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Sesquiterpene lactones from *Centaurea thessala* and *Centaurea attica*. Antifungal activity

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Abstract

The aerial parts of *Centaurea thessala* ssp. *drakiensis* and *C. attica* ssp. *attica* afforded, in addition to several known sesquiterpene lactones, two new eudesmanolides, 4-*epi*-sonchucarpolide and its 8-(3-hydroxy-4-acetoxy-2-methylene-butanoyloxy) derivative and one new eudesmane derivative, named atticin. The in vitro antifungal activity of most compounds was tested against nine fungal species, using the micro-dilution method. All the compounds tested showed great antifungal activity.

Keywords: *Centaurea thessala* ssp. *drakiensis*; *Centaurea attica* ssp. *attica*; Asteraceae; Sesquiterpene lactones; Germacranolides; Elemanolides; Eudesmanolides; Eudesmane derivative; Antifungal activity

1. Introduction

Extensive chemotaxonomic investigations of the large genus *Centaurea* (Mabberley, 1997) have been carried out (Connolly and Hill, 1991; Kaij-a-Kamb et al., 1992). Characteristic constituents of *Centaurea* species, many of which are used in folk medicine, are by order of their abundance, guaianolides, germacranolides, elemanolides and eudesmanolides.

In continuation of our work on the chemical constituents of *Centaurea* sp. (Skaltsa et al., 1999; Cardona et al., 1991, 1992, 1994, 1997; Fernández et al., 1989) we have investigated specimens of two Greek plants, *C. thessala* ssp. *drakiensis* and *C. attica* ssp. *attica*, whose major sesquiterpene lactones, cnicin (**1**), 4′-acetylcnicin (**2**), 8α-[(4-acetoxy-5-hydroxy)-angelate]salonitenolide (**3**), 8α-(3,4-dihydroxy-2-methylene-butanoyloxy)-dehydromelitensin (**4**) and methyl 8α-(3,4-dihydroxy-2-methylene-butanoyloxy)-6α,15-dihydroxyelema-1,3,11 (13)-trien-12-oate (**5**) have been already described (Skaltsa et al., 1999). The evaluation of the antifungal activity of most compounds is also reported in this paper.

2. Results and discussion

The crude extracts of the aerial parts of *C. thessala* ssp. *drakiensis* afforded the dehydromelitensine derivatives, 8α-(3,4-dihydroxy-2-methylene-butanoyloxy) (**4**) (Bruno et al., 1995; Tsankova et al., 1994; Barrero et al., 1988; Bruno and Herz, 1988) and 8α-(3-hydroxy-4-acetoxy-2-methylene-butanoyloxy) (**6**) (Barrero et al., 1997; Bruno et al., 1994, 1995) and the new eudesmanolide **7**. From *C. attica* ssp. *attica* malacitanolide (**8**), recently found in *C. malacitana* (Barrero et al., 1997), as well as the new eudesmanolide **9** and the new eudesmane derivative **10** were isolated.

Compound **7**, showed in its mass spectrum a molecular peak at *m/z* 280.1291, wich agreed with the molecular formula $C_{15}H_{20}O_5$ and the IR spectrum afforded absorptions characteristic of hydroxyl and carbonyl groups. The structure and stereochemistry of **7** was evident from the ^1H and ^{13}C NMR spectra analyzed with

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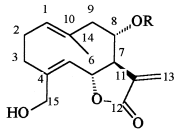
the ^{1}H NMR decoupling experiments, DEPT and ^{1}H–^{13}C correlations (HMQC) (Table 1). The spectral data strongly resembled those of 11β, 13-dihydro-8α-hydroxy-4-*epi*-sonchucarpolide (**12**), previously isolated by us from *Onopordon sibthorpianum* (Lazari et al., 1998), and suggested for **7** the structure of an 11, 13-dehydroderivative of **12**. For compound **7** the coupling patterns and the magnitude of the coupling constants of H-1, H-4 and H-5 to H-8 were in full agreement with the α stereochemistry for H-1, a *cis*-disposition of H-4/H-5 and a *trans*-disposition of H-5/H-6, H-6/H-7 and H-7/H-8 (Rustaiyan et al., 1986). The main differences on the ^{1}H and ^{13}C NMR spectra were the absence of the signals of CH_3–CH of the α-methyl-γ-lactone, as well as the lowfield shift for H-7 (δ 2.53 v δ 1.70 in **12**), and the highfield shifts for C-7 (δ 55.0 v δ 59.8 in **12**) and C-12 (δ 172.5 v δ 178.5 in **12**). New signals in the ^{13}C NMR spectrum of a quaternary carbon (δ 136.5) and a CH_2 (δ 119.2), which exhibited interactions with two doublets at δ 6.19 and 5.97 in the ^{1}H NMR spectrum, indicated the presence of an α-methylene-γ-lactone moiety, and were assigned to C-11, C-13, H-13a and H-13b, respectively. From the above spectral observations compound **7** was identified as the new of 8α-hydroxy-4-*epi*-sonchucarpolide.



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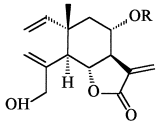
3 R = C



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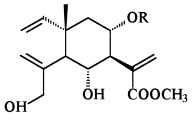
6 R = B



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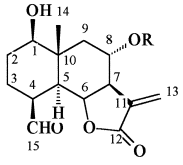

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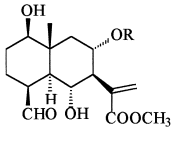
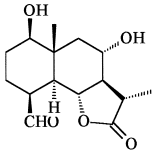
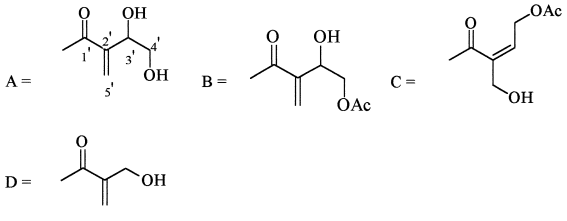


10 R = B
11 R = D



12



The mass spectrum of compound **9** showed a molecular peak at *m*/*z* 436.1730 $[M]^+$, which agreed with the molecular formula $C_{20}H_{28}O_6$. Its ^{1}H and ^{13}C NMR spectra showed typical signals that suggested an eudesmane framework with features common to those of compound **7** and malacitanolide (**8**) (Barrero et al., 1997). The analysis of the NMR spectra with the aid of decoupling experiments, DEPT, ^{1}H–^{1}H COSY and HMQC (Table 2) showed that **9** has an eudesmanolide nucleus with identical functionalization and stereochemistry than compounds **7** and **8**, and an 8α-acyl side chain which strongly resembled that of **8**. Two new signals in the ^{13}C NMR spectra at δ 175.1 (C) and δ 20.9 (CH_3) and a sharp singlet at δ 2.06 (3H) in the ^{1}H NMR spectrum suggested the presence of an acetate group. These assumptions were supported by the mass spectrum fragments at *m*/*z* 376 $[M–AcOH]^+$ and *m*/*z* 262 $[M–C_7H_{10}O_5, M–BOH]^+$. For the acetyl group we assigned the position C-4′ in the basis of the lownfield shift of the signals of the H-4′a (δ 4.29) and H-4′b (δ 4.18) in compound **9** v the same protons in compound **8** (δ 3.85 and 3.61, respectively) (Barrero et al., 1997). A similar lowfield shift is observed for those protons in 4′-acetylcnicin (**2**) (Jakupovic et al., 1986) versus cnicin (**1**) (Rustaiyan et al., 1982; Barrero et al., 1988). Consequently, compound **9** is the new 4′-acetoxymalacitanolide, the 8α-(4-acetoxy-3-hydroxy-2-methylenebutanoyloxy) derivative of compound **7**.

Atticin (**10**) was a very unstable colorless oil, which showed absorptions of hydroxyl and carbonyl groups in the IR spectrum. The ^{1}H and ^{13}C NMR spectra were in part close to those of compound **9** and showed signals confirming an eudesmane framework and the 8α-acyl side chain present in **9** (Table 2). In the ^{1}H NMR spectrum

Table 1
Chemical shifts assignment for compound **7** ($CDCl_3$)

	^{1}H δ (*J* in Hz)	^{13}C (multiplicity)[a]
1	3.35 (4.4, 10.6, *dd*)	76.4 (CH)
2a	1.69 (*m*)	25.6 (CH_2)
2b	1.57 (*m*)	
3a	2.41 (14.1, *br d*)	20.6 (CH_2)
3b	1.40 (*m*)	
4	2.75 (5.6, *br t*)	43.8 (CH)
5	1.94 (5.5, 12.0, *dd*)	46.7 (CH)
6	4.36 (11.0, 12.0, *dd*)	74.4 (CH)
7	2.53 (3.1, 10.6, *tt*)	55.0 (CH)
8	4.07 (4.4, 10.6, *td*)	65.8 (CH)
9a	2.28 (4.5, 12.7, *dd*)	47.3 (CH_2)
9b	1.29 (*m*)	
10	–	41.2 (C)
11	–	136.5 (C)
12	–	172.5 (C)
13a	6.19 (3.1, *d*)	119.2 (CH_2)
13b	5.97 (2.7, *d*)	
14	0.79 (*s*)	11.8 (CH_3)
15	9.90 (*s*)	200.1 (CH)

[a] HMQC allowed assignements of protonated carbons.

Table 2
Chemical shifts assignments for compounds **9** and **10** ($CDCl_3$)

	9		**10**	
	^{1}H δ (J in Hz)	^{13}C (multiplicity)[a]	^{1}H δ (J in Hz)	^{13}C (multiplicity)[a]
1	3.37 (4.6, 11.2, *dd*)	78.0 (CH)	3.31 (5.0, 11.2, *dd*)	78.0 (CH)
2a	1.80-1.70 (*m*)	27.1 (CH_2)	1.69 (*m*)	26.7 (CH_2)
2b	1.64-1.54 (*m*)		1.56 (*m*)	
3a	2.41 (*m*)	22.7 (CH_2)	2.36 (4.6, 12.4, *dd*)	22.3 (CH_2)
3b	1.48-1.40 (*m*)		1.41 (13.3, *t*)	
4	2.77 (5.4, *t*)	44.9 (CH)	2.89 (5.0, *t*)	45.0 (CH)
5	1.99 (5.4, 12.0, *dd*)	48.8 (CH)	1.68 (5.4, 10.8, *dd*)	48.8 (CH)
6	4.50 (11.6, *t*)	76.1 (CH)	4.42 (10.4, 10.8, *dd*)	76.2 (CH)
7	2.86 (2.9, 7.9, 10.8, *dddd*)	53.7 (CH)	2.67 (10.4, 10.8, *dd*)	53.9 (CH)
8	5.27 (4.2, 10.8, *td*)	69.7 (CH)	5.36 (4.6, 11.2, *td*)	70.3 (CH)
9a	2.48 (4.1, 12.8, *dd*)	43.8 (CH_2)	2.36 (4.6, 12.4, *dd*)	44.0 (CH_2)
9b	1.26 (12.8, 13.3, *dd*)		1.23 (12.1, 12.4, *dd*)	
10	–	41.5	–	
11	–	136.4	–	
12	–	171.5	–	
13a	6.17 (3.3, *d*)	120.8 (CH_2)	6.33 *s*	128.7 (CH_2)
13b	5.58 (2.9, *d*)		5.75 *s*	
14	0.88 (*s*)	13.9 (CH_3)	0.89 *s*	11.9 (CH_3)
15	9.91 (*s*)	201.9 (CH)	9.94 *s*	203.6 (CH)
1′	–	164.7	–	
2′	–	138.3	–	
3′	4.70 (3.3, 6.6, *dd*)	69.7 (CH)	4.61 (3.3, 6.6, *dd*)	69.6 (CH)
4′a	4.29 (3.7, 11.6, *dd*)	67.1 (CH_2)	4.18 (3.7, 11.6, *dd*)	67.4 (CH_2)
4′b	4.18 (6.6, 11.6, *dd*)		4.09 (7.4, 11.6, *dd*)	
5′a	6.36 (*s*)	128.1 (CH_2)	6.25 (*s*)	127.6 (CH_2)
5′b	6.03 (*s*)		5.90 (*s*)	
−OAc	2.06 (*s*)	(CH_3), 175.1 (CO)	2.05 (*s*)	20.6 (CH_3)
$-OCH_3$	–	–	3.76 (*s*)	52.1 (CH_3)

[a] HMQC allowed assignements of protonated carbons.

chemical shifts and coupling patterns for H-1, H-4 and H-5 to H-8 were in full agreement with those observed for compound **9**. On the other hand, a methoxy singlet at δ 3.76 (δ 52.1 in the ^{13}C NMR), as well as slightly broadened singlets for exomethylene protons (δ 6.33 and 5.75, δ 128.7 in the ^{13}C NMR) suggested the presence of the methyl ester of the hydroxy acid formed by the hydrolysis of the lactone **9**, similar to 4-*epi*-carmanin (**11**), a metabolite isolated from *Onopordon carmanicum* (Rustaiyan et al., 1986). This assumption was confirmed for a peak at m/z 436 $[M–MeOH]^+$ in the mass spectrum. Further information on the structure of **10** was obtained from the results of the C-H correlation in the HMQC spectrum (Table 2) and allowed us to assign for atticin the structure depicted of the 8α-acetoxy eudesmane derivative **10**.

Eudesmanolides are rare compounds for this genus, being isolated, so far, from *C. ornata* (Navarro et al., 1990), *C. hyssopifolia* (Gonzalez et al., 1977), *C. stoebe* (Huneck et al., 1986), *C. aspera* (Cardona et al., 1991), *C. malacitana* (Barrero et al., 1997) and *C. paui* (Cardona et al., 1997). Additionally, the chemistry of both taxa is characterized by the absence of guaianolides, common metabolites of other *Centaurea* species (Connolly and Hill, 1991; Fraga, 1992, 1993, 1994, 1995, 1996, 1997, 1998, 1999). Furthermore, our results are in agreement with previously studied *Centaurea* species, whose constituents contain mainly a 8α-(3,4-dihydroxy-2-methylene-butanoyloxy) side chain.

The main compounds of both species are cnicin (**1**) and 4′-acetylcnicin (**2**) (Skaltsa et al., 1999), so both taxa, belonging to the section Acrolophus (Cass.) D. C. again shows that cnicin, as well as closely related lactones are characteristic of this genus, as they were previously isolated from *C. cineraria* ssp. *umbrosa* (Bruno and Herz, 1988), *C. diffusa* (Milkova et al., 1993), *C. maculosa* (Kelsey and Locken, 1987), *C. paui* (Cardona et al., 1994, 1997), *C. vallesiaca* (Geppert et al., 1983), all belonging to the same section.

From Tables 3 and 4, it can be seen that all the investigated compounds possess greater antifungal potential than miconazole (commercial fungicide), which was used as a control. The most resistant micromycetes in this experiment were *Penicillium ochrochloron, P. funiculosum* and *Trichoderma viride*, while *Aspergillus ochraceus* was the most susceptible species against compounds investigated. According to MICs and MFCs values (Tables 3 and 4), it can be concluded that the most effective compounds are the germacranolides **3**, **1** and **2**, while the less effective ones are the

Table 3
Minimum inhibitory concentrations (MICs, μg/ml) of different compounds

Fungal species	**1**	**2**	**3**	**4**	**6**	**7**	**8**	**9**	**10**	Miconazole
Aspergillus niger	0.125	0.125	0.03	0.25	0.5	0.25	0.5	0.5	1	1.5
Aspergillus ochraceus	0.06	0.06	0.03	0.125	0.25	0.25	0.5	1	2	1.5
Aspergillus versicolor	0.125	0.125	0.06	0.125	0.5	0.125	1	1	2	2
Aspergillus flavus	0.5	0.125	0.25	1	0.5	0.25	0.25	1	2	0.5
Penicillium ochrochloron	0.25	0.25	0.125	0.25	0.5	0.25	0.5	1	2	2
Penicillium funiculosum	0.5	0.5	0.25	0.5	1	1	1	1	2	2
Trichoderma viride	0.5	0.5	0.25	0.25	0.5	0.25	1	1	2	2
Cladosporium cladosporioides	0.125	0.125	0.125	0.5	0.5	0.5	0.5	1	2	0.03
Alternaria alternata	0.25	0.125	0.125	0.25	0.03	0.5	0.5	0.06	1	0.5

Table 4
Minimum fungicidal concentrations (MFCs, μg/ml) of different compounds

Fungal species	**1**	**2**	**3**	**4**	**6**	**7**	**8**	**9**	**10**	Miconazole
Aspergillus niger	0.25	0.25	0.125	0.5	0.5	0.5	1	1	2	4
Aspergillus ochraceus	0.25	0.25	0.125	0.5	0.5	0.5	1	1	2	4
Aspergillus versicolor	0.25	0.25	0.25	0.25	0.5	1	1	1	2	2
Aspergillus flavus	0.5	0.25	0.5	1	0.5	1	0.5	1	2	2
Penicillium ochrochloron	0.5	0.5	0.5	0.5	1	0.5	1	1	2	5
Penicillium funiculosum	0.5	0.5	1	1	1	1	1	2	4	5
Trichoderma viride	0.5	0.5	1	1	1	0.5	1	2	4	2
Cladosporium cladosporioides	0.25	0.25	0.25	0.5	0.5	1	1	1	1	0.03
Alternaria alternata	0.5	0.5	0.5	0.5	0.25	1	1	0.125	1	0.5

eudesmanolides **9, 8** and **7** and the eudesmane derivative **10**. That can be attributed to different skeletal type and functional groups of the compounds. The higher activity of germacranolides could be related with the lipophility degree required for sesquiterpenoids passing through the fungal cell wall. Since the chemical composition of the fungal cell walls is highly lipophilic, they generally represent large barriers for the penetration of hydrophobic compounds and the transport of polar compounds through the outer lipid layer of fungi is retarded. Recently, it has been supported that the hypothesis of an inverse relationship between polarity and antifungal activity for sesquiterpene lactones, in general. It seems that, besides the α-methylene-γ-lactone, a relative low polarity, instead of a second Michael acceptor group, is actually responsible for an enhanced antifungal activity (Inoue et al., 1995; Barrero et al., 2000). Our results support this hypothesis, since, regarding the retention times of our compounds on a RP-18 column, their polarity is decreasing from eudesmanolides > elemanolides > germacranolides.

3. Experimental

3.1. General procedures

NMR: 400, 200 MHz (^{1}H) and 50.3 MHz (^{13}C). Chemical shifts are reported in δ (ppm) values. MS: Fisons VG Autospec GC 8000 (CI, CH_4); Hewlett-Packard mod. 5988A; (EI, 70eV). FT–IR Spectrometer: Perkin-Elmer Paragon 500. Polarimeter: Perkin-Elmer 341. Vacuum liquid chromatography (VLC): silica gel (Merck; 43–63 μm), CC: silica gel (SDS; 40–63 μm), gradient elution with the solvents mixts indicated in each case; HPLC: CE 1100 Liquid Chromatography Pumb Techsil 10- C18 (250×10 mm).

3.2. Plant material

Aerial parts of *Centaurea thessala* Hausskn. ssp. *drakiensis* (Freyn & Sint.) Georg. were collected on Mountain Pelion (central Greek mainland), in June and of *C. attica* Nym. ssp. *attica* on Mountain Parnes (Attiki-Greece) also in June 1997. Both plants were authenticated by Dr. Th. Constantinidis (Institute of Systematic Botany, University of Patras). A voucher specimen has been deposited in the Herbarium of the above mentioned Institute (*C. thessala* ssp. *drakiensis*: no. Skaltsa & Lazari 105; *C. attica* ssp. *attica*: no. Skaltsa & Lazari 106).

3.3. Bioassays

For the bioassays nine fungi were used: *Aspergillus niger* (ATCC 6275), *Aspergillus ochraceus* (ATCC 12066), *Aspergillus versicolor* (ATCC 11730), *Aspergillus flavus* (ATCC 9643), *Penicillium ochrochloron* (ATCC 9112),

Penicillium funiculosum (ATCC 36839), *Trichoderma viride* (IAM 5061), *Cladosporium cladosporioides* (ATCC 13276) and *Alternaria alternata* (DSM 2006).

The organisms were obtained from the Mycological Laboratory, Department of Plant Physiology, Institute for Biological research "Sinisa Stankovic", Belgrade, Yugoslavia. The micromycetes were maintained on malt agar (MA) the cultures were stored at +4°C and subcultured once a month (Booth, 1971).

In order to investigated the antifungal activity of compounds modified microdilution technique was used (Hanel and Raether, 1988; Daouk et al., 1995). The fungal spores were washed from the surface of agar plates with sterile 0.85% saline containing 0.1% Tween 80 (vol/vol). The spore suspension was adjusted with sterile saline to a concentration of approximately 1.0×10^5 per ml in a final volume of 100 μl per well. The inocula were stored at +4°C for further use. Dilution of the inocula were cultured on solid MA to verify the absence of contamination and to check the validity of the inoculum.

Minimum inhibitory concentrations (MICs) determination was performed by a serial dilution technique using 96-well microtitre plates. Extracts of compounds investigated were dissolved in broth Malt medium with fungal inoculum to achieve concentrations of 0.03–1 μg/ml. The microplates were incubated for 72 h at 28°C. The lowest concentrations without visible growth (at the binocular microscope) was defined as MIC. The minimum fungicidal concentrations (MFCs) was determined by serial subcultivation of a 2 μl into microtitre plates containing 100 μl of broth per well and further incubation for 72 h at 28°C. The lowest concentration with no visible growth was defined as the MFC, indicating $\geqslant$99.5% killing of the original inoculum. Commercial fungicide, miconazole, was used as a control (0.03–5 μl/ml).

3.4. Extraction and chromatography

The fresh plant material was extracted as described previously (Skaltsa et al., 1999).

C. thessala ssp. *drakiensis*. CC of the fr. eluted with cyclohexane–EtOAc 1:3 over silica gel using cyclohexane–MeOH mixts followed by further purification on HPLC (MeOH–H_2O 1:1) yielded **7** (2.2 mg). CC of the fr. eluted from VLC with EtOAc over silica gel using cyclohexane-MeOH mixts followed by further purification on HPLC (MeOH–H_2O 1:1) yielded **4** (5.8 mg) and **6** (3.4 mg).

C. attica ssp. *attica*. CC of the fr. eluted with cyclohexane–EtOAc 1:3 over silica gel using cyclohexane–MeOH mixts and by further purification on HPLC (MeOH–H_2O 1:1) were obtained compounds **9** (11.5 mg) and **10** (3.8 mg). Further purification of the fr. eluted with EtOAc on HPLC (MeOH–H_2O 1:1) yielded compound **8** (2.1 mg).

3.5. 8α-Hydroxy-4-epi-sonchucarpolide (7)

Oil; $[\alpha]_D^{20}+7.1$ ($CHCl_3$ *c*, 0.18); IR ν_{max}^{KBr} cm $^{-1}$: 3600–3300 (OH), 1764 (C=O, γ-lactone), 1720 (C=O, aldehyde); CIMS *m/z* (rel. int.): 280.1291 $[M]^+$ (11) ($C_{15}H_{20}O_5$ requires 280.1311), 263 $[M–OH]^+$ (14), 262 $[M–\mathbf{B}OH]^+$ (67), 244 $[M–H_2O]^+$ (33), 234 $[M–OH–HCO]^+$. ^{1}H NMR and ^{13}C NMR spectral data: see Table 1.

3.6. 8α-(3′-Hydroxy-4′-acetoxy-2′-methylene-butanoyloxy) 4-epi-sonchucarpolide (9)

Oil; $[\alpha]_D^{20}+29.3$ ($CHCl_3$ *c*, 0.15); IR ν_{max}^{KBr} cm $^{-1}$: 3600–3300 (OH), 1773, 1764 (C=O, γ-lactone, ester), 1719, 1712 (C=O, acetate, aldehyde). CIMS *m/z* 436.1730 $[M]^+$ (100) ($C_{22}H_{28}O_9$ requires 436.1733), 420 $[M–MeOH–OH]^+$ (22), 376 $[M–AcOH]^+$ (55), 363 $[M–Ac–H_2CO]^+$ (40), 262 $[M–\mathbf{B}OH]^+$ (20). ^{1}H NMR and ^{13}C NMR spectral data: see Table 2.

3.7. Atticin (10)

Oil; $[\alpha]_D^{20}+21.7$ ($CHCl_3$ *c*, 0.18); IR ν_{max}^{KBr} cm $^{-1}$: 3600–3300 (OH), 1773, 1764 (C=O, γ-lactone, ester), 1719, 1712 (C=O, acetate, aldehyde); MS (EI): 435 $[M–CH_3OH]^+$ (5), 405 $[M–H–CH_3OH–H_2CO]^+$ (9), 378 $[M–AcOH–H_2CO]^+$ (3), 351 $[M–C_6H_9O_3]^+$ (6), 293 $[M–\mathbf{B}–H_2CO]^+$ (8), 256 $[M–\mathbf{B}OH–MeOH–H_2O]$ (11). ^{1}H NMR spectral data: see Table 2.

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