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BIOACTIVE POLAR TRITERPENOIDS FROM *MELILOTUS MESSANENSIS**

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Key Word Index—*Melilotus messanensis*; Leguminoseae; Fabaceae; sweet clover; allelopathy; lupane and nor-lupane triterpenes; messagenic acids F–I; oleanane triterpenes; melilotigenins B–D; *Lactuca sativa*; *Lycopersicon esculentum*; *Allium cepa*; *Hordeum vulgare*.

Abstract—In addition to the known 3-oxoplatanic acid, melilotigenin and soyasapogenol E, two new lupane (messagenic acids F, G), two nor-lupane triterpenic acids (messagenic acids H, I), and three new oleanane triterpenes (melilotigenins B-D) were isolated and characterized from the polar bioactive fractions of *Melilotus messanensis*. The synthesis of messagenic acids D, F, G, betulonic acid and 3,29-dioxolup-20(30)-en-28-oic acid has been carried out from betulinic acid. These compounds exhibited clear selectivity (parameters and species) over germination and growth of monocotyledon species with average of inhibition of −50% on the germination of *Hordeum vulgare*, and average of stimulation of 30% on the germination of *Allium cepa*.

INTRODUCTION

In a continuation of our allelopathic studies on *Melilotus messanensis* L. All [1–3] we now report the isolation and structural elucidation of five lupane carboxylic acids and five oleanane triterpenes from the bioactive polar fractions. The new lupane acids messagenic acid F (**2**), G (**3**), H (**8**), and I (**9**), and 3-oxoplatanic acid (**7**), previously described from *Platanus spp.* as the methyl ester [4], were isolated. The oleananes melilotigenin (**10**) and soyasapogenol E (**14**), previously described from *Melilotus officinalis* [5, 6], and the new compounds melilotigenin B (**11**), melilotigenin C (**12**), and melilotigenin D (**13**) were also obtained. The spectroscopic data of the free acid natural product **7** are reported for the first time in this paper. ^{13}C NMR data of melilotigenin (**10**) and soyasapogenol E (**14**) have been included since they have been unambiguously assigned. The compounds have been identified by spectroscopic techniques (IR, MS, ^{1}H and ^{13}C NMR experiments). The synthesis of **1-3**, **5** and **6** from betulinic acid (**4**) has also been carried out, both to confirm the structures and to obtain sufficient amounts of material to perform the bioassays.

In a continuation of our systematic studies of the bioactivity shown by metabolites isolated from allelopathic active fractions of *M. messanensis* [1–3], we now present the biological activities of eight lupane triterpenes and four oleanane triterpenes isolated or synthesized in this work. We have tested their effects on the germination and growth of the dicotyledons *Lycopersicon esculentum* L. and *Lactuca sativa* L. (var. nigra and var. romana), and the monocotyledons *Hordeum vulgare* L. and *Allium cepa* L. The range of concentrations tested falls between 10^{-4} and 10^{-9} M values (Fig. 1).

RESULTS AND DISCUSSION

Messagenic acid F (**2**) has a molecular ion at *m/z* 470, $C_{30}H_{46}O_4$. The fragmentation pattern is typical of a lupane triterpene with peaks at *m/z* 189 and *m/z* 207, corresponding to C-11,C-14 cleavage.

The most significant signals of the ^{1}H NMR spectrum are those corresponding to the aldehyde proton (δ 9.51, *s*, H-30), the methylene system (δ 6.28, *s*, H-29; δ 5.90, *s*, H-29′), the hydrogen attached to the hydroxyl group at C-3 (δ 3.16, *dd*, $J = 5$ Hz, $J = 11$ Hz,

*Part 7 in the series "Natural Products as Allelochemicals"; for Part 6 see Macias F.A., Simonet, A.M. and Galindo, J.C.G. in *Proceedings of the Plant Growth Regulation Society of America*, (Ed. Duane W. Greene), 22nd annual meeting, Minneapolis, Minnesota 1996, 53.

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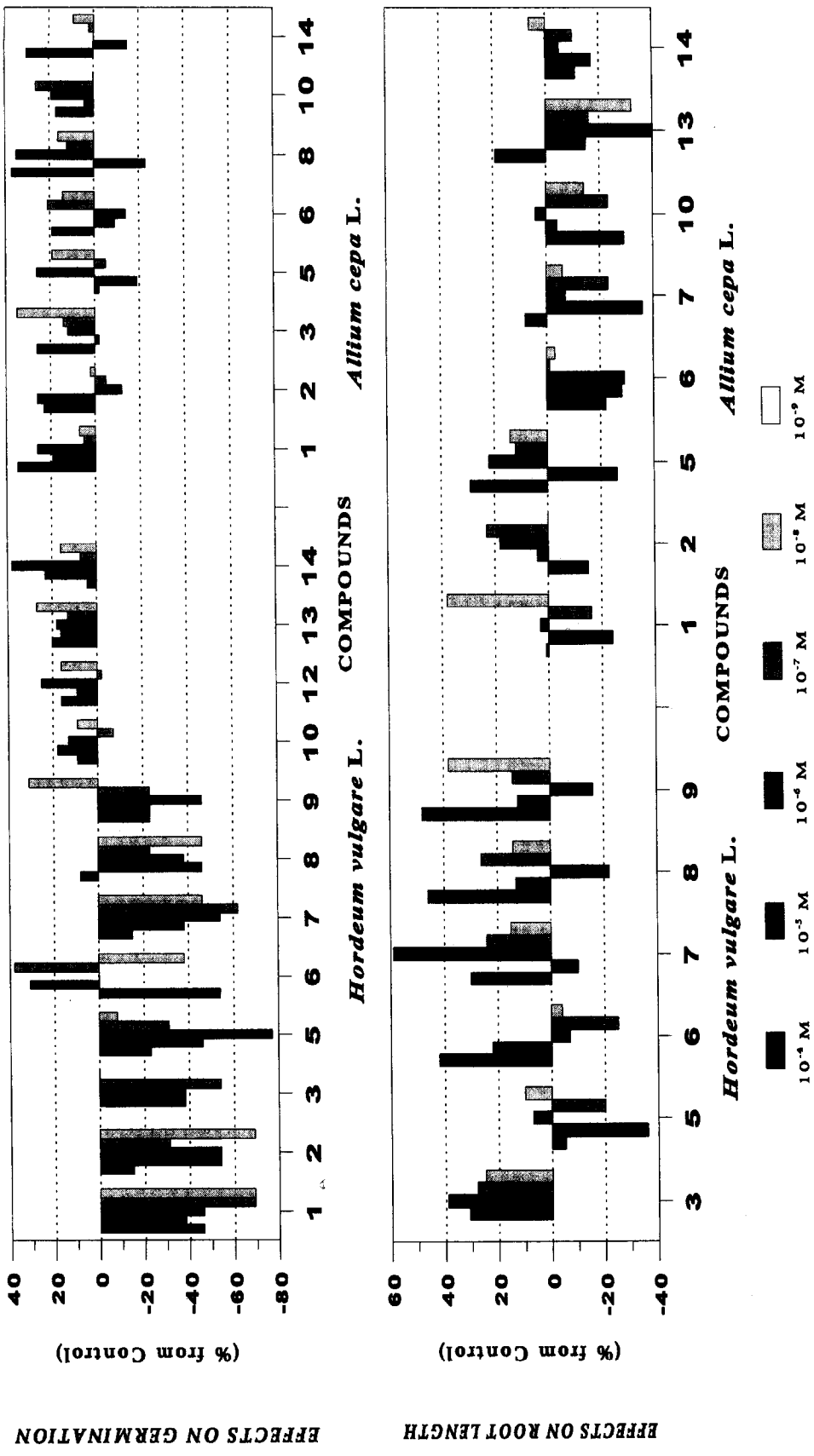


Fig. 1. Effects of selected compounds on germination and growth of monocotyledons.

H-3α), and the H-19 signal (δ 3.31, *ddd*, $J=5$ Hz, $J=12$ Hz, $J=12$ Hz). There are five methyl signals at δ 0.73 (*s*, H-24), 0.79 (*s*, H-25), 0.91 (*s*, H-27), 0.92 (*s*, H-26), and 0.95 (*s*, H-23), assigned by comparison with those of betulinic acid (**4**) [7]. All of these data confirm the proposed structure of 3β-hydroxy-29-oxolup-20(30)-en-28-oic acid for **2**.

Messagenic acid G (**3**) has an EI mass spectrum with $[M]^+$ at *m/z* 470, $C_{30}H_{46}O_4$, and the C-11, C-14 cleavage peak at *m/z* 205, which implies the presence of a carbonyl group on the AB-ring system of a pentacyclic triterpene. The ^1H NMR spectral data are in good agreement with the proposed structure **3**: five non-modified methyl signals at δ 0.92 (*s*, H-26), 0.97 (*s*, H-25), 0.98 (*s*, H-27), 1.01 (*s*, H-24), 1.06 (*s*, H-23), assigned by comparison of those of the gammacer-16-en-3-one [2] and messagenic acid D [3]; a methylene system at δ 4.98 (*s*, H-29) and δ 4.92 (*s*, H-29′), an hydroxymethylene system at δ 4.12 (*s*, 2H, H-30), and the H-19 signal at δ 2.88 (*ddd*, $J=11$ Hz, $J=11$ Hz, $J=5$ Hz). The position of the C-17 signal downfield at δ 55.9, following the same trend of the compounds above described, confirms the position of the acidic group at C-28 and thus, the structure 29-hydroxy-3-oxolup-20(30)-en-28-oic acid is proposed for **3**.

Messagenic acid H (**8**) shows an EI mass spectrum with a $[M]^+$ at *m/z* 472, $C_{29}H_{44}O_5$, and a fragmentation pattern typical of a 20-oxo-nor-lupane: the base peak at *m/z* 43, and the C-11, C-14 cleavage at *m/z* 205, indicating the presence of a carbonyl group on the AB ring system. Peaks at *m/z* 454 and *m/z* 441 are assigned to the $[M-H_2O]^+$ and $[M-CH_2OH]^+$ ions, respectively.

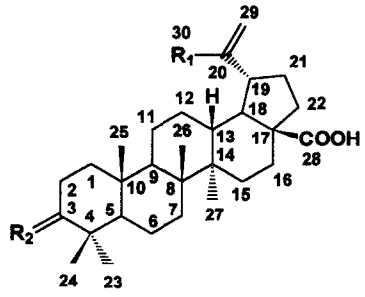
The comparison of its ^1H NMR spectroscopic data with those of platanic acid [8] and compound **1** reveals as major differences the presence of a $CO-CH_2-CH_2$ moiety, correlated by ^1H-^1H NMR COSY 2D experiments (δ 2.47, *ddd*, $J=6$ Hz, $J=8$ Hz, $J=16$ Hz, H-2β; δ 2.40, *ddd*, $J=5$ Hz, $J=8$ Hz, $J=16$ Hz, H-2α; δ 1.87, *ddd*, $J=13$ Hz, $J=6$ Hz, $J=5$ Hz, H-1β; δ 1.36, *m*, H-1α), the lack of the H-29 methyl signal, and the presence of an hydroxymethylene group signal at δ 4.29 (*brs*, 2H, H-29, H-29′) [3], respectively. A signal at δ 3.10 (*brt*) is assigned to the hydroxylic proton at C-29. The position of the signals corresponding to H-19 (δ 3.21, *ddd*, $J=11$ Hz, $J=11$ Hz, $J=5$ Hz) and C-17 (δ 55.9) allow us to allocate the COOH group at C-28. A comparison with the ^{13}C NMR data of compounds **1** and **4** (Table 2) confirms the proposed structure as 29-hydroxy-30-nor-3,20-dioxolupan-28-oic acid for **8**.

Messagenic acid I (**9**) has an EI mass spectrum similar to those described above, with a $[M]^+$ at *m/z* 474 ($C_{29}H_{46}O_5$) and major relevant diagnostic peaks at *m/z*: 456 $[M-H_2O]^+$, 207, and 189 (C-11,C-14 cleavage). Signals corresponding to the protons attached to C-3 (δ 3.18, *dd*, $J=5$ Hz, $J=11$ Hz, H-3α) and to C-19 (δ 3.20, *ddd*, $J=5$ Hz, $J=11$ Hz, H-19β) are overlapping in the ^1H NMR spectrum. Otherwise, the hydroxyl group at C-3 induces a downfield effect on the H-23 methyl signal, which appears at δ 0.95 (*s*, 3H). Comparison of the rest of the signals with those of messagenic acid H leads to the proposed structure of 3β,29-dihydroxy-30-nor-20-oxolupan-28-oic acid for **9**.

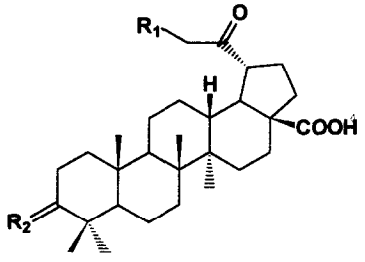
Melilotigenin B (**11**), $C_{30}H_{46}O_3$, has an EI mass spectrum with a peak at *m/z* 424 corresponding to the $[M-CH_2O]^+$ ion. The $[M]^+$ peak is not observed, and the base peak appears at *m/z* 232, corresponding to the retro-Diels-Alder cleavage of the C-ring, two units lower than that of **10**. Comparison of ^1H NMR data of **10** and **11** reveals a great similarity, except for the lack of the proton attached to the C-22 hydroxyl group. Thus, the analysis of the ^1H NMR COSY 2D experiment allow us to establish the presence of the $CO-CH_2-CH_2$ moiety (δ 1.54, *m*, H-1; δ 1.91, *ddd*, $J=5$ Hz, $J=8$ Hz, $J=13$ Hz H-1′; δ 2.44, *ddd*, $J=8$ Hz, $J=8$ Hz, $J=16$ Hz, H-2β; δ 2.55, *ddd*, $J=5$ Hz; $J=9$ Hz, $J=15$ Hz, H-2α) and the $CO-CH_2$ system (δ 2.42, *d*, $J=14$ Hz, H-21; δ 2.05, *dd*, $J=14$ Hz, $J=3$ Hz, H-21′). These data confirm the proposed structure of 24-hydroxy-olean-12-ene-3,22-dione for **11**, previously isolated as a product of the metabolic transformation of saponins in a culture of *Streptomyces* [9]. This represents the first report of this compound as a natural product.

Melilotigenin C (**12**) has an EI mass spectrum with a molecular ion at *m/z* 456, $C_{30}H_{48}O_3$, and a base peak at *m/z* 234 corresponding to the retro-Diels-Alder cleavage of the C-ring. Major diagnostic signals in the ^1H NMR spectrum are those corresponding to a $CO-CH_2-CH_2$ moiety in ring-A as observed by an ^1H NMR COSY 2D experiment (δ 1.55, *m*, H-1; δ 1.89, *m*, H-1′; δ 2.42, *ddd*, $J=8$ Hz, $J=8$ Hz, $J=16$ Hz, H-2β; δ 2.55, *ddd*, $J=4$ Hz, $J=9$ Hz, $J=16$ Hz, H-2α); an hydroxylic group at C-24 (δ 2.88, *dd*, $J=4$ Hz, $J=9$ Hz. C-O*H*, exchangeable with D_2O), which has a hydrogen bond with the C-3 carbonyl group; a hydroxymethylene group (δ 3.97, *dd*, $J=3$ Hz, $J=11$ Hz, H-24; δ 3.47, *dd*, $J=9$ Hz, $J=11$ Hz, H-24′; $CDCl_3+D_2O$: δ 3.96, *d*, δ 3.45, *d*); a vinyl proton (δ 5.27, *dd*, $J=3$ Hz, $J=3$ Hz, H-12); and a proton attached to the hydroxyl group at C-22 (δ 3.44, *m*; $CDCl_3+D_2O$, δ 3.42, *dd*, $J=5$ Hz, $J=5$ Hz, H-22α). Methyl signals have been assigned by comparison with soyasapogenol B [6, 10] and soyasapogenol E [6]. The signal at δ 0.99 is assigned to the C-25 methyl group because of its positive NOE with the H-24 protons. All data are consistent with the proposed structure 22,24-dihydroxyolean-12-en-3-one for **12**.

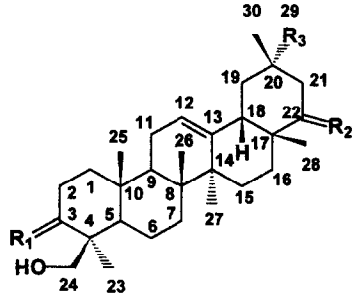
Melilotigenin D (**13**) has an EI mass spectrum with an $[M]^+$ at *m/z* 484, $C_{30}H_{46}O_5$. Its fragmentation pattern is typical of an olean-12-ene skeleton, with the base peak at *m/z* 262, corresponding to the retro-Diels-Alder cleavage of ring-C. This peak also confirms the attachment of the acid function to the D, E-ring system. The ^1H NMR spectrum shows as major diagnostic signals those corresponding to the $CO-CH_2-CH_2$ moiety, [^1H NMR COSY 2D experiment] (δ 1.81, *m*, H-1; δ 1.34, *m*, H-1′; δ 2.81, *ddd*, $J=15$ Hz, $J=14$ Hz, $J=6$ Hz, H-2β; δ 2.36, *ddd*, $J=15$ Hz;



$R_1 = CH_2OH$; $R_2 = OH\beta$, $H\alpha$	**Messagenic acid D (1)**
$R_1 = CHO$; $R_2 = OH\beta$, $H\alpha$	**Messagenic acid F (2)**
$R_1 = CH_2OH$; $R_2 = O$	**Messagenic acid G (3)**
$R_1 = CH_3$; $R_2 = OH\beta$, $H\alpha$	**Betulinic acid (4)**
$R_1 = CH_3$; $R_2 = O$	**Betulonic acid (5)**
$R_1 = CHO$; $R_2 = O$	**3,29-Dioxolup-20(30)-en-28-oic acid (6)**



$R_1 = H$; $R_2 = O$	**3-Oxoplatanic acid (7)**
$R_1 = OH$; $R_2 = O$	**Messagenic acid H (8)**
$R_1 = OH$; $R_2 = OH\beta$, $H\alpha$	**Messagenic acid I (9)**



$R_1 = OH\beta$, $H\alpha$; $R_2 = O$; $R_3 = COOH$	**Melilotigenin (10)**
$R_1 = R_2 = O$; $R_3 = Me$	**Melilotigenin B (11)**
$R_1 = O$; $R_2 = OH\beta$, $H\alpha$; $R_3 = Me$	**Melilotigenin C (12)**
$R_1 = R_2 = O$; $R_3 = COOH$	**Melilotigenin D (13)**
$R_1 = OH\beta$, $H\alpha$; $R_2 = O$; $R_3 = Me$	**Soyasapogenol E (14)**

$J = 5$ Hz, $J = 3$ Hz, H-2α); the hydroxymethylene group (δ 4.32, *d*, $J = 11$ Hz, H-24; δ 3.88, *d*, $J = 11$ Hz, H-24′); and the CH_2-CO system (δ 2.72, *dd*, $J = 14$ Hz, $J = 2$ Hz, H-21α; δ 3.44, *d*, $J = 14$ Hz, H-21β). The assignment of the acid group as C-29 was based on the comparison of their ^{13}C NMR data with those of subprogenins C and D [11] since the ^{13}C NMR data of melilotigenin are not available. The deshielding of C-3 from δ 80.2 in **10** to δ 214.7, as well as C-1 (δ 40.3), C-2 (δ 35.6), and C-4 (δ 55.0), in comparison of those of **10** (δ 39.0, δ 28.5, δ 43.3, respectively) is perfectly consistent with the proposed structure 24-hydroxy-3,22-dioxoolean-12-en-29-oic acid for **13**.

Since the lupane derivatives were obtained only in small amounts, they were prepared from betulinic acid (**4**), which was relatively abundant in *M. messanensis* [1]. Oxidation of **4** with pyridinium chloro chromate (PCC) afforded betulonic acid (**5**) [12, 13]. The allylic oxidation of **5** with selenium dioxide (SeO_2) and *tert*-butyl-hydroperoxide (*t*-ButOOH) as previously described [14, 15] yielded a mixture of **3** and the aldehyde 3,29-dioxolupan-20(30)-en-28-oic acid (ν_{max} 1700, 1686, 1681 cm^{-1}; δ_H 9.52) (**6**). Similarly allylic oxidation of **4** yielded a mixture of compounds **1** and **2**.

Bioassay data and discussion

The effects produced by selected compounds on germination and growth are presented in Table 3 and Fig. 1. The behaviour shown by the compounds is very close to that previously published for other triterpenoid acids [3] with respect to the sensitivity of the different plant target species. Sensitivity increases in the order *Lactuca sativa*, *Lycopersicon esculentum*, *Allium cepa*, and *Hordeum vulgare*. Results obtained with the monocotyledon species *A. cepa* are much lower than those for *H. vulgare* and with opposite behaviour.

Germination data for *Hordeum vulgare* (Fig. 1, Table 3) are homogeneous and deeply inhibitory for every lupane and nor-lupane acids and remain active at the lower concentrations, eg. compounds **1** (from −38%, 10^{-6} M, to −69%, 10^{-8} M and 10^{-9} M), **2** (from −15%, 10^{-5} M, to −69%, 10^{-9} M), and **7** (from −15%, 10^{-5} M, to −62%, 10^{-8} M). These

Table 1. ^{1}H NMR of triterpenes **2**, **3**, **6–13** (399.95 MHz, $CDCl_3$,signal of residual $CHCl_3$, centred at δ 7.25 ppm)*

H	2	3	6	7	8	9	10 †	11	12	13 †
1	1.63 *m*	1.88 *ddd*	1.87	1.87	*β* 1.87	1.62 *m*	1.57	1.91 *ddd*	1.89 *m*	1.81
	0.87 *m*	1.37	1.35	1.37	*α* 1.36	0.88	0.99	1.54	1.55	1.34
2*β*	1.61 *m*	2.47 *ddd*	2.47	2.47	2.47	1.57a	2.01	2.44	2.42	2.81
α	1.53 *m*	2.38 *ddd*	2.37	2.40	2.40	1.52a	1.90	2.55	2.551	2.36
3*α*	3.16 *dd*	—	—	—	—	3.18 *dd*	3.62	—	—	—
5	0.65 *dd*					0.67 *dd*	0.94	0.99 *m*	0.98	0.96
6	1.49 *m*					1.50 *m*	1.69	1.54		1.62
	1.35 *m*					1.37 *m*	1.42	1.25		1.41
7							1.42 *m*	1.43		1.41
							1.26 *m*	1.35		1.29
9							1.60 *m*	1.69 *dd*	1.61	1.65 *m*
11	1.31 *m*	1.43		1.44	1.47	1.49	1.88 (2H)	1.93 (2H)	1.94	1.90
	1.23 *m*	1.27		1.31	1.26	1.24			1.87	(2H)
12	1.31 *m*	1.43		1.44	1.41	1.49	5.34 *brs*	5.32 *dd*	5.27	5.34
	0.87 *m*	1.09		1.08	1.03	0.97				
13	2.19 *m*	2.21	2.23	2.07	2.04	2.03	—	—	—	—
15	1.49 *m*	1.48	1.60	1.47	1.48	1.48	1.68	1.77 *ddd*	1.73 *m*	1.69
	1.19 *m*	1.24	1.20	1.23	1.26	1.22	0.98	1.10	1.02	0.96
16	2.29 *m*	2.27	2.29	2.28 *ddd*	2.32 *m*	2.30	2.20 *ddd*	1.97	1.73 *m*	2.20
	1.49 *m*	1.48	1.48	1.47	1.48	1.48	1.23	1.20	1.21	1.22
18		1.74 *dd*		1.52	2.27	2.25	2.56	2.34	2.09 *brd*	2.56 *dd*
19	3.31 *ddd*	2.88	3.32	3.24	3.21	*β* 3.20	2.87 *dd*	2.10	1.68 *m*	2.87
							1.98 *m*	1.35	1.02	2.00
21	2.09 *ddd*	2.10 *m*		2.07	2.07	2.07	3.44 *d*	2.42	1.43 *m*	*β* 3.44 *d*
	1.72 *m*	1.42		1.52	1.54	1.54	2.72 *dd*	2.05	(2H)	*α* 2.72
22	1.97 *dd*	1.96		1.98	2.01 *d*	2.00 *m*	—	—	*α* 3.44 *m*	—
	1.37 *m*	1.54		1.59	1.63	1.65				
23	0.95 *s*	1.06	1.05	1.06	1.06	0.95	1.54	1.27	1.27	1.47
24	0.73 *s*	1.01	1.00	1.01	1.00	0.74	4.51 *d*	3.97 *dd*	3.97	4.32 *d*
							3.71 *d*	3.47 *dd*	3.47	3.88 *d*
								OH 2.83 *dd*	OH 2.88	
25	0.79 *s*	0.97	0.96	0.95	0.95	0.80	0.93	0.99	0.99	1.18
26	0.92 *sa*	0.92	0.94	0.90	0.90 a	0.91 b	0.94	0.99	0.98	0.96
27	0.91 *sa*	0.98	0.91	1.01	1.00 a	0.99 b	1.22	0.99	1.12	1.21 a
28	—	—	—	—	—	—	1.23 *s*	1.23	1.03	1.22 a
29	9.51 *s*	4.12	9.52	2.17	4.29 *brs*	4.29	—	0.98 *s*	0.91	—
					OH 3.11	OH 3.11				
30	6.28 *s*	4.98	6.28	—	—	—	1.41 *s*	0.85	0.86	1.41
	5.90 *s*	4.92	5.90							

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

†: Spectrum recorded in pyridine-d_6.

a,b: Values within the same column may be interchanged.

J (Hz): **3, 6, 7, 8, 11, 12, 13**: 1*β*,2*β* = 7; **3, 6, 7, 8, 11, 12**: 1*α*,2*α* = 8; 1*α*,2*β* = 9; 1*β*,2*α* = 5; **6, 7, 8, 11, 12, 13**: 2*α*,2*β* = 16; **6, 7, 8, 11, 13**: 1*β*,1*α* = 13; **2, 6, 7, 8, 9**: 18,19 = 11; 19, 21*α* = 11; 19,21*β* = 5; **2, 3, 7, 8, 9**: 22*α*,22*β* = 12; 22*α*,21*α* = 8; **2, 9, 10**: 2*α*,3*α* = 5; 2*β*,3*α* = 11; **3, 7, 8**: 13,18 = 11; **10, 11, 13**: 15*α*,16*α* = 4; 15*β*,16*α* = 16*α*,16*β* = 13; 19*α*,19*β* = 14; 18,19*α* = 4; 18,19*β* = 14; 19,21*α* = 2; 21*α*,21*β* = 14; **10, 12, 13**: 24a,24b = 11; **11, 12, 13**: 11*α*,12 = 11*β*,12 = 3; **2, 9**: 5,6*β* = 10; **11, 12**: 9,11*β* = 11; 24a,OH = 3; 24b,OH = 9; **3**: 13,18 = 11; **7**: 15,16*α* = 15,16*β* = 3, 16*α*,16*β* = 10; **11**: 9,11*α* = 7; 18,19*β* = 13.

results are not in accordance with those previously reported for **1** [3], but now they are homogeneous and follow the same trend of the other tested triterpenoid acids (compounds **3, 5, 6, 8–10,** and **13**). Thus, these new values must be considered as correcting the previous reported ones. There are no significant differences between the behaviour of lupane and nor-lupane acids.

The oleanic triterpenes tested (**10, 12–14**), exert a moderately stimulatory effect on germination. This is in good accordance with the results we have obtained previously for soyasapogenols B and G [3].

Root length (Fig. 1, Table 3) is stimulated for almost the major of the lupane and nor-lupane acids. No significant differences between them are observed, except for messagenic acid G (**3**), which has the most

Table 2. ^{13}C NMR of triterpenes 2, 3, 7-10, 12-14 (100.23 MHz, $CDCl_3$, signal centred at δ 77.0 ppm)

C	2	3	7	8	9	10*	12	13*	14
1	38.8	39.5	39.5	39.5	38.7	39.0	38.8	40.3	38.4
2	27.4	34.0	34.0	34.0	27.4	28.5	34.4	35.6	27.6
3	78.9	217.4	217.9	217.8	78.9	80.2	220.5	214.7	80.9
4	38.7	47.3	47.3	47.3	38.9	43.3	51.1	55.0	42.8
5	55.3	54.8	54.8	54.8	55.3	56.4	55.7	57.8	55.9
6	18.3	19.6	19.6	19.6	18.3	19.1	19.3	20.0	18.4
7	34.3	33.5	33.5	33.5	34.2	33.3	32.7	33.1	32.9
8	40.6	40.5	40.5	40.5	40.6	40.0	39.5	40.0	39.7
9	50.3	49.6	49.6	49.6	50.4	48.0	46.5	47.4	47.6
10	37.2	36.9	36.9	36.9	37.2	37.1	36.4	40.3	36.7
11	20.8	21.3	21.3	21.3	20.8	24.1	23.9	24.2	23.8
12	27.2	28.9	28.9	28.9	28.9	124.7	122.2	124.7	123.6
13	38.2	37.4	37.4	37.4	37.4	141.5	143.9	141.6	141.6
14	42.3	42.3	42.3	42.3	42.3	42.1	42.3	42.1	41.8
15	29.7	29.6	29.6	29.6	29.7	25.4	25.8	25.4	25.3
16	31.9	31.2	31.2	31.2	31.3	27.4	28.2	27.3	27.2
17	56.3	55.9	55.9	55.9	56.0	48.3	37.8	48.3	47.6
18	50.3	49.5	49.5	49.5	49.7	47.1	45.0	47.1	47.6
19	42.9	46.5	46.5	46.5	46.5	41.8	46.5	41.7	46.7
20	151.0	154.8	211.9	213.4	213.1	44.6	30.5	44.6	34.1
21	31.9	27.4	27.4	27.4	27.4	46.6	41.5	46.5	50.9
22	36.8	36.7	36.7	36.7	36.8	213.9	77.2	214.4	216.2
23	28.0	26.7	26.7	26.7	28.0	23.6	22.1	21.0	22.4
24	15.3	21.0	21.0	21.0	15.4	64.6	65.8	65.3	64.5
25	16.1	15.9	15.9	15.9	16.1	16.2	16.3	15.8	16.1
26	16.0	15.7	15.7	15.7	15.9	16.9	16.7	16.9	16.7
27	14.6	14.5	14.5	14.5	14.7	25.4	25.2	25.4	25.4
28	178.8	178.9	179.8	179.3	179.4	21.7	28.2	21.7	25.1
29	194.9	65.3	29.7	68.1	68.1	178.3	32.5	178.9	32.0
30	131.1	68.1	-	-	-	21.0	20.0	21.0	20.5

* Spectrum recorded in pyridine-d_6

homogeneous profile of activity (31% at 10^{-6} M, 39% at 10^{-7} M, 28% at 10^{-8} M, and 25% at 10^{-9} M) and compound **7**, which presents the highest values (30% at 10^{-5} M, 59% at 10^{-7} M, and 24% at 10^{-8} M). The rest of the triterpenoid acids show rather dispersed values. Oleanane triterpenes show no significative activity. The values of activity showed on shoot length are of low significance, excepting some isolated cases, and do not follow any general trend.

Germination of *Allium cepa*. (Fig. 1, Table 3) shows a general stimulation pattern, with moderate values. In particular at 10^{-5}M some compounds show values greater than 20%, for example compounds **1** (35%), **3** (26%) and **8** (37%). The overall effect is lower than that showed on *H. vulgare*.

The behaviour showed by roots is moderate (Table 3, Fig. 1), but with some aspects that deserve comment: for example oleanic triterpenes (**10, 12, 13**) show themselves at first sight as inhibitors of the root growth (**10**: −29%, 10^{-5} M; **12**: −31%, 10^{-7} M; **13**: −40%, 10^{-7} M). Lupane acids **6** and **7** show the highest values among lupane and nor-lupane compounds. The effects on shoot length are low or no significant.

There are no significant activities on the dicotyledons of the other plant species tested, except some isolated examples for *Lycopersicum esculentum*: germination: −25%, 10^{-5} M (**10**), −23%, 10^{-6} M (**10**), −20%, 10^{-6} M (**12,13**); root length: −37%, 10^{-5} M, (**10**).

The work reported leads to the following conclusions. a) These compounds exhibit clear selectivity (parameters and species) over germination and growth of monocotyledon species with average of inhibition of −50% on the germination of *Hordeum vulgare*, and average of stimulation of 30% on the germination of *Allium cepa*. b) Triterpenoid acids show the same behaviour as previously reported [3]: they are active principally on monocotyledon species. As *M. messanensis* has to share its biotope mainly with grasses, which represent the main competitors, the specificity in its action should be the response to its environment. c) Acids show higher levels of activity than the other compounds. As the pH of the solutions is buffered in a range of 6.0–6.5, triterpenoid acids are in their ionized in carboxylate form. It has been proved for other triterpene and saponins that their

Table 3. Germination and growth activity of triterpenes **1–3, 5–10, 12–14**

Compound	Germination (% difference from control)					Root length (% difference from control)				
	10^{-5}M	10^{-6}M	10^{-7}M	10^{-8}M	10^{-9}M	10^{-5}M	10^{-6}M	10^{-7}M	10^{-8}M	10^{-9}M
Hordeum vulgare L.										
1	−46	−38	−46	−69	−69	−8	19	−50	53a	7a
2	−15	−54	−54	−31	69	1	1	−46	45	10b
3		−38	−38	−54	0		31	39	28	25
5	−23	−46	−77	−31	−8	−5	−36	7	−20	10
6	−54	31	0	38	−38	42	22	−7	−25	−4
7	−15	−38	−54	−62	−46	30	−10	59b	24b	15
8	8	−46	−38	−23	−46	46	13	−22	26	14
9	−23	−23	46	−23	31	48	12	−16	14	38
10	9	18	13	−7	9	−2	−11	−26	−3	−4
12	16	9	25	−2	16	−1	−6	8	−26	−23
13	20	16	18	13	27	−10	17	−12	3	4
14	4	23	38b	7	16	1	23	2	−13	1
Allium cepa L.										
1	35	19	26	5	7	1	−24	3	−16	38
2	23	26	−12	−5	2	−15	4	18	23	0
3	26	−2	12	14	35	10	3	9	3	−4
5	−2	−19	26	−5	19	29	−26	22	12	14
6	19	−9	−14	21	14	−22	−28	−29	−1	−3
7	2	−28	0	−19	5	8	−39	−7	−23	−6
8	37	−23	35	12	16	3	−19	30	−7	−13
9	−12	14	19	−23	2	−7	−7	2	−18	15
10	17	4	19	26	0	−29	−4	4	−23	−14
12	4	4	28	2	−34	0	1	−31	2	−23
13	9	11	2b	−13	0	19	−15	−40	−16	−32
14	30	−15	0	2	9	−11	−17	−5	−10	6

Values presented as percentage differences from the control (e.g., 16% means 116% compared with the control). $P > 0.05$ for the Welch's test.

a: $P > 0.01$;

b: $0.01 < P < 0.05$

sodium salts are more active than the non ionized acids [15, 16].

EXPERIMENTAL

Plant material

Melilotus messanensis L. was all collected on 11 April and 2 July 1991 in Trebujena, Cádiz, Spain (Voucher specimen 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. The latter period was selected as it provided the more bioactive aq. extract in the lab bioassay.

Extraction and isolation

Fresh plants with seeds (stage **b**) (382 g) were soaked in deionized H_2O (wt plant: vol. solvent 1:3), for 24 hr at 25° in the dark. The aq. extract was extracted (10 ×) with CH_2Cl_2–H_2O(1:2) and the combined extracts dried over Na_2SO_4 and evapd *in vacuo* to yield 1.2 g of crude extract. Crude extract was separated by silica gel CC using *n*-hexane-EtOAc mixtures of increasing polarity, yielding 214 × 50 ml frs, which were reduced to 13 frs (**A**–**M**) after comparison by TLC.

Previously we reported the results of the studies of fractions **C**, **D**, **E**, **G**, **I**, **K**, **L**, and **M** [1–3]. Here we report the results of the analysis of minor compounds of medium and high polar fractions.

Identification

Known compounds were identified by comparison of their physical and spectroscopic data (mp, [α], IR, MS, ^{1}H and ^{13}C NMR) with those previously reported in the literature. Structures of new compounds were established based on the analysis of their spectroscopic data and, in some cases, chemical correlations.

Messagenic acid F (**2**)

$C_{30}H_{46}O_4$, amorphous solid. $[\alpha]_D^{25}$ +1° ($CHCl_3$, *c*0.2). IR $\nu_{max}^{KBr,neat}$ cm^{-1}: 3312 (OH); 1693 CO–OH); 1693 (α,β unsatured aldehyde). EIMS (70 eV) *m/z* (rel. int.): 470 $[M]^+$ (3); 452 $[M-H_2O]^+$ (5); 437 $[M-H_2O-CH_3]^+$ (4);

233 (22); 207 (52); 189 (83); 135 (50). ^{1}H NMR see Table 1. ^{13}C NMR see Table 2.

Messagenic acid G (**3**)

$C_{30}H_{46}O_4$, amorphous solid. $[\alpha]_D^{25}$ +22° ($CHCl_3$, *c*0.5). IR $\nu_{max}^{KBr,neat}$ cm^{-1}: 3401 (OH); 1710 (CO); 1699 (CO–OH). EIMS (70 eV) *m/z* (rel. int.): 470 $[M]^+$ (0.5); 452 $[M-H_2O]^+$ (3); 205 (15); 55 (100). ^{1}H NMR see Table 1; ^{13}C NMR see Table 2.

Messagenic acid H (**8**)

$C_{29}H_{44}O_5$, amorphous solid. IR $\nu_{max}^{KBr,neat}$ cm^{-1}: 3450 (OH); 1712 (CO); 1699 (CO–OH). EIMS (70 eV) *m/z* (rel. int.): 472 $[M]^+$ (0.3); 454 $[M-H_2O]^+$ (1); 441 $[M-CH_2OH]^+$ (3); 205 (7); 55(100). ^{1}H NMR see Table 1. ^{13}C NMR see Table 2.

Messagenic acid I (**9**)

$C_{29}H_{46}O_5$, amorphous solid. $[\alpha]_D^{25}$ −7° ($CHCl_3$, *c*0.11). IR $\nu_{max}^{KBr,neat}$ cm^{-1}: 3340 (OH); 1714 (CO); 1693 (CO–OH). EIMS (70 eV) *m/z* (rel. int.): 474 $[M]^+$ (0.2); 456 $[M-H_2O]^+$ (1); 207 (11); 189 (17); 44 (100). ^{1}H NMR see Table 1. ^{13}C NMR see Table 2.

Melilotigenin B (**11**)

$C_{30}H_{46}O_3$, white powder. $[\alpha]_D^{25}$ +25° ($CHCl_3$, *c*0.04). IR $\nu_{max}^{KBr,neat}$ cm^{-1}: 3431 (OH); 1708 (CO); 1657 (C=C). EIMS (70 eV) *m/z* (rel. int.): 424 $[M-CH_2O]^+$ (7); 232 (100); 217 (42), 203 (16). ^{1}H NMR see Table 1.

Melilotigenin C (**12**)

$C_{30}H_{48}O_3$, white powder. $[\alpha]_D^{25}$ +57° ($CHCl_3$, *c*0.07). IR $\nu_{max}^{KBr,neat}$ cm^{-1}: 3427 (OH); 1705 (CO). EIMS (70 eV) *m/z* (rel. int.): 456 $[M]^+$ (1); 426 $[M-CH_2O]^+$ (6); 411 (2); 234 (100). ^{1}H NMR see Table 1. ^{13}C NMR see Table 2.

Melilotigenin D (**13**)

$C_{30}H_{44}O_5$, white powder. $[\alpha]_D^{25}$ -7° ($CHCl_3$, *c*0.07). EIMS (70 eV) *m/z* (rel. int.): 484 $[M]^+$ (0.2); 454 $[M-CH_2O]^+$ (4); 410 (3); 262 (100). ^{1}H NMR see Table 1. ^{13}C NMR see Table 2.

Synthesis of lupane triterpenes

Oxidation of **4** with pyridinium chlorochromate (PCC) Compound **4**, 30 mg was stirred with 29 mg of PCC in $CHCl_3$ (30 ml) during 2 hr at room temp. The reaction mixture was then filtered through silica gel yielding 28 mg of **5** (93%) as unique product.

Oxidation of **4** *with* SeO_2/*tert-butyl hydroperoxide* (*t-ButOOH*) Compound **4** (20 mg) was stirred in 15 ml $CHCl_3$ (3mM) with 1.1 mg of SeO_2 and 0.04 ml of *t*-ButOOH during 24 hr at room temp. The crude reaction was filtered and washed through silica gel using $CHCl_3$ and EtOAc. Polar EtOAc fraction was then purified using HPLC (*n*-hexane-Me_2CO 6:1) yielding 17% of **1**, 1% of platanic acid, and 8% of **2**.

Oxidation of **5** *with* SeO_2/*tert-butyl hydroperoxide* (*t-ButOOH*) Compound **5** (28 mg) was stirred in 20 ml $CHCl_3$ (3 mM) with 1.6 mg of SeO_2 and 0.05 ml of *t*-ButOOH during 70 min at room temp. The crude reaction was filtered and washed through silica gel using $CHCl_3$ and EtOAc. Polar EtOAc fraction was then purified using HPLC (*n*-hexane-Me_2CO 6:1) yielding 5% of **3** and 6% of **6**.

3,29-*Dioxolup*-20(30)-*en*-28-*oic acid* (**6**). $C_{30}H_{44}O_4$, amorphous solid. $[\alpha]_D^{25}$ +16° ($CHCl_3$, *c*0.08). IR $\nu_{max}^{KBr.neat}$ cm^{-1}: 1700 (CO); 1686 (CO–OH); 1681 (CHO). ^{1}H-NMR see Table 1.

Seed germination bioassays Seeds of *Lactuca sativa* var. nigra and var. romana, *Lycopersicon esculentum* L., and *Allium cepa* L. were obtained from FITÓ, S. L. (Barcelona, Spain). *Hordeum vulgare* L. was obtained from Rancho La Merced, Junta de Andalucía, Jerez, Cádiz, Spain. All undersized or damaged seeds were discarded and the assay seeds were selected for uniformity.

Germination and growth bioassays for tested species were as follows: *L. sativa* var. romana, 25 seeds per dish, 5 ml test soln, 4 days dark, 25° and four replicates of each conc; *L. esculentum*, 25 seeds per dish, 5 ml test soln, 5 days dark, 25° and four replicates of each conc; *A. cepa*, 25 seeds per dish, 5 ml test soln, 5 days dark, 25° and four replicates of each conc; *H. vulgare*, 10 seeds per dish, 5 ml test soln, 5 days dark, 25° and 10 replicates of each conc [17].

Test solns (10^{-4}M or 10^{-5}M) were prepared using H_2O-MES (2-[N-Morpholino]ethanesulfonic acid, 10 mM), and the rest were obtained by dilution. Parallel controls were performed. All pH values were adjusted to 6.0 before bioassay with MES. Osmotic pressure values were measured on a vapour pressure osmometer (WESCOR 5500) and ranged between 30 and 38 mOsmolar.

Data are presented as percentage differences from control in graphics and tables (Fig. 1 and Table 3). Thus, zero represents the control; positive values represent stimulation of the studied parameter and negative values represent inhibition.

Statistical treatment

Germination and root and shoot length values were tested by the Welch's test; differences between test solns and controls were significant ($P=0.01$) (Table 3).

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REFERENCES

1. Macías, F. A., Simonet, A. M. and Esteban, M. D., *Phytochemistry*, 1994, **36**, 1369.
2. Macías, F. A., Simonet, A. M., Esteban, M. D. and Galindo, J. C. G., *Phytochemistry*, 1996, **41**, 1573.
3. Macías, F. A., Simonet, A. M. and Galindo, J. C. G., *Journal of Chemical Ecology*, 1997, **23**, 1781.
4. Aplin, R. T., Halsall, T. G. and Norin, T., *Journal of the Chemical Society Chemical Communications*, 1963, 3269.
5. Kang, S. S. and Woo, W. S., *Journal of Natural Products*, 1988, **51**, 335.
6. Kang, S. S., Lim, C. H. and Lee, S. Y., *Archives of Pharmacological Research*, 1987, **10**, 9.
7. Winston, F. T. and Lynn, A., Azzam, A., *Journal of Natural Products*, 1992, **55**, 395.
8. Fujioka, T., Kashiwada, Y., Kilkuskie, R. E., Cosentino, L. M., Ballas, L. M., Jiang, J. B., Janzen, W. P. and Chen, I., Lee, K., *Journal of Natural Products*, 1994, **57**, 243.
9. Kaneda, M., Ishimaru, K. and Nakamura, S., *Chemistry and Pharmaceutical Bulletin*, 1984, **32**, 1287.
10. Baxter, R. L., Price, K. R. and Fenwick, G. R., *Journal of Natural Products*, 1990, **53**, 298.
11. Takeshita, T., Yokoyama, K., Yi, D., Kinjo, J. and Nohara, T., *Chemistry and Pharmaceutical Bulletin*, 1991, **39**, 1908.
12. Cole, B. J. W., Bentley, M. D. and Hua, Y., Bu, L., *Journal of Wood Chemistry and Technology*, 1991, **11**, 209.
13. Carpenter, R. C., Sotheeswaran, S., Sulthabawa, U. S. and Ternai, B., *Organic Magnetic Resonance*, 1980, **14**, 462.
14. Macías, F. A., Galindo, J. C. G. and Massanet, G. M., *Phytochemistry*, 1992, **31**, 1969.
15. Haruna, M. and Ito, K., *Journal of the Chemical Society. Chemical Communication*, 1981, 483.
16. Gorski, P. M., Miersch, J. and Plazynski, M. J., *Journal of Chemical Ecology*, 1991, **17**, 1135.
17. Macías, F. A., Castellano, D., and Molinillo, J. M. G., *Journal of Chemical Ecology*, 1998, in press.