DOI:10.1021/jf904596m

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Depsides and Other Polar Constituents from *Origanum dictamnus* L. and Their in Vitro Antimicrobial Activity in Clinical Strains

Aliki Chatzopoulou,[†] Anastasia Karioti,[†,‡] Chrysoula Gousiadou,[†] Vanesa Lax Vivancos,[†] Panagiotis Kyriazopoulos,[§] Stamatina Golegou,[§] and Helen Skaltsa*,[†]

[†]Department of Pharmacognosy and Chemistry of Natural Products, School of Pharmacy, University of Athens, Panepistimiopolis, Zografou, 157 71 Athens, Greece, [‡]Department of Pharmaceutical Sciences, University of Florence, via Ugo Schiff 6, Polo Scientifico, Sesto Fiorentino, 50019 Florence, Italy, and [§]Microbiological Department, Patision General Hospital, Chalkidos 15, 11 143 Athens, Greece

Phytochemical investigation of the polar extracts of the aerial parts of *Origanum dictamnus* afforded 15 secondary metabolites. One new depside was isolated, to which the trivial name salvianolic acid P (**1**) was given, in addition to the known depsides rosmarinic acid (**2**) and rosmarinic acid methyl ester (**3**), as well as two monoterpenes, thymoquinone (**4**) and thymoquinol 2-*O*-β-glucopyranoside (**5**); two simple phenolic acids, oresbiusin A (**6**) and *E*-caffeic acid (**7**); six flavonoids, namely, apigenin (**8**), kaempferol (**9**), quercetin (**10**), eriodictyol (**11**), taxifolin (**12**), naringenin (**13**); and two alicyclic derivatives, that is, 12-hydroxyjasmonic acid (**14**) and its 12-*O*-β-D-glucoside (**15**). The structures of all isolated compounds were established by spectroscopic methods, mainly 1D and 2D NMR, as well as HPLC-DAD-MS and HR-MS spectrometric analyses. The absolute configuration of compound **1** was determined by CD measurements as 7′*R*, 7″*S*, 8″*S*. Compound **1** is interesting as it contains a benzodioxane ring, which is unusual in natural products. Moreover, it has been proved to be active against the Gram-negative clinical strains *Acinetobacter hemolyticus*, *Empedobacter* brevis, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*.

KEYWORDS: *Origanum dictamnus*; Lamiaceae; depsides; salvianolic acids; circular dichroism; phenolic acids; flavonoids; alicyclic derivatives; antimicrobial activity

INTRODUCTION

Origanum dictamnus L. (Lamiaceae) or Cretan dittany is an endemic herb of Crete (Greece) (*1*). Because of its therapeutic properties, known since antiquity, it is also being cultivated. It has been used as a remedy for ailments of the stomach and intestinal tract and rheumatism and is especially effective as an antihemorrhage agent and a stimulant of the nervous system and against difficulties of childbirth (*2*). Due to their importance as culinary herbs and their significance for the Mediterranean diet, species of the genus *Origanum* have been long studied, mainly for their high content in essential oils (*3*−*5*). Studies on their nonvolatile constituents, however, proved them to be a rich source of phenolic compounds (*6*−*9*), with potential use in the food industry and in the development of functional foods (*10*−*12*). The most interesting compounds recently discovered in *Origanum* species are the depsides, a chemical group containing caffeic acid subunits linked together through esteric or ether bonds. Because depsides have shown important biological activities (*13*−*16*), research for plant sources rich in this type of compounds is desirable. Little is known about their presence in *Origanum* species, although it is well documented in other members of the Lamiaceae family. In our previous paper (*8*), we reported the isolation of depsides, such as rosmarinic acid, 10-epi-lithospermic acid, and epi-lithospermic acid B from *Origanum vulgare* ssp. *hirtum*. The objective of the present work was to investigate the secondary metabolites of *O. dictamnus* with emphasis on depsides in order to find other *Origanum* species rich in this chemical group. Moreover, the isolated compounds have been tested against a panel of clinical strains.

*Corresponding author (telephone/fax +30 210 7274593; e-mail skaltsa@pharm.uoa.gr).

MATERIALS AND METHODS

General Procedures. ^{1}H, ^{13}C, and 2D NMR spectra were recorded in DMSO-d_6 on Bruker DRX-400 (100.6 MHz for ^{13}C NMR) and Bruker DRX-600 instruments at 295 and 305 K, respectively. Chemical shifts are given in parts per million (ppm) and were referenced to the solvent signals at 2.50 and 39.5 ppm for ^{1}H and ^{13}C NMR, respectively. 2D spectra were performed using standard Bruker microprograms. IR spectra were obtained on a Perkin-Elmer Paragon 500 FT-IR spectrophotometer. UV spectra were recorded on a Shimadzu UV-160A spectrophotometer, according to the method of Mabry et al.(*17*). ESI mass spectra were measured on a Thermo LTQ Orbitrap (FT-MS^n) (University of Florence, Italy). Optical rotations were measured on a Perkin-Elmer 341 polarimeter. CD spectra of **1** and **2** were recorded using a Jasco spectropolarimeter (J-800) in a cuvette of 1 cm path length. Spectra were taken as the average of four accumulations from 200 to 500 nm. The scan rate was 50 nm/min. Spectra were recorded in MeOH in a range of

 Published on Web 04/27/2010

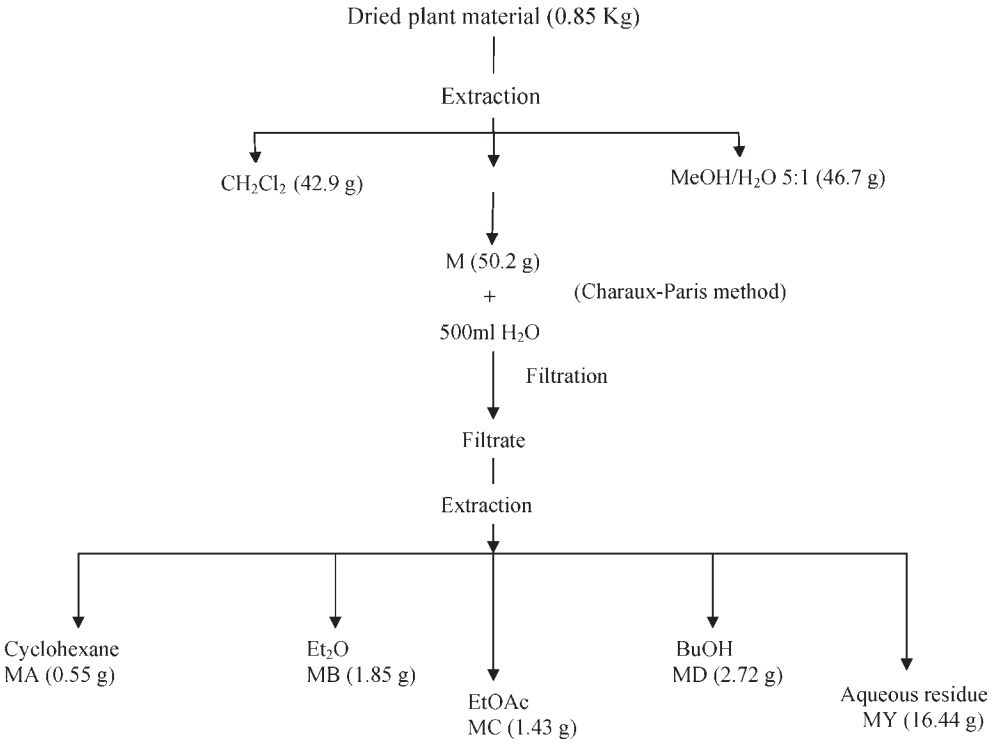

Figure 1. Graph of the initial stages of extraction and Charaux−Paris procedure.

concentrations of 1−0.1 mM. The molecular ellipticity is reported as deg × cm^2 × $dmol^{-1}$.

HPLC-DAD-MS analyses were performed using a HP 1100 liquid chromatograph (Agilent Technologies, Palo Alto, CA) equipped with a HP 1040 diode array detector (DAD), an automatic injector, an autosampler, and a column oven and managed by a HP 9000 workstation (Agilent Technologies). The HPLC system was interfaced with a HP 1100 MSD API-electrospray (Agilent Technologies). The interface geometry, with an orthogonal position of the nebulizer with respect to the capillary inlet, allowed the use of analytical conditions similar to those of HPLC-DAD analysis. Mass spectrometry operating conditions were optimized to achieve maximum sensitivity values: gas temperature, 350 °C, at a flow rate of 10 L/min; nebulizer pressure, 30 psi; quadrupole temperature, 30 °C; and capillary voltage, 3500 V. Full scan spectra from m/z 100 to 1500 in both positive and negative ion modes were obtained (scan time 1 s). Fragmentor was set at 120 and 180. Separations were performed on a reversed-phase column, Purosphers Star RP-18, namely, Hibars Prepacked column RT (250 × 4.6 mm) with a particle size of 5 mm (Merck, Darmstadt, Germany). The eluents were water adjusted to pH 3.2 by formic acid (A) and methanol (B). The mobile phase was a gradient of A from 95 to 0% (100% B) run in 25 min, at a flow rate of 0.8 mL/min. The system was operated with an oven temperature at 26 °C; the injection volume was 5 μL. Chromatograms were recorded at 254, 280, 330, and 350 nm. DAD spectra between 200 and 450 nm were stored for all peaks exceeding a threshold of 0.1 mAu.

Vacuum−liquid chromatography (VLC) was carried out on silica gel 60H (Merck, article 7736). Column chromatography (CC) was carried out on Sephadex LH-20 (Pharmacia Fine Chemicals) and silica gel (Merck, article 9385). TLC: Merck silica gel 60 F_{254} (article 5554); detection, UV light; spray reagents, vanillin−H_2SO_4 on silica gel; Neu 's reagent on cellulose (*18*).

Plant Material. Aerial parts of cultivated *O. dictamnus* were collected in September 2005 from Herakleion (Crete). The wild *O. dictamnus* was identified by Ass. Prof. Th. Constantinidis (Faculty of Biology, University of Athens), and a voucher specimen (Skaltsa and Chatzopoulou, no. 01C, ATHU) has been deposited in the Laboratory of Pharmacognosy and Chemistry of Natural Products, University of Athens.

Extraction, Isolation, and Identification of the Secondary Metabolites of *O. dictamnus* L. The air-dried powdered aerial parts (0.85 kg) of the plant were extracted with dichloromethane (D; 42.9 g), methanol (M; 77.0 g), and methanol/water 5:1 v/v (MH; 46.7 g), successively. Part of the methanol residue (50.2 g) was treated according to the Charaux−Paris procedure (*19*). Briefly, it was dissolved in 500 mL of H_2O and then partitioned in solvents of increasing polarity, using cyclohexane (MA; 0.55 g), Et_2O (MB; 1.85 g), ethyl acetate (MC; 1.43 g), and butanol (MD; 2.72 g). Finally, an aqueous residue (MY; 16.44 g) was yielded (**Figure 1**).

The ether extract (MB; 1.85 g) was purified by CC over Sephadex LH-20 using methanol as eluent. The fractions were combined to 10 groups (MBA−MBK). Further purification of group MBD (18→24, 158.8 mg) by CC over silica gel using as eluents mixtures of CH_2Cl_2/EtOAc of increasing polarity afforded **4** (4.1 mg), **5** (3.3 mg), **10** (2.5 mg), **13** (36.6 mg), and **14** (2.6 mg). Fraction MBI (46→48, 57.8 mg) was subjected to RP-HPLC with MeOH/AcOH 5% 1:1 and yielded **7** (Rt = 57 min, 1.2 mg), **11** (Rt = 40.5 min, 4.0 mg), and **12** (Rt = 17.7 min, 5.4 mg). Fraction MBK (Rt = 49−55 min, 64.1 mg) was purified by CC over silica gel using as eluents mixtures of CH_2Cl_2/MeOH of increasing polarity. The fractions eluted with CH_2Cl_2/MeOH 98:2 (10.1 mg) were identified as **8**. Finally, fraction MBL (Rt = 56−64 min, 69.5 mg) was purified by CC over silica gel using as eluents mixtures of CH_2Cl_2/MeOH of increasing polarity. The fractions eluted with CH_2Cl_2/MeOH 98:2 were combined and identified as **12** (10.1 mg), whereas the fractions eluted with CH_2Cl_2/MeOH 96:4 were also combined and identified as **9** (2.5 mg).

The aqueous residue (MY) was subjected to VLC (5 cm × 8 cm) over reversed phase silica gel (RP-18, Merck, article 10167) using as eluents mixtures of water/methanol of decreasing polarity. Ten fractions of 500 mL have been received. The fraction eluted with H_2O/MeOH 90:10 (1.18 g) was further purified by CC over silica gel using as eluents mixtures of CH_2Cl_2/MeOH/H_2O of increasing polarity and yielded **15** (67.3 mg). Further purification of the fraction eluted from the latter CC with CH_2Cl_2/MeOH/H_2O 70:30:3 (50.9 mg) by CC over Sephadex LH 20 afforded **1** (4.1 mg).

The initial methanol/water 5:1 residue (MH; 46.7 g) was subjected to VLC (5 cm ×8 cm) over silica gel (Merck, article 7736) using as eluents mixtures of CH_2Cl_2/MeOH of increasing polarity. Twelve fractions (MHA−MHL) of 500 mL have been received. Fraction MHE (0.7 g; eluted with CH_2Cl_2/MeOH 30:70) was further purified by CC over silica gel using mixtures of CH_2Cl_2/MeOH/H_2O of increasing polarity, and the fraction eluted with CH_2Cl_2/MeOH/H_2O 90:10:1 was identified as **3** (19.0 mg). Fraction MHI (322.0 mg; eluted with CH_2Cl_2/MeOH/H_2O

Table 1. ^{1}H (305 K, 600 MHz) and ^{13}C NMR (295 K, 100.6 MHz) Spectroscopic Data of Compound **1** (in DMSO-d_6; δ in ppm, J in Hz)

position	δ_H	δ_C		HMBC	ROESY
1		127.9	C		
2	7.21 (1H, d, 1.8)	116.7	CH	1, 3, 4, 6	7, 7″, 2″ 6″
3		143.0	C		
4		145.8	C		
5	6.85 (1H, d, 8.5)	116.9	CH	1, 3, 4	6
6	7.15 (1H, dd, 8.5, 1.9)	122.2	CH	4	5, 7, 8
7	7.47 (1H, d, 15.9)	144.8	CH	1, 2, 6, 9	2, 6, 8′
8	6.39 (1H, d, 16.0)	115.3	CH	1, 7, 9	2, 6, 8′
9		165.9	C=O		
1′		126.8	C		
2′	6.67 (1H, d, 2.0)	116.5	CH	3′	7′a, 7′b
3′		145.2	C		
4′		143.9	C		
5′	6.61 (1H, d, 8.0)	115.4	CH	1′, 3′	6′
6′	6.51 (1H, dd, 8.1, 2.0)	120.0	CH	2′, 4′	2′, 7′a, 7′b
7′a	2.99 (1H, dd, 14.3, 3.9)	36.4	CH_2	1′, 2′, 6′, 8′	2′, 6′
7′b	2.86 (1H, dd, 14.3, 8.8)			9, 1′, 2′, 6′, 8′	2′, 6′
8′	4.99 (1H, dd, 8.9, 3.8)	73.8	CH	1′, 9, 9′	2′, 7′a, 7′b, 7
9′		171.7	C=O		
1″		128.7	C		
2″	6.76 (1H, d, 1.6)	114.4	CH	3″, 6″	7″, 8″, 2
3″		145.8[a]	C		
4″		144.9[a]	C		
5″	6.66 (1H, d, 8.4)	115.4	CH	1″, 3″	6″
6″	6.64 (1H, dd, 8.4, 1.7)	118.0	CH	4″, 5″	5″, 7″, 8″, 2
7″	5.31 (1H, d, 3.5)	75.3	CH	3, 1″, 2″, 6″	2″, 6″, 2
8″	4.64 (1H, br s)	77.1	CH		2″, 6″
9″		169.3	C=O		

[a] Signal assignment may be opposite.

87:13:1.3) was subjected to CC over silica gel using as eluent CH_2Cl_2/MeOH/H_2O. The fraction eluted with CH_2Cl_2/MeOH/H_2O 97:3:0.3 was identified as **6** (10.0 mg), whereas the fraction eluted with CH_2Cl_2/MeOH/H_2O 96:4:0.4 was identified as **2** (19.8 mg).

The structures of compounds **2**−**15** were established by spectroscopic methods, mainly 1D and 2D NMR, as well as UV−vis and HPLC-DAD-MS spectrometric analyses.

7′R,7″S,8″S-Salvianolic acid P (*I*)*:*. amorphous yellow powder; $[\alpha]_D^{23}$ +14.74 (MeOH *c*, 0.2); UV−vis (MeOH), λ_{max} nm 236, 244sh, 286, 324; IR (film), ν_{max} cm^{-1} 3728 (O—H), 2936 (C—H), 1727, 1712 (C=O); ^{1}H and ^{13}C NMR spectra, see **Table 1**; HR-ESI-MS *m*/*z* 537.10324 $[M - H]^-$ (calcd for $C_{27}H_{21}O_{12}$ 537.10323). CD (MeOH) $\Delta\varepsilon$ (nm) +1.58 (350), −1.70 (324), +0.75 (297), −7.50 (278), −9.67 (240), +5.17 (218).

Bioassays. Samples were dissolved at 10.0 mg/mL in dimethyl sulfoxide (DMSO, Merck, article 2951) and diluted with the nutrient medium to a concentration of 1000 μg/mL. Final concentrations of 50.0, 25.0, 12.5, 6.25, and 3.12 μg/mL have been used. The proportion of DMSO never exceeded 1% in the medium. The following organisms (clinical isolates) were used: *Acinetobacter hemoliticus*, *Empedobacter brevis*, *Enterobacter cloacae*, *Morganella morganii*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Proteus mirabilis*, and *Klebsiella pneumoniae*. The antibacterial assays were carried out by the microdilution technique, according to a previously reported method (*20*). Minimum inhibitory concentration (MIC) determination was performed by a serial dilution technique using 96-well microtiter plates. The lowest concentrations without visible growth (at the binocular microscope) were defined as concentrations that completely inhibited bacterial growth (MICs). DMSO was used as a control, whereas commercial antibiotics were used as positive controls. The experiments for each sample were conducted in duplicate, and the results were expressed as average values (**Table 2**).

RESULTS AND DISCUSSION

Compound **1** was obtained as an amorphous yellowish powder. The IR spectrum of **1** contained absorption bands characteristic of hydroxyl (3728 cm^{-1}), aliphatic groups (2936 cm^{-1}), and carbonyl groups (1727, 1712 cm^{-1}). The ^{1}H NMR spectrum

Table 2. Minimum Inhibitory Concentrations (Millimoles per Milliliter) of Compounds **1**−**15**

	A. hemolyticus	*E. brevis*	*Ps. aeruginosa*	*Kl. pneumoniae*
1	0.012	0.012	0.012	0.012
2	–[a]	–	–	–
3	0.134	–	0.134	–
4	0.30	–	–	–
5	0.152	–	–	–
6	0.236	–	–	–
7	0.28	–	0.28	–
8	–	–	–	–
9	–	–	–	–
10	–	–	–	–
11	0.174	–	0.174	–
12	0.164	–	–	–
13	0.183	–	–	–
14	0.22	–	–	–
15	–	–	–	–
ampicillin	>0.040	0.040	>0.040	>0.040
tetracyclin	0.018	>0.018	>0.018	0.018

[a] No activity.

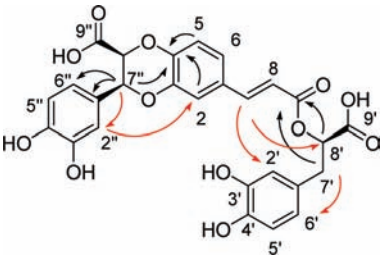

Figure 2. Diagnostic HMBC and ROE correlations for compound **1**.

(**Table 1**) showed olefinic signals in the aromatic region characteristic of three ABX systems, three protons belonging to oxymethines, and finally a pair of aliphatic, benzylic protons.

The COSY spectrum permitted the assignment of the aliphatic protons and displayed the following connectivities: H-7″ (δ_H 5.31)/H-8″ (δ_H 4.64) and H-8′ (δ_H 4.99), H-7′a (δ_H 2.99), and H-7′b (δ_H 2.86). Correlations of the olefinic protons H-7 and H-8 with the quaternary carbon C-1 (δ_C 127.9) and the carbonyl carbon at δ_C 165.9 in the HMBC spectrum confirmed the presence of a caffeic acid moiety (see the Supporting Information). Due to signal overlapping in the HMBC, ROESY correlations were used to distinguish which aliphatic protons belonged to each aromatic system: interactions of protons H-7′a, H-7′b, and H-8′ with aromatic protons H-2′ and H-6′ revealed the presence of a 3,4-dihydroxyphenyllactic acid moiety, whereas interactions of protons H-7″ and H-8″ with the protons H-2″ and H-6″ confirmed the presence of a 3-(3,4-dihydroxyphenyl)glyceric acid group. In the latter, diagnostic crosspeaks between H-7″ and the aromatic carbons C-1″ and C-2″ proved its α position to the aromatic ring (**Figure 2**). The HMBC spectrum gave substantial evidence for the linkage between the three subunits: a crosspeak between proton H-8′ and the carbonyl at δ_C 165.9 (C-9) indicated that position 8′ was an esterification site, whereas key correlations of H-7″, H-2, and H-5 with the aromatic carbon C-3 revealed that the 3-(3,4-dihydroxyphenyl)glyceric acid is linked through an ether type linkage to the caffeic acid. This was further confirmed in the ROESY experiment through the crosspeak between H-2 and H-2″. Similarly, in the same spectrum, H-8″ showed interaction with H-7 (see the Supporting Information). Mass spectral data suggested the presence of an extra dioxane ring formed by the linkage of the two groups. A literature survey revealed the presence of a similar compound, salvianolic acid J, isolated from

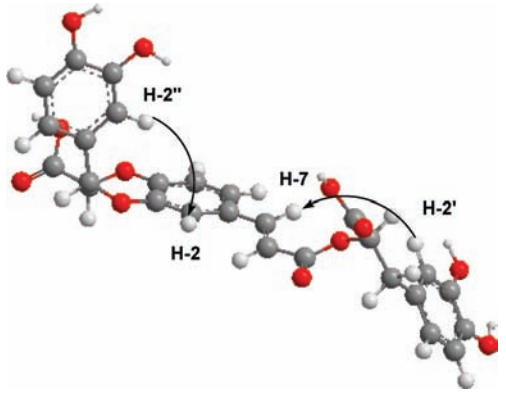

Figure 3. Minimized energy model (ChemDraw 3D) and diagnostic ROE correlations for compound **1**.

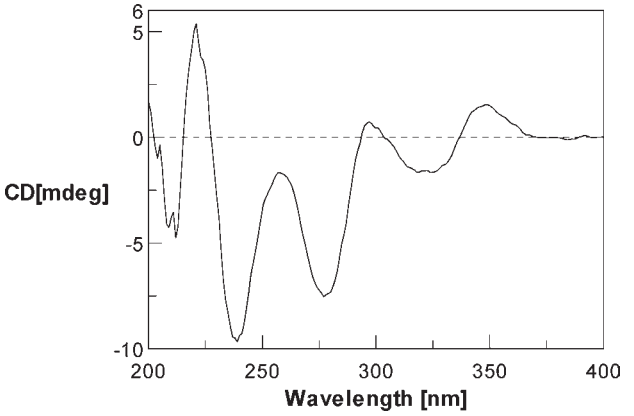

Figure 4. Circular dichroism (CD) spectrum (MeOH, 25 °C) of compound **1** (0.1 mM).

Salvia flava (*21*) with opposite substitution pattern at positions 7″ and 8″. Due to the similarity of their structures, compound **1** was given the trivial name salvianolic acid P.

The energy-minimized structure models (ChemDraw 3D) of both 7″*R*,8″*R* or 7″*S*,8″*S* stereomers showed that in both cases (**Figure 3**) the aromatic ring of the 3-(3,4-dihydroxyphenyl)glyceric acid is folded almost axially either below or above the aromatic ring of the caffeic acid. The circular dichroism spectrum of compound **1** (**Figure 4**) was in complete accordance with previously reported data for other benzodioxane derivatives (*22*−*24*). It showed a very strong negative Cotton effect at a diagnostic wavelength of 240 nm, indicating the *β*,cis-orientation of the aryl and carboxyl substitutes, that is, a 7″*S*,8″*S* and an 8′*R* configuration. Comparison of the CD spectrum of compound **1** with the CD spectrum of (*R*)-rosmarinic acid (**2**) in the same concentration (0.1 mM) showed a red shift (from 232 to 240 nm) in the CD maximum (*22*), which is due to the presence of the *β*,cis-substitution of the benzodioxane ring and also an amplification of the negative signal from −7.6 to −9.7 due to the coexistence of a (*R*)-rosmarinic acid unit with the 7″*S*,8″*S* 3-(3,4-dihydroxyphenyl)-glyceric acid moiety. Therefore, the absolute stereochemistry of compound **1** was established as (2*S*,3*S*)-6-((*E*)-3-((*R*)-1-carboxy-2-(3,4-dihydroxyphenyl)ethoxy)-3-oxoprop-1-enyl)-3-(3,4-dihydroxyphenyl)-2,3-dihydrobenzo[*b*][1,4]dioxine-2-carboxylic acid, or as 7′*R*,7″*S*,8″*S*-salvianolic acid P.

The presence of benzodioxane rings in natural products is unusual. A few examples of compounds containing this group are the flavonolignans of *Silybum marianum* and the neolignans from *Licaria*, *Virola*, and *Juniperus* species (*22*−*24*).

This is only the fourth case of the presence of depsides in the genus, while all previous reports concern solely *Origanum vulgare* (*25*, *26*). This may be partly attributed to the polar nature of these types of constituents, as they can be easily discarded in the course of phytochemical analyses together with the sugar-containing fractions. Another reason for their misinterpretation is their UV spectra, which are similar to those of caffeic acid, and therefore constituents of this kind could be easily confused and characterized generally as caffeic acid derivatives.

Methanol extracts of cultivated and wild *O. dictamnus* have been previously reported (*27*) to possess interesting antimicrobial activity. For this reason, it was decided to test the antibacterial activity of the isolated compounds. Interestingly, salvianolic acid P (**1**) was the most potent, as it was shown to possess almost similar activity as commercial antibiotics against *A. hemolyticus*, *E. brevis*, *Ps. aeruginosa*, and *Kl. pneumoniae*. *A. hemolyiticus* was found to be the most sensitive strain, as it was also inhibited by the methyl ester of rosmarinic acid (**3**), the monoterpenes **4** and **5**, the phenolic acids **6** and **7**, the dihydroflavonoids **11**−**13**, and the alicyclic derivative **14**. Also, compounds **3**, **7**, and **11** inhibited *Ps. aeruginosa* (**Table 2**). All compounds were inactive against *E. cloacae*, *M. morganii*, *Ps. fluorescens*, and *Pr. mirabilis*.

This is the first report of antimicrobial activity of these types of constituents, and it could be suggested that they also contribute to the antimicrobial potential of the plant.

ACKNOWLEDGMENT

We are grateful to Dr. Elena Michelucci (Mass Spectrometry Center, University of Florence − CISM) for recording the HRESI mass spectra. We thank Prof. Anna Rita Bilia, University of Florence, for the NMR facilities (Bruker 600 MHz), Dr. Chiara Gabbiani, University of Florence, for measuring the CD spectra, and Assistant Prof. Theophanis Constantinidis (University of Athens) for the identification of the wild plant.

Supporting Information Available: Structural and spectral information. This material is available free of charge via the Internet at http://pubs.acs.org.

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Received for review January 5, 2010. Revised manuscript received March 24, 2010. Accepted April 20, 2010.