Structure Elucidation, Conformational Analysis and Thermal Effects on Membrane Bilayers of an Antimicrobial Myricetin Ether Derivative

C. Demetzos*, D. Angelopoulou

School of Pharmacy, Department of Pharmacognosy. Panepistimiopolis, Zografou 15771, University of Athens, Greece

A. Kolocouris†, I. Daliani, and T. Mavromoustakos

Institute of Organic and Pharmaceutical Chemistry, National Hellenic Research Foundation,
Vas. Constantinou 48, 11635 Athens, Greece

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The membrane perturbing 3,7,4',5'-tetramethyl ether of myricetin **1** was isolated from *Cistus monspeliensis* L. Its structure was elucidated and its conformational properties were explored using a combination of 2D NMR spectroscopy and computational chemistry. The obtained results showed that compound **1** adopts four enantiomeric pairs of low energy conformers characterized: (a) by an aromatic ring B twisted through rotation about C2–C1' bond from the rigid isoflavone ring; (b) a 4'-O–CH_3 bond oriented out of the plane with equal probability upwards or downwards the phenyl ring B, while all the other O–CH_3 bonds are oriented in the plane of the aryl ring. Two of these enantiomeric pairs are lowest in energy. These possible bioactive conformers are possibly stabilized by van Der Waals interactions. The 3',5-diacetyl derivative **2** of compound **1** was synthesized and its structure elucidation was achieved based on the chemical shift assignment of the parent compound **1**. The Differential Scanning Calorimetry (DSC) results revealed that the degree of the thermal effects exerted by the flavonoids at dipalmitoylphosphatidyl choline (DPPC) bilayers followed the order **1** > **2** > myricetin. Their antimicrobial activity against Gram positive bacteria followed the same order.

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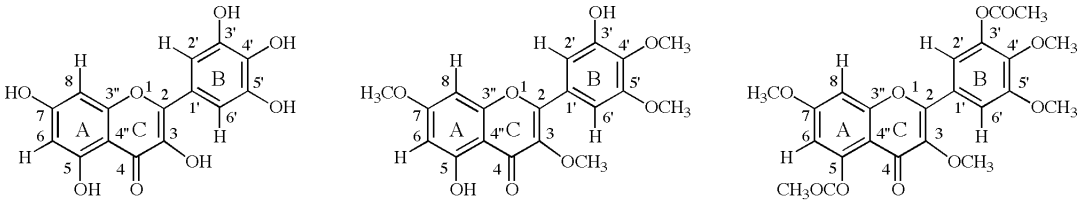
Introduction.

Flavonoids are one of the classes of heterocyclic natural products widely distributed in plants as glycosides or as free aglycons, which are far less abundant. It has been found that compounds of this kind have pharmacological properties like antimicrobial, antitumor, antiviral, enzyme inhibiting and central vascular system activities [1-3] that may be due to the membrane perturbing effects of these compounds. Several flavonoid aglycons were isolated and studied from the genus *Cistus*, the members of which have predominant habitats in the Mediterranean region [4]. In the recent years our laboratory contributed in the chemical and pharmacological investigation of *Cistus*, a genus found in Spain, Greece, Cyprus and geographic areas located between Morocco and Tunisia [5-9]. We have also performed comparison studies of active and inactive membrane perturbing compounds of natural origin using various biophysical methods [10].

Whereas myricetin [4] is a very widely distributed flavonoid, its methyl ethers are rare natural products. Myricetin 3,4',5',7-tetramethyl ether **1** (Figure 1) was first isolated several years ago from leaves of *C. monspeliensis* and later from other plants and its structure was assigned on the basis of UV-vis, IR and ^{1}H-NMR spectroscopy [11-12].

The lack of a detailed and unambiguous structure interpretation of ^{1}H and ^{13}C-NMR spectra and a conformational study for the pharmacologically important compound **1** prompted us to apply modern 2D NMR techniques and computational chemistry for providing this valuable information. The results confirm the structure of compound **1** and are believed to provide lead information for the structure assignment of other flavonoid derivatives and analogues that are planned to be synthesized in the future, and for further biophysical studies. The diacetyl derivative **2** was synthesized through acetylation of flavonoid **1**. The antimicrobial activity and thermotropic properties in dipalmitoylphosphatidyl choline (DPPC) bilayers of compound **1** its 3',5-diacetyl derivative **2** and myricetin were also studied and compared.



τ_1 = C6-C7-O-CH_3, τ_2 = C4"-C5-O-H
τ_3 = C4-C3-O3-CH_3, τ_4 = C3-C2-C1'-C6'
τ_5 = C4'-C5'-O5'-CH_3, τ_6 = C3'-C4'-O4'-CH_3
τ_7 = C4'-C3-O-H

Figure 1. Chemical structures of flavonoids **1**, **2**, myricetin and torsion angles for compound **1**.

Results and Discussion.

Structure Elucidation.

The ^1H and ^{13}C NMR spectra of compound **1** are shown in Figures 2 and 3. The strategy followed for its structural assignment is explained below. From the ^1H NMR spectrum H6 and H8 could be easily distinguished from the H2' and H6' protons based on previously reported data [11]. The two phenolic hydroxyl groups were also easily distinguished based on their chemical shifts. The phenolic hydroxyl group attached to C-5 was shifted downfield and resonated at 12.54 ppm due to its hydrogen bonding with the carbonyl group at C-4, whereas the phenolic hydroxyl group attached to C-3' resonated at 5.93 ppm. 7-Methoxy protons were assigned at 3.85 ppm due to their NOE correlation with H-6 and H-8. In addition, the 5'-methoxy protons were assigned at 3.92 ppm and H6' at 6.35 ppm due to their spatial proximity. A 2D COLOC experiment provided ^3J couplings and allowed the assignments of the methoxy carbons connected through oxygen to carbons C-7 and C-5'. From the COLOC results (Table 2) C-7 was assigned unambiguously at 165.8 ppm since it was ^2J correlated with protons H-6 and H-8. The resonance at 162.2 ppm was assigned to C-5 because it showed correlation with H-6 and 5'-OH protons as is expected. The resonance corresponding to C-3' is assigned to 160.0 ppm because it showed the expected ^2J correlation with H-8. Similarly, C-1' appeared to resonate at 155.6 ppm because it was correlated through ^2J couplings with H-2' and H-6'. C-5' could be distinguished from other carbons and was assigned to the resonance at 152.3 ppm based on its ^3J coupling with the corresponding methoxy group

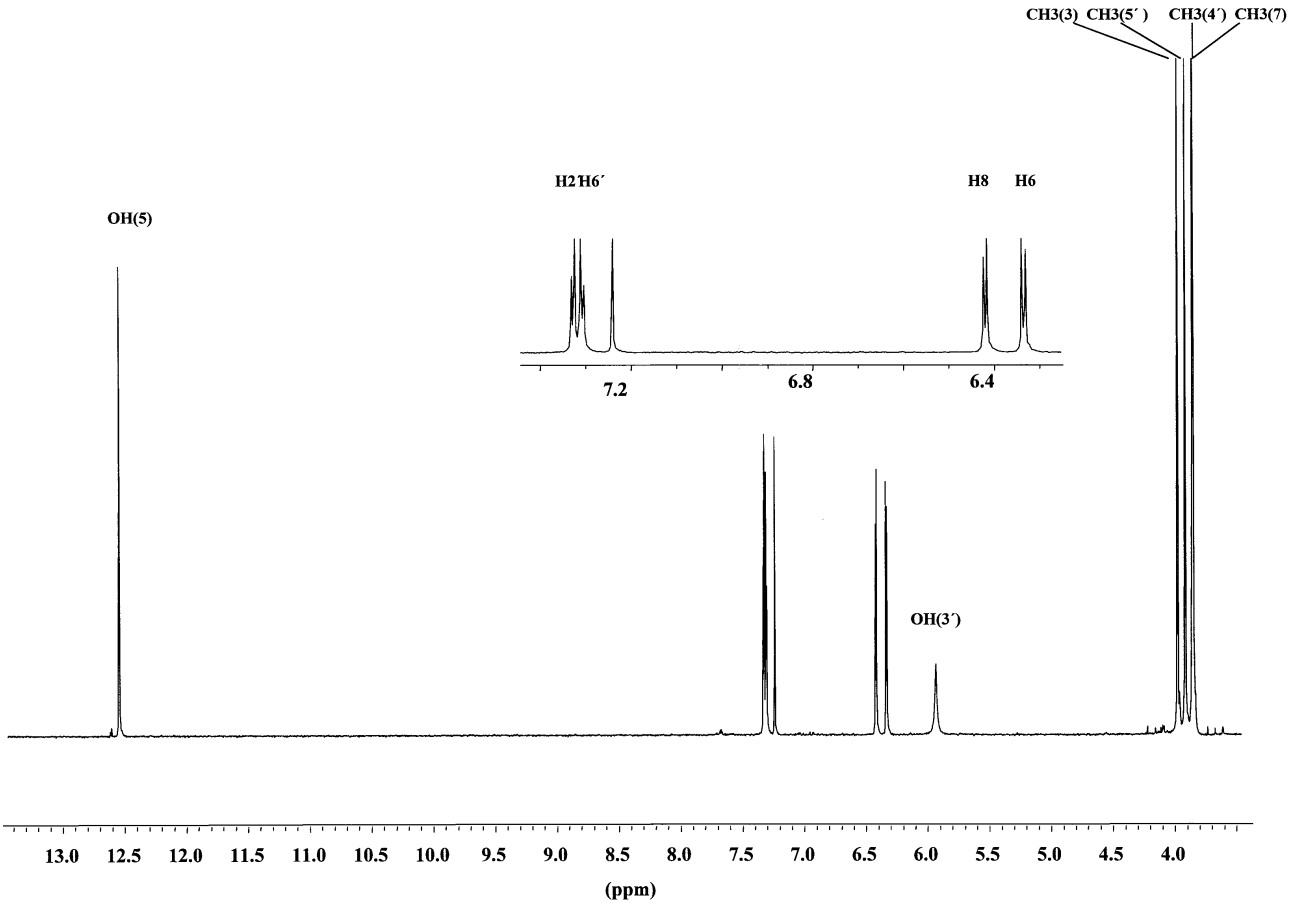


Figure 2. ^1H-NMR spectrum of flavonoid **1** in $CDCl_3$ at 298 K recorded on a Bruker AC 300 MHz.

Table 1

^1H Chemical Shift Assignments of Compound **1** in $CDCl_3$ at 298 K Recorded on a Bruker AC 300 MHz

Assignment	Chemical Shift
CH_3(7) (s)	3.85
CH_3(4') (s)	3.86
CH_3(5') (s)	3.92
CH_3(3) (s)	3.98
OH(3') (bs)	5.93
H6 (d)	6.33
H8 (d)	6.42
H6' (d)	7.31
H2' (d)	7.33
OH(5)	12.54

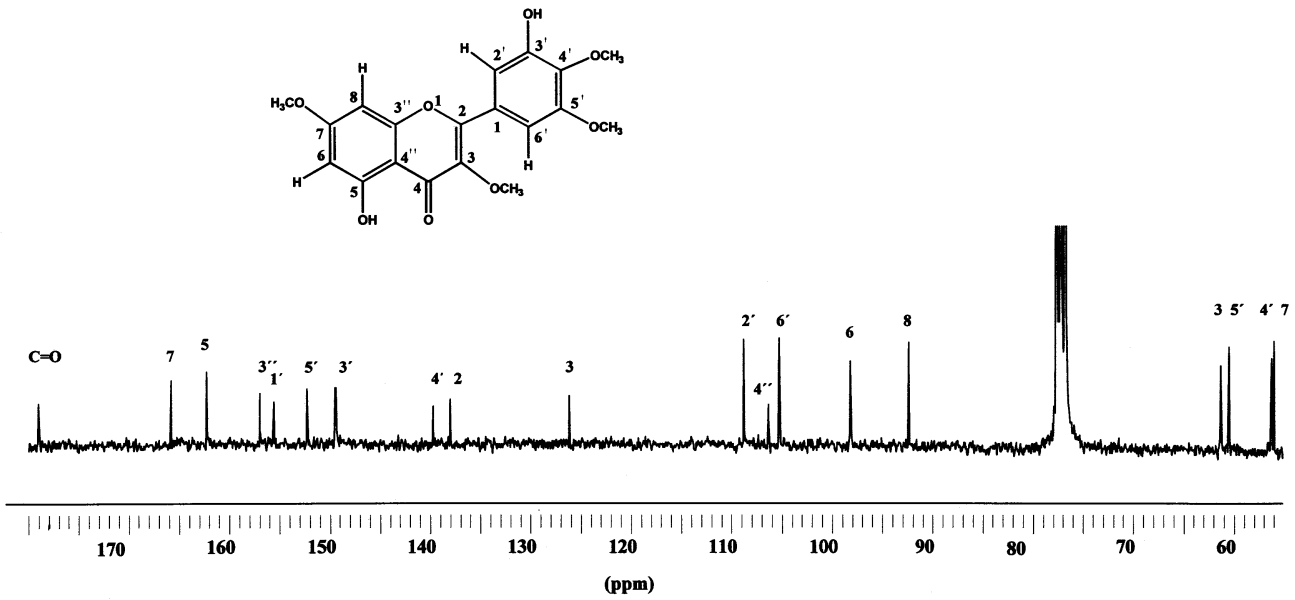


Figure 3. ^{13}C-NMR spectrum of flavonoid **1** in $CDCl_3$ at 298 K recorded on a Bruker AC 300 MHz.

Table 2

^{13}C Chemical Shift Assignment of Compounds **1** and **2** and 2D HETCORR Results in $CDCl_3$ at 298 K Recorded on a Bruker AC 300 MHz

Assignment	Chemical Shifts (ppm)		Dipolar correlations observed in HETCORR experiments
	Compound 1	Compound 2	
7 (OCH_3)	56.1	56.2	H-7 (OCH_3)
4′ (OCH_3)	60.5	60.3	H-4′ (OCH_3)
5′ (OCH_3)	56.3	56.4	H-5′ (OCH_3)
3 (OCH_3)	61.3	61.0	H-3 (OCH_3)
8	92.4	98.7	H-6
6	98.2	108.4	---------
2'	105.3	115.6	H-2' ,H-6′
4"	106.3	111.5	H-6, H-5'(O*H*)
6'	108.8	115.6	H-6',H-2'
3	126.2	125.8	----------
2	138.0	141.6	H-2' ,H-6', H-3 (OCH_3)
4'	139.7	143.3	H-4'
3'	149.4	144.0	H-2' ,H-6', H-3' (O*H*)
5'	152.3	150.6	H-5'(OCH_3)
1'	155.6	153.0	H-2' ,H-6'
3"	160.0	153.5	H-8
5	162.2	157.9	H-6 ,H-5'(O*H*)
7	165.8	163.9	H-7 (OCH_3)
C=O	179.1	169.8,173.2	----------

protons. C-3' was easily assigned to the resonance at 149.4 ppm due to its correlations with 3'-phenolic proton and H-2'. C-6′ was established to resonate at 105.3 ppm through its ^{1}J correlation with H-6'. C-4'' was assigned to the resonance at 106.3 ppm because it was correlates with the 5-phenolic proton and H-6. The remaining proton bearing C-6 and C-8 were assigned based on their ^{1}J with H-6 and H-8 whereas the remaining tertiary carbons C-4', C-2 and C-3 were assigned at 139.7 ppm, 138.0 ppm and 126.2 ppm correspondingly based on their expected chemical shifts from literature data [13-16]. A combination of COLOC and CHCORR experiments confirmed the assignment of 4'- methoxy carbon at 60.5 ppm. Interestingly, the methoxy group attached at C-4' showed a downfield shift of about 4 ppm relative to the other two methoxy groups attached to C-5' and C-7. This is attributed to the fact that the methoxy group attached at C-4' is flanked between two *ortho* substituents which impose it to deviate from being planar with the aromatic system. Details on this subject are given in a publication by Makriyannis and Knittel [17]. The only remaining methoxy carbon at C-3 was assigned at 61.3 ppm. C-2 showed a ^{4}J correlation with 3-methoxy protons and ^{3}J dipolar correlations with H-2' and H-6'. C-3 did not show any dipolar correlation. The results of this analysis are shown in Tables 1 and 2.

The assignment of compound **2** was based solely on the 1D ^{13}C NMR spectrum because it showed peaks resonating at similar chemical shifts. Four additional peaks at 20.9 pp., 21.3 ppm (CH_3CO), 169.8 ppm and 173.2 ppm (*C*O) define unambiguously its structure.

Computational Chemistry.

A strategy that uses exhaustive grid scan in combination with dynamics and experimental NOE data was applied in an attempt to explore the possible bioactive low energy conformers of compound **1** [18].

A preliminary structure was constructed that fits the observed NOEs. This was energy minimized to reach the local minimum **A** (Figure 5, Table 3). In this low energy conformer the chromone and phenyl B rings deviate from being coplanar. The 4'-OCH_3 bond points out of the plane (τ_4=52°) relative to the B phenyl ring whereas all others O–CH_3

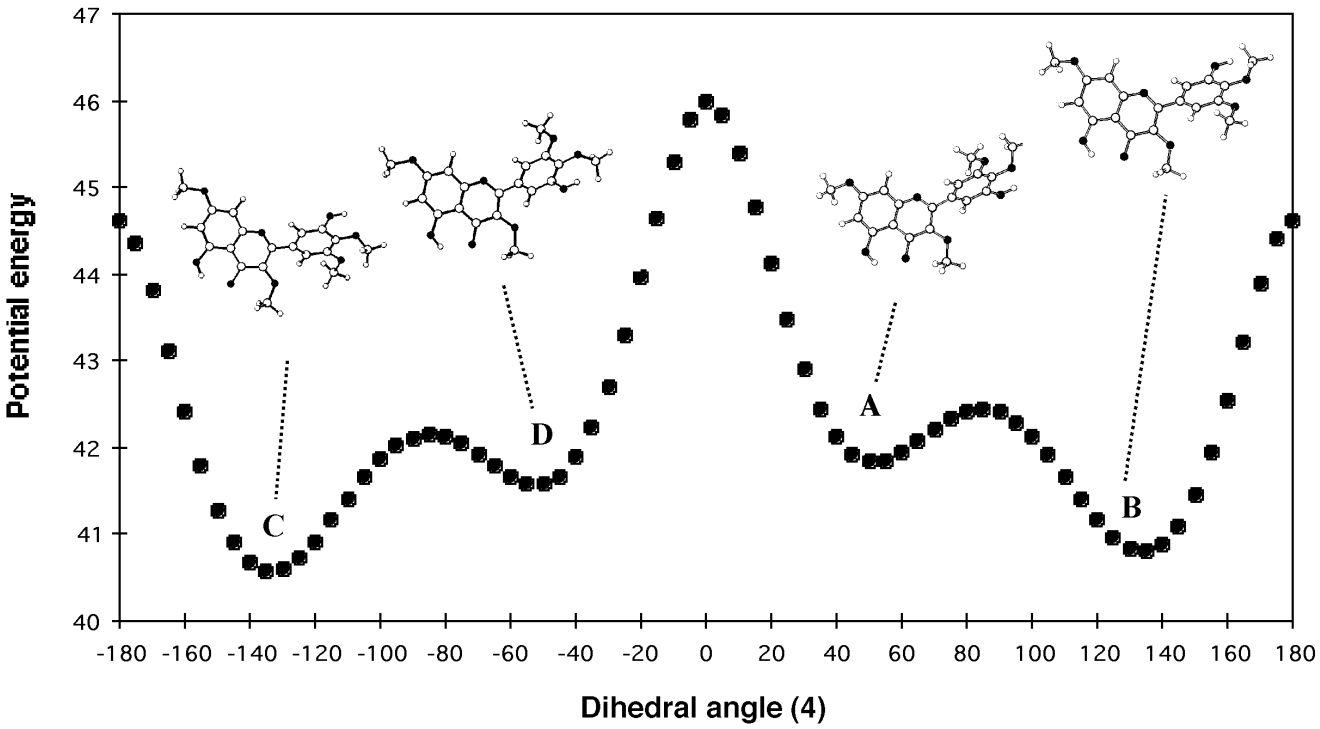


Figure 4. Conformational energy profile (kcal mole^{-1}) as a function of dihedral angle C4 (C3-C2-C1'-C6').

Table 3

Critical Dihedral Angles of Compound **1**, Calculated Most Important H-H Distances and Relative Potential Energy Values (kcal◊mol^{-1}) for Conformers **A-D**, **A'-D'**

Conformer	τ_3	τ_4	τ_5	τ_6	3'-OH 4'-OCH_3	4'-OCH_3 5'-OCH_3	5'-OCH_3 6'-H	4'-OCH_3- (6'-H/2'-H)	Relative Energy
A	1.3	51.6	-177.7	101.2	3.3	4.1	2.7	4.1	1.26
B	1.8	134	-172.2	104.2	3.4	4.2	2.7	4.0	0.22
C	-1.5	-133.8	175.4	103.4	3.4	4.0	2.7	4.0	0.00
D	-1.6	-52.0	-179.2	102.8	3.4	4.1	2.7	4.1	1.00
A'	-1.3	-51.6	177.7	-101.2	3.3	4.1	2.7	4.1	1.26
B'	-1.8	-134	172.2	-104.2	3.4	4.2	2.7	4.0	0.22
C'	1.5	133.8	-175.4	-103.4	3.4	4.0	2.7	4.0	0.00
D'	1.6	52	179.2	-102.8	3.4	4.1	2.7	4.1	1.00

bonds are oriented in the plane, in agreement with obtained NOE data. 4'-OCH_3 and 3-OCH_3 groups are oriented in the opposite site relative to B phenyl ring plane. 7-OCH_3 group can assume two in plane possible orientations, the former observed in the **A** conformer is oriented towards H-6 (τ_1=0°) and the latter (τ_1=180°) being higher in energy by 1 kcal·mol^{-1} is oriented towards H-8. Such in plane orientations explain the NOEs observed between the 7-OCH_3 and H-6, H-8 protons. H-5 is restricted to be in plane because of its hydrogen bonding with the 4-carbonyl group of the isoflavone ring (τ_2=180°). Due to steric interactions between 3-OCH_3 and aromatic ring B the 3-O–CH_3 bond is oriented in plane towards the 4-carbonyl group. Thus, τ_3 torsion is limited to adopt only one value (τ_3=1°). This is consistent with the absence of NOE between 3-OCH_3 and H-6'. A small NOE between the 3'-OH and 4'-OCH_3 group causes restraints to the possible low energy values of τ_7. Indeed, the grid scan around the τ_7 torsion angle confirms that the lowest energy orientation of 3'-OH corresponds to τ_7 equal to 0°. As a consequence of this, the phenolic hydroxyl is in

plane with the aromatic ring B. This orientation is the one adopted by conformer **A**.

Using conformer **A** as a starting structure a 2D grid scan around τ_5 and τ_6 dihedral angles showed that the two lowest energy structures have the 5'-O–CH_3 bond oriented in plane towards H-6' (τ_5=180°) and 4'-O–CH_3 out of plane with τ_6 value to adopt either a value of 101 or -101°. 4'- and 3-OCH_3 groups are oriented at the same site relative to B phenyl ring plane (corresponds to **C'** conformer). The presence of NOE between 5'-OCH_3 and H-6' and the absence of any NOE between 4'-OCH_3 and 5'-OCH_3 are in agreement with these structures.

Using these restraints and **A**, **C'** as starting structures two grid scan searches around τ_4 were performed in order to detect any other low energy conformers. Each grid scan generated four low energy minima **A, B, C, D** (τ_4=52, 134, -134, -52) and **A'**, **B'**, **C'**, **D'** (τ_4=-52, -134, 134, 52). Conformers **A'**, **B'**, **C'**, **D'** are the enantiomers of structures **A**, **B**, **C**, **D**. All the structures were found to be consistent with the NOE data. In Table 3 are shown the most critical dihedral angles and interproton distances which support the observed (2.5-3.5 Å) or absent NOEs (>4 Å). In all eight low energy conformers the two aromatic rings are not coplanar. Representative molecular models of the four low energy conformers **A**, **B**, **C**, **D** are plotted in Figure 5 and the energy profile generated using conformer **A** as starting structure is shown in Figure 4. The lowest in energy conformations were found to be structures **B**, **C** and their enantiomers **B'** and **C**'. This may be attributed to the favorable van der Waals interactions between 3-OCH_3 and 5'-OCH_3 (Figure 5). Figure 6 shows a representative enantiomer pair of flavonoids (**C** and **C**'). The above results were confirmed with a dynamics simulation experiment.

Antimicrobial Activity.

Flavonoids **1** and **2** were tested for their antimicrobial activity against Gram positive and Gram negative bacteria

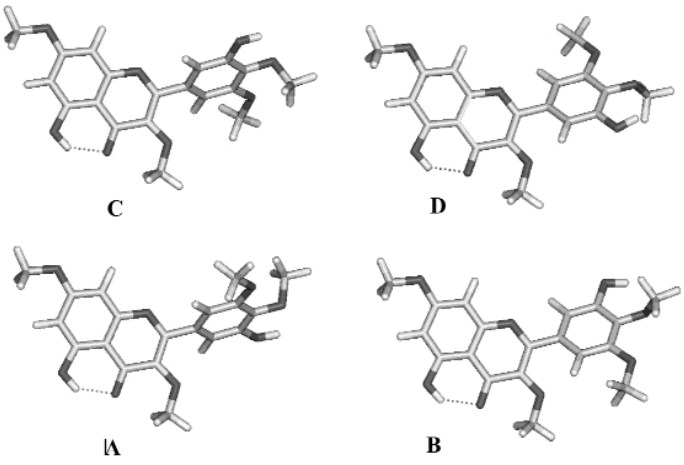


Figure 5. Low energy of four conformers (A, B, C, D) derived after a combination of exhaustive grid scan search, dynamics and use of the observed NOE data.

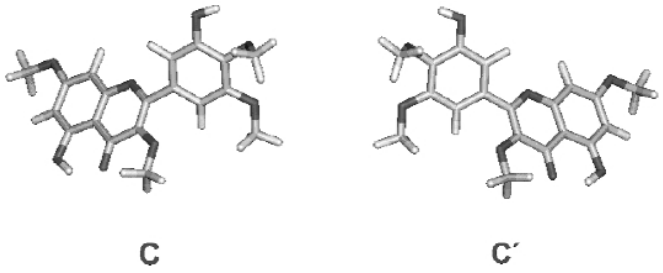


Figure 6. Low energy enantiomeric conformers (**C**, **C**').

[19]. The parent compound myricetin was used for comparison purposes and the well known antimicrobial agents carvacrol and streptomycin were used as controls. The results show that compound **1** exhibited a comparable potency to carvacrol against Gram positive bacteria. The activity of diacetyl derivative **2** was significantly smaller and comparable to that of myricetin. All the tested compounds were inactive against Gram negative bacteria (Table 4).

Table 4

Minimum Inhibitory Concentrations (μg/ml) of **1**, **2** and Myricetin

Microorganism	**1**	**2**	myricetin	Carvacrol	Streptomycin
S. aureus	500	1500	>2000	300	100
S. epidermidis	450	1500	>2000	300	100
K. pneumonia	>1000	>2000	>2000	300	100
E. coli	>1000	>2000	>2000	300	100
Ps. aeruginosa	>1000	>2000	>2000	300	100

Differential Scanning Calorimetry.

Differential scanning calorimetry (DSC) was applied in order to compare the thermotropic properties of compounds **1**, **2** and myricetin in membrane bilayers (Figure 7) [10]. These properties would then be compared with antimicrobial activity.

The DPPC bilayers exist in the gel phase for temperatures lower than 35 °C and in the liquid crystalline phase for temperatures higher than 42 °C. The transition is accompanied by several structural changes in the lipid molecules as well as systematic alterations in the bilayer geometry, but the most prominent feature is the *trans-gauche* isomerization taking place in the acyl chain conformation. The average number of *gauche* conformers indicates the effective fluidity, which depends not only on the temperature, but also on perturbations due to the presence of a drug molecule intercalating between the lipids. The DDPC bilayers show the characteristic pretransition with a low enthalpy-change and a sharp main transition, both at the expected transition temperatures of 35.1 and 41.3 °C. When 1%-mol (x=0.01) of compound **1** was incorporated into DPPC bilayers the most significant changes on the thermal properties of DPPC bilayers were observed. Thus, at this low concentration the presence of compound **1**

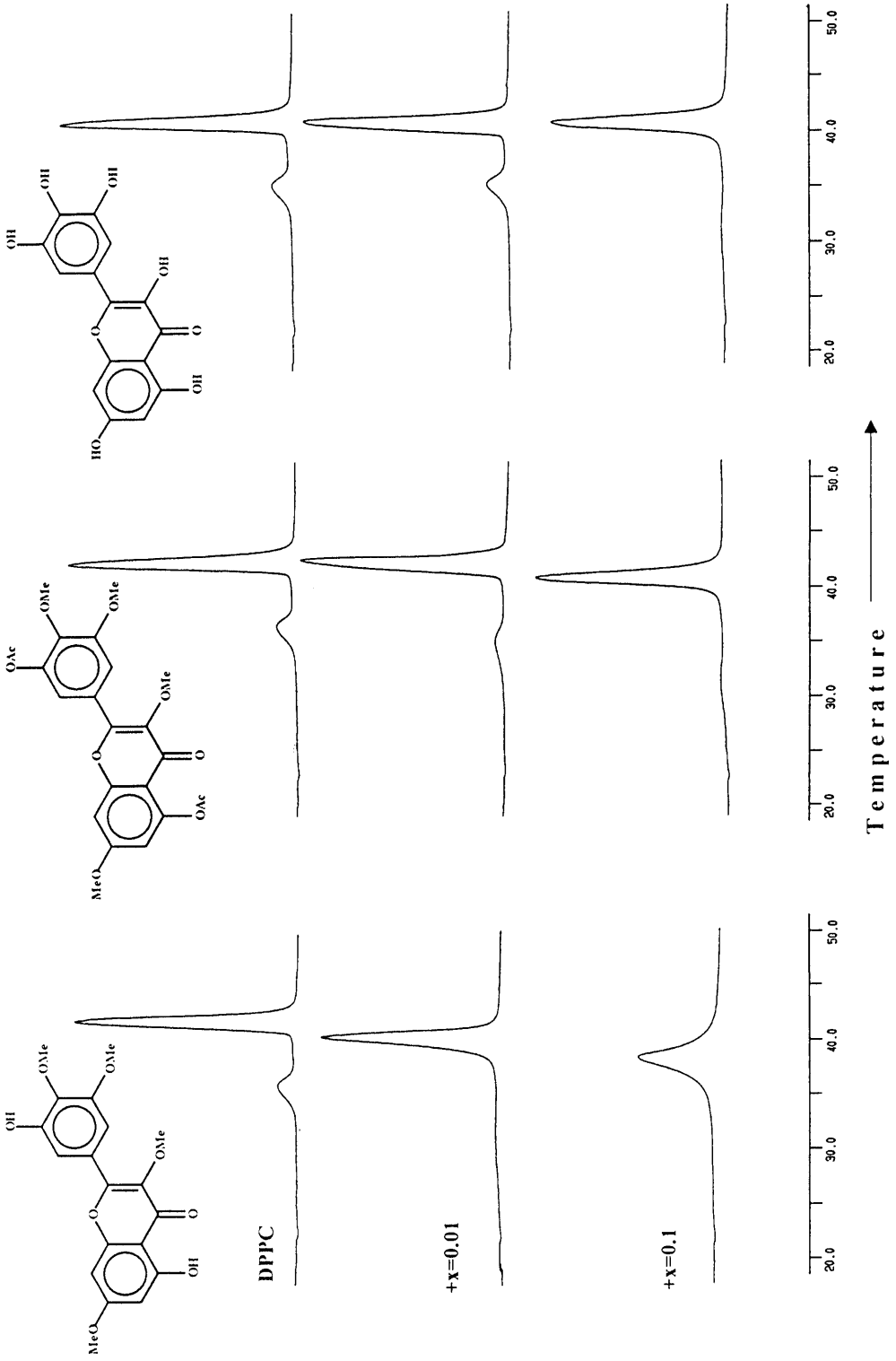


Figure 7. DSC scans of DPPC/1 (x=0.01) and DPPC/1 (x=0.1) (left), DPPC/2 (x=0.01) and DPPC/ 2 (x=0.1) (middle) and DPPC/3 (x=0.01) and DPPC/ 3 (x=0.1) (right).

abolished the pretransition temperature of DPPC bilayer and lowered its phase transition temperature by 1.3 °C. Compound **2** caused only broadening of the pretransition temperature and lowered the main phase transition temperature only by 0.4 °C (Table 5). Myricetin did not cause any significant changes on the thermal properties of DPPC bilayers. At higher concentration (x=0.1) **1** caused significant broadening of the main phase transition and further lowering of the main phase transition temperature (Tc = 39.3 °C). In addition, its incorporation resulted in lowering of the ΔH by

Table 5

Values of Transition Temperature (Tc), Half-Width ($Tc_{1/2}$) and Enthalpy-Change (ΔH) of DPPC and DPPC/Compound **1** or Compound **2** or Myicetin in the three Preparations Studied

Sample	T_c (^oC)	$T_{c1/2}$	ΔH (J/g)
DPPC	35.9,42.1	2.1,1.1	6.7,52.3
DPPC+(*x*=0.01) **1**	40.9	1.3	41.7
DPPC+(*x*=0.01) **2**	34.3, 41.7	3.3, 1.3	4.2,43.3
DPPC+(*x*=0.01) myricetin	36.0, 41.9	2,1.3	6.0,43.5
DPPC+(*x*=0.1) **1**	39.3	2.1	33.9
DPPC+(*x*=0.1) **2**	41.1	1.3	42.0
DPPC+(*x*=0.1) myricetin	41.2	1.4	41.5

ca. 20%. This expresses the significant perturbing effect that takes place during the melting of alkyl chains of the phospholipid bilayer. Such an effect was missing from the other two compounds. Compounds **2** and **3** caused broadening of the pretransition and the main phase transition temperature by 1 °C. These results are in agreement with their antimicrobial activity against Gram positive activity.

Conclusions.

A unambiguous structure elucidation and detailed NMR structure analysis on the membrane active flavonoid **1** using modern 2D NMR techniques in an attempt to study its conformational properties was performed. Such an analysis is missing on the literature. Flavonoid **1** isolated from *C. monspeliensis* L. was found to possess important antimicrobial activity. Therefore, its structure elucidation and conformational properties are of some biological importance. In addition, the complete and unambiguous ^1H and ^{13}C NMR assignments will facilitate further biophysical and synthetic studies. For example, using the information resulted from the complete NMR analysis of compound **1** the NMR assignment of derivative **2** can be realized by performing of only 1D ^{13}C-NMR experiment (see Table 2).

To explore the conformational properties of compound **1** a computational analysis is performed. In this analysis four low energy pairs of enantiomers were derived compatible with experimental data obtained by 2D NOESY spectroscopy. These conformers are characterized by a flexibility of the aromatic ring B, since it was found to be twisted in four orientations relative to isoflavone ring. In addition, there is flexibility of the methoxy group attached to C-4' pointing with equal probability upwards or downwards relative to B phenyl ring. All other critical dihedral angles that characterize methoxy and phenolic hydroxyl groups adopt fixed values that establish their planarity with the rigid isoflavone ring or aromatic ring B.

In the derived low energy conformers **A**, **B** and their enantiomers 3-OCH_3 and 4'-OCH_3 are located in opposite site relative to B-phenyl ring, while in **C**, **D** and their enantiomers are located in the same site. The lowest energy compatible with NOE data among eight observed conformers and therefore most possible likely are **B**, **C** and their enantiomers. A possible reason for this *vis a vis* the other low energy conformers is that τ_4 dihedral angle imposes 3-OCH_3 and 5'-OCH_3 to a spatial promixity and optimum Van der Waals interactions.

Compounds **1** and **2** were tested in terms of their antimicrobial activity and compared with that of myricetin. The antimicrobial results for **1**,**2** and myricetin followed the order **1**>**2**>myricetin.

In order to examine any structure activity relationships and effects of the flavonoids on the membrane bilayers their effects on the thermal properties on the model bilayer DPPC were examined. Using as diagnostic parameters the phase transition temperature (Tc), enthalpy change (ΔH) and the linewidth change (ΔTc1/2), DSC results revealed that the degree of the thermal effects exerted by the flavonoids at DPPC bilayers followed the same order. An interesting result is the far more perturbing effect observed by compound **1** compared to compounds **2** and myricetin. Thus, flavonoid **1** lowered ΔH by almost 20% while the other two affected it only marginally. It also broadened and lowered more effectively the phase transition temperature of DPPC. These results point out that the studied molecules may exert their antimicrobial activity through membrane perturbation. This proposition is validated from the fact that the three compounds under study have only small structural differences. However, their thermal effects are clearly distinctive. In particular, the effects of agent **1** are far more pronounced compared to those of **2** and **3.** Interestingly, the most effective flavonoid **1** has an intermediate polarity compared to **2** and myricetin. This shows that optimum lipophilicity may account for its effectiveness on membrane bilayers.

EXPERIMENTAL

Materials.

Dipalmitoyl-glycero-*sn*-3-phosphatidylcholine (DPPC) was obtained from Avanti Polar Lipids, Inc. AL, USA. Compound **1** was isolated from *Cistus monspeliensis* L. and flavonoid **2** was synthesized in our laboratory. Flavonoid **3** was purchased from Sigma.

Plant Material.

The aerial parts of C. *monspeliensis*, were collected in July 1997 on the island of Crete. A Specimen was identified by Dr. D. Perdetzoglou (Dept. of Pharmacognosy, School of Pharmacy, University of Athens, Greece). A Voucher specimen has been deposited in the Dept. of Pharmacognosy, School of Pharmacy, University of Athens, Greece).

Extraction of Plant Material. Isolation of Compound **1.**

The air-dried and powdered leaves of C. *monspeliensis*, were extracted with hexane at room temperature. The hexane extract was fractionated by column chromatography (silica 230-400 mesh, Merck), using hexane, hexane-CH_2Cl_2 and CH_2Cl_2-EtOAc, mixtures of increasing polarity. Seven fractions were obtained (200 mL each), and tested by TLC. The fifth fraction was further fractionated by column chromatography, using hexane, hexane-Et_2O mixtures of increasing polarity. Eleven fractions were obtained (100 mL each),

the purity of which were tested by TLC. From the seventh fraction, compound **1** was isolated by preparative TLC (CH_2Cl_2-MeOH 98:2).

Synthesis of Compound **2**.

Compound **1** (10 mg) was treated with a mixture of acetic anhydride in pyridine. The resulting mixture was left for 48 hours at room temperature to yield compound **2** (8 mg).

Methods.

Differential Scanning Calorimetry.

An appropriate amount of the phospholipid with or without the flavonoid under study was dissolved in spectroscopic grade chloroform. The solvent was then evaporated by passing a stream of O_2-free nitrogen over the solution at 50 °C and the residue was placed under vacuum (0.1 mmHg) for 12 hour. For measurements this dry residue was dispersed in appropriate amounts of bidistilled water by vortexing. After dispersion in water (50% w/w), portions of the samples (*ca.* 5 mg) were sealed in stainless steel capsules obtained from Perkin-Elmer. Thermograms were obtained on a Perkin-Elmer DSC-7 calorimeter. All samples were scanned at least twice until identical thermograms were obtained, using a scanning rate of 2.5 °C/minute. The temperature scale of the calorimeter was calibrated with indium (Tm=156.6 °C). Thermograms from samples stored at freezer temperatures (-15 °C) for a few days were identical to those run immediately after sample preparation [10].

Antimicrobial Activity.

The microbial strains (Table 4) used were from the American Type Culture Collection. Culture media were purchased from Oxoid (U.K). MIC of compounds **1**, **2**, myricetin carvacrol and streptomycin were determined by a microdilution assay essentially as recommended by NCCLS [19]. Microbial cells were suspended in Mueller Hinton broth to give a final density of 5×10^5 - 10^6 CFU/ml and incubated at 37 °C for 18 hours under aerobic conditions with a methanolic solution of the compounds under study. Control microbial cultures were incubated with methanol under the same conditions. Methanol was found not to be toxic under these experimental conditions. MIC was defined as the lowest concentration that inhibited visible growth.

High Resolution NMR Spectroscopy.

1D and 2D NMR spectra were obtained using programs available in the library of a Bruker AC 300 MHz spectrometer. All spectra were run in $CDCl_3$ (for ^1H spectra the $CHCl_3$ peak at 7.24 ppm was used as an internal reference and for ^{13}C the central $CDCl_3$ line was set at 77.7 ppm). All experiments were run at ambient temperatures. NOESY experiments were performed using different mixing times (0.3-2.0 s) in order to ensure that observed NOEs were not due to the spin diffusion. An optimum mixing time of 0.5 s was found.

Computational Chemistry - Molecular Modeling.

Computer calculations were performed on a Silicon Graphics O2 using the QUANTA software package. Molecular mechanics calculations were carried out using the CHARMm force field. The various torsion angles of the three dimensional structure of flavonoid **1** were suitably manipulated and optimized first with conjugate gradient and then with Newton-Raphson minimization algorithms, using an energy gradient tolerance of 0.01 kcal mol^{-1} $Å^{-1}$, to reach a local minimum that agrees with the observed NOEs. To find the preferred torsion angles that correspond to the lowest energy conformers and energy barriers of compound **1**, bond rotatory searches (systematic grid scan searches) were used. Intervals of 5° were applied for single bond rotation, and 10° for two-bond rotation. During bond rotation searches the predetermined torsion angle remained constant while minimization using 200 steps of conjugate gradient algorithm was applied to relax the whole molecule. The lowest energy conformers found were further minimized to reach local minima and used as starting structures for the next grid scan search.

Molecular dynamics simulations on agent **1** were carried out at 1000.0 K with time steps of 1 fs for 300 ps using an output frequency of 1 ps to sample 300 frames of conformers. After cluster analysis, the obtained low energy conformers of each cluster were then further minimized. These conformers were compared and agreed with those obtained using a combination of exhaustive grid scan search and molecular mechanics calculations [14].

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REFERENCES AND NOTES

* C. Demetzos: tel. +30-1-727-4596; fax. +30-221-82210; e-mail: demetzos@galenos.pharm.uoa.gr

† Present address: School of Pharmacy, Department of Pharmaceutical Chemistry, Panepistimiopolis, Zografou 15771 University of Athens, Greece.

[1] D. Pathak, K. Pathak, and A. K. Singla, *Fitoterapia*, **5**, 371 (1991).

[2] M. Cushman and D. Nagarathnan, *J. Nat. Prod.*, **59**, 1656 (1991).

[3] N. Kaneda, J. M. Perruto, D. D. Soejarto, A. D. Kinghorn, N. R. Farnsworth, and P. Tuchinda, *Planta Med.*, **56**, 672 (1990).

[4] T. Vogt, P. Proksch, and P. G. Gulz, *J. Plant Physiol.*, **131**, 25 (1987).

[5] W. Greuter and R. Raus, Med-Checklist Notule, *Willdenowia*, **4**, 275 (1981).

[6] C. Demetzos, C. Harvala, S. M. Philianos, and A. L. Skaltsounis, *J. Nat. Prod.*, **53**, 1365 (1990).

[7] C. Demetzos, S. Mitaku, M. Couladis, C. Harvala, and D. Kokkinopoulos, *Planta Med.*, **60**, 590 (1994).

[8] C. Demetzos, S. Mitaku, A. L. Skaltsounis, M. Couladis, and F. Libot, *Phytochemistry*, **35**, 979 (1994).

[9] C. Demetzos, B. Stahl, T. Anastassaki, M. Gazouli, L. Tzouvelakis, and M. Rallis, *Planta Med.*, **65**, 76 (1999).

[10a] T. Mavromoustakos, E. Theodoropoulou, and D.-P. Yang, *Biochim. Biophys. Acta*, **1328**, 65 (1997); [b] T. Mavromoustakos, I. Daliani, *Biochim. Biophys. Acta*,. **1420**, 252 (1999).

[11] G. Berti, O. Livi, D. Segnini, and I. Cavero, *Tetrahedron*, **23**, 2295 (1967).

[12] E. Wollenweber and K. Mann, *Z. Naturforsch*, **39c**, 303 (1984).

[13] H. Wagner, V. M. Chari, and S. Johann, *Tetrahedron Letters*, **21**, 1799 (1976).

[14] C. J. Chang, *Lloydia*, **41**,17 (1978).

[15] C. D. Hufford and L. Lasswell, *Lloydia*, **41**, 151 (1978).

[16] K. R. Markham, Techniques of Flavonoid Identification, Academic Press Inc (London) Ltd. (1982).

[17] A. Makriyannis and J. J. Knittel, *Tetrahedron Letters*, **30**, 2753 (1979).

[18] A. E. Howard and P. A. Kollman, *J. Med. Chem.*, **31**, 1669 (1988).

[19] National Committee for Clinical Laboratory Standards Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; National Committee for Clinical Laboratory, Standards, Wayne, Pa., (1995) Vol. 15, p. 14, Approved standard no M7-A3.