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Polar Constituents from the Aerial Parts of *Origanum vulgare* L. Ssp. *hirtum* Growing Wild in Greece

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From the polar extracts of *Origanum vulgare* L. ssp. *hirtum* 19 compounds have been isolated. The structures and relative stereochemistry have been elucidated by spectroscopic analysis and determined as apigenin, luteolin, chrysoeriol, diosmetin, quercetin, eriodictyol, cosmoside, vicenin-2, caffeic acid, *p*-menth-3-ene-1,2-diol 1-*O*-β-glucopyranoside, thymoquinol 2-*O*-β-glucopyranoside, thymoquinol 5-*O*-β-glucopyranoside, thymoquinol 2,5-*O*-β-diglucopyranoside, 12-hydroxyjasmonic acid, 12-hydroxyjasmonic acid 12-*O*-β-glucopyranoside, lithospermic acid B, rosmarinic acid, 10-*epi*-lithospermic acid, and *epi*-lithospermic acid B. The three latter products display unusual stereochemistry of the 3,4-hydroxyphenyllactic acid unit(s), which to the authors' best knowledge has never been reported before in similar compounds. Moreover, lithospermic acid B (and its stereoisomers), *p*-menth-3-ene-1,2-diol 1-*O*-β-glucopyranoside, 12-hydroxyjasmonic acid, and 12-hydroxyjasmonic acid 12-*O*-β-glucopyranoside were isolated for the first time from *Origanum* species.

KEYWORDS: ***Origanum vulgare* L. ssp. *hirtum*; Lamiaceae; Nepetoideae; phenolic acids; monoterpene glucosides; phenolic glucosides; hydroxyjasmonic acid**

INTRODUCTION

Origanum vulgare L. ssp. *hirtum* Ietswaart (Lamiaceae, subfamily Nepetoideae) is an annual herb widely distributed in Eurasia and North Africa. Introduced by humans, this species has also been encountered in North America (*1*). It is commonly used throughout the Mediterranean as a spice under the name "oregano", and from the morphological point of view can be distinguished by its small green bracts and white flowers (*2*). Most studies have been focused on its essential oil. Quantitative and qualitative essential oil analyses show that the major constituents are carvacrol and/or thymol, accompanied by *p*-cymene and γ-terpinene (*3*).

As there are no data concerning the nonvolatile secondary metabolites of Greek *O. vulgare* L. ssp. *hirtum*, in this study we dealt with the isolation and structural elucidation of its constituents. In particular, our interest was focused on the isolation of the polar constituents (**Chart 1**), because our previous study revealed that the polar extracts of the plant have promising potential for preventing diabetes complications (*4*). From the methanolic extract eight flavonoids were isolated, namely, apigenin (**1**), luteolin (**2**), chrysoeriol (**3**), diosmetin (**4**), eriodictyol (**5**), quercetin (**6**), cosmoside (**7**), and vicenin-2 (**8**), together with two phenolic compounds, caffeic acid (**9**) and rosmarinic acid (**10**), and two phenolic glucosides, thymoquinol 2-*O*-β-glucopyranoside (**15**) and thymoquinol 5-*O*-β-glucopyranoside (**16**). From the aqueous methanol extract (MeOH/H_2O 5:1) one phenolic glucoside, thymoquinol 2,5-*O*-β-diglucopyranoside (**17**), one monoterpene glucoside, *p*-menth-3-ene-1,2-diol 1-*O*-β-glucopyranoside (**14**), and two hydroxyjasmonic derivatives, 12-hydroxyjasmonic acid (**18**) and 12-hydroxyjasmonic acid 12-*O*-β-glucoside (**19**), were isolated, together with one known depside lithospermic acid B (**12**) and two new depsides, 10-*epi*-lithospermic acid (**11**) and *epi*-lithospermic acid B (**13**) (the latter could not be purified above 75%, being contaminated primarily by **11**; however, this was considered to be sufficient for the spectroscopic investigation described below).

Finding lithospermic acid analogues in this plant extract raised our interest, owing to the potent anti-HIV activity recently reported for these products. In fact, they are being targeted for the development of so-called functional foods, that is, conventional foods with added health benefits (*5*).

Our spectral data on compounds **10**, **11**, and **13** initially appeared to be fully compatible with well-known carbon skeletons. After a closer look, the NMR shifts of **11** and **13** did

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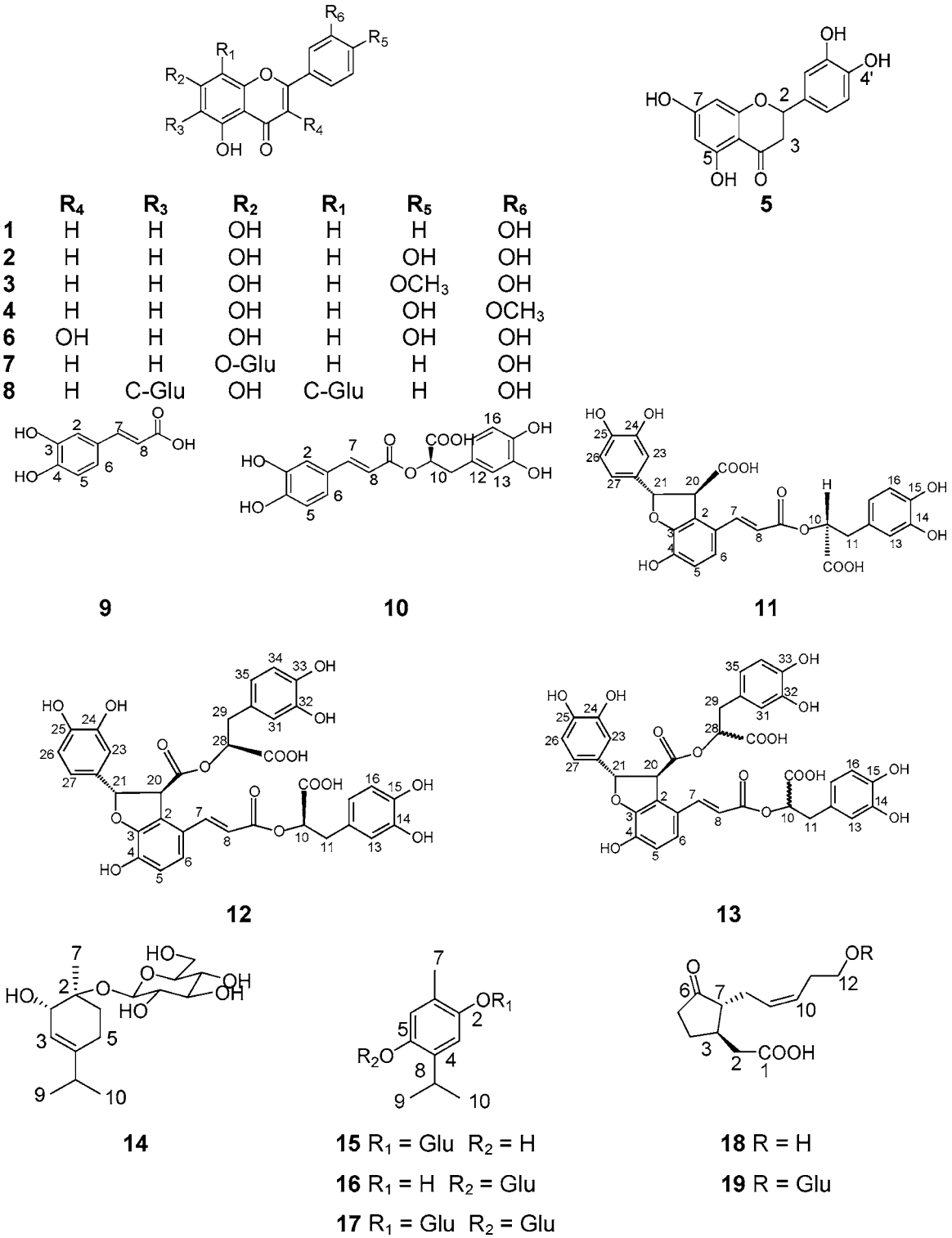
Chart 1. Structures of Polar Constituents Isolated from *O. vulgare* L. Ssp. *hirtum*



not exactly match those reported in the literature for lithospermic acid and lithospermic acid B, which demonstrated that we dealt indeed with diastereomers of the lithospermic acid motif. Moreover, a careful quantitative analysis of the circular dichroism (CD) spectrum of rosmarinic acid (**10**) revealed that this compound is recovered as a scalemic mixture. These findings demonstrate that this plant produces some polyphenolic compounds, incorporating (*S*)-dihydroxyphenyllactic acid, in contrast with the common (*R*)-motif. We believe that this is especially relevant for at least two reasons: (1) it may uncover a new biosynthetic pathway for this moiety; (2) these products may display interesting biological activity, which need to be further investigated.

MATERIALS AND METHODS

General Procedures. NMR spectra were recorded in CD_3OD on Bruker AC 200 (50.3 MHz for ^{13}C NMR), Bruker DRX 400 (399.95 MHz for ^{1}H NMR and 2D-NMR), and Bruker AC 500 instruments (500.1 MHz for ^{1}H NMR and 2D-NMR and 125.7 MHz for ^{13}C NMR) and on a Varian Inova 600 (599.684 MHz for ^{1}H NMR and 2D-NMR and 150.805 MHz for ^{13}C NMR). Chemical shifts are reported in δ (parts per million) values. IR spectra were recorded in a Perkin-Elmer

Table 1. ^{1}H NMR (CD_3OD, 600 MHz, *J* in Hertz) and ^{13}C NMR (CD_3OD, 150 MHz) Data of Compound **11** in Neutral and Low pH and of Compound **13**

	neutral pH		low pH		lithospermic acid[a]		compound **13**	
position	δ_H	δ_C	δ_H	δ_C	δ_H[c]	δ_C[c]	δ_H	δ_C[b]
11b	2.94 dd (J = 10.5, 13.8)	38.8	2.94	38.4	3.02	37.6	2.90	ND[c]
11a	3.01 dd (J = 2.4, 13.8)		3.03		3.11		3.04	
20	4.29 d (J = 5.6)	61.0	4.30	60.1	4.40	57.2	4.26 d (J = 4.0)	58.5
10	4.88 dd (J = 2.4, 10.5)	78.0	4.91	78.0	5.22	74.7	5.01[d]	77.7
21	5.88 d (J = 5.4)	89.9	5.87	89.9	5.95	88.6	5.99 d (J = 4.0)	87.9
8	6.28 d (J = 16.1)	116.2	6.30	116.3	6.36	116.4	6.11 (J = 15.8)	117.0
17	6.60 dd (J = 2.0, 8.1)	119.9	6.59	119.9	6.65	118.3	6.58 d (J = 8.4)	120.5
16	6.65 d (J = 8.0)	115.7	6.65	116.1	6.72	116.2	6.62 d (J = 8.4)	115.8
26	6.75 dd (J = 1.6, 8.4)	115.5	6.75	116.1	6.75	116.3	6.74 s	118.0
5	6.75 d (J = 8.0)	118.1	6.75	118.2	6.83	118.2	6.72	115.9
27	6.76 d (J = 8.0)		6.75		6.80	121.9	6.74 s	117.1
23	6.85 d (J = 2.0)	113.1	6.84	113.5	6.84	117.4	6.82 s	121.6
13	7.09 d (J = 2.0)	118.0	7.00	117.7	6.76	113.3	7.08 s	118.3
6	7.13 d (J = 8.6)	120.0	7.15	120.3	7.20	120.9	7.10	120.6
7	7.96 d (J = 16.1)	143.1	7.90	143.0	7.84	143.0	7.22 d (J = 15.8)	141.8
28							5.00[d]	78.6
29a							3.02 m	ND
29b							2.69 d (J = 13.7)	
31							6.40 s	116.9
34							6.44 d (J = 8.7)	116.2
35							6.22 d (J = 8.7)	120.9

[a] ^{1}H NMR (CD_3OD, 500 MHz) and ^{13}C NMR (CD_3OD, 125 MHz) data of lithospermic acid (14) are reported for comparison. [b] Signals assigned through HMQC experiment. [c] Not determined. [d] Signal pattern unclear due to overlapping.

Paragon model 500 (FT-IR) spectrometer. ESI mass spectra were provided by the University of Notre Dame, Department of Chemistry and Biochemistry, Notre Dame, IN. EI mass spectral data were recorded on an HP 1100 MSD API electrospray (using Na as reagent). UV spectra were recorded on a Shimadzu UV-160A spectrophotometer, according to the method of Mabry et al. (*6*). The optical values were determined at 25 °C in MeOH on a Perkin-Elmer 341 polarimeter. CD spectra were recorded with a Jasco J715 spectropolarimeter, using a 0.01 cm cell, on two solutions of about 1 and 4 mM. The HPLC system consisted of an HP 1100L instrument with a diode array detector and managed by an HP 9000 workstation. The HPLC system was interfaced with an HP 1100 MSD API electrospray. Conditions were as follows: vacuum liquid chromatography (VLC), silica gel 60H (Merck, art. 7736); MPLC, Büchi 668, RP-silica gel 60 (Merck, art. 10167); HPLC, Sykam S1021 solvent delivery system, UV−vis detector S3200, column, Kromasil C18; column chromatography (CC), silica gel 60 (Merck, art. 9385), gradient elution with the solvents mixtures indicated in each case; Sephadex LH-20 (Pharmacia) TLC, Merck silica gel 60 F254 (art. 5554); Merck cellulose (art. 5552); detection, UV light, spray reagent [vanillin−H_2SO_4 on silica gel; Neu 's reagent on cellulose (*7*)].

Plant Material. Aerial parts of *O. vulgare* ssp. *hirtum* were collected from Pogoni-Ioannina (Epirus, northwestern Greece) in July 2002 and authenticated by Dr. Th. Constantinidis (Institute of Systematic Botany, Agricultural University of Athens). A voucher specimen is deposited in the Herbarium of the Institute of Systematic Botany, Agricultural University of Athens (ACA), under the code Lazari 1.

Extraction, Isolation, and Identification of the Polar Constituents of *O. vulgare* L. Ssp. *hirtum*. The air-dried powdered aerial parts of *O. vulgare* L. ssp. *hirtum* (0.27 kg) were successively extracted at room temperature with petroleum ether, CH_2Cl_2, MeOH, and MeOH/H_2O 5:1. The dried MeOH extract (34.1 g) was subjected to VLC over silica gel (10 × 8 cm) with CH_2Cl_2/MeOH/H_2O mixtures of increasing polarity to yield 16 fractions (A−P). Further purification of fraction H (0.91 g; eluted with CH_2Cl_2/MeOH/H_2O 85:15:1.5) by CC over silica gel with mixtures of CH_2Cl_2/MeOH afforded 14 fractions (HA−HN). Fractions HF and HG were further applied to CC on silica gel using mixtures of CH_2Cl_2/MeOH/H_2O and yielded compounds **5** (3.2 mg), **4** (5.9 mg), and **6** (2.6 mg), respectively. Fraction HGF (9.3 mg; eluted with CH_2Cl_2/MeOH:H_2O 96:4:0.4−90:10:1) was purified by TLC on silica gel (CH_2Cl_2/MeOH/H_2O 90:10:1) to give compound **2** (1.0 mg). Fraction J (1.99 g; eluted with CH_2Cl_2/MeOH/H_2O 75:25:2.5) was fractionated by CC on silica gel with mixtures of CH_2Cl_2/MeOH/H_2O and afforded compounds **8** (5.2 mg) and **9** (46.5 mg). Further purification of fraction JG (610.0 mg; eluted with CH_2Cl_2/MeOH/H_2O 87:13:1.3) by CC and over silica gel followed by RP-18 HPLC (MeOH/H_2O 20:80) allowed the isolation of compounds **15** (6.9 mg; t_R 8.8 min) and **16** (13.5 mg; t_R 11.0 min). Fraction K (1.5 g; eluted with CH_2Cl_2/MeOH/H_2O 70:30:3) was subjected to successive CC on silica gel with mixtures CH_2Cl_2/MeOH/H_2O to afford compound **3** (3.7 mg). Fraction N (2.41 g; eluted with CH_2Cl_2/MeOH/H_2O 30:70:7) was fractionated by CC on silica gel and afforded compound **10** (13.8 mg). Fraction NL (1.2 g; eluted with MeOH 100%) was subjected to successive CC over Sephadex LH-20 (MeOH) and silica gel to yield compound **1** (2.0 mg). The MeOH/H_2O (5:1) (17.5 g) extract was subjected to RP-MPLC (RP-18 silica gel) with H_2O/MeOH mixtures of decreasing polarity to yield 17 fractions (A−Q) of 300 mL. Fraction B (1.6 g; eluted with H_2O 100%) was further applied to RP-MPLC (RP-18 silica gel) with H_2O/MeOH mixtures of decreasing polarity to yield 16 fractions (A′−P′). Fractions B′ and C′ were identified as compound **19** (89.6 mg) and compound **18** 12-hydroxyjasmonic acid (1.5 mg), respectively. Purification of fraction BI (34.8 mg; eluted with H_2O/MeOH 99:1) by RP-18 HPLC (MeOH/H_2O 20:80) allowed the isolation of compound **17** (3.7 mg; t_R 8.2 min). Fraction D (0.5 g; eluted with H_2O 100%) was applied to Sephadex LH-20 (MeOH) and yielded a mixture (97.2 mg), which was further separated by RP-18 HPLC (MeOH/H_2O 20:80) and afforded compound **11** (10.2 mg) and compound **13** (3.2 mg). Fraction E (0.28 g; eluted with H_2O/MeOH 95:15) was applied to CC on silica gel with mixtures of CH_2Cl_2/MeOH/H_2O (50:50:5) and yielded compound **12** (31.4 mg) and 12-hydroxyjasmonic acid (**18**) (1.6 mg). Purification of fraction G (148.5 mg; eluted with H_2O/MeOH 90:10) by RP-18 HPLC (MeOH/aq AcOH 2% 30:70) yielded compound **3** (10.9 mg; t_R 11.6 min). Purification of fraction I (99.5 mg; eluted with H_2O/MeOH 85:15−80:20) by RP-18 HPLC (CH_3CN/H_2O 20:80) allowed the isolation of compound **14** (5.6 mg; t_R 14.9 min).

10-*epi*-Lithospermic acid (11) ($C_{27}H_{22}O_{12}$): $[a]_D^{20}$ +99.4 (MeOH, *c* 0.06); UV λ_{max}/nm (approximately ϵ_{max}), in CH_3OH, 314 (14000), 290 (16000), 253 (17000), 230 (sh), 201 (86000); CD λ_{max}/nm (approximately $\Delta\epsilon_{max}$), in CH_3OH, 333 (+3.0), 307 (+3.3), 283 (−1.4), 252 (+8.3), 232 (+8.7), 205 (−19.7); ^{1}H and ^{13}C NMR data, see **Table 1**.

***epi*-Lithospermic acid B (13) ($C_{39}H_{39}O_{16}$):** UV λ_{max}/nm (approximately ϵ_{max}), in CH_3OH, 321 (12000), 286 (13000), 254 (13000), 228 (sh), 200 (81000); CD λ_{max}/nm (approximately $\Delta\epsilon_{max}$), in CH_3OH,

333 (+2.5), 299 (+2.6), 281 (−1.0), 251 (+8.3), 228 (+8.2), 205 (−20.6); ^{1}H and ^{13}C NMR data, see **Table 1**.

Rosmarinic acid (10) ($C_{20}H_{22}O_8$): UV λ_{max}/nm (approximately ϵ_{max}), in CH_3OH, 330 (15000), 291 (12000), 249 (sh), 233 (sh), 219 (17000); CD λ_{max}/nm (approximately $\Delta\epsilon_{max}$), in CH_3OH, 329 (+0.9), 297 (+1.1), 280 (−1.1), 234 (−1.8).

RESULTS AND DISCUSSION

Phenolic acids **9** and **10** are common constituents of the Lamiaceae family (*8*). Caffeic acid (**9**) plays a central role in the biochemistry of this family; in *Salvia* species it is the building block of a variety of plant metabolites from simpler monomers to multiple condensation products giving rise to a variety of oligomers (*9*). Rosmarinic acid (**10**) is an excellent chemotaxonomic marker, present in the subfamily Nepetoideae and absent in the subfamily Lamioideae in Erdtmann's (1945) two-subfamily system (*10*). Its isolation from *O. vulgare* L. has been previously reported (*11*−*14*).

By comparing the absolute value of the CD spectrum of compound **10** with the literature data, (*15*) we determined that we deal with a scalemic mixture, with an enantiomeric ratio of about 70:30 in favor of (*R*)-(+)-rosmarinic acid. One might challenge this finding claiming that (partial) racemization may have occurred before analysis. This point will be discussed below.

The oligomers of caffeic acid **11** and **13** clearly display ^{1}H and ^{13}C NMR strictly compatible with the connectivity of the known products lithospermic acid and lithospermic acid B (**12**). For both compounds, the vicinal *J* couplings demonstrate that stereochemistry of the double bond is *E* and that the two substituents on the dihydrobenzofuran ring must be *trans*, that is, that the ring is either 20*S*, 21*S* or 20*R*, 21*R*. Of course, at this stage we cannot determine the absolute stereochemistry of this moiety. Unfortunately, NMR is unable to provide direct information on the relative configuration at C-10, which is too remote from the other stereogenic elements of the molecule. Recently, lithospermic acid has been the object of total synthesis, and its NMR spectrum is thus certainly and accurately known (*16*). By comparison of the ^{1}H and ^{13}C data in our hands, it turns out that there are several differences, as shown **Table 1**.

The observed resonance shifts, especially for H-10 and C-10, are well beyond any reasonable experimental error and demonstrate that the overall molecular structure of the two compounds must be different, thus indicating that we deal with two diastereomers. Because lithospermic acid is 10*R*, 20*S*, 21*S* and because we have already determined the *trans* substitution in the dihydrobenzofuran ring, our compound (**11**) is either 10*S*, 20*S*, 21*S* or 10*R*, 20*R*, 21*R*.

The final configurational assignment can be performed through CD spectroscopy. **Figure 1** shows the absorption and CD spectra of **11** in methanol. The spectra are dominated by the $\pi-\pi^*$ transitions of the aromatic chromophores, namely, caffeic acid and 1,2-dihydroxybenzene (two moieties). In the presence of several electric dipole allowed transitions, the CD is largely determined by the exciton or coupled dipole mechanism (*17*). Looking into the structure of **11**, we inferred that the molecular portion comprising caffeic acid and the closest 1,2-dihydroxybenzene, attached to the furanyl ring, would be chiefly responsible of the observed chiro-optical properties because of the vicinity and relative rigid arrangement between the two chromophores.

In fact, the strength of the exciton coupling is inversely proportional to the square of the interchromophoric distance. Moreover, the remaining dihydroxybenzene moieties are connected to this molecular portion through flexible chains; therefore, they will assume a large manifold of reciprocal arrangements, with positive and negative chiralities, averaging the relative exciton couplings to almost negligible values. This

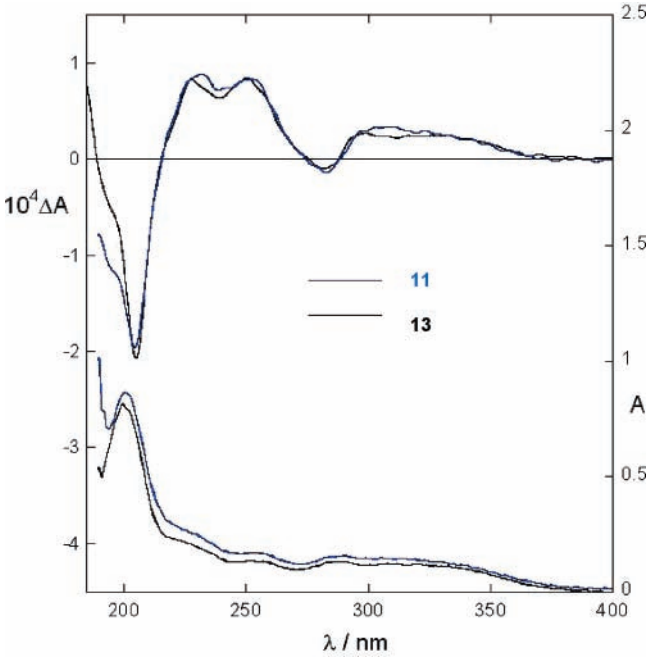


Figure 1. CD spectra of compounds **11** (blue line) and **13** (black line).

is especially true in methanol, which disrupts the framework of intramolecular hydrogen bonds, as confirmed by means of molecular mechanics conformational searches and NMR experiments in CD_3OD. The mobility of the side chains containing the caffeic acid units can be probed through a ROESY experiment. If the molecule is stiff in a well-defined conformation, possibly held together by hydrogen bonds, long-range Overhauser effects should be expected. On the contrary, the ROESY spectrum of **11** in methanol-d_4 reveals only short-range connectivity, which may be taken as a proof of the molecular flexibility.

As a conclusion, CD spectroscopy can (only) effectively probe the stereochemistry of the benzofuran moiety, and it is largely insensitive to the configuration of C10. This is demonstrated by the close similarity of the CD spectrum of **11** (**Figure 1**) and of the dimethyl ester of prolithospermic acid, a methanolysis product of lithospermic acid, reported in ref *18* in the 230−350 nm region. Thus, the configuration of the rigid core of **11** is 20*S*, 21*S*, and therefore the complete product is 10*S*, 20*S*, 21*S*.

The rigid core of **13** originates a CD spectrum (**Figure 1**) closely similar to the one described above for **11**, and we must conclude that also in this case we deal with a 20*S*, 21*S* configuration. Similarly to what was described above for **11**, we detect significantly shifted resonances with respect to literature data (**Table 1**), remarkably in the case of C10 and C28, resonating at about 77.7 and 78.6 ppm, against a value of about 74−75 ppm commonly reported. Unfortunately, in this case a safe configurational assignment is impossible, because we have two stereocenters at C10 and C28.

It is noteworthy that for **11** we could not detect any significant amount of the previously reported 10*R*, 20*S*, 21*S* diastereomer. This sheds light on the point raised above concerning a possible partial racemization of rosmarinic acid: if the C^α of the phenyllactic moiety would invert during extraction/isolation, it would produce epimeric mixtures at C10 in **11**, which we do not observe at all. This leads us to exclude the possibility that our results are the outcome of a stereochemical shuffling occurring during the isolation of **10** and **11**. The recovery of analogues of lithospermic acid, which moreover incorporate the less common (*S*)-3,4-hydroxyphenyllactic motif, is noteworthy.

Finally, it is worth observing that the presence of the lithospermic acid B motif in this plant is an interesting finding

in itself, as this tetramer of caffeic acid is the most common component in *Salvia* species (*19*, *20*), but, to the best of our knowledge, has not yet been reported in *Origanum*.

Among flavonoids, apigenin and its 7-*O*-*β*-glucoside (cosmoside), luteolin, and diosmetin have been found before in *O. vulgare* L. (*21*−*23*). Apigenin and luteolin were also isolated from *O. dictamnus* L. (*24*), *O. majorana* L. (*25*), and *O. majoricum* Camb. (*26*), whereas eriodictyol and cosmoside have been isolated from *O. dictamnus* (*24*). Vicenin-2 was found to be present in all species of section Majorana of the genus *Origanum* (*27*).

Phenolic glucosides **15**−**17** were previously isolated from *O. syriacum* L. (*28*); however, this is the first report in *O. vulgare*. Monoterpene glucoside **14**, recently found in thyme (*29*), is described for the first time in *O. vulgare*. As far as we know, there are no references in the literature concerning the isolation of hydroxyjasmonic acid (**18**) and its 12-*O*-*β*-glucoside (**19**) from *Origanum*. Hydroxyjasmonic acid derivatives have been noted for their defense responses in higher plants (*30*). This could explain the fact that compound **19** is one of the most abundant secondary metabolites of the plant.

Therefore, the polar extract of *O. vulgare* L. ssp. *hirtum* reveals the presence of several compounds that have not been previously described in closely similar species.

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