CLEROSTEROL AND 3-HYDROXY-HEXADECANOIC ACID METHYL ESTER FROM *AJUGA IVA* LEAVES,

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(Soumis en février 1998, accepté en mai 1998)

Abstract : Two known compounds have been isolated for the first time from the leaves of *Ajuga iva* (Labiatae) : (24S)-28-methyl-25-methylene cholest-5-en-3-ol (clerosterol) and 3-hydroxy-hexadecanoic acid methyl ester. This study involves the structural elucidation of Clerosterol by one and two-dimensional NMR techniques.

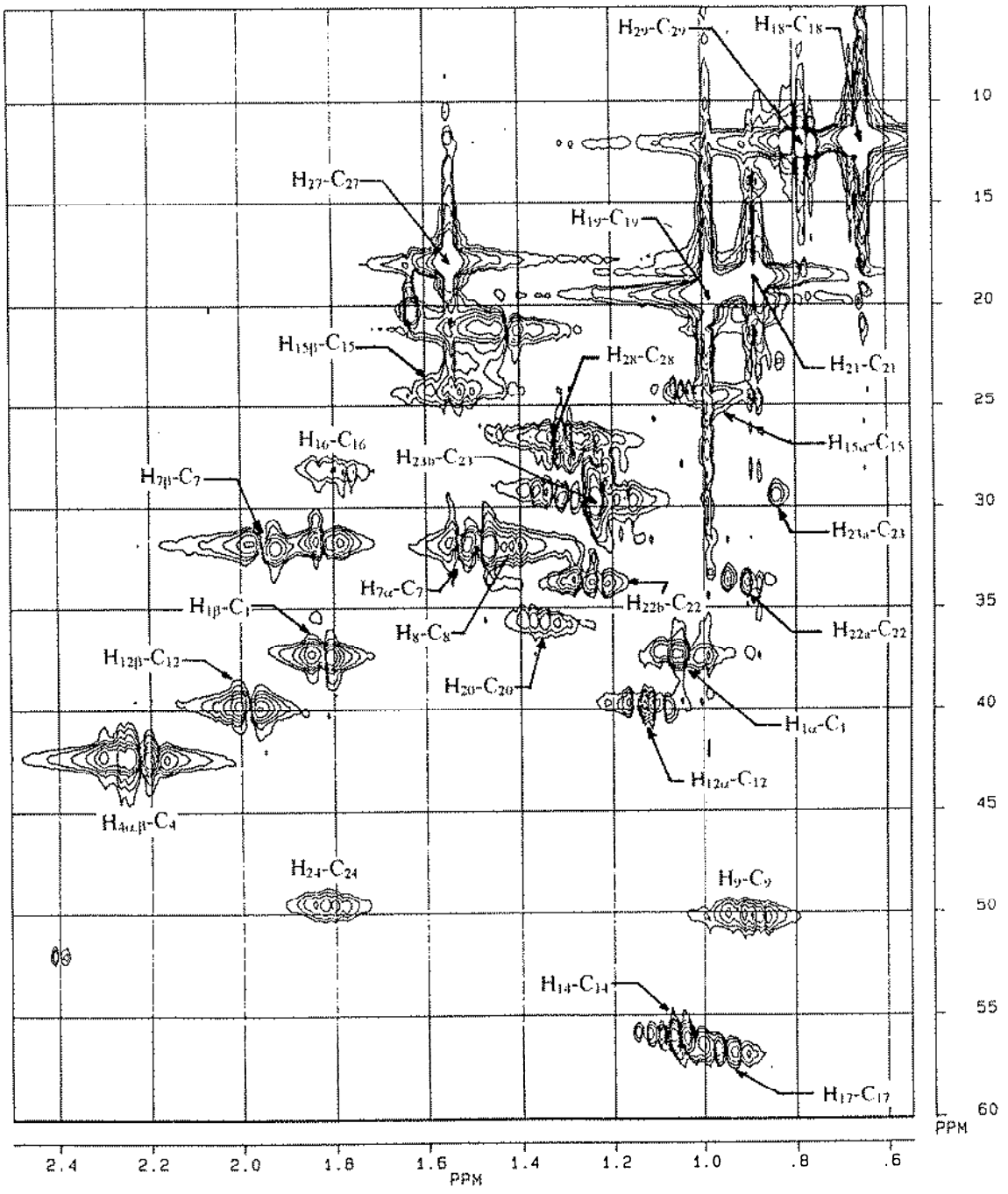
Résumé : Deux composés connus ont été isolés pour la première fois à partir des feuilles d'*Ajuga iva* (Labiées) : le (24S)-28-méthyl-25-méthylène cholest-5-én-3-ol (clérostérol) et l'ester méthylique de l'acide 3-hydroxy-hexadécanoïque. La structure du clérostérol a été élucidée par les techniques de RMN 1D et 2D.

Keywords : *Ajuga iva*, Labiatae, steroid, ester of fatty acid.

Introduction : Our research work fits into the contribution of the chemical study of medicinal plants growing in Tunisia (1-4). One of the latter, *Ajuga iva* (Labiatae), is a medicinal plant used in folk medicine by the inhabitants of North Africa. It has several interesting properties: it is antifebrile, anthelmintic, hypoglycaemic, therefore useful to diabetic people, vulnerary effects have been attributed to it and it is also used to treat inflammation (5-7). Following our studies on this plant, we report in this paper the isolation and the structure elucidation of a steroid (clerosterol) and the methyl ester of a 3-hydroxy fatty acid which have been isolated for the first time from *Ajuga iva* leaves.

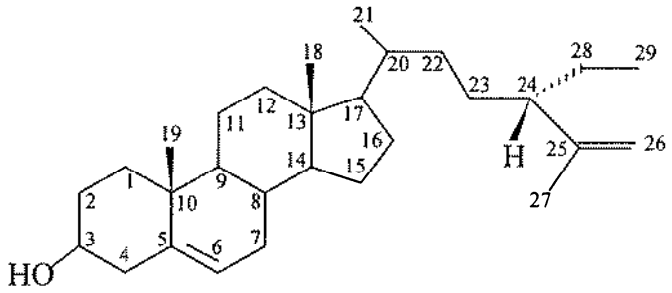
We have recently reported the isolation of three new diglyceride compounds (8) from the leaves of this plant, previous chemical studies which have been made on *Ajuga iva* showed the isolation of clerodane diterpenoids (5), flavonoids (9) and ecdysteroids (10-12).

fig 1. Portion of the HMQC spectrum of compound 1 showing CH correlation via one-bond carbon-proton coupling



Results and discussion :

Compound 1 : Sigel cc of the acetone extract of the dried leaves afforded four fractions. The third one after extraction with hexane and repeated chromatography of polar fraction provided clerosterol ((24S)-28-methyl-25-methylene cholest-5-en-3-ol), obtained as a white powder. The elemental analysis and the MS which contained the molecular ion peak at m/z 412, indicated a molecular formula of $C_{29}H_{48}O$ and six degrees of insaturation. The significant fragment ion at m/z 84 is a typical McLafferty rearrangement, diagnostic of terminal Δ^{25} double bond in the side chain. The fragment ion at m/z 366 ($M-H_2O-C_2H_2$) indicated the presence of a side chain containing one double bond. The peak at m/z 239 corresponded to the loss of a part of ring A. The peak at m/z 149 (100%) confirms the steroidal structure and is also used to assign the Δ^5 insaturation in ring B. The ^1H NMR spectrum (table I) showed the presence of a terminal methylene group by the observation of olefinic signals at at δ 4.61 (1H, br s) and δ 4.70 (1H, br s) (13, 14). This was reinforced by the IR spectrum showing an absorption band at 1374 cm^{-1}. Furthermore, the ^1H NMR spectrum displayed a signal at δ 5.34 (1H, m) characteristic of a Δ^5 vinyl proton coupled with $H_{7\text{-}\alpha}$ and $H_{7\text{-}\beta}$. The enlargement of the 0.6-2.4 ppm zone of the ^1H NMR spectrum allowed us to attribute the five methyl groups.



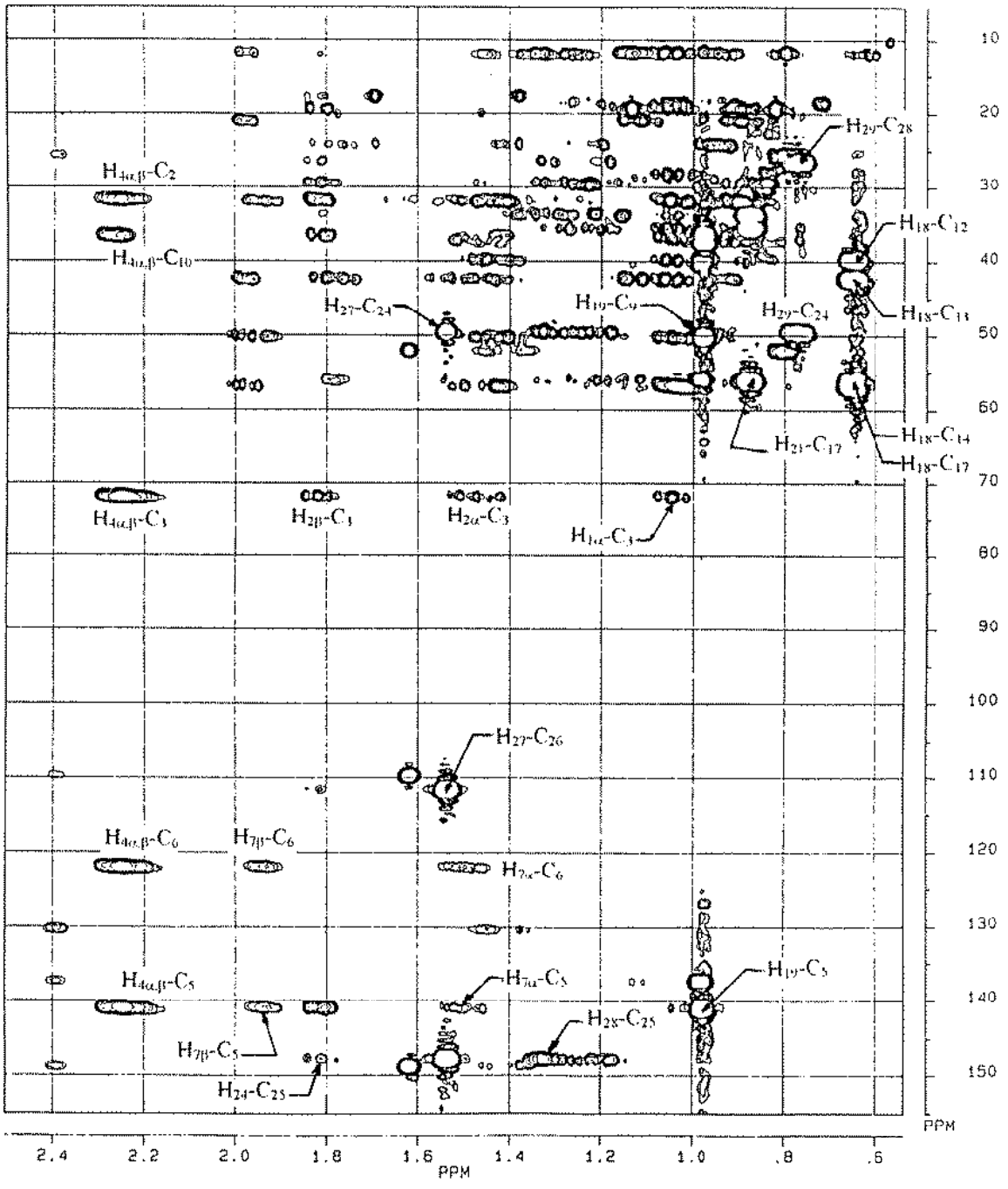
Clerosterol

2D homo and heteronuclear experiments (HMQC fig1, HMBC fig2 and fig3) were also carried out to confirm the proposed structure.

The stereochemistry of C_{24} was identified as 24S by comparison of the physical constants (mp and $[\alpha]_D^T$) of the studied compound with the same one isolated from *Ajuga reptans* (15). The measures carried out were mp = 133 °C, $[\alpha]_D^{20}$ = -41.88° (c=0.97, $CHCl_3$) . They were in keeping with those of the literature (15).

Compound 2 : It is identified as a novel autoregulator controlling virulence in Ralstonia Solanacearum (16). The elemental analysis and the mass spectrum indicated a molecular formula of $C_{17}H_{34}O_3$ (Mr 286) with only one degree of insaturation. The ion at m/z 103

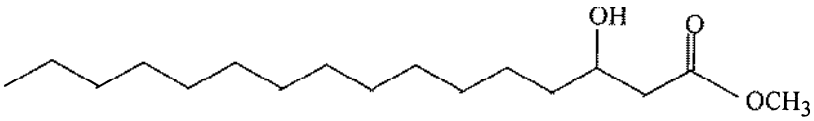
fig 2. Portion of the HMBC spectrum of compound 1 showing CH correlation via long-range coupling



(100%) corresponds to the typical fragment ($^{+}OH=CH-CH_2-COO-CH_3$) (16) showing the presence of a hydroxyl group in position 3 in relation to the carbonyl of the ester function, m/z 255 ($M-OCH_3$). This confirms that this compound is a methyl ester, the peak at m/z 74 corresponds to a McLafferty rearrangement which confirms on the other hand the methyl ester structure. Peaks at 29, 43, 57, 71, 85, 99, 113, 127, 141, 155, 169 are easily attributable to alkyl ions obtained further to the split in the C-C link along the hydrocarbon chain. The IR spectrum showed bands for hydroxyl 3217 cm^{-1}, and carbonyl of an ester function 1729 cm^{-1}. The ^1H NMR spectrum (table II) displayed signals at δ 0.86 (3H, t, J=6.9 Hz) and at δ 1.29 (m) relative to a terminal methyl group of hydrocarbon chain and $(CH_2)_n$, respectively. The signal at δ 2.84 (1H, s) was identified as the proton of the hydroxyl. The signal at δ 3.62 (3H, s) was assigned to the methoxy protons. More information was obtained from the enlargement of the 2.3-2.5 ppm zone which allowed us to see two dd 2.36 (1H, dd, J1=15.4 Hz, J2=8.1 Hz) and 2.46 (1H, dd, J1=15.4 Hz, J3=8.1 Hz), these two gem protons coupled with another proton resonating at 3.97 ppm (1H, m) (CHOH).

The ^{13}C NMR spectrum showed in particular the presence of a carbonyl of an ester function (172.9 ppm), a hydroxyl carbon (68.6 ppm) and a methoxy group (51.5 ppm). The same spectrum confirms the hypothesis of the presence of a long hydrocarbon chain.

The physical constants measured are mp = 69 °C, $[\alpha]_D^{20}$ = -35.2 (c=0.3, $CHCl_3$)



3-hydroxy-hexadecanoic acid methyl ester

Experimental :

General experimental procedure : NMR spectra were recorded on Bruker WM 250 or Bruker AM 400 spectrometers operating at 250.13 MHz or 400.13 MHz for ^1H and 62.53 MHz or 100.6 MHz for ^{13}C. ^1H NMR chemical shifts were referenced to residual hydrogen absorptions of $CDCl_3$ (7.24 ppm) or of $(CD_3)_2CO$ (2.03 ppm), coupling constants are given in Hz. ^{13}C nmr chemical shifts were referenced to $CDCl_3$ (77.0 ppm) or to $(CD_3)_2CO$ (30.1 ppm). High resolution mass spectra were determined on AEI MS 9 or AEI MS 902. IR spectra

were recorded on a Bio-Rad win-IR PRO Spectrophotometer. Melting point determination was performed on a Büchi 510 apparatus using capillary tubes.

Microanalysis were performed by the service of elemental analysis at the School of Chemical Sciences, University of East Anglia, Norwich, England.

Rotary power determination was performed in $CHCl_3$ on a Perkin elmer 241 MC polarimeter.

Plant material : The leaves were collected in June 1995 in Monastir (Tunisia) and voucher specimens were deposited at the herbarium of the Ecole Supérieure d'Horticulture, Chott Mirriam and identified by Dr F. Skiri.

Extraction : Dried and finely powdered leaves of *Ajuga iva* (300g) were extracted with acetone (4l) at room temperature for seven days. Filtration and evaporation of the solvent yielded 18.1g of crude extract.

Isolation :

Compound 1 : The acetone extract was chromatographed on a column [1] over sigel using a petroleum ether/EtOAc/MeOH gradient elution system. The chromatographic simplification of the mixture of fractions 53-79 collected from column [1] gave 64 fractions, Clerosterol was isolated from fractions 12-13 after their precipitation in methanol. Otherwise, purification and crystallisation of the residue in EtOAc/MeOH (5 :95) finally yielded a white powder (11mg).

Compound 2 : The hexane extract of fractions 80-115 obtained from column [1] was precipitated in acetone, the filtrate was chromatographed on a column over sigel using a n-hexane/EtOAc/MeOH gradient elution system which led to fractions 46-49 from which a residue was obtained by precipitation in acetone. Purification on a column over sigel using a n-hexane/EtOAc/MeOH (1 :12 :3) elution system allowed the isolation of compound 2 (7.2 mg).

Spectral data :

Compound 1 : Powder : mp 133 °C ; $[\alpha]_D^{20}$ = -41.88° (c=0.97, $CHCl_3$) ; IR ν_{max} cm^{-1} (KBr) 3434, 2931, 2854, 1716, 1374 ; ^1H NMR, ^{13}C NMR , HMBC and COSY data see table I ; MS m/z $[M]^+$ 412, 397, 395, 366, 353, 324, 279, 239, 149, 84.

Calcd for $C_{29}H_{48}O$, C 84.46, H 11.65 ; found C 84.25, H 11.60.

Compound 2 : Powder : mp 69 °C ; $[\alpha]_D^{20}$ = -35.2° (c=0.3, $CHCl_3$) ; IR ν_{max} cm^{-1} (KBr) 3217, 2942, 1729, 1160 ; ^1H NMR and ^{13}C NMR see table II ; MS m/z $[M]^+$ 286, 268, 255, 236, 103, 74, 29, 43, 57, 71, 85, 99, 113, 127, 141, 155, 169.

Calcd for $C_{17}H_{34}O_3$, C 71.32, H 11.88 ; found C 71.32, H 11.87.

fig 3. ^{1}H-^{13}C long range correlation from HMBC experiments for compound 1

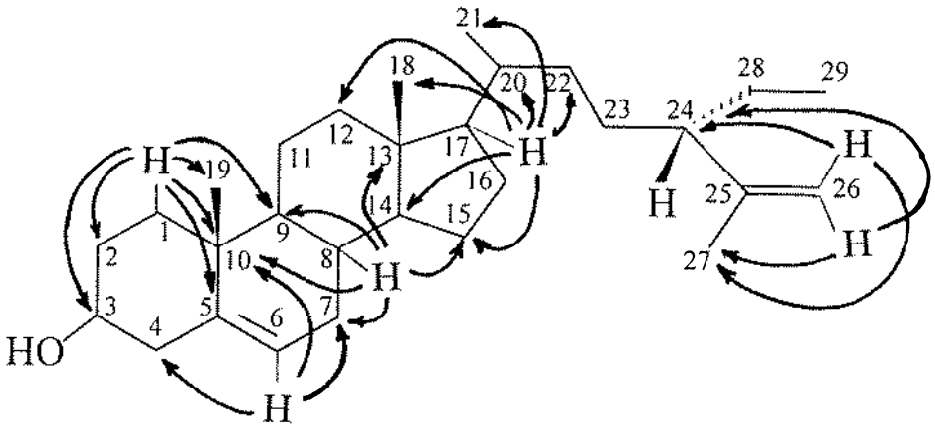


Table I. ^{13}C and ^{1}H spectral data of compound 1

atom	δ ^{13}C	δ ^{1}H (J (Hz))		HMBC	COSY
1α 1β	37.24	1.06 1.85	m	2-3-5-9-10-19	2α-2β 2α
2α 2β	31.85	1.50 1.83	m	1-3-4	1α-1β-3-4α 1α-3-4β
3	71.87	3.50	m		2α-2β-4α-4β
4α 4β	42.28	2.25 2.29	m	2-3-5-6-10	2α-3-6-7α-7β 2α-2β-3-6
5	141.0				
6	121.8	5.34	m	4-7-10	4α-4β-7α-7β
7α 7β	31.78	1.50 1.94	m	5-6-8-9	6-4α
8	31.67	1.41	m	7-9-10-13-15	
9	50.10	0.9	m	1-7-8-10-11-19	
10	36.52				
11	21.08	1.45	m	8-9-12-13	12β
12α 12β	39.67	1.15 1.98	m	9-11-13-14-17-18	12β-18 11-12α
13	42.30				
14	56.08	1.05	m	7-8-9-12-13-16-18	
15α 15β	24.34	0.99 1.55	m	8-13-14-16-17	15β 15α
16	28.25	1.80	m	13-14-15	
17	56.76	0.95	m	12-14-15-18-20-21-22	20
18	11.95	0.66	s	12-13-14-17	12α
19	19.50	0.99	s	1-5-9-10	

20	35.87	1.35	m	22-23	
21	18.70	0.88	d (6.33)	17-20-22	
22a 22b	33.70	0.90 1.24	m	20-21-23-24	
23a 23b	29.54	1.20 1.30	m	20-22-24-28	
24	49.60	1.81	m	22-23-27-28-29	
25	147.6				
26α 26β	111.5	4.61 4.70	br s	24-27	27
27	17.70	1.55	s	24-25-26	26
28	26.60	1.32	m	23-24-29	29
29	12.17	0.78	t (7.18)	24-28	28

Table II. ^{1}H- and ^{13}C NMR data of compound 2

Position	^{1}H nmr	^{13}C nmr
C_1		172.90
C_2	2.36 dd (15.4, 8.1) 2.46 dd (15.4, 4.6)	43.03
C_3	3.97 m	68.60
C_4	1.45 m	37.90
C_5		32.60
C_6-C_{13}	1.29 m	30.80
C_{14}		26.30
C_{15}		23.30
C_{16}	0.86 t (6.9)	14.30
$C_{1'}$	3.62 s	51.50
OH	2.84 s	

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