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POTENTIAL ALLELOPATHIC LUPANE TRITERPENES FROM BIOACTIVE FRACTIONS OF *MELILOTUS MESSANENSIS**

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Key Word Index—*Melilotus messanensis*; Leguminoseae; Fabaceae; sweet clover; lupane triterpenes; messagenin; 30-nor-lupane-3β,28-diol-20-one; allelopathy; *Lactuca sativa*; *Lepidium sativum*; *Hordeum vulgare*; *Triticum aestivum*.

Abstract—The aerial parts of *Melilotus messanenis* (sweet clover) afforded, from the medium polar bioactive fractions, in addition to the known lupane triterpenes lupeol, betulin, betulin aldehyde and betulinic acid, the new norlupane messagenin (30-norlupane-3β,28-diol-20-one) which have been tested as allelochemicals. Structures and their stereochemistries were elucidated by spectral methods and chemical transformations. Messagenin has been synthesized from betulinic acid. The effect of a series of aqueous solutions at 10^{-4}–10^{-9} M of eight natural and synthetic lupane derivatives were tested for their effects on the germination and growth of the dicotyledon species *Lactuca sativa* and *Lepidium sativum* and the monocotyledon species *Hordeum vulgare* and *Triticum aestivum*. All eight lupane triterpenes possess potential allelopathic activity in particular over dicotyledon species and they are likely to be significantly involved in the allelopathic action of *Melilotus messanensis*.

INTRODUCTION

Melilotus species are native from the temperate and subtropical regions in Eurasia and North Africa, some of them are cultivated as forage [1]. Nevertheless, their content in coumarins or *o*-hydroxycinnamic acid in these species represents a limitation for their use as forage [2]. If 'high-coumarin' sweet clover becomes mouldy during harvesting or storage, then toxic levels of dicoumarol can develop rapidly [3]. Dicoumarol, a potent anticoagulant, is a product of microbial action which interferes with the synthesis of vitamin K dependent coagulation factors and may result in extensive haemorrhaging in cattle and spontaneous abortion in bred cows, a syndrome know as 'sweet clover bleedings disease' [4].

Melilotus messanensis is a small shrub (less than 50 cm tall) endemic to the Mediterranean Basin [5], a member of the only 23 species that comprise the genus *Melilotus*. Recently, the potentiality of different *Melilotus* species as *M. segetalis* or *M. messanensis* ecotypes from S. W. Spain, able to grow in saline soils, has been evaluated as forage resources, green manure and as a source of biocide compounds [1, 6–8], because *ca* 15 million ha in the Mediterranean Basin are affected by salinity. A diversified use of this 'marginal land' must include extensive grazing.

Biochemical investigations on *Melilotus* reveal that these species are a rich source in phenol derivatives and phenolic acids [9, 10], coumarins [11–13], flavonoids [14–16], monoterpenes [10], oleanic triterpenes [17–20] and steroids [21] with a wide spectrum of biological activities.

The chemical constitution of *M. messanensis* has been investigated previously showing the presence of low levels of flavonols, simple coumarins [12] and diosgenin [21], but no *o*-hydroxycinnamic acid was found [22].

Several studies have shown that *Melilotus* species, such as *M. alba*, can actively influence the growth of corn [23], tomato and radish seedling roots [14] where the presence of *o*-coumaric acid, coumarin and melilotic acid is related with the inhibitory activity observed.

Preliminary bioassays of *M. messanensis* crude extracts of two different stages of plant development a and b (see Experimental) showed them to be active on germination of *Lactuca sativa*, the medium polar fractions obtained from the crude extract of the second stage b were also active.

From these fractions, we isolated four known lupane triterpenes, lupeol (**1**) [24, 25], betulin (**2**) [26], betulinaldehyde (**3**) [27, 28] and betulinic acid (**4**) [29] and a new nor-lupane, which were identified by spectroscopic techniques (IR, MS and 1D, 2D NMR experiments) and chemical correlation. The structure elucidation of the new nor-lupane which was named messagenin (**5**) is described below.

As part of our research on bioactive natural products (coumarins, terpenoids, lignans, phenolics, etc.), we are

*Part 3 in the series 'Natural Product Models as Allelochemicals' for Part 2 see: Macías, F. A., Galindo, J. C. G., Massanet, G. M., Rodríguez-Luis, F. and Zubía, E. (1993) *J. Chem. Ecol.* **19**, 1371.

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conducting a systematic study of their potential activity [30]. Thus, we are evaluating the regulatory effects of tested compounds on *Lactuca sativa* and *Lepidium sativum* as dicotyledon species and *Hordeum vulgare* and *Triticum aestivum* as monocotyledon species.

In order to evaluate their potential allelopathic activity and to obtain information about the specific structural requirements needed for their biological activity, we have studied the effect of a series of aqueous solutions at 10^{-4}–10^{-9} M of eight natural and synthetic lupanic triterpenes **1**–**6** and **4a**, **4m** on root and shoot lengths of *Lactuca sativa* and *Lepidium sativum* seedlings (dicotyledons) and *Hordeum vulgare* and *Triticum aestivum* seedlings (monocotyledons).

RESULTS AND DISCUSSION

Extraction of the fresh *M. messanensis* (stage b) aqueous extract with methylene dichloride afforded, after chromatography and following the levels of bioactivity exhibited by the fractions with *Lactuca sativa* (Table 3, Fig. 1), five lupane-type triterpenes of increasing polarity. These were lupeol (**1**), betulin (**2**), betulinaldehyde (**3**), betulinic acid (**4**) and a new nor-lupane, messagenin (**5**). This is the first report of lupane-type triterpenes from the genus *Melilotus*. Since the ^{13}C NMR spectral data of **4** and **4a** had not been previously reported, they are included in Table 2. Also, the ^{1}H NMR spectral data for triterpenes **1**, **3**, **4**, **4a** [31], **4m** [32] and **6** [33] are given in Table 1, since they include revisions of previous assignments or new unambiguous assignments.

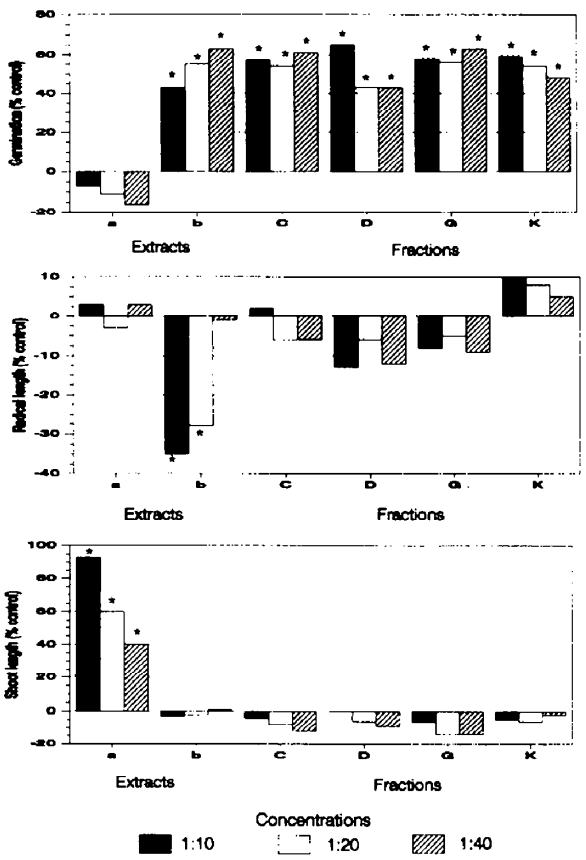


Fig. 1. Effect of aqueous extracts a and b and fractions C–K on the germination, root and shoot length of *Lactuca sativa*.

Messagenin (**5**) was isolated as needles with a $[M]^+$ at m/z 444 which together with the ^{1}H and ^{13}C NMR data (Tables 1 and 2) was in agreement with the molecular formula $C_{29}H_{48}O_3$. Additional mass spectral peaks at m/z 426 $[M-H_2O]^+$, 207 $[B]^+$ (**7**) and 189 $[A]^+$ (**8**) were diagnostic of a lupane-type triterpene structure [34]. The base peak m/z 43 $[Me-C=O]^+$ as well as ^{1}H NMR resonances at δ2.60 (*ddd*, H-19β) and 2.15 (3H, *s*, H-29) and ^{13}C NMR signals at δ210.4 (C-20) and 27.2 (C-29) were a clear diagnostic of a 20-keto-30-nor-lupane. Strong IR absorptions at 3046 and 1672 cm^{-1} corroborated the presence of hydroxyls and a methylketone group, respectively. The ^{1}H NMR data of **5** (Table 1) were similar to those of structurally related lupanes as **1**–**4** [24–29] with two hydroxyl groups at C-3 and C-28, as indicated by a doublet of doublets at δ3.18 (H-3α) and two doublets at 3.77 (H-28a) and 3.23 (H-28b), respectively. The stereochemistry at C-3 was derived from the couplings between H-3, H-2a and H-2e ($J_{2a,3\alpha}=11.1$; $J_{2e,3\alpha}=5.0$ Hz) which were in agreement with an α-orientation for H-3.

The complete unambiguous assignments of the ^{1}H NMR (Table 1) and ^{13}C NMR (Table 2) were made on the basis of ^{1}H NMR 2D COSY and NOE difference experiments (Fig. 2) and the aid of heteronuclear multipulse APT experiments and ^{1}H–^{13}C correlations using the trace analysis on bi-dimensional spectra, assuming the most probable conformation of **5** resulting from molecular mechanics calculations (MMX) [35] (Fig. 2), helped with the observed NOE difference effects.

To confirm the proposed structure **5** for messagenin, a series of chemical transformations was performed using as starting material betulinic acid (**4**) which was isolated in large amounts from *M. messanensis* (Scheme 1). Compound **4** was reduced ($LiAlH_4$–THF) to give **2** (90%) and **3** (5%). Ozonolysis of **2** dissolved in CH_2Cl_2–MeOH (9:1) at −76° using O_3 and Me_2S, as reductive reagent, produced a product identical to **5** (70%) and a secondary product **6** (10%), thus confirming the structure of **5** as 30-nor-lupane-3β,28-diol-20-one, which has been synthesized on a 67% overall yield. The chemical correlation between **2**–**5** has been established.

Compound **6** was identified by comparison of physical data (mp, [α], IR, MS, ^{1}H and ^{13}C NMR) with those reported in the literature for lup-20(29)ene-3β,28,30-triol which has been isolated from *Maytenus canariensis* [33].

In spite of the large number and wide variety of naturally occurring triterpenes that have been chemically characterized (1500 new triterpenes between 1977–1987 [36–40]), little work has been done on their biological activity [41–45] and ecological significance (antimicrobial [46], herbicidal [47] and allelopathic activity [49, 50]).

In the literature, only oleanic-type triterpenes such as ursolic acid [49], medicagenic acid and their glycoside

Table 1. ^{1}H NMR of lupane triterpenes **1**, **3**–**6**, **4a**, **4m** (399.95 MHz, $CDCl_3$, signal of residual $CHCl_3$ centred at δ7.25 ppm)*

H	1	3	4†	4a	4m	5	6
1a	0.89	0.90	0.90		0.85	0.85	0.88
e	1.66	1.65	1.58	1.65	1.66	1.63	1.64
2a	1.53	1.54	1.82	0.99	1.51	1.49	1.55
e	1.59	1.59	1.82	1.59	1.51	1.59	1.61
3	3.18 *dd*	3.17	3.45	4.46	3.13	3.18	3.17
5	0.67 *dd*	0.67	0.80	0.78	0.65	0.68	0.67
6a	1.37	1.36	1.40	1.37	1.32	1.35	1.39
e	1.49	1.49		1.48	1.48	1.54	1.51
7			1.36	1.19	1.33	1.40	1.39
9		1.16	1.36		1.33	1.27 *dd*	1.19
11a		1.42	1.36	1.41	1.38	1.40	1.29
e		1.24	1.15	1.28	1.22	1.40	1.24
12a		1.03	1.18	1.03	0.97	1.15	0.88
e		1.74	1.84	1.69	1.64	0.97	1.55
13		2.01 *ddd*	2.71	2.18	2.16	1.54 *ddd*	1.62
15a	1.88		1.73	1.51		1.67 *ddd*	1.68
e	1.18	1.17	1.19	1.18	1.10	1.04	1.06
16a	1.29	1.42	1.53	1.42	1.33	1.91	1.22
e	1.37	2.06 *ddd*	2.63	2.27	2.19	1.26	1.92
18	1.32	1.71	1.70	1.60	1.54	2.02	1.67
19	2.37 *ddd*	2.85	3.51	3.00	2.96	2.60	2.28
21α	1.92	1.87	2.20	1.96	1.84	2.05	2.09 *dddd*
β	1.32	1.45	1.52	1.42	1.33	1.50	1.39
22α	1.65	1.74	2.20	1.96	1.86	1.91	1.86 *ddd*
β	1.01	1.33	1.54	1.42	1.40	1.14	1.09
23	0.95 *s*	0.95	1.20	0.97	0.94	0.96	0.95
24	0.75 *s*	0.74	0.98	0.84	0.73	0.75	0.74
25	0.81 *s*	0.80	0.79	0.83	0.79	0.81	0.81
26	1.02 *s*	0.90	1.03	0.85	0.89	0.99	1.00
27	0.95 *s*	0.96	1.04	0.93	0.94	0.98	0.97
28	0.77 *s*	9.66 *d*		—	—	a 3.77 *d*	3.78
						b 3.23 *d*	3.30
29a	4.67 *d*	4.74	4.92	4.74	4.68	2.15 *s*	4.94 *d*
b	4.55 *dq*	4.62	4.74 *d*	4.61 *dq*	4.56	—	4.89 *s*
30	1.67 *q*	1.68 *s*	1.76	1.69	1.66	—	4.10 *m*
Me				2.04 *s*	3.64		

*Multiplicities are indicated when the coupling constant can be measured and they are not repeated if identical with those in the preceding column.

†Spectrum in pyridine-d_5.

J(Hz): **1**, **2**, **3**, **4a**, **4m**, **5**, **6**: 2a, 3 = 11.1; 2e,3 = 5.0; 18,19 = 19,21α = 11.1; 19,21β = 5.4; **1**, **4m**, **5**, **6**: 5,6a = 9.8; **3**, **4a**: 12a,13 = 13,18 = 12.4; 12e,13 = 3.7; **3**, **4a**, **6**: 16a,16e = 12.6; 16e,15e = 2.7; **5**, **6**: 28a,28b = 11; **1**, **3**, **4**, **4a**, **4m**, **6**: 29a,29b = 1.9; **1**, **4a**, **4m**: 29a,30 = 1.5; **3**: 22,28 = 1.6; **4**: 2a,3 = 8.3; 2e,3 = 7.5; 18.19 = 19,21α = 8.5; 19,21β = 4.1; **5** (obtained by NOEs experiments): 9,11a = 12.2; 12a,13 = 13,18 = 12.1; 12e,13 = 2.3; 15a,15e = 15a,16a = 13.4; 15a,16e = 4.0; **6**: 21α,22β = 11.1; 21α,22α = 21α,22β = 2.6; 22α,22β = 12.6; 22α,21β = 8,3.

derivatives [51, 52] or soyasapogenol B [53] have been reported that can actively influence the growth of surrounding plants where the effect was inhibition for the acid derivatives and stimulation for soyasapogenol B.

In this study we are evaluating some natural and synthetic lupane-type triterpenes with different functionalization at C-17: Me, CH_2OH, CHO, COOH and COOMe (**1**–**6**, **4a** and **4m**); different attached groups at C-20: methyl and methylene (**1**–**4**, **4a** and **4m**), methyl and ketone (**5**) and CH_2OH and methylene (**6**), and with a blocked hydroxyl group at C-3 (**4a**), the three reaction sides of the lupane molecule.

The experimental results on the germination, radical length and shoot length are summarized in Tables 3–7 and Figs 1, 3–5, where the numbers are expressed as per cent units from the control, consequently 0 represents an observed value identical to the control, a positive value represents stimulation and a negative value represents inhibition.

Table 2. ^{13}C NMR of lupane triterpenes **4**, **4a**, **5** (100.23 MHz, $CDCl_3$, signal of residual $CHCl_3$ centred at δ 77.00 ppm)*

C	4†‡	4a	5†
1	38.5 *t*	38.4	38.6
2	28.2 *t*	23.7	27.3
3	78.1 *d*	80.9	78.8
4	39.4 *s*	37.8	38.8
5	55.9 *d*	55.4	55.1
6	18.7 *t*	18.1	18.2
7	34.7 *t*	34.2	34.1
8	41.0 *s*	40.7	40.7
9	50.9 *d*	50.4	50.2
10	37.5 *s*	37.1	37.1
11	21.1 *t*	20.8	20.8
12	26.0 *t*	25.4	27.2
13	39.2 *d*	38.4	36.2
14	42.8 *s*	42.4	42.5
15	30.2 *t*	29.7	26.9
16	32.8 *t*	32.1	28.8
17	56.6 *s*	56.3	47.8
18	49.7 *d*	49.2	49.6
19	47.7 *d*	46.9	52.0
20	151.4 *s*	150.4	213.5
21	31.1 *t*	30.5	27.6
22	37.4 *t*	37.0	33.9
23	28.5 *q*	27.9	27.9
24	16.2 *q*	16.5	15.4
25	16.3 *q*	16.2	16.0
26	16.2 *q*	16.0	15.9
27	14.8 *q*	14.6	14.6
28	179.0 *s*	181.1	60.5 *t*
29	110.0 *t*	109.7	29.4 *q*
30	19.4 *q*	19.3	—

Compound **4a**: Me: 21.3, CO: 171.0.

*Degree of protonation was obtained by APT heteronuclear multipulse programs; multiplicities are not repeated if identical with those in the preceding column.

†The assignments were obtained by ^{13}C–^{1}H correlation.

‡Spectrum obtained in pyridine-d_5.

As observed with the extract b and the fractions C, D, G and K (Table 3, Fig. 1) from which they were isolated, **1–5** showed (Table 4, Fig. 3) a high stimulatory activity on the germination of *Lactuca sativa* seeds in high and low concentration, pointing out **2**, **5** and **6** (10^{-4}, 10^{-7}, 10^{-9} M **2**: +38%; 10^{-6} M **5**: +73%; 10^{-6} M **6**: +75%). The effects on the radical and shoot length are, in general, of little or no significance.

These compounds have a small effect on the germination and growth of *Lepidium sativum* (Table 5, Fig. 4), a very useful dicotyledon species to analyse allelopathic effects on the root and shoot length [54]. The most powerful stimulatory effects on the radical and shoot length are those shown by **5** with a CH_2OH group at C-17 (10^{-4} M: +44%, radical length and +36% shoot length).

The compounds have small effects on the germination and growth of *Hordeum vulgare* and *Triticum aestivum* seeds (Tables 6, 7, Fig. 5), except for **3** and **4m** with *H. vulgare*. Compound **3** had an inhibitory effect on the shoot length (10^{-6} M, −42%; 10^{-7} M, −44%) and there are stimulatory effects on germination promoted by **3** (10^{-9} M, +30%) and **4m** (10^{-7} M, +36%). The data suggest that the bioactivity of these compounds can be related to the presence of a free hydroxyl group at C-3, a CH_2OH at C-17 as shown by **2**, **5** and **6**, and this is increased when a methyl and ketone groups and CH_2OH and methylene are attached at C-20.

From the isolated lupane per cents of *M. messanensis*, the molar concentrations of these compounds in the 1:10

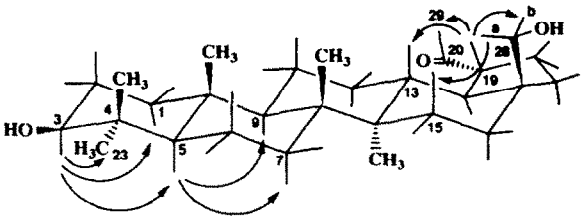


Fig. 2. NOE difference experiments on messagenin (**5**).

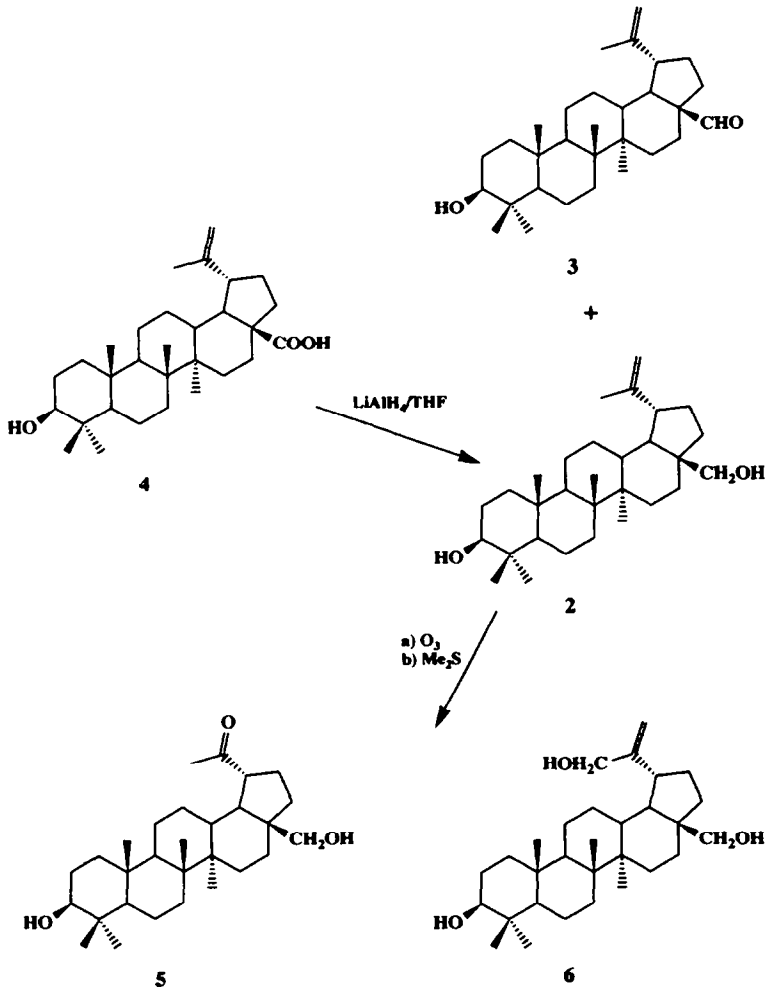
Table 3. Statistical results of allelopathic bioassays (using *L. sativa*) of aqueous extracts and fractions of *Melilotus messanensis**

	(%) Germination			(%) Radical length			(%) Shoot length		
	1:10	1:20	1:40	1:10	1:20	1:40	1:10	1:20	1:40
Extract a	−7[b]	−11[b]	−16[b]	3[b]	−3[b]	3[b]	93	60	40
Extract b	43	55	63	−35	−28	−1[b]	−3[b]	−2[b]	1[b]
Fraction C	57	54	61	2[b]	−6[b]	−6[b]	−4[b]	−8[b]	−12[b]
Fraction D	65	43	43	−13[b]	−6[b]	−12[b]	0[b]	−6[b]	−9[b]
Fraction G	58	56	63	−8[b]	−5[b]	−9[b]	−6[b]	−14[b]	−14[b]
Fraction K	59	54	48	10[b]	8[b]	5[b]	−5[b]	−6[b]	−2[b]

*Values are expressed as per cents from the control and are significantly different with $P < 0.01$ for student's *t*-test.

[a]Values significantly different with $0.01 < P < 0.05$.

[b]Values significantly different with $0.05 > P$.



Scheme 1. Chemical transformations used in the structural elucidation of lupanic triterpenes from *Melilotus messanensis*.

aqueous extract (obtained by diluting the original aqueous extract with de-ionized water 1:10, 1:20 and 1:40) are: lupeol (**1**), 1.1×10^{-6} M; betulin (**2**), 2.4×10^{-6} M; betulinaldehyde (**3**), 2.8×10^{-6} M; betulinic acid (**4**), 5.5×10^{-5} M and messagenin (**5**), 3.1×10^{-6} M.

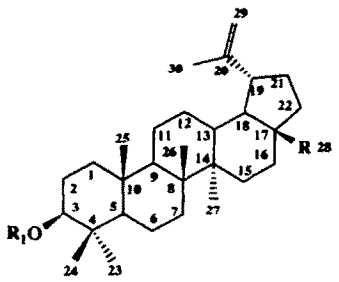
The concentrations of **1**–**5** in the 1:10 aqueous extract are in the same range as those that were active in the bioassay. The above findings suggest that the lupane triterpenes **1**–**5** are very likely responsible for the allelophatic activity of *M. messanensis* aqueous extract with a certain specificity for dicotyledon species.

EXPERIMENTAL

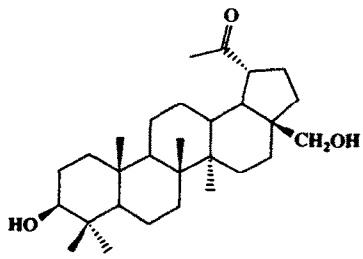
Plant material. Melilotus messanensis was collected on 11 April and 2 July 1991 in Trebujena, Cádiz, Spain (the voucher is deposited at the University of Seville Herbarium, Spain, SEV-7992) during two different plant development stage periods, (a) plants at the beginning of flowering and (b) plants with seeds. Selection of period (b) was based on the established bioactivity of the different aqueous extracts corresponding to stage a and b (Table 3 and Fig. 1).

Extraction and isolation. Fresh plants at stage b (382 g) were soaked with H_2O (wt plant: V. solvent 1:3) for 24 hr at 25° in the dark. The H_2O extracts were re-extracted (10 ×) with 0.5 l of CH_2Cl_2 per 1.0 l of H_2O, and the combined extracts were dried over Na_2SO_4 and evapd *in vacuo* to yield 1.2 g of crude extract termed H_2O–CH_2Cl_2 extract which was sepd by CC on silica gel using *n*-hexane–EtOAc mixts of increasing polarity yielding 214 × 50 ml frs which were reduced to 13 frs (A–M) after comparison by CCF.

By following the bioactivity exhibited by the medium polar frs on *Lactuca sativa*, frs C, D, G and K provided 5 lupane triterpenes, lupeol (**1**), betulin (**2**), betulinaldehyde (**3**), betulinic acid (**4**) and messagenin (**5**). Frs 15–20 (C), after HPLC (*n*-hexane–EtOAc, 9:1) provided 5 mg of **1**. Chromatography of frs 21–34 (D) by HPLC (*n*-hexane–EtOAc, 5:1, 9:1) gave 14 mg of **3**. Frs 40–47 (G)



1	R=CH_3	R_1=H
2	R=CH_2OH	R_1=H
3	R=CHO	R_1=H
4	R=COOH	R_1=H
4a	R=COOH	R_1=Ac
4m	R=$COOCH_3$	R_1=H



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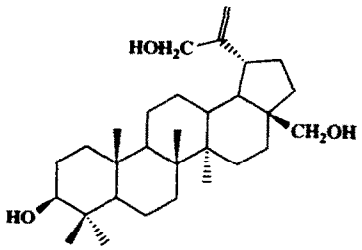

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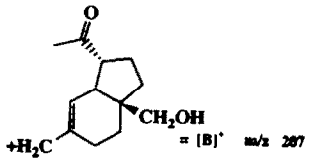

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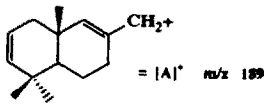

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Scheme 2.

after CC on silica gel with N_2 pressure (CH_2Cl_2, CH_2Cl_2–EtOAc, 9:1) gave **2** (12 mg) and **4** (290 mg). Messagenin (**5**, 16 mg) was obtained from frs 100–121 (K) by HPLC ($CHCl_3$–MeOH, 99:1). The known compounds were identified by comparison of physical data (mp, [α], IR, MS, ^1H, and ^{13}C NMR) with those reported in the literature.

Preparation of **2** *and* **3**. Compound **4** (250 mg) was dissolved in dry THF (25 ml) and stirred with reflux for 4 hr with $LiAlH_4$ (4 eq.) under N_2 atm., and allowed to reach room temp. followed by acidification with H_2SO_4 (5%), and extracted the aq. phase with Et_2O (3 × 30 ml). The Et_2O solns were combined, dried with $MgSO_4$. Purification by CC on silica gel with *n*-hexane–CH_2Cl_2, 1:4 as eluent gave **2** (90%) and **3** (5%). Compound **3**; ^1H NMR: Table 1.

Preparation of **4a**. Acetylation of **4** (10 mg) with 1 ml of Ac_2O–pyridine (3:1) for 24 hr at room temp. followed by usual work-up and CC on silica gel using *n*-hexane–EtOAc, 4:1 gave 5 mg of **4a** as needles; IR $\nu_{max}^{KBr,\,neat}$ cm^{-1}: 1734 (COOH), 1687 (OCOCMe), 1684 (C=CH_2); EM (70 ev) *m/z* (rel. int.): 498 $[M]^+$ (3); 452 $[M-HCOOH]^+$ (4); 438 $[M-AcOH]^+$ (19); 423 $[M-HOAc-Me]^+$ (8); 395 (12); 248 (18); 234 (9); 203 (20); 189 (91); 175 (25); 43 (100); ^1H NMR: Table 1; ^{13}C NMR: Table 2.

Preparation of **4m**. Triterpene **4** (10 mg) was dissolved in dry Et_2O (5 ml) and stirred at room temp. with an excess of CH_2N_2. After 4 hr the solvent was evapd under red. pres. Purification by CC on silica gel with *n*-hexane–CH_2Cl_2, 1:4 gave 8 mg of **4m**; ^1H NMR: Table 1; ^{13}C NMR: Table 2.

Messagenin (**5**). $C_{29}H_{48}O_3$, needles, mp 213–215° ($CHCl_3$): $[\alpha]_D^{25}$ −17.81° ($CHCl_3$); *c* 0.87; IR $\nu_{max}^{KBr,\,neat}$ cm^{-1}: 3406 (OH), 1672 (C=O); EM (70 ev) *m/z* (rel. int.): 444 $[M]^+$ (6); 426 $[M-H_2O]^+$ (5); 411 $[M-H_2O$

Table 4. Statistical results of allelopathic bioassays (using *L. sativa*) of lupane triterpenes **1–6**, **4a** and **4m***

	(%) Germination						(%) Radical length						(%) Shoot length					
	10^{-4} M	10^{-5} M	10^{-6} M	10^{-7} M	10^{-8} M	10^{-9} M	10^{-4} M	10^{-5} M	10^{-6} M	10^{-7} M	10^{-8} M	10^{-9} M	10^{-4} M	10^{-5} M	10^{-6} M	10^{-7} M	10^{-8} M	10^{-9} M
1	20	40	33	23	10[b]	0[b]	−6[b]	1[b]	−3[b]	3[b]	−2[b]	−1[b]	6[b]	17[a]	8[b]	12[b]	13[b]	8[b]
2	59	47	45	51	31	48	6[b]	4[b]	−1[b]	7[b]	0[b]	9[b]	3[b]	1[b]	3[b]	10[b]	9[b]	10[b]
3	38	25	33	38	33	38	8[b]	−3[b]	4[b]	0[b]	0[b]	−3[b]	3[b]	−4[b]	5[b]	10[b]	4[b]	−2[b]
4	37	37	31	12[b]	29	−14[b]	7[b]	5[b]	3[b]	−10[b]	9[b]	9[b]	9[b]	3[b]	5[b]	−8[b]	3[b]	−9[b]
4a	28	33	44	28	−5[b]	−13[b]	8[b]	5[b]	10[b]	8[b]	−3[b]	−6[b]	19	2[b]	12[b]	16[b]	16[b]	16[b]
4m	30	30	42	27	27	5[b]	5[b]	3[b]	3[b]	5[b]	3[b]	0[b]	13[b]	13[b]	12[b]	8[b]	13[b]	11[b]
5	55	31	73	31	51	49	7[b]	2[b]	4[b]	7[b]	7[b]	−11[b]	11[b]	11[b]	0[b]	9[b]	9[b]	−8[b]
6		54	75	43	52	52	—	4[b]	4[b]	5[b]	9[b]	2[b]	—	−1[b]	−14	−3[b]	−7[b]	−3[b]

*Values are expressed as per cents from the control and are significantly different with $P<0.01$ for student's t-test.
[a]Values significantly different with $0.01<P<0.05$.
[b]Values significantly different with $0.05>P$.

Table 5. Statistical results of allelopathic bioassays (using *L. sativum*) of lupane triterpenes **2**, **4** and **5***

	(%) Germination						(%) Radical length						(%) Shoot length					
	10^{-4} M	10^{-5} M	10^{-6} M	10^{-7} M	10^{-8} M	10^{-9} M	10^{-4} M	10^{-5} M	10^{-6} M	10^{-7} M	10^{-8} M	10^{-9} M	10^{-4} M	10^{-5} M	10^{-6} M	10^{-7} M	10^{-8} M	10^{-9} M
2	−20[b]	−24[b]	−8[b]	−20[b]	−8[b]	−27[b]	15[b]	21[b]	19[b]	28[b]	21[b]	15[b]	24[b]	32[a]	25[a]	24[b]	26[b]	17[b]
4	−17[b]	−20[b]	−13[b]	−11[b]	−10[b]	−18[b]	40[b]	28[b]	10[b]	20[b]	18[b]	31[b]	26[b]	24[b]	22[b]	7[b]	29[b]	20[b]
5	−11[b]	−23[b]	−17[b]	−27[b]	−20[b]	−25[b]	44[a]	18[b]	26[b]	18[b]	28[b]	11[b]	36	22[b]	22[b]	19[b]	19[b]	−3[b]

*Values are expressed as per cents from the control and are significantly different with $P<0.01$ for student's t-test.
[a]Values significantly different with $0.01<P<0.05$.
[b]Values significantly different with $0.05>P$.

Table 6. Statistical results of allelopathic bioassays (using *H. vulgare*) of lupane triterpenes **1–6**,**4a** and **4m***

	(%) Germination						(%) Radical length						(%) Shoot length					
	10^{-4} M	10^{-5} M	10^{-6} M	10^{-7} M	10^{-8} M	10^{-9} M	10^{-4} M	10^{-5} M	10^{-6} M	10^{-7} M	10^{-8} M	10^{-9} M	10^{-4} M	10^{-5} M	10^{-6} M	10^{-7} M	10^{-8} M	10^{-9} M
1	—	13[b]	13[b]	19[a]	6[b]	30	—	−3[b]	6[b]	−1[b]	6[b]	7[b]	—	−12[b]	−23	−11[b]	−12[b]	−19[a]
2	14[b]	9[b]	3[b]	16[b]	7[b]	14[b]	−7[b]	13[b]	−2[b]	−15[b]	2[b]	2[b]	15[b]	15[b]	10[b]	2[b]	7[b]	10[b]
3	—	6[b]	21	21	30	15[b]		−21	−1[b]	−4[b]	1[b]	−12[b]	—	−31	−42	−44	−35	−38
4	12[b]	16[b]	9[b]	10[b]	−3[b]	2[b]	−1[b]	3[b]	0[b]	−8[b]	−19[b]	3[b]	6[b]	0[b]	3[b]	3[b]	−7[b]	−2[b]
4a	—	11[b]	−9[b]	13[b]	8[b]	13[b]	—	5[b]	0[b]	5[b]	7[b]	16[b]	—	−3[b]	6[b]	−11[b]	2[b]	−2[b]
4m	—	19[a]	23	36	23	32	—	1[b]	6[b]	23	21	19[a]	—	7[b]	−5[b]	−8[b]	−13[b]	−5[b]
5	19[a]	12[b]	5[b]	31	9[b]	24	1[b]	30	3[b]	1[b]	9[b]	17[b]	14[b]	14[b]	16[b]	13[b]	15[b]	13[b]
6	—	−4[b]	20	−9[b]	5[b]	4[b]		20	−7[b]	9[b]	3[b]	9[b]		20[a]	−6[b]	11[b]	−2[b]	2[b]

*Values are expressed as per cents from the control and are significantly different with $P<0.01$ for student's t-test.
[a]Values significantly different with $0.01<P<0.05$.
[b]Values significantly different with $0.05>P$.

Table 7. Statistical results of allelopathic bioassays (using *T. aestivum*) of lupane triterpenes **2**, **4** and **5***

	(%) Germination						(%) Radical length						(%) Shoot length					
	10^{-4} M	10^{-5} M	10^{-6} M	10^{-7} M	10^{-8} M	10^{-9} M	10^{-4} M	10^{-5} M	10^{-6} M	10^{-7} M	10^{-8} M	10^{-9} M	10^{-4} M	10^{-5} M	10^{-6} M	10^{-7} M	10^{-8} M	10^{-9} M
2	11[b]	−11[b]	13[b]	13[b]	−18[b]	−7[b]	13[b]	0[b]	−9[b]	−14[b]	−14[b]	4[b]	18[b]	20[b]	2[b]	−8[b]	−4[b]	8[b]
4	7[b]	7[b]	2[b]	0[b]	9[b]	9[b]	7[b]	15[b]	−13[b]	−7[b]	−10[b]	−1[b]	14[b]	6[b]	−2[b]	−3[b]	−11[b]	−4[b]
5	15[b]	−7[b]	7[b]	−2[b]	−12[b]	−4[b]	−6[b]	20[b]	8[b]	−8[b]	11[b]	24[b]	4[b]	14[b]	15[b]	−2[b]	0[b]	29[b]

*Values are expressed as per cents from the control and are significantly different with $P<0.01$ for student's t-test.
[a]Values significantly different with $0.01<P<0.05$.
[b]Values significantly different with $0.05>P$.

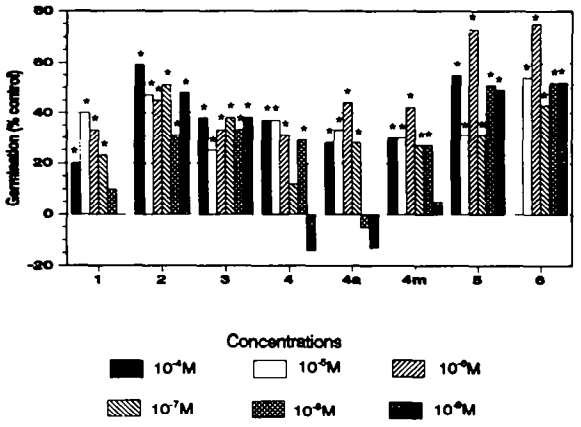

Fig. 3. Effect of lupane triterpenes **1**–**6**, **4a** and **4m** on the germination of *Lactuca sativa*.

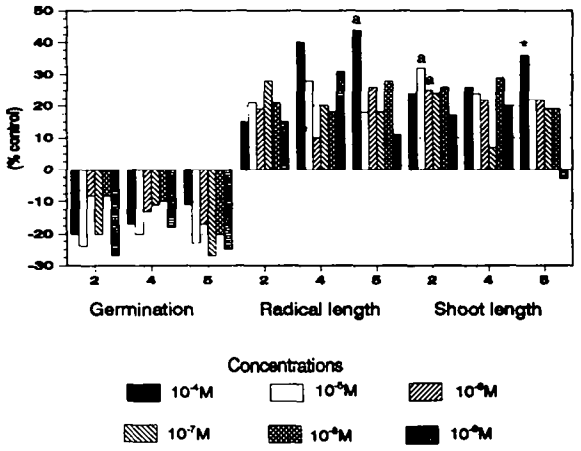

Fig. 4. Effect of lupane triterpenes **2**, **4** and **5** on the germination, root and shoot length of *Lepidium sativum*.

$-Me]^+$ (2); 395 (6); 383 (3); 207 (19); 189 (41); 177 (11); 43 (100); ^{1}H NMR: Table 1; ^{13}C NMR: Table 2.

Preparation of **5** *and* **6**. Betulin (**2**) (100 mg) was dissolved in 20 ml CH_2Cl_2–MeOH (9:1). The mixt. was cooled to $-76°$ using a solid CO_2–Me_2CO bath, then a current of O_3 was introduced into the soln until a consistent blue colour persisted. The reductive work-up with 0.3 ml of dimethylsulphide and sepn by CC on silica gel (CH_2Cl_2–EtOAc, 19:1), purification by HPLC gave **5** (70%) and **6** (10%). Compound **6**; ^{1}H NMR: Table 1; ^{13}C NMR: Table 2.

Weight per cents (weight of isolated lupanes/weight of plant × 100) of lupanes isolated from *Melilotus messanensis* were the following: lupeol (**1**), 0.0013%; betulin (**2**), 0.0031%; betulinaldehyde (**3**), 0.0037%; betulinic acid (**4**), 0.076% and messagenin (**5**), 0.0042%.

Bioassays. Seed germination bioassay. Seeds of *Lactuca sativa*, *Lepidium sativum*, *Hordeum vulgare* and *Triticum*

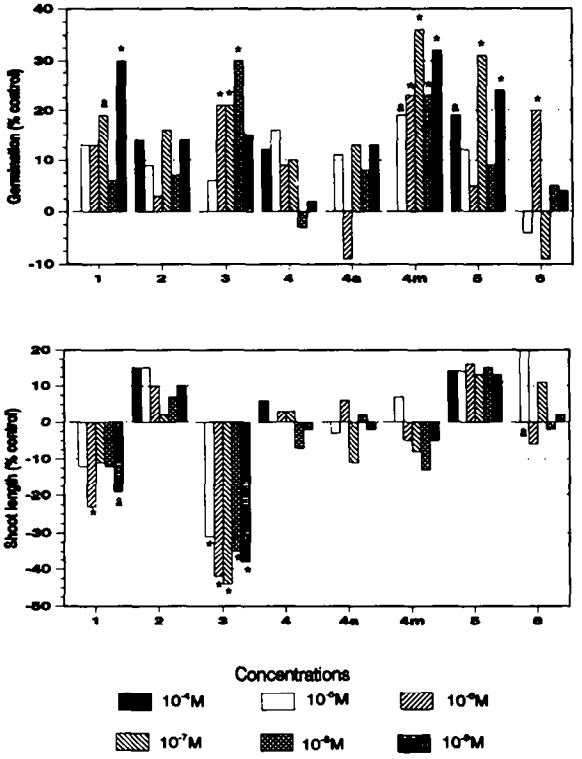

Fig. 5. Effect of lupane triterpenes **1**–**6**, **4a** and **4m** on the germination and shoot length of *Hordeum vulgare*.

aestivum 1991 crop, were obtained from Rancho La Merced, Junta de Andalucia, Jerez, Cádiz, Spain. All undersized and damaged seeds were discarded and the assay seeds were selected for uniformity of size.

Germination bioassays consisted of germinating 25 lettuce seeds and *Lepidium sativum* for 5 (3 for germination and 2 for root and shoot growth) and 2.5 days, respectively, and 5 *Triticum aestivum* and *Hordeum vulgare* seeds for 3 days, in the dark at 25° in 9-cm plastic Petri dishes containing a 10-cm sheet of Whatman no. 1 filter paper and 10 ml of a test or control soln for lettuce and *L. sativum* and 5 ml for *H. vulgare* and *T. aestivum*.

Test solns of water extract a and b were prepd by diluting the original extract to 1:10, 1:20 and 1:40 (V. extract: V. H_2O) using de-ionized H_2O and for the frs by diluting the appropriate amount of each fr. to obtain a similar concn to 1:10, 1:20 and 1:40 aq. extract. Test solns (10^{-4} M) were prepd using de-ionized H_2O and test solns 10^{-5}–10^{-9} M were obtained by diluting the previous soln. There were 3 replicates for *L. sativa* and *L. sativum* and 19 for *H. vulgare* and *T. aestivum* of each treatment and of parallel controls. The number of seeds per replicate, time and temp. of germination were chosen in agreement with a number of preliminary experiments, varying the number of seeds, vol. of test soln per dish and the incubation period.

All the pH values were adjusted to 6.0 before the bioassay using MES (2-[N-morpholino]ethanesulphonic acid, 10 mM). The osmotic pressure values were

measured on a Vapour Pressure Osmometer WESCOR 5500 and range between 30 and 38 mosmolar.

Statistical treatment. The germination, root and shoot length values were tested by the student's *t*-test; the differences between the experiment and the control were significant at a value of $P=0.01$ (Tables 3–7).

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