STRUCTURE OF A NEW NEO-CLERODANE DITERPENOID FROM *AJUGA PSEUDOIVA* LEAVES AND ITS INSECT ANTIFEEDANT AND ANTIBACTERIAL ACTIVITIES

H. BEN JANNET *, M. H. OUESLATI *, A. CHAARI *,
M. T. MARTIN **, A. LOUKACI **, M. J. SIMMONDS ***, Z. MIGHRI *

* *Laboratoire de Chimie des Substances Naturelles et de Synthèse Organique, Faculté des Sciences de Monastir, Université du Centre, 5000 Monastir, Tunisie*
** *Institut de Chimie des Substances naturelles, CNRS, Gif-sur-Yvette, France*
*** *Jodrell Laboratory, Royal Botanic Gardens, Kew, Richmond, Surrey, UK*

(Soumis en février 2002, accepté en mai 2002)

ABSTRACT: From the leaves of the plant *Ajuga pseudoiva* a new neo-clerodane diterpenoid, Hativene D, has been isolated. Its structure was established by spectroscopic means. The antifeedant activity of this compound has been studied against Egyptian cotton leafworm *Spodoptera littoralis* (Boisd.) (Lepidoptera) by application of the leaf disk method. This substance exhibited an interesting activity even at 1 ppm. Structure-activity relationship is discussed. The antibacterial effect of this neo-clerodane has been also tested.

Key words: *Ajuga pseudoiva*, Lamiacea, clerodane diterpenoid, Hativene D, anti-insect, antibacterial.

RESUME: Un nouveau diterpène clérodane, Hativene D, a été isolé des feuilles de la plante *Ajuga pseudoiva*. Sa structure a été établie à l'aide de techniques spectroscopiques. Son activité anti-appétante vis-à-vis du *Spodoptera littoralis* (Lepidoptera), insecte nuisible au cotonnier, a été étudiée en utilisant la méthode de disques. Cette nouvelle substance naturelle a montré un pouvoir anti-appétant intéressant même à la concentration 1 ppm. La relation structure-activité a été discutée. L'activité antibactérienne de ce diterpène clérodane a été aussi étudiée.

Mots clés : *Ajuga pseudoiva*, Lamiacées, diterpène clérodane, Hativene D, anti-insecte, antibactérien.

INTRODUCTION

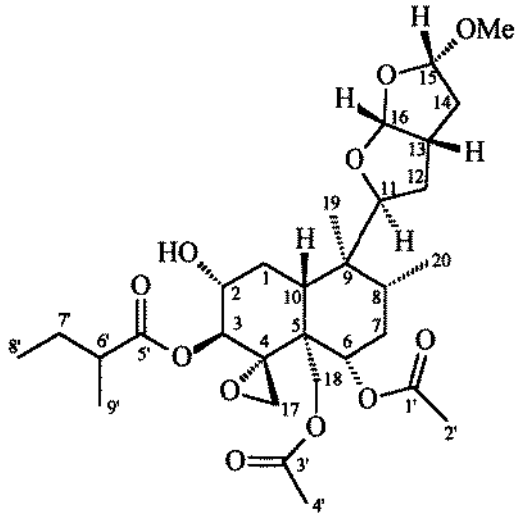
In our search for new bioactive natural compounds in some plants growing in Tunisia [1-12], we continued to investigate the plant *Ajuga pseudoiva* (Lamiacea), a species which grows throughout North Africa. It has been used in folk medicine in various cultures and several interesting medicinal properties, such as antidiabetic, anthelmintic, hypoglycaemic and vulnerary effects have been attribuated to it [13].

Recently, we have isolated from this plant three new neo-clerodanes Hativenes A-C [9]. They have been shown to have potent antifeedant activity against *Spodoptera littoralis* larvae [12]. The results of these studies prompted us to isolate and caracterise other bioactive natural compounds.

We report in this paper the isolation and the structural elucidation of a new clerodane diterpenoid Hativene D (**1**), epimer of Lupulin A previously isolated from the same source. Its stereochemistry was established by interpretation of the corresponding NMR (1 and 2D) spectra and by comparison with previously related data [9]. We also report in this paper the effects of Hativene D (**1**) on the feeding behaviour of larvae of *Spodoptera littoralis* and its antibacterial activity towards *Pseudomonas aeruginosa, Escherichia coli, Salmonella typhimurium* and *Staphylococcus aureus*.

RESULTS AND DISCUSSION

Hativene D (**1**) was isolated as an oil from the same crude fraction from which we have isolated Hativenes A-C [9]. Its IR spectrum displayed absorptions corresponding to free hydroxyl (3435 cm^{-1}), ester (1730 cm^{-1}) groups and oxirane ring (3030 cm^{-1}).



Compound **1**

The ^1H NMR spectrum of **1** showed signals for two acetate groups (δ 1.91 and 2.10) and for a 2-methylbutyric ester function (δ 2.33, 1H, m; 1.09, 3H, d, J=7.0 Hz; 1.43 and 1.66, 2H, m; 0.87, 3H, t, J=7.2 Hz). The presence of these last three ester groups was supported by signals due to protons on carbon bearing oxygen atoms at δ 5.21 (1H, d, J=9.8 Hz), 4.67 (1H, dd, J_1=11.5 Hz, J_2=4.5 Hz) and 4.38 (AB system, J=12.2 Hz). The position of these systems in relation to the rings was proved by some ^2J correlations of protons with tertiary and quaternary carbons observed in the HMBC spectrum.

The AB system at 2.53 (1H, d, J=4.3 Hz) and 2.78 (1H, d, J=4.3 Hz) was assigned to the C-17 oxirane protons in agreement with the presence of (C-4)-(C-17) epoxide [9]. This position is proved by ^2J and ^3J correlations observed in the HMBC spectrum between protons resonating at 2.53 and 2.78 and the quaternary carbons (δ 74.5 and 62.8) identified to be C-3 and C-4, respectively (Table 1).

The relevant cross-peaks at δ 3.60 (1H, m) and 5.21 (1H, d, J=9.8 Hz) in the ^1H-^1H COSY NMR spectrum of **1** pointed to the presence of a hydroxyl group and an ester function (2-methylbutyric) attached at C-2 and C-3, respectively.

The occurrence of the hexahydrofuranofuran moiety was confirmed by the appearance in the ^1H NMR spectrum of **1** of an acetalic proton at δ 5.79 (1H, d, J=5.4 Hz) and of signals at δ 4.36 (1H, dd, J_1=11.3 Hz, J_2=5.7 Hz) and 2.78 (1H, m) attributable to H-11 and H-13, respectively, and of

signals at δ 109.1, 82.8 and 40.5 relative to C-16, C-11 and C-13, respectively [9]. This result was confirmed in an other hand by the HMQC spectrum.

The appearance of a methyl group signal at δ 3.31 (3H, s) in the ^{1}H NMR spectrum and a ^{13}C NMR resonance at δ 104.8, confirmed by the HMQC spectrum, suggested the presence of a methoxyl group at C-15, as found previously for Lupulin A and Hativenes A-C [9]. This location was confirmed by the HMBC spectrum displaying a ^{2}J correlation between methylic protons (δ 3.31) and tertiary carbon identified to be C-15. Elucidation of the relative stereochemistry of compound **1** was mostly based on the close similarity of its ^{1}H ($CDCl_3$) NMR data (Table 1) to those of Hativenes B and C [9]. The 2D NOESY spectrum of **1** showed the correlation of H-11 with the OMe protons at C-15 which reinforces the α-orientation of methoxyl group. The compared chemical shifts of H-2 and H-3 with those of Hativene B [9] and the NOE between H-3 and H-18 as well as dipolar coupling of H-2 to H-10 and H-2 to H-17 confirmed the proposal α-orientation of the hydroxyl group attached at C-2 and β-orientation of the 2-methylbutyric ester moiety attached at C-3.

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

Atom	δ ^{13}C	δ ^{1}H (J(Hz))	Atom	δ ^{13}C	δ ^{1}H (J(Hz))
1ax	30.2	1.72 m	16	109.1	5.79 d (5.4)
1eq		2.59 m	17a	42.4	2.53 d (4.3)
2	71.8	3.60 m	17b		2.78 d (4.3)
3	74.5	5.21 d (9.8)	18a	61.5	4.38 d (12.2)
4	62.8		18b		4.78 d (12.2)
5	45.5		19	13.8	0.91 s
6	71.3	4.67 dd (11.5; 4.5)	20	16.3	0.87 d (7.0)
7ax	33.2	1.42 m	1'	170.0	
7eq		1.56 m	2'	20.9	1.91 s
8	35.8	1.48 m	3'	170.9	
9	40.0		4'	20.9	2.10 s
10	43.4	1.70 m	5'	175.6	
11	82.8	4.36 dd (11.3; 5.7)	6'	41.1	2.33 m
12a	32.6	1.62 m	7'	26.6	1.43 m
12b		1.75 m			1.66 m
13	40.5	2.78 m	8'	11.2	0.87 t (7.2)
14a	39.4	1.79 m	9'	15.8	1.09 d (7.0)
14b		2.25 m	OMe	54.5	3.31 s
15	104.8	4.94 d (5.5)			

The result of the behavioural responses of *Spodoptera littoralis* to the clerodane **1** shows its high antifeedant activity at 100, 10 and 1 mg/ Litre. This activity starts to disappear at 0.1 mg/ Litre (Table 2). This data is compared to those of the other clerodanes Hativenes A-C, Lupulin A and 14,15-dihydroajugapitin that we have isolated from the same plant and we have tested against the same insect and reinforced our previous studies conclusions [12] and the comments reported by other authors [14,16] showing that feeding inhibition was to be associated with the presence of a perhydrofuranofuran moiety at C-9, a spiroepoxide ring at C-4 and two acetate groups at C-6 and C-18 in the decaline ring system.

Comparison of the antifeedant index at 1 and 0.1 mg/Litre of Hativene D and its epimer Lupulin A [12] shows clearly that the orientation (α or β) of the methoxyl group attached at C-15 did not modify considerably the activity but the presence of this group in the molecule decreases the activity by comparison with that of 14,15-dihydroajugapitin more active at 0.1 mg/Litre and which does not have this group. This observation reinforces, no doubt, our previous comments [12] and that reported by Belles et al [16].

Table 2 Behavioural response of *Spodoptera littoralis* larvae to Hativene D 1

Compound	Antifeedant index at concentration (mg/l)[a]			
	100	10	1	0.1
Hativene D	100±10.1	100±5.6	75.8±11.2	47.3±7.2

[a] Antifeedant index (%): see experimental. Values are mean±S.E.M., n = 10.

The antibacterial activity of Hativene D (**1**) was tested by using a paper-diffusion method. Compound **1** showed high activity towards *Escerichia coli* (inhibitory zone 5-7 mm), *Pseudomonas aeruginosa* (4-6 mm) and *Salmonella typhimurium* (5-7 mm), and weak activity against *Staphylococcus aureus* (1.5 mm).

MATERIALS AND METHODS

- General experimental procedures :

1 and 2D ^{1}H and ^{13}C NMR spectra were recorded on Bruker AM 400 spectrometer operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C using TMS as internal standard and $CDCl_3$ as solvent. Coupling constants are given in Hz. IR spectrum was carried out on a Nicolet 205 IR-FT spectrophotometer.

- Plant material :

Aerial part of the plant *Ajuga pseudoiva* was collected in June 1995 in Monastir, Tunisia. A voucher specimen was deposited at the herbarium of the Ecole Supérieure d'Horticulture, Chott Meriem, Sousse, Tunisia.

- Extraction and isolation :

Dried and finely powdered *Ajuga pseudoiva* leaves (343 g) were extracted with acetone at room temperature for seven days. The chromatographic simplification (Merck 7734, hexane-EtOAc-MeOH) of the crude extract gave four fractions; the last one (6.9 g) was divided between hexane and MeOH. The hexane layer showing antifeedant effect towards *Spodoptera littoralis* and antibacterial activity against bacteria indicated above was simplified by cc silica gel (Merck 7734, hexane-$CHCl_3$-EtOAc). Further activity-guided cc separation afforded a fraction (95 mg) which is simplified into five fractions (Merck 7734, CH_2Cl_2-MeOH 98:2). The last three fractions were further analysed by HPLC using phase HPLC column (Lichrosorb^{R} RP-select B 10 µm) eluted with MeOH-H_2O 6:4 applying a flow rate of 3 mL/mn to give among other compounds Hativene D (9 mg).

- Insect :

Larvae of S. littoralis collected from a laboratory culture (25 ± 1°C) were used into their final stadium under a 16:8 light/dark photoperiod [14].

- Behavioural bioassay :

This method allowed us to see the ability of *S. littoralis* larvae to seize the samples and to choose between a control and treated disc.

Larvae of *S. littoralis* were deprived of food for 4 h before being placed individually in petri dishes (8.5 cm in diameter) with two glass-fibre discs (Whatman GF/A 2.1 cm in diameter) saturated with a sucrose solution (0.05 M; 100 µl). A control disc contained only the sucrose solution, and the treated disc was treated additionally with 100 µl of the solution containing the test sample (extract, fraction or pure compound) dissolved in the appropriate solvent. The discs were

left to dry and then weighed before being presented to the larvae . the duration of the experiment was usually 8-24 h, the time required for the insect to eat approximately 50% of one of the discs. At the conclusion of the feeding trial, larvae were removed and the discs dried and reweighed. The antifeedant index $[(C-T)/(C+T)] \times 100$ was calculated, where C and T represent the amount eaten of control and treated discs, respectively.

- Antibacterial assay :

A paper disc diffusion method was used. Bacteria were prepared according to standard techniques [15]. The sample was dissolved in acetone and its concentration was adjusted to 0.03 mg/mL. Then, paper discs (5 mm diameter) were saturated with the solution and placed into the petri dishes after seeding the test organism into the cooled specific medium. The inhibitory zones (mm) were compared with the control disc treated with acetone used to dissolve the sample.

Compound **1**: colourless oil, IR ($CHCl_3$) ν_{max} cm^{-1} 3435 (OH), 3030 (oxirane ring), 1730 and 1250 (ester groups), ^1H and ^{13}C data see Table 1.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. A. Bakhrouf for assistance in antibacterial essays. Thanks are also due to Dr. F. Harzallah-Skhiri for botanical classification of the plant material.

REFERENCES

[1] B. Ben Hassine, A.M. Bui, Z. Mighri, *J. Soc. Chim. Tunisie*, **1982**, *1(7)*, 3-10.
[2] B. Ben Hassine, A.M. Bui, Z. Mighri, A. Cave, *Plant. Med. Phytother.*, **1982**, *16(3)*, 197-205.
[3] M. Askri, A.M. Bui, Z. Mighri, *J. Soc. Chim. Tunisie*, **1982**, *1(8)*, 23-28.
[4] M. Askri, Z. Mighri, A.M. Bui, B.C. Das, P.J. Hylands, *J. Nat. Prod.*, **1989**, *52(4)*, 792-796.
[5] H. Ben Jannet, Z. Mighri, L. Serani, O. Laprevote, J.C. Jullian, F. Roblot, *Nat. Prod. Letters*, **1997**, *10*, 157-164.
[6] H. Ben Jannet, A. Chaari, M.T. Martin, Z. Mighri, *J. Soc.Chim. Tunisie*, **1998**, *4(4)*, 179-187.
[7] A. Chaari, H. Ben Jannet, M.T. Martin, Z. Mighri, *J. Soc. Chim. Tunisie*, **1998**, *4(4)*, 257-265.
[8] H. Ben Jannet, A. Chaari, M.T. Martin, Z. Mighri, *J. Soc. Chim. Tunisie*, **1998**, *4(6)*, 483-488.
[9] H. Ben Jannet, A. Chaari, Z. Mighri, M.T. Martin, A. Loukaci, *Phytochemistry*, **1999**, *52*, 1541-1545.
[10] H. Ben Jannet, A. Al Mourabit, A. Gateau-Olesker, C. Marazano, Z. Mighri, *Tetrahedron Asymmetry*, **1999**, *10*, 2381-2386.
[11] H. Ben Jannet, A. Chaari, A. Bakhrouf, M.T. Martin, Z. Mighri, *J. Soc. Chim. Tunisie*, **1999**, *4(6)*, 503-515.
[12] H. Ben Jannet, F. Harzallah-Skiri, Z. Mighri, M.S.J. Simmonds, W.M. Blaney, *Fitoterapia*, **2000**, *71*, 105-112.
[13] E. le Floc'h, Contribution à une étude ethnobotanique de la flore tunisienne, Publications Scientifiques Tunisiennes, Imprimerie Officielle de la République tunisienne, **1983**, 203-204.
[14] W.M. Blaney, M.S.J. Simmonds, S.V. Ley, P.S. Jone, *Entomol. Exp. Appl.*, **1988**, *46*, 267-274.
[15] C. Booth, Methods in Microbiology, Academic Press: London, **1971**, *4*, 36-39.
[16] X. Belles, F. Camps, J. Coll, M.D. Piulachs, *J. Chem. Ecol.*, **1985**, *11*, 1439-1445.