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# Labdane diterpenes from *Marrubium thessalum*

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ABSTRACT

From the aerial parts of *Marrubium thessalum*, four labdane diterpenes, 13*S*-preperegrinine, 3α-hydroxymarrubiin, 9α,13*R*-15,16-bisepoxy-15β-methoxy-3-oxo-labdan-6β,19-olide and 15-methoxyvelutine C, have been isolated together with four known diterpenes and one methoxylated flavone, ladanein. The structures of the isolated compounds were established by means of NMR (COSY, HSQC, HMBC, NOESY, and ROESY) and MS spectral analyses. Complete NMR assignments are reported for 13*R*-preperegrinine. Characteristic compounds of the plant were peregrinine and ladanein.

## 1. Introduction

The genus *Marrubium* belongs to the Lamiaceae family. It comprises approximately 30 species, indigenous in Europe, the Mediterranean area and Asia (Mabberley, 1997). *Marrubium* sp., including *Marrubium vulgare*, which is the most known representative, are traditionally used as expectorants and for their antispasmodic properties, in acute or chronic bronchitis, coughs, asthma and in general for respiratory infections. Also, they are used in cases of lack of appetite and dyspepsia. Externally, they have been used in ulcers and wounds (Blumenthal, 1998; Bradley, 1992; PDR, 2000). In particular, *M. vulgare* has been listed by the Council of Europe as a natural source of food flavouring.

Characteristic constituents of the genus are considered to be the diterpenes, including marrubiin as the main compound (Piozzi et al., 2006). These could be classified according to their structure to different types (furanolabdanes, prefuranoid labdanes which include hemiacetals/spiroethers, butenolide rings). In general, the labdane skeleton shown by marrubiin is a typical marker of the genus *Marrubium*. Previous investigations on *Marrubium velutinum* and *Marrubium cylleneum* revealed that these plants are a rich source of diterpenes, phenylethanoid glycosides and flavonoids with interesting biological activities (Karioti et al., 2003, 2005a,b). Immunomodulating effects of a series of diterpenes (Karioti et al., 2007a) and the possible synergistic inhibitory activity against mushroom tyrosinase of polar constituents (Karioti et al., 2007b) stimulated us to continue our research on this genus, with the phytochemical investigation of *Marrubium thessalum*.

*M. thessalum* Boiss. and Heldr. is endemic to central (Thessalia) and northern (Macedonia) Greece and possibly to southern Albania (Baden, 1991). In this paper, as a tribute to the chemotaxonomy of the genus we report on the isolation and identification of four new and four known labdane-type diterpenes from *M. thessalum*, as well as one flavone, ladanein. The present investigation represents the first chemical study on this species.

## 2. Results and discussion

From the dichloromethane extract of the aerial parts of *M. thessalum* four (**1**, 2, **4**, **5**) known and four (**3**, **6**–**8**) new diterpenes were isolated. Compounds **1**–**3** are the major metabolites of the plant, while the others were isolated in smaller amounts (Fig. 1).

Compounds **2**, **3** appeared on the TLC plate as a single spot. $^{1}$H and $^{13}$C NMR spectra showed several split signals, indicating the presence of a mixture (2.5:1) of two closely related compounds. Further 2D spectra confirmed this assumption. Separation of this mixture was achieved with the use of RP-HPLC and a new series of spectra were measured for the pure isolates. Based on spectroscopic evidence, compound **2** was assigned as the previously known prefuranic labdane diterpene, 13*R*-preperegrinine, while compound **3** as its C-13 epimer, which is a new naturally occurring compound. It should be mentioned that also premarrubiin was found to be a mixture of the two C-13 epimers (Piozzi et al., 2006). The HR-ESI-MS mass spectrum of compound **3** revealed a pseudomolecular peak at *m/z* 369.1662 [M + Na]$^+$, indicating a molecular weight corresponding to a molecular formula

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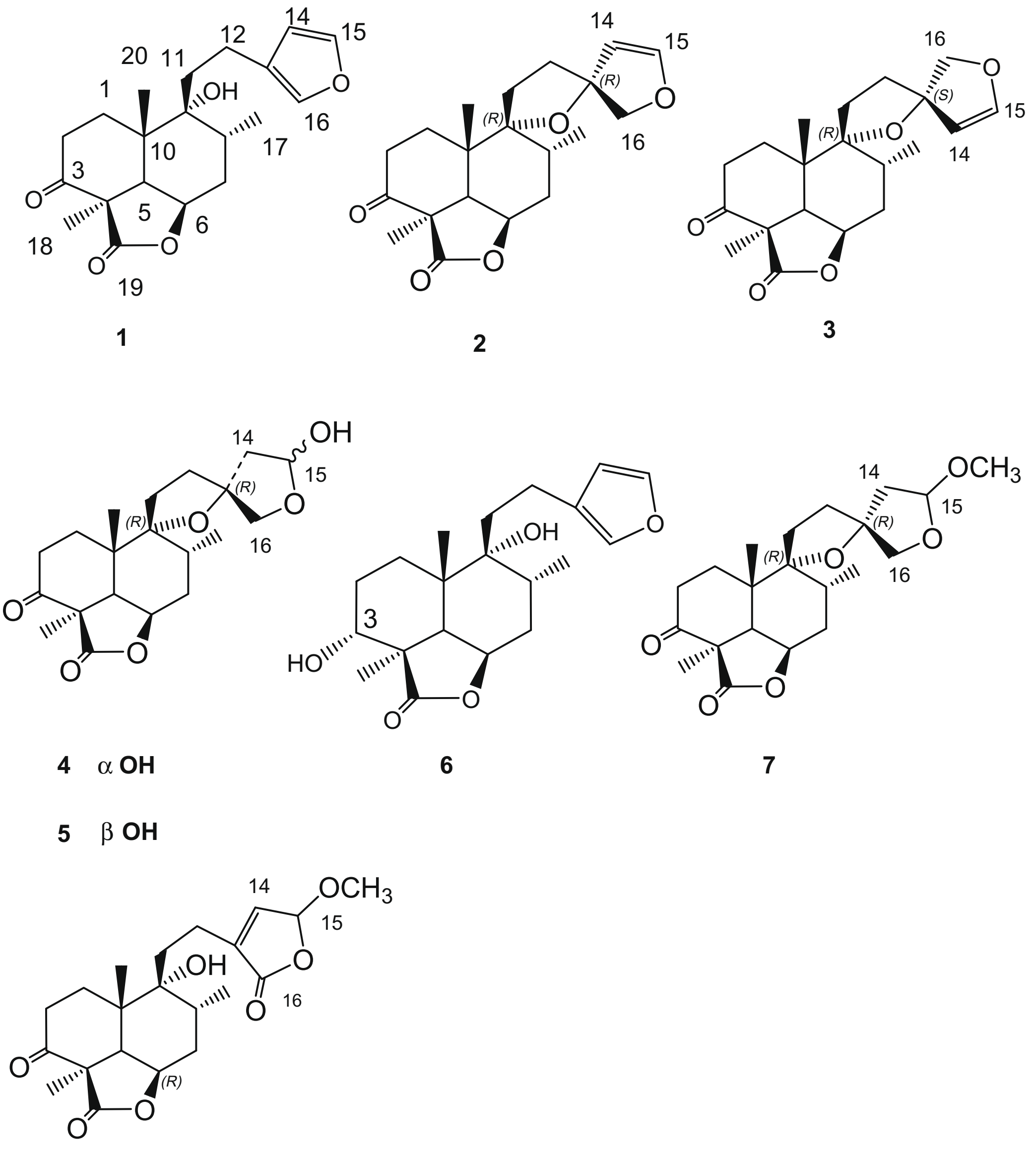


1 2 3

4 α OH 6 7

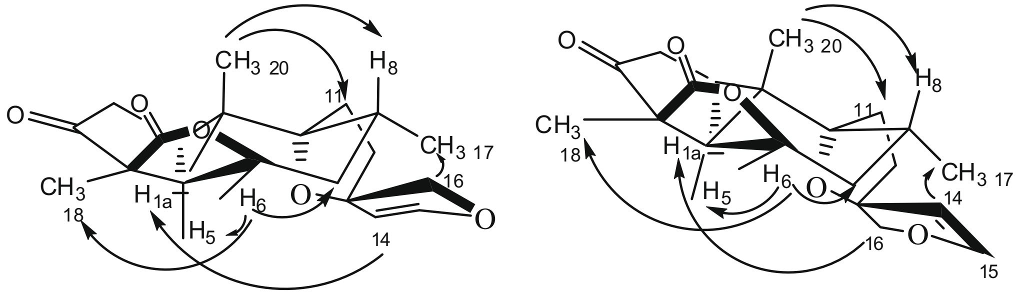
5 β OH

8

**Fig. 1.** Compounds isolated from *M. thessalum*.

$C_{20}H_{26}O_5$. Its IR spectrum contained absorption bands characteristic of γ-lactone (1780 $cm^{-1}$), keto (1715 $cm^{-1}$) and enol-ether (3100, 1615 $cm^{-1}$) groups. The $^1H$ NMR spectrum of **3** was almost identical to that of **2**, described by Savona et al. (1984). It showed signals of two tertiary methyl groups (at δ 1.44 *s* and 0.91 *s*), one secondary methyl group (at δ 0.94 *d*, $J = 6.2$ Hz), H-6 (at δ 4.58 *t*, $J = 5.1$ Hz) and H-5 (at δ 2.63 *d*, $J = 4.7$ Hz). Two protons formed an enol-ether system at δ 6.49 ($J = 2.4$ Hz) and at δ 5.10 ($J = 2.4$ Hz) and two other gave raise to an AB system at δ 4.39 ($J = 10.2$ Hz) and 4.04 ($J = 10.2$ Hz). Diagnostic NOE between H-14/H-17 (Fig. 2) and H-16a,b/H-1a proved that compound **3** has opposite stereochemistry at C-13 compared to **2** (H-16a/H-17 and H-14/H-1a), (Fig. 2) and thus is considered to be its 13*S* epimer. It must be noted that H-16b of compound **2** gave a NOE crosspeak at an area where overlapping protons (H-12a,b, H-11a and H-8) appeared, therefore it was not possible to clarify with which proton it had this peak. However, this signal also supports its 13*R* configuration. This data is in contrast to Savona et al. (1984), who supported that both H-16 give a NOE with H-17. The above NOE crosspeaks also explain a difference between the two compounds concerning the chemical shifts of H-1a ($\Delta\delta = -0.18$) and C-12 ($\Delta\delta = +0.90$) at compound **3**, which appear affected by the magnetic field of the double bond $\Delta^{14}$. Detailed $^1H$ and $^{13}C$ NMR data are reported for compounds **2** and **3** (Table 1).

Compound **6** exhibited a pseudomolecular peak at $m/z = 371.1823$ $[M + Na]^+$ in its mass spectrum, consistent with the molecular formula $C_{20}H_{28}O_5$. Its IR spectrum had absorption bands typical of hydroxyl (3550, 3410 $cm^{-1}$), γ-lactone (1782 $cm^{-1}$) and furan (872 $cm^{-1}$) groups. The $^{13}C$ NMR spectrum exhibited resonances of 20 carbons: two tertiary methyl groups (at δ 14.8 and 23.2), one secondary methyl group (at δ 16.3), five methylenes (at δ 28.3, 26.8, 31.4, 34.9, and 21.1) and seven methines (at δ 66.5, 46.3, 77.1, 32.3, 110.6, 143.1, and 138.6). Of the latter, two were oxygenated (at δ 66.5 and 77.1) and three were olefinic (at δ 110.6, 143.1 and 138.6). Finally it displayed five quaternary carbons (at δ 49.0, 75.8, 39.2, 124.8, and 184.2), one belonging to an ester-carbonyl group (at δ 184.2). The above data along with $^1H$ NMR spectrum indicated the presence of a furanic type diterpene



**2** **3**

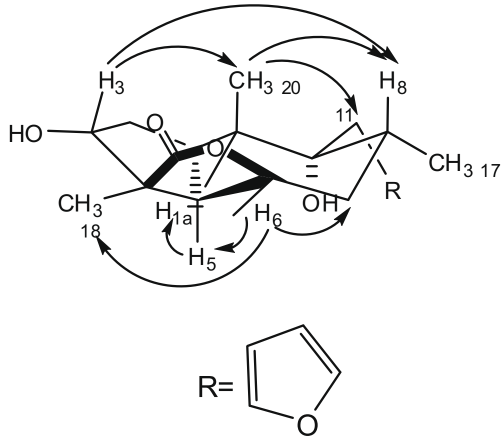
**Fig. 2.** Selected NOE correlations of compounds **2** and **3**.

**Table 1**
$^1$H NMR data of compounds **2**, **3**, **6**–**8** (400 MHz, CDCl$_3$, *J* in Hz).

| | **2** | **3** | **6** | **7** | **8** |
|---|---|---|---|---|---|
| H-1a | 2.11* | 1.93* | 1.83* | 1.95* | 2.18 *ddd* (*J* = 14.8, 12.1, 5.9) |
| H-1b | 1.65* | 1.64* | 1.41* | 1.64* | 1.60* |
| H-2a | 2.62–2.50* | 2.60–2.50* | 1.81* | 2.57–2.51* | 2.62–2.54* |
| H-2b | 2.62–2.50* | 2.60–2.50* | 1.55* | 2.57–2.51* | 2.62–2.54* |
| H-3 | – | – | 4.53 *dd* (J = 12.5, 4.3) | – | – |
| H-5 | 2.58 *d* (*J* = 4.3) | 2.63 *d* (*J* = 4.7) | 2.35 *d* (*J* = 4.7) | 2.61 *d* (*J* = 4.7) | 2.79 *d* (*J* = 4.3) |
| H-6 | 4.56 *t* (*J* = 5.1) | 4.58 *t* (*J* = 5.1) | 4.75 *dd* (*J* = 5.8, 5.5) | 4.57 *t* (*J* = 5.1) | 4.61 *t* (*J* = 5.1) |
| H-7a | 2.05* | 2.09* | 2.13* | 2.09* | 2.08* |
| H-7b | 1.74* | 1.74* | 1.69* | 1.70* | 1.76* |
| H-8 | 2.05* | 2.02* | 2.08* | 2.04* | 2.03* |
| H-11a | 2.07* | 2.04* | 1.87* | 2.04* | 1.81* |
| H-11b | 1.78* | 1.87* | 1.75* | 1.88* | 1.65* |
| H-12a | 2.15–2.00* | 2.21* | 2.51 *dd* (*J* = 9.0, 7.8) | 2.15* | 2.51–2.41* |
| H-12b | 2.15–2.00* | 1.96* | 2.51 *dd* (*J* = 9.0, 7.8) | 1.90* | 2.51–2.41* |
| H-14a | 5.13 *d* (*J* = 2.4) | 5.10 *d* (*J* = 2.4) | 6.25 brs | 2.18–2.13* | 6.78 brs |
| H-14b | – | – | – | 2.18–2.13 | – |
| H-15 | 6.48 *d* (*J* = 2.4) | 6.49 *d* (*J* = 2.4) | 7.34 brs | 4.97 *dd* (*J* = 5.9, 3.5) | 5.73 brs |
| H-16a | 4.44 *d* (*J* = 10.6) | 4.39 *d* (*J* = 10.2) | 7.21 brs | 3.91 *d* (*J* = 8.6) | – |
| H-16b | 4.09 *d* (*J* = 10.6) | 4.04 *d* (*J* = 10.2) | – | 3.60 *d* (*J* = 8.6) | – |
| H-17 | 0.88 *d* (*J* = 6.3) | 0.94 *d* (*J* = 6.2) | 0.94 *d* (*J* = 6.6) | 0.85 *d* (*J* = 6.2) | 0.96 *d* (*J* = 5.1) |
| H-18 | 1.44 *s* | 1.44 *s* | 1.28 *s* | 1.44 *s* | 1.46 *s* |
| H-20 | 0.89 *s* | 0.91 *s* | 1.01 *s* | 0.90 *s* | 0.89 *s* |
| –OCH$_3$ | – | – | – | 3.38 *s* | 3.58 *s* |

* Signal pattern unclear due to overlapping.

(Piozzi et al., 2006), bearing two oxygen groups, as suggested by the presence of two oxymethine protons at $\delta$ 4.53 (*dd J* = 12.5, 4.3 Hz, corresponding to an oxygenated carbon at $\delta$ 66.5) and at $\delta$ 4.75 (*dd J* = 5.5, 5.8 Hz, corresponding to an oxygenated carbon at $\delta$ 77.1). Key correlations in the $^1$H–$^1$H COSY spectrum, between the latter oxymethine and protons H-5, H-7a,b, allowed its unambiguous assignment as H-6. Therefore, the extra oxygen group was possibly situated at ring A. This assumption was further confirmed by an HMBC experiment, where common correlations of H-1a, H-2b and H-18 with its corresponding carbon at $\delta$ 66.5 were observed. Similarly, correlations in the COSY experiment of the oxymethine proton at $\delta$ 4.53 with protons H-2a,b proved that carbon C-3 is the one bearing a secondary hydroxyl function. This assignment also explained the deshielding of C-2, C-4 and H-5 and the shielding of C-18. NOESY experiment as well as chemical shifts and coupling constants of the non-overlapped protons established the relative stereochemistry of compound **6**. NOE crosspeaks between H-20/H-8 and H-5/H-1a on the one hand and the lack of NOE between H-5 and H-20 on the other, proved the *trans* fusion of rings A and B. Proton H-6 (*dd J* = 5.5, 5.8 Hz) had an equatorial position, as shown by its coupling constants and the $\gamma$-lactone ring is in a diaxial arrangement. NOE crosspeaks between H-5/H-6, H-6/H18, H-6/H-7b, H-5/H-18, H-5/H-1a, and H-7b/H-17 indicated that these are on the same side ($\alpha$). Observed NOEs between H-8/H-20, H-1b/H-20 proved they share common orientation ($\beta$). In such a planar system NOE crosspeaks between H-11/H-20 are expected in case of equatorial orientation of C-11. Moreover, NOESY experiment established the relative stereochemistry at ring A and proved the configuration of H-3. In particular, observed NOE between H-3/H-8, H-20 proved that H-3 has the same orientation with these protons ($\beta$) and hence the hydroxyl group is oriented in opposite direction. Moreover, coupling constants of H-3 (*dd J* = 12.5, 4.3 Hz) and NOE crosspeaks between H-3/H-2a and H-2b indicated that H-3 should have an axial position, H-2a an axial ($\alpha$) orientation, while H-2b an equatorial ($\beta$) position. The observed crosspeaks, especially the one between H-3/H-20, led us to the conclusion that ring A takes a twisted form rather than a chair form compared to ring B (Fig. 3). The above feature is totally in agreement with the available literature data (Iida et al., 1995). The relative stereochemistry of the hydroxyl group on C-3 justifies the large shielding effect on C-18 (same orientation). Likewise the introduction of the ketone-carbonyl at C-3 of peregrinine causes a shielding of C-18 compared to the assignments of the carbons of marrubiin (Hussein et al., 2003; Knöss and Zapp, 1998). A detailed search in the literature revealed the synthesis of **6** from Canonica et al. (1968), the authors however do not describe the



**6**

**Fig. 3.** Selected NOE correlations of compound **6**.

relative stereochemistry of the 3-hydroxyl group. A closer look at the critical signals of protons H-3 and H-6 in the $^{1}$H NMR data showed notable differences in chemical shifts that should be attributed to opposite stereochemistry: H-3 resonates at $\delta$ 3.69 (vs 4.53 in **6**) and H-6 at $\delta$ 4.92 (vs 4.75 in **6**). Therefore, to our opinion, the structure of compound **6** is deduced as 3α-hydroxymarrubiin and that synthetised by Canonica et al. (1968) as 3β-hydroxymarrubiin.

Compound **7** gave a pseudomolecular peak at *m*/*z* $[M + Na]^{+}$ 401.1929, consistent with the molecular formula $C_{21}H_{30}O_{6}$. Its IR spectrum revealed the absence of hydroxyl groups and the presence of γ-lactone (1775 $cm^{-1}$) and keto (1710 $cm^{-1}$) functionalities. The compound is characterised by the presence of a labdane skeleton with a 6, 19 lactone function, a carbonyl group at C-3 and an oxygen group at C-9. The introduction of the carbonyl group at position 3 causes a significant deshielding to protons H-2, H-5, H-18 and to carbon C-4. The $^{1}$H spin systems H-2/H-1a/H-1b and H-5/H-6/H-7/H-8/H-17 were assigned by COSY. Long $^{1}$H–$^{13}$C correlations (HMBC) between H-18/C-3, C-4, C-5, C-19 and H-20/C-9, C-10 established the planar structure on compound **7**. $^{1}$H NMR spectra gave resonances characteristic of a 13-14-15-16 tetrahydrofuran ring carrying a methoxy function. Particularly, the singlet at $\delta$ 3.38 should be attributed to the methoxy group, the AB system at $\delta$ 3.91 ($J$ = 8.6 Hz) and $\delta$ 3.60 ($J$ = 8.6 Hz) to protons H-16 and the oxymethine proton at $\delta$ 4.97 ($J$ = 5.9, 3.5 Hz) is characteristic of proton H-15. The position of the methoxy group was also proved by long $^{1}$H–$^{13}$C correlations (HMBC) between protons of methoxy group and C-15. COSY experiment revealed that protons H-14 are situated at $\delta$ 2.18 and 2.13. HMBC spectrum showed the presence of a second tetrahydrofuran ring and thus it was evident that protons H-11 and H-12 should be placed between two tertiary oxygenated carbons. The relative stereochemistry of compound **7** was established by ROESY experiment and is similar to the relative stereochemistry of the rest of the labdane diterpenes of *M. thessalum*. Due to lack of definitive ROEs, caused by poor amount of compound **7**, the C-13 and C-15 stereochemistry was assigned by comparison with literature data for compounds having an identical C-11/C-16 moiety (Rigano et al., 2006b). More precisely, comparing our findings with those of Rigano et al. (2006b) we observed that H-14a resonates at $\delta$ 2.22 (vs 2.18–2.13 in **7**), H-15 at $\delta$ 4.97 (vs 4.97 in **7**), H-16a at $\delta$ 3.92 (vs 3.91 in **7**), H-16b at $\delta$ 3.60 (vs 3.60 in **7**), $-OCH_{3}$ at $\delta$ 3.38 (vs 3.39 in **7**), H-17 at $\delta$ 0.81 (vs 0.85 in **7**). According to these data **7** was assigned as 9α,13*R*-15,16-bisepoxy-15β-methoxy-3-oxo-labdan-6β,19-olide.

**Table 2**
$^{13}$C NMR data of compounds **2**, **3**, **6**–**8** (50.3 MHz, $CDCl_{3}$, at 295 K).

| | **2** | **3** | **6** | **7*** | **8*** |
|---|---|---|---|---|---|
| 1 | 29.1 | 29.2 | 28.3 | 29.0 | 28.7 |
| 2 | 34.2 | 34.1 | 26.8 | 33.7 | 33.8 |
| 3 | 206.7 | 206.3 | 66.5 | 206.2 | 207.0 |
| 4 | 53.5 | 53.5 | 49.0 | 53.1 | 52.6 |
| 5 | 47.3 | 47.2 | 46.3 | 46.9 | 46.5 |
| 6 | 75.0 | 75.0 | 77.1 | 74.7 | 74.5 |
| 7 | 31.4 | 31.0 | 31.4 | 31.2 | 31.3 |
| 8 | 31.3 | 30.9 | 32.3 | 31.9 | 31.7 |
| 9 | 89.2 | 89.5 | 75.8 | 88.7 | 74.2 |
| 10 | 39.0 | 39.3 | 39.2 | 38.9 | 39.9 |
| 11 | 29.8 | 29.7 | 34.9 | 28.5 | 31.0 |
| 12 | 36.9 | 37.8 | 21.1 | 39.0 | 20.8 |
| 13 | 93.0 | 93.4 | 124.8 | n.o | 138.3 |
| 14 | 107.0 | 106.7 | 110.6 | 46.8 | 141.9 |
| 15 | 148.6 | 148.8 | 143.1 | 104.8 | 102.6 |
| 16 | 80.4 | 81.3 | 138.6 | 75.1 | 171.7 |
| 17 | 16.9 | 16.9 | 16.3 | 16.4 | 16.0 |
| 18 | 20.3 | 20.5 | 14.8 | 20.2 | 20.3 |
| 19 | 174.6 | 174.6 | 184.2 | 174.1 | 174.3 |
| 20 | 18.7 | 19.1 | 23.2 | 18.8 | 17.8 |
| 21 | – | – | – | 55.0 | 57.2 |

* $^{13}$C NMR chemical shifts were assigned on the basis of HSQC and HMBC experiments; n.o: not observed.

Compound **8** showed in its mass spectrum a pseudomolecular ion $[M + Na]^{+}$ at *m*/*z* 415.1718, compatible with a molecular formula $C_{21}H_{28}O_{7}$. Its IR spectrum showed absorptions due to hydroxyl (3600 $cm^{-1}$), γ-lactone (1780, 1760 $cm^{-1}$) and saturated keto (1710 $cm^{-1}$) functionalities. Detailed examination of the spectra showed that compound **8** bears a labdane skeleton, with a keto-group at C-3, a hydroxyl group on C-9, and a 19, 6 lactone function. Other functionalities that were apparent from the spectral data included a α,β-unsaturated γ-lactone group: two protons at $\delta$ 5.73 (br*s*, corresponding to an oxygenated carbon at $\delta$ 102.6) and at $\delta$ 6.78 (br*s*, corresponding to an olefinic carbon at $\delta$ 141.9) and two quaternary carbons at $\delta$ 138.3 and 171.7. Finally, a singlet at $\delta$ 3.58 (*s*, corresponding to an oxygenated carbon at $\delta$ 57.2), which integrated for three protons, was indicative of the presence of a methoxy function. Signals at $\delta$ 6.78 and 5.73 were assigned to protons H-15 and H-14, respectively, which showed COSY correlations to each other. The methoxy group was positioned at C-15, with which it had long $^{1}$H–$^{13}$C correlations (HMBC). The presence of a butenolide ring, in which the carbonyl group was at C-16, was therefore evident. Furthermore, HMBC correlations between methylene protons H-11 and olefinic carbon C-13, showed that the butenolide ring is linked to the rest of the labdane skeleton through H-11 and H-12 methylene groups. The relative stereochemistry of the chiral centers within compound **8** was proposed from the data obtained in the NOESY spectrum. NOE crosspeaks between H-5/H-1a, H-5/H-6, H-5/H-18, H-6/H-7b, H-6/H-18 and H-7b/H-17 showed that these are orientated in the same side (α), while interactions between H-8/H-20, H-11/H-20 indicated that these are on the opposite side (β). The trivial name 15-methoxy-velutine C was given to it in accordance with velutine C isolated by Karioti et al. (2005a).

On the basis of $^{1}$H and $^{13}$C NMR, UV, IR and MS data compounds **1**, **4**, **5**, **9** were identified as peregrinine (**1**) (Salei et al., 1966), 9α,13*R*-15,16-bisepoxy-15α-hydroxy-3-oxo-labdan-6β, 19-olide (**4**) (Khalil et al., 1996), 9α,13*R*-15,16-bisepoxy-15β-hydroxy-3-oxo-labdan-6β, 19-olide (**5**) (Khalil et al., 1996), ladanein (**9**) (Seshadri and Sharma, 1973). HSQC and HMBC experiments in peregrinine supported the reported data by Karioti et al. (2005a) that C-1 should be placed at $\delta$ 28.7 and C-11 at $\delta$ 34.6 instead of 34.6 and 28.7, respectively, assigned by other authors (Khalil et al., 1996; Savona et al., 1976).

## 3. Concluding remarks

Labdane diterpenes which possess a C-9 hydroxy group and a furan ring in the side chain are considered by some authors to be the final products in the biosynthetic pathway, while some others suggest that they are artifacts which arise from their corresponding prefuranoid labdanes during or after the extraction or isolation process, by cleavage of the 9–13 epoxide bridge (Henderson and McCrindle, 1969; Rodríguez and Savona, 1980; Tasdemir et al., 1995, 1997). However, according to our experience, the isolation scheme that was followed in our case was gentle and most importantly rapid. Especially the application of Sephadex LH-20 gel instead of silica gel permits faster separations and is considered to be a mild material compared to silica gel. Under the experimental conditions used it enables separation by both size exclusion and adsorption chromatography. Nevertheless, monitoring of the fractionations before and after the columns and the RP-HPLCs did not show any chemical alteration. Furthermore, the prefuranoid labdanes isolated were stable under the experimental conditions even when $CDCl_3$ was used as a solvent. Therefore, transformation of prefuranoids to furanoids should occur earlier in the extraction or even during the drying process. Preperegrinine is considered to be precursor of peregrinine. Many hemiacetals, like compounds **4** and **5**, have been isolated from *Marrubium* genus and are considered prefuranic compounds. Noteworthy, is the identification of compounds **7** and **8**, since to date only two more metabolites carrying a methoxy function have been reported for *Marrubium* genus (Karioti et al., 2007a). Similar compounds however have been isolated from *Leonurus sibiricus* (Boalino et al., 2004) and *Leonurus persicus* (Tasdemir et al., 1998) showing the close relation of the two genera. They could also be classified as prefuranic labdane diterpenes, which occur even before preperegrinine. However, compound **6** differs from all other diterpenoids of this species and its occurrence in the plant may be of interest from a biogenetic point of view. It bears a furan ring but the ketone-carbonyl at position 3 has been substituted by a hydroxyl. It could be suggested that it follows peregrinine in the biosynthetic pathway. This is the first time that this point of view is raised.

Despite structural modifications which occur in the C-10 to C-16 ring systems, the rest of the labdane skeleton appears to be repeatable among compounds **1**–**8**. All diterpenes isolated share a common feature: a C-3 oxygenation pattern which is similar to previous studies from *M. peregrinum* (Salei et al., 1966, 1970), *M. incanum* (Canonica et al., 1968), *M. friwaldskyanum* (Savona et al., 1984) and *M. velutinum* (Karioti et al., 2005a). It must be noted that *M. peregrinum* (Hennebelle et al., 2007), *M. velutinum* (Karioti et al., 2005a) and *M. incanum* (Canonica et al., 1968) also biosynthesize metabolites which do not possess oxygen group on C-3. However, at least in the last two cases, substances having an oxygenated C-3 surpass quantitatively. *M. vulgare* (Fulke et al., 1968; Henderson and McCrindle, 1969; Popa et al., 1968; Popa and Pasechnik, 1975), *M. alysson* (Savona et al., 1979), *M. sericeum* (Savona et al., 1979), *M. supinum* (Savona et al., 1979), *M. astracanicum* (Iida et al., 1995), *M. polydon* (Hatam et al., 1995), *M. anisodon* (Sagitdinova et al., 1996), *M. globosum* ssp. *globosum* (Takeda et al., 2000), *M. trachyticum* (Çitoğlu and Aksit, 2002), *M. cylleneum* (Karioti et al., 2005a, 2007a), *M. globosum* ssp. *libanoticum* (Rigano et al., 2006a,b), *M. ayardii* (Piozzi et al., 2006), *M. catariifolium* (Piozzi et al., 2006), *M. heterocladum* (Piozzi et al., 2006), *M. leonuroides* (Piozzi et al., 2006), *M. parviflorum* (Piozzi et al., 2006), *M. praecox* (Piozzi et al., 2006) and *M. propinquum* (Piozzi et al., 2006) instead, are characterised by the presence of marrubiin instead of peregrinine. Maybe this preference of each *Marrubium* sp. to a C-3 oxygenated or non-oxygenated pattern reflects infrageneric differentiation within this small genus. The occurrence of marrubiin and peregrinine in the genus could be of chemotaxonomic significance.

The occurrence of the 5-methoxylated flavone ladanein in the plant is also worth noticing, since it is considered to be important chemotaxonomic marker for the genus and for Lamiaceae as well (Harborne et al., 1975). It has been isolated from *M. trachyticum* (Çitoğlu and Aksit, 2002), *M. friwaldskyanum* (Savona et al., 1984), *M. peregrinum* (Hennebelle et al., 2007), *M. velutinum* (Karioti et al., 2005a) and *M. cylleneum* (Karioti, unpublished results).

## 4. Experimental

### 4.1. General experimental procedures

$^1H$, $^{13}C$ and 2D NMR spectra were recorded in $CDCl_3$ on Bruker DRX 400 and Bruker AC 200 (50.3 MHz for $^{13}C$ NMR) instruments at 295 K. Chemical shifts are given in ppm ($\delta$) and were referenced to the solvent signals at 7.24 and 77.0 ppm for $^1H$ and $^{13}C$ NMR, respectively. Mass Spectra were recorded on a Thermo LTQ Orbitrap (FT-MSn) (University of Florence, Italy). The $[\alpha]_D^{20}$ values were obtained in $CH_2Cl_2$ on Perkin–Elmer 341 Polarimeter. FT-IR spectra were recorded on a Perkin–Elmer PARAGON 500 spectrophotometer. Vacuum liquid chromatography (VLC): silica gel 60H (Merck, Art. 7736). HPLC: Jasco PU-2080 Plus, RID detector Shimadzu 10A, Column: Kromasil $C_{18}$ ($250 \times 10$ mm). Column chromatography (CC): silica gel 60 (SDS, 40–63 μm), gradient elution with the solvent mixtures indicated in each case; Sephadex LH-20 (Pharmacia). TLC: Merck silica gel 60 $F_{254}$ (Art. 5554). Detection: UV-light, spray reagent (vanillin-$H_2SO_4$ on silica gel).

### 4.2. Plant material

Aerial parts of *M. thessalum* Boiss. and Heldr. were collected from Olympus mountain (Thessalia, Central Greece; altitude ca. 650 m) in July 2006. A voucher specimen has been kept in the Herbarium of the Institute of Systematic Botany, Agricultural University of Athens (ACA), under the number Skaltsa and Argyropoulou 02.

### 4.3. Extraction and isolation

The air-dried powdered aerial parts of the plant (1.06 kg) were successively extracted at room temperature with petroleum ether, dichloromethane, MeOH and MeOH:$H_2O$ 5:1 (2 l of each solvent, twice, 48 h). The dried dichloromethane extract (32.6 g) was subjected to VLC over silica gel ($10 \times 6$ cm) with cyclohexane:EtOAc:MeOH:$H_2O$ mixtures of increasing polarity to yield 15 fractions (A–O) of 500 ml as follows: cyclohexane:EtOAc 95:5–30:70 (A–K); EtOAc 100% (L); EtOAc:MeOH 50/50 (M); MeOH 100% (N); MeOH/$H_2O$ 50:50 (O). Fractions I and J (481.2 mg; eluted with cyclohexane:EtOAc 60:40–50:50) were combined and further applied to Sephadex LH-20 ($CH_2Cl_2$:MeOH 20:80) to yield five fractions ($I'_1 - I'_5$). Purification of fraction $I'_4$ on Sephadex LH-20 ($CH_2Cl_2$:MeOH 20:80) and on RP-HPLC (ACN:$H_2O$ 45:55) finally yielded compounds **4**/**5** (12.6 mg; $R_t$ 9.3 min), **7** (1.3 mg; $R_t$ 32.4 min), **3** (4.2 mg; $R_t$ 43.9 min) and **2** (4.3 mg; $R_t$ 45.7 min). Fraction L (1.2 g; eluted with EtOAc 100%) was subjected to CC over Sephadex LH-20 ($CH_2Cl_2$:MeOH 20:80) and yielded 11 fractions ($L_1$–$L_{11}$). Fraction $L_6$ was applied to CC over silica gel with cyclohexane:EtOAc and yielded 17 fractions ($L_{6a}$–$L_{6q}$). Fractions $L_{6e}$ (eluted with cyclohexane:EtOAc 75:25–70:30) and $L_{6h}$ (eluted with cyclohexane:EtOAc 70:30) were identified as compounds **1** (46.1 mg) and **6** (11.9 mg), respectively. Fraction $L_{6l}$ (eluted with cyclohexane:EtOAc 50:50–30:70) was further purified by RP-HPLC (ACN:$H_2O$ 45:55) and afforded compound **8** (1.2 mg; $R_t$ 16.6 min).

Fraction M (1.5 g; eluted with EtOAc:MeOH 50:50) was purified over Sephadex LH-20 (cyclohexane:$CH_2Cl_2$:MeOH 7:4:0.5) and afforded ladanein [=7,4′-scutellarein-dimethylether] (1.7 mg).

### *4.3.1. 13S-preperegrinine (**3**)*

Colourless oil (4.2 mg); $[\alpha]_D^{20}$ + 19.00° ($CH_2Cl_2$, *c* 0.10). HR-ESI-MS *m*/*z* 347.1845 $[M+H]^+$ (calc. for $C_{20}H_{27}O_5$ 347.1851), 369.1662 $[M+Na]^+$, (calc. for $C_{20}H_{26}O_5Na$ 369.1678, Δmmu: −0.82); $^1H$ and $^{13}C$ NMR spectral data (see Tables 1 and 2).

### *4.3.2. 3α-hydroxymarrubiin (**6**)*

Colourless oil (11.9 mg); $[\alpha]_D^{20}$ + 20.00° ($CH_2Cl_2$, *c* 0.11). HR-ESI-MS *m*/*z* 371.1823 $[M+Na]^+$, (calc. for $C_{20}H_{28}O_5Na$ 371.1835, Δmmu: −0.62); $^1H$ and $^{13}C$ NMR spectral data (see Tables 1 and 2).

### *4.3.3. 9α,13R-15,16-bisepoxy-15β-methoxy-3-oxo-labdan-6β,19-olide (**7**)*

Colourless oil (1.3 mg); $[\alpha]_D^{22}$ − 40.00° ($CH_3OH$, *c* 0.03). HR-ESI-MS *m*/*z* 379.2112 $[M+H]^+$, 401.1929 $[M+Na]^+$, (calc. for $C_{21}H_{30}O_6Na$ 401.1941, Δmmu: −0.59); $^1H$ and $^{13}C$ NMR spectral data (see Tables 1 and 2).

### *4.3.4. 15-methoxyvelutine C (**8**)*

Colourless oil (1.2 mg); $[\alpha]_D^{20}$ + 13.33° ($CH_2Cl_2$, *c* 0.06). HR-ESI-MS *m*/*z* 393.1900 $[M+H]^+$, (calc. for $C_{21}H_{29}O_7$ 393.1937, Δmmu: −0.81); *m*/*z* 415.1718 $[M+Na]^+$, (calc. for $C_{21}H_{28}O_7Na$ 415.17334, Δmmu: −0.96); $^1H$ and $^{13}C$ NMR spectral data (see Tables 1 and 2).

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2009.03.011.

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