Cytotoxic Constituents of Roots of *Chaerophyllum hirsutum*

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Two new lignan derivatives (**1** and **2**), along with the known scopoletin (**3**), methyl caffeate (**4**), falcarindiol (**5**), 3,5-dicaffeoylquinic acid, 3,5-dicaffeoylquinic methyl ester, chlorogenic acid, *p*-hydroxyphenylethyl-*trans*-ferulate, and vanillin, were isolated from extracts of the roots of *Chaerophyllum hirsutum*. Structure elucidation of the new compounds was carried out by 1D and 2D NMR experiments and by HRMS analysis. Several of the isolated compounds were tested for their cytotoxic activity against the HL-60, HT-1080, LoVo, and LoVo/Doxo cell lines.

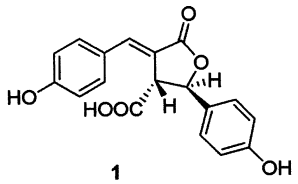
Chaerophyllum hirsutum L. (Apiaceae) is a perennial herb widespread in northern Italy. Little information has been published about the chemical composition of the genus *Chaerophyllum*. Earlier studies reported information on flavonoids from *C. sylvestre*[1] and coumarins from *C. prescottii*.[2] *C. maculatum* is known to contain the diarylbutyrolactone lignan kaerophyllin.[3] The taxonomically related species, *Anthriscus sylvestris* Hoffm., has been studied for the presence of cytotoxic lignans related to podophyllotoxin, such as deoxypodophyllotoxin[4−6] and angeloylpodophyllotoxin, in their roots.[7]

In our ongoing research for new bioactive lignans from Italian plants, the powdered roots of *C. hirsutum* were extracted with petroleum ether, chloroform, and methanol. These three extracts were tested for their cytotoxicity against the LoVo (human intestinal adenocarcinoma) cell line: only the chloroform extract (TCE) showed moderate activity (IC_{50} 57 μg/mL), whereas no activity (>200 μg/mL) was observed against a resistant clone of the LoVo LoVo/Doxo cell line. Fractionation of TCE led to the isolation of scopoletin (**3**), methyl caffeate (**4**), falcarindiol (**5**), phenylethyl ferulate, and vanillin.

Fractionation of the methanol extract led to the isolation and characterization of two new compounds (**1** and **2**) in addition to known phenolic compounds scopoletin (**3**), methyl caffeate (**4**), 3,5-dicaffeoylquinic acid, 3,5-dicaffeoylquinic methyl ester, and chlorogenic acid.

Compound **1** was obtained as a light yellow solid. The UV spectrum showed peaks at 227 and 320 nm. The HRAPITOFMS spectrum displayed a protonated molecular ion $[M + H]^+$ at *m/z* 327.0859, indicating a molecular formula of $C_{18}H_{14}O_6$. The ^{1}H NMR spectrum in CD_3OD of **1** showed four doublets, ascribable to *ortho*-coupled aromatic protons at δ 7.57 (J = 9.0 Hz), 7.14 (J = 8.5 Hz), 6.80 (J = 9.0 Hz), and 6.71 (J = 8.5 Hz). Also evident were a doublet at δ 7.55 (J = 2.5 Hz), a doublet at δ 5.62 (J = 2.6 Hz), and a doublet of doublets at δ 3.82 (J = 2.5, 2.6 Hz) (Table 1).

On the basis of DQF-COSY, HMQC, and HMBC experiments, two *p*-disubstitued aromatic rings were detected. Diagnostic long-range correlations in the HMBC experiment (Figure 1) were observed between H-2/H-6 at δ_H 7.57 and C-7 at δ_C 138.0, between H-7 (δ_H 7.55) and C-2/C-6 at δ_C 133.0, C-8′ at δ_C 57.8, and C-9 at δ_C 175.0. Other long-range correlations were observed between H-8′ at δ_H 3.82 and C-1′ (δ_C 125.5), C-9 (δ_C 175.0) and C-9′ (δ_C 178.2) and H-7′ at δ_H 5.62, and C-2′/C-6′ (δ_C 126.2), C-9 (δ_C 175.0), and C-9′ (δ_C 178.2). The geometry of the double bond was deduced to be *E* on the basis of the value (δ = 2.5 Hz) of the long-range coupling constant between H-7 and H-8′. This was confirmed by the NOESY experiment, which showed the correlation between H-8′ (δ 3.82) and H-2/H-6 (δ 7.57), consistent only with the configuration *E* of the vinyl group. Furthermore, the signal at δ 5.62 (H-7′) exhibited a correlation with the aromatic signal at δ 7.14 (H-2′, H-6′) and a weak correlation with the signal at δ 3.82 (H-8′), indicating an *anti* configuration, in good agreement



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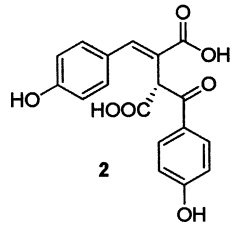

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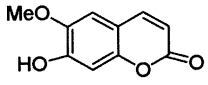

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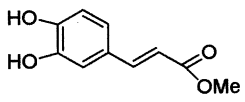

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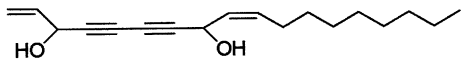

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Table 1. ^{1}H and ^{13}C NMR Data of Compounds **1** and **2** in CD_3OD[a]

	1		2	
position	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C
1		125.5		126.0
2	7.57 d (9.0)	133.0	7.16 d (9.0)	130.6
3	6.80 d (9.0)	114.1	6.72 d (9.0)	115.8
4		158.2		159.6
5	6.80 d (9.0)	114.1	6.72 d (9.0)	115.8
6	7.57 d (9.0)	133.0	7.16 d (9.0)	130.6
7	7.55 d (2.5)	138.0	7.84 d (2)	142.3
8		126.2		124.3
9		175.0		169.3
1′		125.5		126.0
2′	7.14 d (8.5)	126.2	7.86 d (8.0)	126.3
3′	6.71 d (8.5)	115.0	6.75 d (8.0)	117.0
4′		160.0		159.6
5′	6.71 d (8.5)	115.0	6.75 d (8.0)	117.0
6′	7.15 d (8.5)	126.2	6.75 d (8.0)	126.3
7′	5.62 d (2.6)	83.2		197.2
8′	3.82 dd (2.6−2.5)	57.8	4.20 d (2)	37.6
9′		178.2		168.6

[a] Assignments were confirmed by DQF-COSY, HMQC, and HMBC experiments.

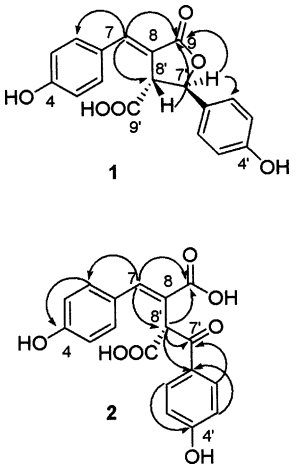

Figure 1. HMBC correlations for compounds **1** and **2**.

with the low value of the coupling constant between these protons (2.6 Hz). Thus, **1** was established to be (2*R**,3*R**)-2-(4-hydroxyphenyl)-4-[(*E*)-1-(4-hydroxyphenyl)methylidene]-5-oxotetrahydro-3-furancarboxylic acid.

Compound **2** was obtained as a pale yellow solid. The UV spectrum showed a peak at 285 nm and a shoulder at 320 nm. The HRAPITOFMS showed a sodiated molecular ion $[M + Na]^+$ at m/z 365.0637, yielding the molecular formula $C_{18}H_{14}O_7$. The ^{1}H NMR spectrum in CD_3OD of **2** indicated two *p*-hydroxydisubstituted aromatic rings. The main difference from the ^{1}H NMR spectrum of **1** was the absence of the signal at δ 5.62. In the ^{13}C NMR spectrum of **2**, the signal at δ 83.2 in **1** was replaced by a signal at δ 197.2, clearly indicating a keto group. Diagnostic long-range correlations were observed in the HMBC spectrum between H-8′ at δ_H 4.20 and C-8 at δ_C 124.3, C-7 (δ_C 142.3), C-9′ (δ_C 168.6), and C-7′ (δ_C 197.2). On the basis of these data, **2** clearly differed from **1** due to the opening of the lactone ring and later oxidation of the alcoholic function at C-7′. Thus, **2** was identified as 2-(4-hydroxybenzeoyl)-3-[(*E*)-1-(4-hydroxyphenyl)methylidene]succinic acid.

The in vitro cytotoxicity of compounds **1**−**5** was evaluated against the HL-60 human (promyelocytic leukemia), HT-1080 (human fibrosarcoma), LoVo (human intestinal adenocarcinoma), and LoVo/Doxo cell lines, in comparison to the reference drug, doxorubicin hydrochloride (Table 2). The LoVo/Doxo cell line expresses the *mdr* factor and thus presents the P-glycoprotein-mediated mechanism of multidrug resistance (*mdr*) to a number of intercalating agents such as various anthracyclines, mitoxantrone, and ametantrone.[8] Although less potent than the internal standard, some of the test compounds inhibited proliferation at micromolar concentration. Interestingly, compounds **2** and, to a lesser extent **3**, inhibited the growth of the LoVo subclone. In fact, the resistant indexes (the ratios between IC_{50} of LoVo/Doxo and that of its correspondent wild type) of **2** and **3** were 1.2 and 2.5, respectively. Considering that doxorubicin has a much higher resistance factor, these data suggest that these compounds can partially overcome the *mdr* mechanism operating in the LoVo cell line induced by doxorubicin. Caffeic acid methyl ester (**4**) and falcarindiol (**5**) were previously studied for their cytotoxic properties but against other cell lines.[4,5,9,10] The cytotoxic activity of scopoletin was previously evaluated against human epidermoid carcinoma (KB cell) by two different research groups, indicating a significant effect on this cell line.[11,12]

Table 2. IC_{50} Values for Compounds **1**−**5** against the HL-60 Human (promyelocytic leukemia), HT-1080 (human fibrosarcoma), LoVo (human intestinal adenocarcinoma), and LoVo/Doxo Cell Lines

	cytotoxicity (μM)[a]			
compound	HL-60	HT-1080	LoVo	LoVo/Doxo
1	11.3 ± 1.8	36.0 ± 4.0	13.9 ± 2.2	>40
2	11.5 ± 1.3	30.6 ± 5.8	16.4 ± 5.4	19.6 ± 2.7
3	9.5 ± 1.3	12.5 ± 0.1	7.3 ± 1.1	18.5 ± 1.5
4	7.5 ± 1.6	11.1 ± 3.5	5.8 ± 1.1	>40
5	8.9 ± 0.4	13.0 ± 0.6	4.3 ± 0.2	> 40
doxo[b]	0.01 ± 0.007	0.012± 0.005	0.20 ± 0.06	13.2 ± 0.01

[a] Data are expressed as mean ± SEM of three independent experiments. [b] Doxorubicin hydrochloride was used as a reference compound.

Experimental Section

General Experimental Procedures. Optical rotations were measured on an Atago Polax/L 300 polarimeter. UV spectra were obtained on a Perkin-Elmer lambda 25 spectrophotometer. IR spectra were recorded on a Perkin-Elmer 1600 FT-IR spectrometer. NMR spectra in CD_3OD were obtained using a Bruker AMX-300, spectrometer, operating at 300.13 MHz for ^{1}H NMR and 75.03 MHz for ^{13}C NMR. 2D experiments, ^{1}H−^{1}H DQF-COSY, and inverse-detected ^{1}H−^{13}C HMQC and HMBC spectra were performed using UXNMR software. Exact masses were measured by an API-TOF spectrometer (Mariner Biosystems). Samples were diluted in a mixture of H_2O−AcCN (1:1) with 0.1% formic acid and directly injected at a flow rate of 10 μL/min. Dianon HP20, Sephadex LH 20, and silica gel 60 were used for column chromatography. Silica gel plates were used for analytical and preparative TLC (Merck cat. 5717 and 5715).

Plant Material. *Chaerophyllum hirsutum* L. (Apiaceae) was collected in July 2002 at Monte Bondone (province of Trento, Italy). The plant was identified by one of the authors (E.M.C.), and a voucher specimen was deposited at the Botanical Garden of the University of Padova, no. 42-73.

Extraction and Isolation. Air-dried and powdered roots of *C. hirsutum* (50 g) were exhaustively extracted in a Soxhlet apparatus with petroleum ether, chloroform, and methanol, to obtain three extracts. The solvents were removed under vacuum. Yields in weight of residue, referring to the weight of dry material extracted, were as follows: petroleum ether 0.45 g, chloroform 0.6 g, and methanol 10.5 g.

A portion of the MeOH extract (7 g) was dissolved in a methanol−water mixture (1:9, 100 mL), applied to a Dianon HP20 column (250 mL), and eluted with 400 mL of water (fraction I), 300 mL of methanol (fraction II), and 200 mL of *n*-hexane (fraction III).

Fraction II (1.5 g) was suspended in a mixture of water−methanol (100 mL) and extracted with petroleum ether,

chloroform, and ethyl acetate (all 3 × 30 mL); the latter two were combined (180 mg) according to their chromatographic behavior. The fractions were further chromatographed on silica gel plates and eluted with $CHCl_3$−MeOH (90:10), and scopoletin (**3**) (5.0 mg) and methyl caffeate (**4**) (3.5 mg) were isolated.

The residual water−methanol mixture (fraction II) was evaporated under vacuum, yielding an oily residue of 1.1 g, diluted in methanol, applied to a Sephadex LH 20 column (150 mL), and eluted with methanol. Fifty-five 5 mL fractions were obtained and combined on the basis of their chromatographic behavior into eight fractions. Further chromatographic steps over a silica gel column (eluted with $CHCl_3$−MeOH in increasing ratios) and preparative TLC (BuOH−HOAc−H_2O, 20:5:1, and $CHCl_3$−MeOH−H_2O, 10:5:1) yielded new compounds **1** (6.5 mg, from fraction 5) and **2** (7.3 mg, from fraction 4), in addition to 3,5-dicaffeoylquinic acid (3.3 mg, from fraction 6), 3,5-dicaffeoylquinic methyl ester (8.2 mg), and chlorogenic acid (6.7 mg, from fraction 7).

The chloroform extract (0.60 g) was chromatographed on a silica gel column (200 mL) using mixtures of chloroform−methanol in increasing polarity from 2% to 20%. The fractions were pooled into four fractions, A−D, and tested for their cytotoxicity. Only fractions B and C showed some activity (IC_{50} of 50.7 and 77.3 μg/mL for the LoVo cell line, respectively). Fraction B also showed activity against the LoVo/Doxo cell line. The active fractions were subjected to further chromatographic steps on a silica gel column (eluted with $CHCl_3$−MeOH in increasing ratios) and silica gel plates (cyclohexane−EtOAc, 2:1 and 1:1), affording the isolation of scopoletin (**3**) (6.6 mg) and methyl caffeate (**4**) (10.2 mg) from fraction B and falcarindiol (**5**) (6.0 mg), phenylethylferulate (8.4 mg), and vanillin (7.6 mg) from fraction C.

(2*R,3*R**)-2-(4-Hydroxyphenyl)-4-[(*E*)-1-(4-hydroxyphenyl)methylidene]-5-oxotetrahydro-3-furancarboxylic acid (1):** yellow solid; $[\alpha]_D^{22}$ +68.8° (*c* 0.16, EtOH); UV (EtOH) λ_{max} (log ϵ) 320 (4.10) and 227 (3.98) nm; IR (KBr) ν_{max} 3397, 1730 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; HRAPITOFMS *m*/*z* 327.0859 $[M + H]^+$ (calcd for $C_{18}H_{15}O_6$, 327.0868).

2-(4-Hydroxybenzoyl)-3-[(*E*)-1-(4-hydroxyphenyl)methylidene]succinic acid (2): pale yellow solid; $[\alpha]_D^{22}$ +52.1° (*c* 0.12, EtOH); UV (EtOH) λ_{max} (log ϵ) 285 (4.11) nm; IR (KBr) ν_{max} 3390, 1732, 1700 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; HRAPITOFMS *m*/*z* 365.0637 $[M + Na]^+$ (calcd for $C_{18}H_{14}O_7$-Na, 365.0637).

Scopoletin (**3**), chlorogenic acid, and vanillin were identified on the basis of their UV and mass spectra by comparison with authentic samples purchased from Sigma-Aldrich Corp. Methyl caffeate (**4**),[13] falcarindiol (**5**),[14] 3,5-dicaffeoylquinic acid,[15] 3,5-dicaffeoylquinic methyl ester,[16] and *p*-hydroxyphenylethyl-*trans*-ferulate[17] were identified by comparison of their spectral data (UV, HRMS, ^1H NMR, and ^{13}C NMR) with those reported in the literature for the same compounds.

Antiproliferative Activity. Extracts were dissolved in 20 mg/mL DMSO as stock solutions. Compounds were dissolved in 1 mg/mL DMSO and as stock solutions. Cell lines were from American Type Culture Collection (ATCC), HL-60 human promyelocytic leukemia, HT-1080 human fibrosarcoma, and LoVo human intestinal adenocarcinoma factor cell lines. The LoVo/Doxo human intestinal adenocarcinoma cell lines expressing the *mdr* factor was a kind gift.

Exponentially growing HL-60 leukemia cells were suspended at a density of 1×10^5 cells/mL in a complete medium (RPMI 1640 containing 10% fetal bovine serum, 100 UI/mL penicillin G, and 100 μg/mL streptomycin). Cell viability was determined by the MTT method[18] 72 h later. LoVo and LoVo/Doxo[8,19] were cultured in Ham's F12 medium (Sigma); HT-1080 cells were grown in DMEM medium containing 10% fetal bovine serum, 100 UI/mL penicillin G, and 100 μg/mL streptomycin.

Activities against cell lines derived from solid tumors were evaluated in exponentially growing cultures seeded at 5×10^4 cells/mL, which were allowed to adhere for 18 h to culture plates before addition of drugs. Cell viability was determined as described above 72 h later. Tumor cell growth at each drug concentration was expressed as percentage of untreated controls, and the concentration resulting in a 50% growth inhibition (IC_{50}) was determined by linear regression analysis.

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