heart infusion broth (Bio Mérieux 51009) supplemented with 7% horse serum (Biochrom, S9133) and 1% Isovitalex (Oxoid SR090A), and the tests were performed on 96-well microtiter plates cultured microaerobically for 3 days at 37 °C in an anaerobic jar (Bio Mérieux, 96127, supplemented with Genbox microaer, Bio Mérieux, 96125). Dilutions of the inocula were subcultured on Pylori agar (Bio Mérieux 43263) to verify the absence of contamination and to check the validity of the inoculum. The MIC was taken at the lowest concentration of each compound that inhibited visible growth. DMSO was used as a control, while ampicillin was used as a positive control. The experiments for each sample were conducted in duplicate. To compare the anti-HP activity to other bacteria, the following organisms were used: Gram-positive *Staphylococcus aureus* (ATCC 6538), *Bacillus cereus* (clinical isolates), *Micrococcus flavus* (ATCC 10240); Gram-negative *Pseudomonas aeruginosa* (ATCC 27853), *Proteus mirabilis* (clinical isolates), *Escherichia coli* (ATCC 35218).

Bacterial species were cultured overnight at 37 °C in TSB (tryptone soya broth, Oxoid CM 129). Suspensions contained $\sim 10^9$ cells/mL. The antibacterial assays were carried out by the microdilution method.[19,20] Suspensions were adjusted with sterile saline to a concentration of approximately 1.0×10^5 in a final volume of 100 μL per well. Dilutions of the inocula were subcultured on TSA (tryptone soya agar, Oxoid CM 131) to verify the absence of contamination and to check the validity of the inoculum. Minimum inhibitory concentration (MIC) determination was performed by a serial dilution technique using 96-well microtiter plates. The plates were incubated for 36 h at 37 °C. The lowest concentrations without visible growth (at the binocular microscope) were defined as concentrations that completely inhibited bacterial growth (MICs). DMSO was used as a control, while streptomycin was used as a positive control.

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