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Structure Elucidation and Conformational Properties of a Novel Bioactive Clerodane Diterpene Using a Combination of High Field NMR Spectroscopy, Computational Analysis and X-ray Diffraction

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Abstract—The structure of a novel CC clerodane type diterpenoid, namely (+)-19-acetoxy-*cis*-clerodan-3-ene-15-oic acid **1** was elucidated and its conformational properties were explored using a combination of high field NMR spectroscopy and computational analysis. The structural analysis provided results consistent with those obtained by a single X-ray diffraction study of its dicyclohexylammonium salt. The new clerodane type diterpene isolated in large quantities from *Cistus monspeliensis* L. leaves was found to exhibit significant antibacterial activity against *Staphyloccoci* ($MIC_{50} = 0.085$ mM) and therefore represents a promising lead compound. Interestingly the deacetylated derivative **2** of compound **1** was inactive.

Introduction

The isolation and identification of potential lead bioactive compounds from natural sources is a desirable task. Our on going program focused on the identification of new bioactive natural products, hopefully in fair amounts, from a widely distributed species of the Mediterranean area, i.e., *Cistus monspeliensis* L. (Cistaceae). In folk medicine its flower branches were used for asthma, while infusion of leaves replaces tea.[1]

Clerodane diterpenoids are a family of secondary metabolites. Some of them were found to possess considerable biological activity. As a result of this biological potentiality, an intensive and competitive research was triggered. Four types of clerodanes are known with respect to the stereochemistry of ring fusion and C-8, C-9 substitution, i.e., types TC, TT, CC and CT. Hitherto, the three quarters of isolated clerodanes have *trans*-ring fusion.[2,3] The relative stereochemistry of the substituents provides a means for applying SAR studies in order to explore the stereoelectronic requirements for their activity.

The CC subclass of these bicyclic compounds is structurally characterized by a *cis*-decalin ring bearing at 8 and 9 positions two methyl groups *cis* to each other. In addition, the position 9 is substituted by another group of varied structure that is *cis* with H10. Few examples of molecules are reported in the literature falling in this subclass.[2,3] In this paper, we report the identification and structure determination of two new CC clerodane diterpenoids which were isolated from *C. monspeliensis* L. leaves. To the best of our knowledge compound **1** is one of the simplest in structure of *cis* clerodanes and one of the most active natural products against *Staphylococci*. The deacetylated derivative of compound **1**, i.e., compound **2**, exhibit modest activity.

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Table 1. ^{1}H and ^{13}C chemical shifts (ppm) of clerodane **1** obtained in CD_2Cl_2 solution at 298 K (600 MHz)

Protons	Chemical shifts	Protons	Chemical shifts	Carbons	Chemical shifts	Carbons	Chemical shifts
8 CH_3	0.74 d (7)	8	1.45 m	8 CH_3	16.03	5	36.59
9 CH_3	0.76 s	1α, 1β	1.98 m, 1.82 br dd (14, 8)	1	17.52	11	37.50
13 CH_3	0.97 d (7)	13	1.88 m	9 CH_3	17.55	8	37.68
5 CH_3	1.09 s	CH_3CO	2.05 s	13 CH_3	20.00	9	40.34
12,12′	1.13 m, 1.27 m	2	2.15 m, 2.20 m	CH_3CO	21.31	14	41.60
11,11′	1.27 m, 1.94 m	14	2.19, 2.40 dd (14, 8)	2	24.32	10	45.37
7α,β[a]	1.27 m	4-CH_2OAc	4.55 s	7	29.18	4 CH_2OAc	66.81
6α, 6β	1.27 m, 1.44 m	3	5.65 br t (3)	12	29.51	3	129.39
10	1.37 br d (5)			13	31.30	4	138.89
				5 CH_3	34.73	4 CH_2OCOCH_3	171.00
				6	35.37	14 CO_2H	178.70

[a]α and β are used to designate axial and equatorial positions, respectively.

Results and Discussion

Natural product material

The plant material (100 g) was powdered and extracted with hexane to yield the nonpolar compounds, followed by methanol. The hexane extract (7.5 g) of *C. monspeliensis* L. leaves was subjected to chromatography on silica gel (230–400 mesh ASTM, Merck) using hexane/CH_2Cl_2, CH_2Cl_2/EtOAc mixtures of increasing polarity, to yield nine fractions (1–9). Fraction 7 (3.8 g) (CH_2Cl_2/EtOAc 1:1) was further fractionated using hexane/ether mixtures of increasing polarity to yield compound **1** as an oil (800 mg) (hexane/ether 4:6) with $[\alpha]_D$ +14° ($c = 20.75$ g/100 mL, $CHCl_3$). Compound **2** (30 mg) was isolated from the methanolic extract or was yielded after sodium methoxide/methanol treatment of compound **1**.

Structure determination—conformational analysis

The biological significance of this molecule prompted us to perform its structure determination and conformational analysis studies using a combination of high field NMR spectroscopy, X-ray diffraction and computational analysis.

The ^{1}H NMR spectrum of the new compound at 298 K contained peak patterns that resembled those reported for structurally similar compounds.[4] Therefore, the assignments in the literature became the starting point in the structure elucidation. However, total structure assignment could not be made based solely on these reports, because they lacked detailed information on the relative stereochemistry of the chiral centers. For this, a complete assignment of protons and carbons was sought based on the additional information provided by the chemical shifts as well as the successive bond connectivities using a combination of 2D ^{1}H–^{1}H COSY, 2D ^{1}H–^{1}H NOESY, ^{1}J C–H HSQC and ^{3}J C–H HMBC correlation spectroscopy and 300, 500 and 600 MHz spectrometers. The combined information of these experiments led to the unambiguous structural formula of the new 19-acetoxyclerodan-3-ene-15-oic acid **1** (Fig. 1). In Table 1 are provided the ^{1}H and ^{13}C chemical shifts of compound **1** obtained in CD_2Cl_2 solution at 298 K.

A combination of 2D NOESY and ROESY spectroscopy using various mixing times was applied in order to provide information about the conformational properties of compound **1** and resolve the relative orientation of the three methyl groups and the 3-methyl-pentanoic acid moiety attached to the decalin system. The *cis* decalin ring system was established from the strong NOE between 5CH_3 and 10H.[5] The NOEs between 11H and 1Ha, 8CH_3 and 10H as well as the NOE between 9CH_3 and 7Ha protons support axial orientation of 9CH_3 and equatorial position of the 3-methyl-pentanoic acid moiety. The strong NOEs between 8CH_3 with 11H and 7Hα,β as well as the moderate NOE with 13CH_3 confirm its equatorial position.

In order to further confirm the structure of (+)-19-acetoxy-*cis*-clerodan-3-ene-15-oic acid **1** its dicyclohexylammonium salt was crystallized from a CH_2Cl_2/pentane solution and an X-ray analysis was undertaken. The

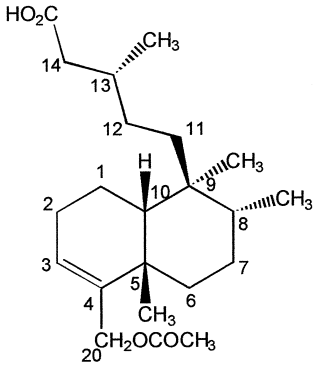

Figure 1. Molecular structure of compound **1**.

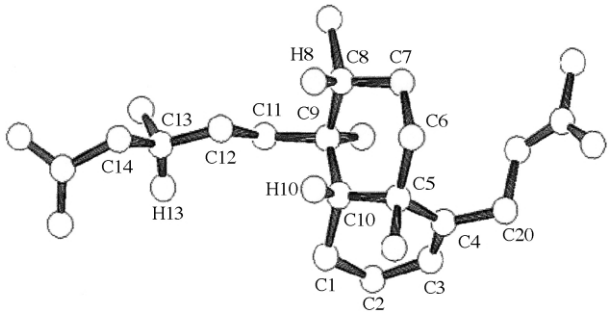

Figure 2. X-ray structure of the anion form of (+)-19-acetoxy-*cis*-clerodan-3-ene-15-oic acid **1**.

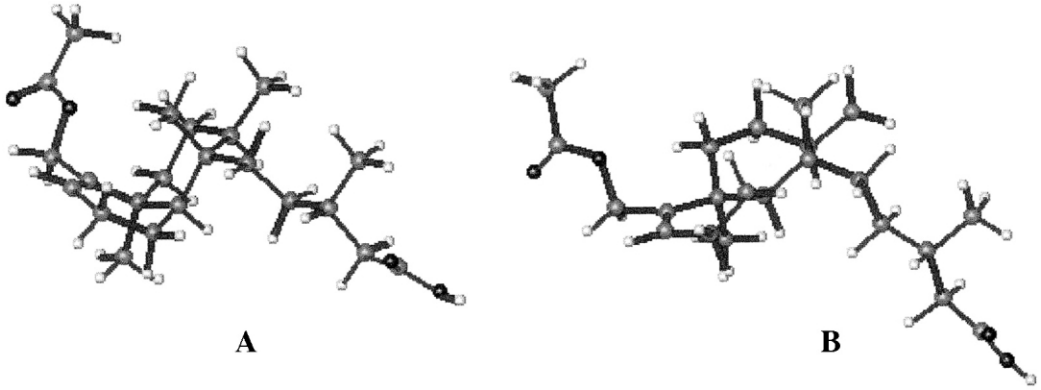

Figure 3. Low energy possible bioactive conformers (**A**, left; **B**, right) of clerodane **1** as they were depicted from a molecular dynamics experiment and molecular mechanics calculations.

cyclohexane ring was found to adopt a chair conformation and cyclohexene ring a sofa. In sofa conformation all the carbons of cyclohexene ring except 10C are in plane. The X-ray structure of compound **1** and the lowest energy conformation using a combination of computational analysis and NOESY/ROESY experiments are shown in Figures 2 and 3 (structure **A**).

A stochastic search of the conformational space of **1** using a molecular dynamics simulation was undertaken in order to explore possible low energy conformers consistent with NOE correlations. A starting structure was constructed using X-ray data and by imposing the most important NOE constraints described in the Table 2. The lowest energy conformer **A** accounted for all observed NOE data, and its decalin ring consisted of the cyclohexane and cyclohexene rings adopting a chair and sofa conformations, respectively (Fig. 3, Table 2) in agreement with X-ray analysis. The dynamics experiment followed by molecular mechanics analysis revealed a highly unfavored conformer **B** which was higher by 3.42 kcal mol^{-1} in energy,[6] explained by the diaxial steric interactions between $8CH_3$ and $9CH_3$ as well as 9-carbon chain and $5CH_3$ groups. This is confirmed by observing no significant spectral changes at a temperature range of $-20\,^{\circ}C$ to $-80\,^{\circ}C$ in ^1H and ^{13}C spectra. The decalin ring of conformer **B** consisted of a twist-boat cyclohexane and a distorted half-chair cyclohexene rings. These twist-boat, half chair conformations of the two rings resulted through inversion of conformation **A** chair ring.

The chair conformation of cyclohexane found in conformation **A** supports the broad doublet ($J = 5$ Hz) observed for 10H and assigned at 1.35 ppm. If cyclohexane was adopting a twist-boat conformation as found in **B**, a doublet with a coupling constant of ~10–12 Hz should be expected reasoned by the *trans* relationship between 10H with one of the 1H protons of cyclohexene.

Table 2. Most important interproton distances that establish relative orientation of the three methyl groups and 3-methyl-pentanoic moiety attached to the decalin ring as depicted from the combination of X-ray diffraction, NMR spectroscopy and computational analysis

Interproton distances[a]	According to X-ray structure	According to lowest energy structure	Observed NOE
11H–$9CH_3$	2.08	2.19	s
11H–$8CH_3$	2.50	2.39	s
11′H–10H	2.82	3.20	m
11′H–1(eq)H	1.82	2.13	s
$8CH_3$–7(eq)H	2.38	2.42	s
$8CH_3$–$13CH_3$	3.57	3.41	m
$8CH_3$–$9CH_3$	2.17	2.21	[b]
10H–6(ax)H	2.62	2.57	s
$5CH_3$–6(ax)H	2.34	2.41	m
$5CH_3$–1(ax)H	2.00	2.20	m
$9CH_3$–7(ax)H	1.96	2.19	s
$5CH_3$–10H	2.49	2.39	s
10H–8H	2.32	2.38	s
$4OCH_2$–$5CH_3$	2.26	2.41	s
$OCOCH_3$	3.60	3.52	l
$OCOCH_3$	2.70	2.67	m

[a]When the distances between protons were not unique the shortest was chosen.
[b]Not detectable due to the resolution of the ^1H spectrum.

Biological activity evaluation

The antimicrobial activity of **1** and **2** is shown in Table 3. Compound **1** was found to be very active against *Staphylococci* ($MIC_{50} = 0.085$ mM) and had a moderate activity against Gram negative bacteria ($MIC_{50} = 1.37$ mM). To the best of our knowledge compound **1** seems to be one of the most active natural products

Table 3. Antimicrobial activity of compounds **1** and **2**

Microorganisms	MIC_{50}[a] (mM)			
	1	2	Sclareol	Streptomycin[b]
S. aureus (ATCC 6538)	0.085	1.24	0.32	0.0021
S. epidermidis (ATCC 12228)	0.085	1.24	0.32	0.0021
S. hominis (ATCC 27844)	0.085	1.24	0.32	0.0021
K. pneumoniae (ATCC 13883)	1.37	0.93	>1623	0.0006
E. coli (ATCC 25922)	1.37	0.93	>1623	0.0006
P. aeruginosa	1.37	0.93	>1623	Not tested

[a]MIC_{50} was defined as the lowest concentration that inhibited visible growth. All data represent mean values for at least two separate experiments. For in vitro assays see ref 7.
[b]Sulfate salt.

against *Staphylococci*. It is four times more active than sclareol, a labdane type diterpenoid with significant activity against *Staphylococci*[7] and its activity is close to that of streptomycin (Table 3). In addition, the new isolated compound has the simplest in structure of molecules falling in the subclass CC and therefore can serve as a lead compound for further SAR studies. Compound **2** showed a moderate activity, much lower than that of compound **1**. The presence of the hydroxyl group at C-20 reduced dramatically its activity against *Staphylococci* while its activity against Gram negative bacteria remained comparable to that of compound **1** (Table 3). None of the new compounds exhibit significant cytotoxic or cytostatic activity against human leuchemic cell lines, i.e., CCRF-CEM, MOLT4, HUT78, HL60 and K562.

Conclusion

Compound **1** with a novel structure and potent antimicrobial activity against Gram positive bacteria was isolated from leaves of *C. monspeliensis* L. in large quantities (0.8% w/w yield). Its deacetylated analogue **2** was inactive. The combination of high field NMR spectroscopy, computational chemistry and X-ray analysis show that compound **1** is of a CC type clerodane diterpenoid. These results open an avenue for the development of SAR studies and the synthesis of new derivatives with better biological profile.

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References and Notes

1. Polumin, O. *Flowers of Europe, A Field Guide*. Oxford University Press: Oxford, 1969; p 254.
2. Tokoroyama, T. *Synthesis*, **2000**, 611 and references cited therein.
3. For useful reviews see: Hanson, J. R. *Nat. Prod. Rep*. **1995**, *12*, 207; **1996**, *13*, 59; **1998**, *15*, 93; **1999**, *16*, 209
4. The resonances at 5.65 ppm (t, $J=3$ Hz), 4.55 ppm (dd, $J=2$, 5 Hz) and 2.08 ppm (s) were correspondingly assigned as 3, $4OCH_2$ and acetate CH_3 protons and represent the characteristic clerodane functionality $-CH{=}C\text{-}CH_2OCOCH_3$. The resonances at 0.97 and 0.74 ppm (d, $J=7$ Hz) correspond to 13 and $8CH_3$. The signals observed at 2.19 and 2.40 ppm assigned to $14CH_2$ are characteristic of a clerodane-15-oic acid.
5. $5CH_3$ resonance at 1.09 ppm and the 10H resonance (d, $J=5$ Hz) at 1.37 ppm agree with those reported for a *cis* structure by Jakupovic, J.; Baruah, R. N.; Zdero, C.; Eid, F.; Pathak, V. P.; Chau-Thi, T. V.; Bohlmann, F.; King, R. M.; Robinson, H. *Phytochemistry* **1986**, *25*, 1873.
6. According to Boltzmann equation $\Delta G^{\circ} = -RT\ln(N_1/N_2)$.
7. Demetzos, C.; Stahl, B.; Anastassaki, M.; Gazouli, M.; Tzouveleki, L.; Rallis, M. *Planta Medica* **1999**, *65*, 76.