Cytotoxic Germacranolides from *Inula verbascifolia* subsp. *methanea*

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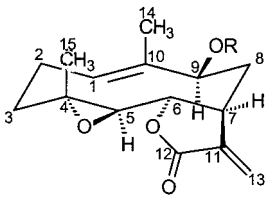
The aerial parts of *Inula verbascifolia* subsp. *methanea* yielded three new epoxygermacranolides, compounds **1**−**3**, in addition to the previously known 9β-hydroxyparthenolide. The structures of **1**−**3** have been elucidated on the basis of their spectral data (MS, CD, 1D and 2D NMR) and by quantum mechanical calculations. The in vitro cytotoxic activity of compounds **1**−**3** was evaluated against six human solid tumor cell lines.

Inula verbascifolia (Willd.) Hausskn. subsp. *methanea* (Hausskn.) Tutin (Asteraceae) is an endemic plant of central and southern Greece,[1] which has not been previously studied. The present note reports the isolation and the structure elucidation of three new epoxygermacranolides (**1**−**3**) in addition to the known lactone 9β-hydroxyparthenolide, from the aerial parts of this plant. All compounds were identified by means of spectral data (IR, ^{1}H NMR ^{13}C NMR, HRFABMS, and CIDMS). The stereochemistry of these compounds was established using a combination of 1D and 2D NMR spectroscopy, computational analysis, and analysis of CD spectra. The in vitro cytotoxic activities of compounds **1**−**3** were also evaluated against six human solid tumor cell lines.



1: R = $COCH_2C(CH_3)(OH)CH_2CH_3$

2: R = $COCH_2C(CH_3)(OH)CH_3$

3: R = $COCH(CH_3)CH(OH)CH_3$

A lipophilic extract of the aerial parts of *Inula verbascifolia* subsp. *methanea* was chromatographed on a Si gel column using CH_2Cl_2 containing increasing amounts of MeOH. Further chromatography of the main fractions on Si gel columns yielded compounds **1**−**3** and 9β-hydroxyparthenolide, a germacranolide, isolated previously from *Anvillea garcinii*.[2,3]

Compound **1** was obtained as colorless gum. The molecular mass of 378 was deduced from the mass spectrum (CI, isobutene, *m*/*z* 379 $[M + H]^+$). Moreover, fragment ions at *m*/*z* 306 and 247 indicated the occurrence of an *O*-C_6-acyl moiety.[4] Its molecular formula was deduced to be $C_{21}H_{30}O_6$ on the basis of the protonated molecular ion at *m*/*z* 379.4423 $[M + H]^+$ in the HRFABMS. The IR spectrum showed an absorption band corresponding to an α,β-unsaturated γ-lactone (1765 cm^{-1}). The ^{1}H and ^{13}C NMR spectral data of this compound demonstrated general features similar to those of 9β-hydroxyparthenolide (Tables 1 and 2, respectively). The major differences in the ^{1}H NMR spectrum of **1** were the downfield shift of H-9, at δ 5.22 (dd, J = 10.9, 2.1 Hz), and the presence of signals corresponding to a 3-hydroxy-3-methylpentanoyloxy unit [at δ 2.53 (H-2′a, d, J = 15.7 Hz), 2.43 (H-2′b, d, J = 15.7 Hz), 1.55 (H-4′, q, J = 7.5 Hz), 0.91 (H-5′, t, J = 7.5 Hz), and 1.21 (H-6′, s)]. The ^{13}C NMR spectrum displayed 21 carbon signals, which were assigned by HMQC and DEPT 135° experiments as the resonances of six quaternary, five methine, six methylene, and four methyl carbon atoms. Six signals could be readily assigned to a 3-hydroxy-3-methylpentanoyloxy unit, namely, two quaternary (172.0 and 71.0 ppm), two methylene (44.4 and 34.6 ppm), and two methyl (26.2 and 8.3 ppm). The remaining 15 carbons indicated the occurrence of an epoxygermacranolide skeleton.[2] The presence of a α-methylene-γ-lactone moiety was confirmed by the ^{13}C NMR signals at δ 169.0 (OCO), 137.9, and 122.0 ppm ($C{=}CH_2$). The COSY experiments enabled the spectral assignment and the identification of the gross structure of **1**. For example, the COSY analysis from the low-field signals of the α-methylene-γ-lactone moiety (δ 6.25 and 5.65 for H-13a and H-13b, respectively) established the assignment of the H-7 at 2.91, according to its allylic couplings to H-13a (d, J = 3.4 Hz) and H-13b (d, J = 3.4 Hz). Moreover, the coupling constants between H-7 and H-6 (t, J = 8.5 Hz) indicated a *trans* attachment of the α-methylene-γ-lactone.[5] Compound **1** is thus a 9-hydroxyparthenolide-type germacranolide, esterified at C-9 by a 3-hydroxy-3-methylpentanoyloxy unit. The conformational behavior and the relative stereochemistry were proved using J couplings and NOEs derived from 1D ^{1}H and 2D NOESY spectra as well as with molecular mechanics calculations. The stereochemistry of C-9 was deduced by the coupling constants of $J_{8\alpha,9}$ = 10.9 Hz and $J_{8\beta,9}$ = 2.1 Hz, which agreed with an axial orientation of H-9, whereas an equatorial orientation of H-9 would give values of $J_{8\alpha,9}$ about 6.0 Hz and $J_{8\beta,9}$ about 1 Hz.[2] The lack of any NOE between H-1 and H-14 (methyl group) and the occurrence of a NOE between the H-15 and H-14 methyl groups (Table 1) indicated the *trans* configuration of the 1,10-double bond.[6] The presence also of a strong cross-peak in the NOESY spectrum between H-1 and H-5/H-9 on one hand and H-7 and H-9/H-5, on the other hand, suggested that

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Table 1. ^{1}H NMR Data and NOESY Correlations of Compounds **1**−**3** (400 MHz, $CDCl_3$)

	1				**2**		**3**	
position	δ (mult)	NOESY	J^a	J^b	δ (mult)	J^b	δ (mult)	J^b
1	5.51 (dd)	9, 5, 2b, 3b	13.3/3.4	12.3/3.1	5.49 (dd)	12.3/3.1	5.51 (dd)	12.3/3.1
2a	2.45 (m)[c]	2b, 14, 15			2.43 (m)		2.45 (m)	
2b	2.25 (m)	1, 2a, 3b			2.20 (m)		2.20 (m)	
3a	2.04 (m)				2.10 (m)		2.05 (m)	
3b	1.24 (m)	1, 5, 2b			1.25 (m)		1.25 (m)	
5	2.68 (d)	1, 7, 3b	8.5	8.5	2.70 (d)	8.7	2.70 (d)	9.2
6	3.83 (t)	8b, 15	8.5	8.5	3.81 (t)	8.7	3.81 (t)	9.2
7	2.91 (m)	5, 9, 8a			2.92 (m)		2.90 (m)	
8a	2.40 (m)	13b, 9, 7			2.44 (m)		2.44 (m)	
8b	2.15 (m)	6			2.20 (m)		2.20 (m)	
9	5.22 (dd)	1, 7, 8a	11.3/1.4	10.9/2.1	5.23 (dd)	10.7/2.0	5.23 (dd)	10.9/2.2
13a	6.25 (d)	13b	3.4	3.4	6.36 (dd)	3.4	6.36 (d)	3.4
13b	5.65 (d)	13a, 8a	3.4	3.4	5.66 (d)	3.4	5.66 (d)	3.4
14	1.71 (s)	2a, 15			1.70 (s)		1.70 (s)	
15	1.30 (s)	6, 2a, 14			1.30 (s)		1.30 (s)	
2′a	2.53 (d)	4′, 5′		15.7	2.51 (s)		2.50 (q)	6.7
2′b	2.43 (d)			15.7	2.51 (s)			
3′							3.88 (q)	6.7
4′	1.55 (q)	5′, 2′a	7.5	7.5	1.30 (s)		1.28 (d)	6.7
5′	0.91 (t)	4′, 2′a	7.5	7.5	1.30 (s)		1.22 (d)	6.7
6′	1.21 (s)							

[a] Calculated coupling constants from model structures (in Hz). [b] Experimental coupling constants (in Hz). [c] Assignment determined by HMQC and COSY experiments. Multiplicity not determined (signal overlap).

Table 2. ^{13}C NMR Data of Compounds **1**−**3** (50 MHz, $CDCl_3$)

position	**1**	**2**	**3**
1	128.1	128.2	128.0
2	23.8	23.8	23.8
3	35.9	36.0	35.9
4	61.3	62.0	61.4
5	65.9	65.9	66.0
6	81.5	81.5	81.6
7	44.0	44.0	44.0
8	36.0	35.9	36.0
9	81.0	81.0	80.9
10	132.6	132.6	132.8
11	137.9	137.8	137.9
12	169.0	168.8	168.7
13	122.0	122.1	122.1
14	11.7	11.7	11.7
15	17.3	17.3	17.3
1′	172.0	171.9	174.8
2′	44.4	46.4	47.4
3′	71.0	69.1	69.6
4′	26.2	29.7	21.4
5′	34.6	29.7	14.7
6′	8.3		

these protons are oriented on the same side of the molecule and supported also an axial orientation for these three methine protons, which is in full agreement with the observed coupling constants of these protons. It was observed that no significant spectral changes were observed at a temperature range of −10 to −50 °C in ^{1}H, ^{13}C, and NOESY NMR spectra.

Computational conformation analysis was performed using molecular mechanics calculations, and their conclusions were in good agreement with the above experimental results. First, low-energy conformations of compound **1** were generated using the Low-Mode/Monte Carlo[7] search protocol with the MM2 force field as it is implemented in the molecular modeling software Macromodel 6.5.[8] After a 5000-step search, the conformer with the lower energy was found to adopt the expected, for the 12,6-lactonized germacrolides, UU ($^{15}D_5$, $_1D^{14}$) conformation.[6,9] This conformer is consistent with the NOE observed between H-15 and H-14 and the absence of NOE between H-1 and H-14. Moreover, the orientation of H-1, H-5, H-7, and H-9 is in agreement with the observed cross-peak in the NOESY data. Finally, the theoretical values of some ^3J coupling constants were calculated using Macromodel 6.5 and found to be in very good agreement with the experimental values (Table 1).

The absolute configuration of compound **1** (except C-3′) was established by comparison of its CD spectrum with those of 9-hydroxyparthenolide and parthenolide.[10] In this spectrum, a negative Cotton effect at 230 nm was observed. Thus, compound **1** was identified as 9β-(3-hydroxy-3-methylpentanoyloxy)parthenolide.

The molecular mass of 364 of compound **2** was deduced from the mass spectrum (CI, isobutene m/z 365 $[M + H]^+$). Moreover, fragment ions at m/z 306 and 247 indicated the occurrence of an *O*-C_5-acyl moiety.[11] Its molecular formula was determined by HRFABMS as $C_{20}H_{28}O_6$. The ^{1}H and ^{13}C NMR spectra of this compound were seen to be very close to those of compound **1** (Tables 1 and 2), except that in **2** the 3-hydroxy-3-methylpentanoyloxy side chain was replaced by a 3-hydroxy-3-methylbutyryloxy unit (absence of the methylene group of ^{1}H and ^{13}C NMR signals at δ 1.55 and 34.6, respectively).[11] The relative stereochemistry, the $^{15}D_5$, $_1D^{14}$ conformation, and the absolute stereochemistry of compound **2** were identical with those of compound **1**, as shown by coupling constants, NOESY data, computational conformation analysis using molecular mechanics calculations, and its CD spectrum (231 nm, negative Cotton effect), respectively. Accordingly, compound **2** was assigned as 9β-(3-hydroxyisovaleryloxy)parthenolide.

The mass spectrum (CI, isobutene) of compound **3** showed that its molecular mass was identical with that of **2**. Its molecular formula was determined by HRFABMS as $C_{20}H_{28}O_6$, and it was recognized as an esterified derivative of 9β-parthenolide, as shown by the downfield signal of H-9 at δ 5.23 in the ^{1}H NMR spectrum, the ^{13}C NMR spectrum, and its NOESY data, and CD spectrum. The ^{1}H NMR spectrum of **3** was also characterized by the presence of two doublets and two multiplets, at δ 1.28, 1.22, 2.50, and 3.88, which corresponded to two methyl groups and two methine groups, respectively. Thus compound **3** was assigned as 9β-(3-hydroxy-2-methylbutyryloxy)parthenolide.

The cytotoxicity of the crude CH_2Cl_2 plant extract and of compounds **1**−**3** was evaluated (Table 3). The three new compounds showed the most potent activity against the

Table 3. Cytotoxic Activity (IC_{50} values, μg/mL) of Compounds **1**−**3** Against a Tumor Cell-Line Panel[a]

compound	cell type					
	colon HT-29	colon HCT-116	colon HRT-18	breast MCF-7	prostate LNCaP	prostate PC-3
1	11.7	7.1	8.75	>40	30.2	15.7
2	17.5	14.1	14.9	>40	34.2	18.2
3	11.7	0.39	13.4	>40	25.4	11.3
CH_2Cl_2 extract[b]	35	17	37	40	40	30
daunorubicin HCl	0.072	0.28	0.11	0.12	0.011	0.23

[a] For protocols used, see Experimental Section. [b] Extract obtained from *I. verbascifolia* subsp. *methanea*.

three colon cancer and the PC-3 androgen insensitive cells, but only marginal or no significant activity against the MCF-7 and LNCaP cells, which express estrogen and androgen receptors, respectively. The most active compound was **3**, against the HCT-116 colon carcinoma cell line (IC_{50}, 0.39 μg/mL).

Experimental Section

General Experimental Procedures. Optical rotations were measured on a Perkin-Elmer 341 polarimeter. IR spectra were obtained on a Perkin-Elmer Paragon 500 instrument. CD spectra were obtained on a JASCO J-715 spectropolarimeter. ^{1}H NMR (400 MHz) and ^{13}C NMR (50 MHz) data were recorded on a Bruker DRX400 spectrometer and a Bruker AC200 spectrometer, respectively (using TMS as an internal standard). COSY, HMQC, HMBC, and NOESY (mixing time 950 ms) NMR data were performed using standard Bruker microprograms. CIMS (using isobutane as reagent gas) and HRFABMS were recorded with a Nermag R 10-10C spectrometer and an AEI MS-902 spectrometer, respectively. Column chromatography was carried out using silica gel 60 (Merck, 0.015−0.040 mm) with an applied pressure of 300 mbar. TLC was carried out on glass precoated silica gel 60 F_{254} plates (Merck).

Molecular Mechanics Calculations. All calculations were performed on a R5000 Silicon Graphics workstation. For the molecular mechanics calculations the MM2 force field was used as it is implemented in Macromodel 6.5.[7] The Truncated Conjugate Gradient (TNCG) minimization method with an energy convergence criterion of 0.01 kcal mol^{-1} was also used for geometry optimization.

Plant Material. The aerial parts of *Inula verbascifolia* (Willd.) Hausskn. subsp. *methanea* (Hausskn.) Tutin were collected near Mount Imittos Attiki, Greece, in June 1999 and were identified by Dr. Th. Konstantinidis. A voucher specimen has been deposited at the Herbarium of the University of Athens, Departement of Pharmacognosy (NEK005).

Extraction and Isolation. The whole plant, dried and pulverized (1.1 kg), was extracted with cyclohexane (2 L × 2), CH_2Cl_2 (2 L × 3), and then MeOH (2 L × 4). The CH_2Cl_2-soluble extract was concentrated under reduced pressure to give a residue (10 g), which was subjected to vacuum-liquid chromatography on Si gel (0.015−0.04 mm). Elution with a CH_2Cl_2−MeOH gradient yielded seven fractions. Fractions 5 and 6 were chromatographed (0.32 and 0.17 g, respectively) over a flash silica gel column using a hexane−AcOEt gradient to afford compounds **1** (15 mg), **2** (22 mg), **3** (14 mg), and 9β-hydroxyparthenolide (27 mg).[2,3,10]

Cell Culture and Assessment of Cytotoxicity. Compounds **1**−**3** were tested for their cytotoxic activity on the following human solid tumor cell lines: MCF-7, derived from a mammary adenocarcinoma of a 69-year old Caucasian (ATCC: American Type Culture Collection, Rockville); LNCaP clone FGC, derived from a prostate carcinoma of a 50-year old Caucasian (ATCC); PC-3, derived from a prostate adenocarcinoma of a 62-year old Caucasian (ATCC); HT-29, derived from a colorectal adenocarcinoma of a 44-year old Caucasian female (ECACC: European Collection of Cell Cultures, Salisbury, UK); HRT-18 (also designated as HCT-8), derived from an ileocecal colorectal adenocarcinoma of a 67-year-old male (ECACC); HCT 116, derived from a human colorectal carcinoma (ECACC).[12]

All cell lines were routinely cultured in Dulbecco's minimal essential medium supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL), and 10% fetal bovine serum (media and antibiotics from Biochrom KG, Berlin, Germany), in an environment of 5% CO_2 and 85% humidity at 37 °C. Adherent cells were subcultured using a trypsin 0.25%−EDTA 0.02% solution. Cytotoxicity was estimated by a modification of the MTT assay.[13] Briefly, cells were plated in 96-well flat-bottomed microplates at a density of 5000 cells/well. Next, 24 h after the plating, test compounds were added, appropriately diluted in DMSO. After a 48 h incubation, the medium was replaced with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma, St. Louis, MO) dissolved at a final concentration of 1 mg/mL in serum-free, phenol-red-free RPMI (Biochrom KG), for a further 4 h incubation. Then, the MTT-formazan was solubilized in 2-propanol, and the optical density was measured at a wavelength of 550 nm and a reference wavelength of 690 nm. In every experiment, daunorubicin HCl was included as a positive control.

9β-(3-Hydroxy-3-methylpentanoyloxy)parthenolide (1): colorless gum; $[\alpha]^{20}_D$ −8° (*c* 0.3, $CHCl_3$); CD (*c* 5 × 10^{-6}, MeOH) $\Delta\epsilon$ −8 (230 nm); IR ν_{max} 1765, 1730 cm^{-1}; ^{1}H NMR data, see Table 1; ^{13}C NMR data, see Table 2; DCIMS (isobutene) *m/z* 379 $[M + H]^+$, 306 $[M + H - C(CH_3,OH)CH_2CH_3]^+$, 247 $[M + H - HOOCCH_2C(CH_3,OH)CH_2CH_3]^+$; HRFABMS *m/z* $[M + H]^+$ 379.4423, calcd for $C_{21}H_{31}O_6$, 379.4427.

9β-(3-Hydroxyisovaleryloxy)parthenolide (2): colorless gum; $[\alpha]_D$ −1° (*c* 0.3, $CHCl_3$); CD (*c* 5 × 10^{-6}, MeOH) $\Delta\epsilon$ −7 (231 nm); IR ν_{max} 1765, 1725 cm^{-1}; ^{1}H NMR data, see Table 1; ^{13}C NMR data, see Table 2; DCIMS (isobutene) *m/z* 365 $[M + H]^+$, 306 $[M + H - C(CH_3,OH)CH_3]^+$, 247 $[M + H - HOOCCH_2C(CH_3,OH)CH_3]^+$; HRFABMS *m/z* $[M + H]^+$ 365.4165, calcd for $C_{20}H_{29}O_6$, 365.4162.

9β-(3-Hydroxy-2-methylbutyryloxy)parthenolide (3): colorless gum; $[\alpha]_D$ +1° (*c* 0.3, $CHCl_3$); CD (*c* 5 × 10^{-6}, MeOH) $\Delta\epsilon$ −9 (230 nm); IR ν_{max} 1765, 1735 cm^{-1}; ^{1}H NMR data, see Table 1; ^{13}C NMR data, see Table 2; DCIMS (isobutene) *m/z* 365 $[M + H]^+$, 320 $[M + H - CH(OH)CH_3]^+$, 247 $[M + H - HOOCCH(CH_3)CH(OH)CH_3]^+$; HRFABMS *m/z* 365.4160, calcd for $C_{20}H_{29}O_6$, 365.4162.

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