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New cytotoxic saturated and unsaturated cyclohexanones from *Anthemis maritima*

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Abstract—Two new cyclohexenones (antheminones A and B) and a new cyclohexanone, (antheminone C) along with five known compounds were isolated from the leaves of *Anthemis maritima* L. The structures were mainly deduced from extensive 1D and 2D NMR spectroscopy and mass spectrometry. The new compounds were tested in vitro for their cytotoxic activity against adherent and non-adherent cancer cell lines. Antheminones A and C exhibited significant antiproliferative activity against leukemia cells with IC_{50} values ranging from 3.2 to 14 μM.

The genus *Anthemis* is represented by 130 accepted taxa and is known to contain sesquiterpene lactones and flavonoids.[1] *Anthemis maritima* L. (Asteraceae) is an aromatic herb which grows on sandy beaches along the western Mediterranean coasts.[2] There are no reports in the literature regarding the chemical constituents of this plant. As part of a continuing search aimed at the discovery of novel cytotoxic compounds from Sardinian plants belonging to the Anthemideae tribe,[3] it was found that the EtOAc extract of the leaves of *A. maritima* showed cytotoxic activity. The phytochemical analysis resulted in the isolation of two new cyclohexenones (**1** and **2**) and a new cyclohexanone (**3**). From the petroleum ether extract three known flavonoids, salvigenin (**4**), cirsimaritin (**5**), and eupatilin (**6**) and the triglyceride 2-*trans*,*trans*-sorbo-1,3-dimyristin (**7**) were also isolated.

The dried and powered leaves of *A. maritima*[4] (580 g) were ground and extracted with petroleum ether (5 L) by percolation. The remaining plant material was then extracted with EtOAc (4 L) giving 27.9 g of dried extract. An aliquot (20 g) of the EtOAc extract was subjected to VLC (silica gel) using a step gradient of petroleum ether–CH_2Cl_2–EtOAc (9:1:0–0:1:9, 500 mL each) to yield 53 fractions. Homogeneous fractions were pooled to give seven major fractions (F1–F7). A portion of fraction F2 (0.5 g) was subjected to open-column chromatography over Sephadex LH-20 using methanol as eluent to give a mixture of two compounds. Subsequent purification by semi preparative RP HPLC with water–acetonitrile (60:40) as eluent yielded compounds **1** (8.4 mg) and **2** (26.9 mg). Fraction F2 (0.6 g) was fractionated by Sephadex LH-20 using methanol as eluent and then with RP HPLC using a mixture of water–acetonitrile–methanol (50:40:10) to give compound **3** (8.3 mg). From the petroleum extract, by using similar fractionation procedure, the known compounds **4**–**7** were isolated. Compounds **4**–**7** were identified by comparing their physical and spectroscopic data with those reported in the literature.[5] ^{13}C NMR data for compound **7** are reported here for the first time.[6]

The ^{13}C NMR (Table 1) spectrum of compound **1** showed 15 carbon signals, which were sorted by DEPT 90 and 135 experiments into three CH_3, four CH_2, four CH, and four quaternary carbons. This corresponds to a molecular formula of $C_{15}H_{24}O_4$, in agreement with a $[M+H+Na]^+$ at *m*/*z* 291 in the ESI-MS. Elemental analysis confirmed the proposed empirical formula giving C = 66.98% (theoretical = 67.14%) and H = 9.02%

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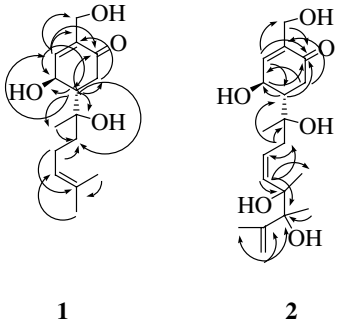
Table 1. ^{1}H (400 MHz) and ^{13}C (100 MHZ) NMR spectral data of antheminones A–C (CD_3OD, δ in ppm)

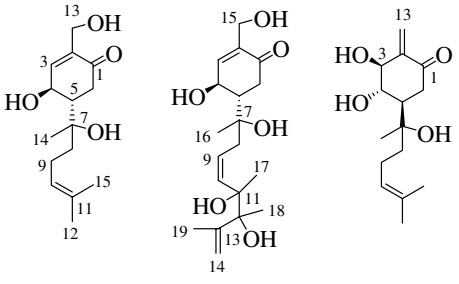
Position	Antheminone A (**1**)		Antheminone B (**2**)		Antheminone C (**3**)	
	δ_C, multiplicity[a]	δ_H, multiplicity[b]	δ_C, multiplicity[a]	δ_H, multiplicity[b]	δ_C, multiplicity[a]	δ_H, multiplicity[b]
1	202.4, s		202.4, s		201.2, s	
2	140.4, s		140.4, s		147.8, s	
3	143.6, d	7.05, dt (1.6, 6)	143.7, d	7.02, dt (1.5, 6)	85.8, d	4.68, d (1.6)
4	65.1, d	4.75, dd (3.6, 6)	65.1, d	4.71, dd (3.5, 6)	80.8, d	4.46, dd, br (1, 1.6)
5	46.0, d	2.18, dt (3.6, 13.6)	46.1, d	2.13, dt (3.5, 13.6)	48.4, d	2.52, dt (2.8, 4.1)
6_{ax}	35.2, t	2.88, dd (13.6, 16.7)	35.1, t	2.88, dd (13.6, 16.8)	43.5, d	2.80, dd (2.8, 19.2)
6_{eq}		2.53, dd (3.6, 16.7)		2.55, dd (3.5, 16.8)		2.61, dd (4, 19.2)
7	75.4, s		75.0, s		87.2, s	
8	41.2, t	1.64, m	44.4, t	2.38, m	42.4, t	1.49, m
9	23.7, t	2.10, m	126.5, d	5.72, m	25.2, t	2.15, m
10	125.6, d	5.22, t (7.5)	139.7, d	5.74, dt (7.2)	125.3, d	5.17, dt (7.2)
11	132.8, q		82.7, s		133, s	
12	26.1, q	1.80, s	90.8, s		26.1, q	1.76, s
13_a	59.9, t	4.31, dd (14.5, 1.6)	146.0, s		122.8, t	5.98, d (1.2)
13_b		4.32, d (14.5)				5.45, d (1.2)
14_a	25.1, q	1.46, s	114.5, t	4.96, br s	26.9, q	1.60, s
14_b				5.01, br s		
15_a	18.0, q	1.71, s	59.9, t	4.29, dd (1.6, 14.5)	18.0, q	1.69, s
15_b				4.31, d (14.5)		
16			25.1, q	1.45, s		
17			25.5, q	1.44, s		
18			25.2, q	1.38, s		
19			17.5, q	1.67, s		

[a] Multiplicity was determined by analysis of the DEPT spectra.
[b] J values (Hz) in parentheses.

(theoretical = 9.01%). Infrared absorption bands at 3420 and 1682 cm^{-1} suggested the presence of a hydroxyl group and α,β-unsaturated ketone, respectively. In the ^{1}H NMR (Table 1) spectrum the methine signals at δ_H 7.05 (1H, dt, $J = 1.6$, 6 Hz), and 5.22 (1H, t, $J = 7.5$ Hz) were assigned as olefinic protons whereas the methine at 4.75 (1H, dd, $J = 3.6$, 6 Hz) ppm must possess an oxygen substituent (δ_C 65.1). In addition to these signals, the ^{1}H NMR spectrum exhibited resonances for one hydroxymethylene function [δ_H 4.31 (dd, $J = 14.5$, 16 Hz) and 4.32 (d, $J = 14.5$ Hz)] and alkenyl chain. A HSQC experiment was utilized to assign the protons to their attached carbons. In the DQF-COSY spectrum, H-5 (δ_H 2.18 [dt, $J = 13.6$, 3.6 Hz)] showed cross-peaks with H-6 methylene protons [δ_H 2.88 (dd, $J = 13.6$, 16.7 Hz) and 2.53 (dd, $J = 3.6$, 16.7 Hz)] and H-4 (δ_H 4.75) while H-4 correlated with H-5 and H-3 (δ_H 7.05). HMBC interactions between H-3 and C-1 (δ_C 202.4), C-2 (δ_C 140.4), C-4 (δ_C 65.1), C-5 (δ_C 46.0) and between H-5 and C-1, C-3 (δ_C 143.6), C-4, and C-6 (δ_C 35.2), (Fig. 1), suggested the presence of a cyclohexenone ring. The structure of the alkenyl chain and its position on the cyclohexanone moiety were unambiguously established by HMBC experiments. In particular, HMBC correlations between the methine proton at δ_H 2.18 and C-4, C-6, C-7 (δ_C 75.4), C-8 (δ_C 41.2), and C-14 (δ_C 25.1) fixed the hexenyl chain at position 5 of the cyclohexenone. The key HMBC connectivities are displayed in Figure. 1. The relative stereochemistry of compound **1** was determined by ROESY experiments and analyzing scalar ($^{3}J_{HH}$) coupling of the protons. Namely, from the coupling patterns of adjacent proton signals in the ^{1}H NMR spectrum, the coupling constants values of $J_{6ax\text{-}5}$ and $J_{4\text{–}5}$ were calculated to be 13.6 and 3.6 Hz, respectively. Therefore, the hexenyl chain and the hydroxyl group at the 5- and 4-positions must be in the pseudoequatorial and pseudoaxial orientation, respectively. This observation was supported on the basis of the ROESY spectrum, which showed correlations between H-4 and H-5. Consequently, the structure of compound **1** was established as, 4-hydroxy-5-(1-hydroxy-1,5-dimethyl-4-hexenyl)-2 (hydroxymethyl)-2-cycloh-



1 **2**



1 **2** **3**

Figure 1. Main HMBC correlations of antheminones A (**1**) and B (**2**).

exen-1-one and accorded the trivial name, antheminone A (**1**).[7]

Antheminone B (**2**) was obtained as a colorless oil. The molecular formula was established as $C_{19}H_{30}O_6$ through a $[M+H]^+$ peak at *m/z* 355 displayed in the positive ESI-MS and by analysis of ^1H and ^{13}C NMR spectral data. Elemental analysis confirmed the proposed empirical formula giving C = 64.51% (theoretical = 64.39%), and H = 8.52% (theoretical = 8.53%). The ^1H and ^{13}C NMR spectra (Table 1) of **2** resembled to those of **1** suggesting the presence of a cyclohexenone moiety. However, in the ^1H NMR spectrum two additional vicinal olefinic methines at δ_H 5.72 (1H, m) and δ_H 5.74 (1H, d, J = 7.2 Hz) and a terminal methylene at δ_H 4.96 (br, s) and δ_H 5.01 (br, s) instead of the methine at δ_H 5.22 and of the methylene at δ_H 2.10 in **1** were observed in **2**. A comparison of the 2D NMR spectra of **2** with those of **1** showed that the structural differences are restricted to the alkenyl chain located at position 5 of the cyclohexenone ring. The cis relative configuration of the vicinal olefinic methines at δ_H 5.72 and δ_H 5.74 was judged from their coupling constants (J = 7.2 Hz). The nature of the side chain was deduced by HMBC experiments showing correlations of H-8 with C-5 (δ_C 46.1), C-7 (δ_C 75.0), and C-9 (δ_C 126.5), of H-10 with C-8 (δ_C 44.4), C-11 (δ_C 82.7), C-12 (δ_C 90.8), and C-17 (δ_C 25.5), of H-18 with C-11 (δ_C 82.7), C-12 (δ_C 90.8), and C-13 (δ_C 146.0), of H-14 with C-12 (δ_C 90.8), C-13 (δ_C 146.0), and C-19 (δ_C 17.5) (Fig. 1). On the basis of coupling constants and ROESY correlations, the relative stereochemistry at C-5 and C-6 in **2** was assumed to be the same as in **1**. Hence compound **2** was assigned as, 4-hydroxy-2-(hydroxymethyl)-5-[(3*Z*)-1,5,6-trihydroxy-1,5,6,7-tetramethyl-3,7-octadienyl]-2-cyclohexen-1- one and named antheminone B (**2**).[8]

Antheminone C (**3**) was isolated as a colorless oil. The molecular formula was determined to be $C_{15}H_{24}O_4$ from the molecular ion peak $[M+H+Na]^+$ at *m/z* 291 in the ESI-MS and by analysis of ^1H and ^{13}C NMR spectral data. Elemental analysis confirmed the proposed empirical formula giving C = 67.44% (theoretical = 67.14%) and H = 9.02% (theoretical = 9.01%). It followed therefore, that antheminones A and C possessed the same molecular formula. The ^1H and ^{13}C NMR spectra (Table 1) of compound **3** showed that its structure is related to that of compound **1**, with the exception that the hydroxymethylene protons at δ_H 4.31 and 4.32, and the olefinic signal at δ_H 7.05 were replaced by a terminal methylene at δ_H 5.45 (d, J = 1.2 Hz) and 5.98 (d, J = 1.2 Hz), and by a methine proton bearing a hydroxyl function at δ 4.68 (d, J = 1.6 Hz). HMBC correlations between the two methylene protons at δ_H 5.45 and 5.98 and C-1 (δ_C 201.2), C-2 (δ_C 147.8), and C-3 (δ_C 85.8) confirmed the presence of an α-methylenecyclohexanone ring instead of a 2-(hydroxymethyl)-2-cyclohexene-1-one moiety. The coupling constants values of $J_{6ax\text{-}5}$ and $J_{4\text{–}5}$ were calculated to be 2.8 and 1 Hz, respectively, suggesting that the side chain at the 5-position must have a pseudoaxial orientation while the hydroxyl group at position 4 possessed a pseudo-equatorial orientation. Correlations observed in the ROESY spectrum between H-3 and H-4 demonstrated that the hydroxyl functions at C-3 and C-4 were on the opposite face of the cyclohexanone ring. Compound **3** was determined to be 3,4-dihydroxy-5-(1-hydroxy-1,5-dimethyl-4-hexenyl)-2-methylenecyclohexanone and named antheminone C (**3**).[9]

The absolute configuration of antheminones A–C could not be determined due to the fact that a crystalline derivative suitable for an X-ray structure determination could not be obtained from the compounds isolated.

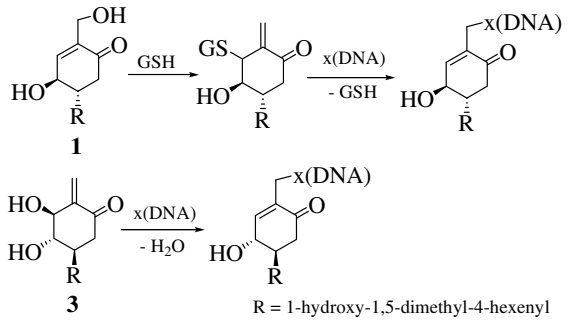
Antheminones **1**–**3** were evaluated for their cytotoxic activity against a panel of adherent and non-adherent human cancer cell lines (Table 2) as previously reported.[10] Compounds **1** and **3** were more active with respect to compound **2** against all the cell lines and showed significant antiproliferative activity toward the cells related to the immune system (HL-60, U-937, and Jurkat T) with antheminone C being the most active one with an IC_{50} value of 3.2 μM for HL-60 cells.

Cyclohexanones derivatives are relatively rare compounds in plants and usually isolated from fungi, bacteria, worms, and mushrooms.[11] Most often, cyclohexenones are produced by endophytic fungi plants and it seems that the role played by these compounds in the fungus–plant relationship is to provide protection to the plant. This possible role of the cyclohexenones is supported by the finding that they showed activity against phytopathogenic fungi.[12] Cyclohexenones showed interesting biological properties, such as, antibacterial and anti-tumor activities.[11] As regards the anticancer activity, in recent years cyclohexenones related to 2-crotonyloxymethyl-cyclohex-2-enone (COMC) have received great attention because of their peculiar mechanism of action.[13–15] This latter involves initial conjugation of glutathione (GSH) to cyclohexenone derivatives, a reaction catalyzed by glutathione transferase (GST), leading to the generation of a glutathioylated exocyclic enone.[16] The alkylation of intracellular proteins and/or nucleic acids by this intermediate leads to cell death.[17] Cyclohexenones have been suggested to be a potentially

Table 2. Cytotoxic activities of antheminones A–C (IC_{50} ± SD) determined after 72 h

Compound	IC $_{50}$ (μM) of cell proliferation after 72 h incubation					
	HCT-116 (colon)	CaCo-2 (colon)	MCF-7 (breast)	HL-60 (leukemia)	U-937 (leukemia)	Jurkat T (leukemia)
Antheminone A (**1**)	15 ± 2	11 ± 1	21 ± 2	7.6 ± 0.6	6.2 ± 3	9.0 ± 0.4
Antheminone B (**2**)	29 ± 4	24 ± 3	29 ± 5	11 ± 0.9	12 ± 0.4	14 ± 2
Antheminone C (**3**)	19 ± 2	9.4 ± 1.1	15 ± 1	3.2 ± 0.6	7.4 ± 1.3	8.4 ± 0.3
Parthenolide[a]	5.9 ± 0.8	10.4 ± 1.0	9.4 ± 0.9	0.9 ± 0.08	2.2 ± 0.3	1.9 ± 0.5

[a] Positive control substance capable of alkylating sulfhydryl moieties.



Scheme 1. Proposed mechanism of action of compounds **1** and **3** according to Hamilton et al.[16]

important new class of prodrugs, which can specifically target multidrug-resistant tumors overexpressing human glutathione transferase.[13]

Antheminone A (**1**) showed a cytotoxic activity comparable with that of the antitumoral compound COMC.[18] Since **1** is closely structurally related to COMC, it can be assumed that antheminone A reacts with GSH like COMC (Scheme 1). Interestingly, antheminone C (**3**) contains a cyclohexanone ring instead of a cyclohexenone one but it is equipotent or even more active (HL-60 cells) than **1**. This observation apparently contrasts with the mechanism proposed by Hamilton et al.[16] which request the conjugation of GSH to cyclohexenone, by a Michael addition, to give an electrophilic exocyclic enone (Scheme 1). The explanation why antheminone C (**3**) is cytotoxic could be related to its exocyclic enonic structure. As a result, antheminone C does not request an activation by GSH and could directly alkylate nucleic acids (Scheme 1).

Acknowledgment

The authors thank Prof. Bruno De Martis of the Dipartimento di Scienze Botaniche, Università di Cagliari for identification of plant material.

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6. *2-trans,trans-Sorbo-1,3-dimyristin* (**7**). $C_{37}H_{66}O_6$. ^{13}C NMR (100 MHz, $CDCl_3$) δ: 173.3 (C-1′, C-1‴), 166.1 (C-1″), 146.1 (C-3″), 140.1 (C-5″), 129.7 (C-4″), 118.0 (C-2″), 68.8 (C-2), 62.1 (C-1, C-3), 34.0 (C-2′, C-2‴), 31.9 (C-12′, C-12‴), 29.6 (C-6′, C-6‴, C-7′, C-7‴, C-8′, C-8‴, C-9′, C-9‴, C-10′, C-10‴), 29.3 (C-5′, C-5‴), 29.2 (C-11′, C-11‴), 29.1 (C-4′, C-4‴), 24.8 (C-3′, C-3‴), 22.7 (C-13′, C-13‴), 18.6 (C-6″), 14.1 (C-14′, C-14‴).
7. *Antheminone A* (**1**). Colorless oil, $C_{15}H_{24}O_4$; $[\alpha]_D^{25}$ −6.0° (*c* 0.03, MeOH); UV (MeOH) λ_{max} (log ε) 227 nm (1.35), 238 (0.97); FT-IR (KBr) ν_{max} 3420, 1682, 1095, 1080, 920 cm^{-1}; ESI-MS (positive-ion mode) *m/z*: 291 $[M+H+Na]^+$; ^1H and ^{13}C NMR, see Table 1.
8. *Antheminone B* (**2**). Colorless oil, $C_{19}H_{30}O_6$; $[\alpha]_D^{25}$ −12.0° (*c* 0.03, MeOH); UV (MeOH) λ_{max} (log ε) 229 nm (1.16), 242 (0.75); ESI-MS (positive-ion mode) *m/z*: 355 $[M+H]^+$; ^1H and ^{13}C NMR, see Table 1.
9. *Antheminone C* (**3**). Colorless oil, $C_{15}H_{24}O_4$; $[\alpha]_D^{25}$ +50.0° (*c* 0.03, MeOH); UV (MeOH) λ_{max} (log ε) 230 nm (1.54), 235 (1.27), 240 (0.93); ESI-MS (positive-ion mode) *m/z*: 291 $[M+H+Na]^+$; ^1H and ^{13}C NMR, see Table 1.
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