Cytotoxic Compounds from *Polygala vulgaris*

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To search for antitumor agents from plants, we studied *Polygala vulgaris* since cytotoxic lignans are known to occur in some *Polygala* species. Preliminary data on plant petrol ether, chloroform, and methanol extracts from the roots and aerial parts, showed *in vitro* cytotoxic activity against the solid tumor LoVo cell line. Fractionation of the active extracts led to the isolation of three new compounds, a derivative of aucuparine and two xanthones, as well as a known methylsinapate. All compounds were tested for *in vitro* cytotoxic activity using two cell lines, LoVo and its strain, which express resistance to common antitumor agents.

Key words *Polygala vulgaris*; cytotoxic activity; xanthone; LoVo; multidrug resistance

Members of Polygalaceae are known to contain a variety of different chemical classes, many of which exhibit significant biological activity. Previous phytochemical investigations on different *Polygala* species yielded several compounds, including cytotoxic lignans,[1—4] xanthones,[5—11] and styrylpyrones.[12] With the aim of searching for antitumor agents from plants, we studied *Polygala vulgaris* L., a species not previously investigated from this point of view.

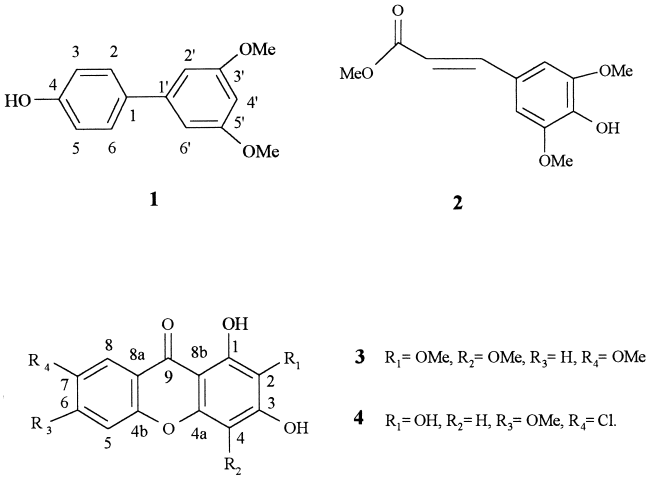
Petroleum ether, chloroform, and methanol extracts were prepared from the roots and the aerial parts of the plant and successively fractionated. The extracts and the newly isolated compounds were tested for cytotoxic activity. The roots and aerial parts of *P. vulgaris* were extracted with solvents of increasing polarity and the cytotoxic activity against the LoVo cell line was evaluated on the residues obtained after solvent removal. The petroleum ether and chloroform extracts from both the roots and aerial parts were active. The chloroform extracts showed the highest activity, with IC_{50} values of 40.0 and 63.4 μg/ml for the aerial parts and roots, respectively.

The chloroform extracts yielded compounds **1**—**4**, although the petroleum ether extracts did not yield any compound because of the low amounts present. Chloroform extracts from both the roots and aerial parts yielded compound **1**. The high resolution (HR)-MS [atmospheric pressure ionization-time of flight (API-TOF)] spectrum showed a molecular ion $[M+H]^+$ at *m/z* 231.1077, indicating a molecular formula of $C_{14}H_{14}O_3$. The complete structure could be elucidated by 1D- and 2D-NMR spectroscopic experiments. The ^{1}H-NMR spectrum showed two *ortho*-coupled aromatic signals at δ 7.42 and 6.88 (each 2H, J=8.4 Hz); two *meta*-coupled signals δ 6.67 (2H) and 6.42 (1H) were observed. The correlation spectroscopy-double quantum filtered (COSY-DQF) spectrum confirmed the coupling patterns. A methoxy signal (δ 3.90, 6H) was also observed. Assignments of all ^{1}H- and ^{13}C-NMR spectroscopic signals were based on ^{1}H-detected heteronuclear multiple quantum coherence (HMQC) and heteronuclear multiple bond connectivity (HMBC) experiments. Long-range correlations in the HMBC spectrum were observed between the proton signal at δ 7.42 and carbon signals at δ 155.8 (C-4) and 143.4 (C-1′). The same was observed between proton δ 6.88 and carbon signals δ 134.3 (C-1). Other long-range correlations were observed between proton signals at δ 6.67 and carbon at δ 99.1 (C-4′) and 134.3 (C-1). Further correlation was obtained from the methoxy signal at δ 3.90 and carbon at δ 161.4.

Compound **1** was identified as 3′,5′-dimethoxybiphenyl-4-ol, an isomer of aucuparin, a phytoalexin isolated for the first time from *Sorbus aucuparia* (L.).[13] All data were in agreement with those reported in the literature for similar biphenyl compounds.[13,14] This compound had not been previously isolated from natural sources to the best of our knowledge.

Compound **2** was obtained as a yellow powder from the chloroform root extract and was identified by UV, ^{1}H-, ^{13}C-NMR and MS spectra as methylsinapate ($C_{12}H_{14}O_5$). All NMR data were in agreement with those in the literature.[15] This compound has not previously been reported from *Polygala* species.

Compound **3** was isolated from the aerial parts as a yellow powder. The electron impact (EI)-MS spectrum (70 eV, positive ion) showed a molecular ion $[M]^+$ at *m/z* 318; HR-MS (API-TOF) yielded a molecolar ion $[M+Na]^+$ at *m/z* 341.0667, indicating a molecular formula of $C_{16}H_{14}O_7$. The UV spectrum showed the typical behavior of a xanthone nucleus.[16] The ^{1}H-NMR spectrum showed a *meta*-coupled doublet at δ 7.60 (J=1 Hz), an *ortho*-coupled doublet at δ 7.46 (J=9 Hz), and a doublet of doublets at δ 7.30 coupled with both protons. Signals of three methoxy groups were also present. The signal at δ 12.50 suggested the presence of a



1 **2**

3 R_1= OMe, R_2= OMe, R_3= H, R_4= OMe

4 R_1= OH, R_2= H, R_3= OMe, R_4= Cl.

Chart 1. Structures of Isolated Compounds

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Table 1. IC_{50} Values (μM) for Compounds **1**—**4** and Doxorubicin against LoVo and LoVo/Doxo Cell Lines

Compound	IC_{50} (μM)	
	LoVo	LoVo/Doxo
1	>40	ND
2	>40	ND
3	34.6±2.25	39.5±1.80
4	8.30±0.09	6.70±0.40
Doxorubicin	0.04±0.005	10.2±0.10

ND, not determined.

Table 2. NMR Data for Compounds **3** and **4**. Numbers in parentheses are coupling constants in Hz

Position	Compound **3** ($CDCl_3$)		Compound **4** (CD_3OD)	
	δ ^{1}H	δ ^{13}C	δ ^{1}H	δ ^{13}C
1		150.1		153.8
2		132.0		132.1
3		146.5		151.7
4		135.0	6.23 s	96.1
4a		150.1		159.8
4b		153.2		154.6
5	7.46 d (9.0)	119.1	6.52 s	104.0
6	7.30 dd (9.0, 1.0)	124.0		149.7
7		154.1		164.9
8	7.60 d (1.0)	109.1	7.33 s	107.8
8a		120.2		106.7
8b		105.1		100.0
9		181.8		178.1
Methoxy 2	3.95 s	61.1		
Methoxy 4	3.94 s	62.0		
Methoxy 7	4.12 s	56.6	3.85 s	56.1
OH 1	12.50 s			
OH 2	6.10 s			

chelated hydroxyl proton with a carbonyl group. The signal at δ 6.10 was assigned to the hydroxyl group in C-3. On the basis of the HMQC experiment, protonated carbons were assigned. By long-range correlation in the HMBC experiment, the complete skeleton of the nucleus was assigned. Diagnostic long-range correlations were observed between the proton signal at δ 7.60 and carbon resonances δ 153.2 (C-4b) and 124.0 (C-6). Further correlation between the proton signal at δ 7.46 and carbon signals at δ 120.2 (C-8a) and 154.1 (C-7) was found. The methoxy position could also be elucidated on the basis of the HMBC correlations between the proton signals at δ 3.94, 3.95, and 4.12 and the respective carbon signals at δ 135.0, 132.0, and 154.1. Positions 1, 3, 4a, and 9a were assigned based on data obtained from the nuclear Overhauser effect spectroscopy (NOESY) experiment and confirmed by ^{13}C spectral and literature data for polyoxygenated xanthones.[6—8] The NOESY experiment showed peaks between the phenolic proton at δ 12.50 and the methoxy group at δ 3.95, while both methoxy groups (δ 3.94, 3.95) showed a correlation with the C-3 phenol proton (δ 6.10). On the basis of its spectral data, compound **3** was characterized as 1,3-dihydroxy-2,4,7-trimethoxyxanthone, a new xanthone derivative.

Compound **4** was obtained from the chloroform extract of the aerial parts as a yellow powder. The UV spectrum showed typical behavior of xanthones.[16] The mass spectrum of compound **4** with a characteristic isotopic pattern supported the presence of chlorine. We observed a molecular ion $[M]^+$ at *m/z* 308.0103, corresponding to $[C_{14}H_9O_6\ ^{35}Cl]$. The ^{1}H-NMR spectrum showed three singlets in the aromatic region (δ 6.23, 6.52, 7.33). Protonated carbons were assigned based on the HMQC spectrum. In the HMBC spectrum, diagnostic correlations were observed between the signal at δ 7.33 and carbon resonances at δ 154.6, 149.7, and 178.1. Further long-range correlations yielded the complete structural assignment. Thus compound **4** was 7-chloro-1,2,3-trihydroxy-6-methoxyxanthone, a novel chloroxanthone derivative. The presence of chloroxanthones in higher plants was first reported in *Hypericum ascyron* by Hu *et al.*[17]

The antiproliferative activity of the isolated compounds was evaluated against the human intestinal adenocarcinoma cell line LoVo and its drug-resistant subclone LoVo/Doxo.[18] Doxorubicin hydrochloride was used as a reference. As shown in Table 1, compounds **1** and **2** are devoid of antiproliferative activity, whereas the two xanthones (**3**, **4**) showed significant activity. In particular, the maximum activity in compound **4** can be correlated with the presence of a chlorine atom and also with three adjacent phenolic hydroxyl groups as observed by Inoue *et al.*[19] for gallic acid.

The antiproliferative activity of the two xanthones was also evaluated against the LoVo subclone resistant to doxorubicin and other intercalating drugs such as MX. This cell line also altered the level of topoisomerase (Topo II) expression.[20] Interestingly, compounds **3** and **4** proved to inhibit the growth of this cell line completely, suggesting that their activity does not interfere with DNA synthesis by affecting the Topo II-catalyzed step.

Experimental

The instruments and materials used in this investigation were as follows: Büchi B-811 as an extraction system; Bruker AMX 300 for NMR spectra; Perkin Elmer lambda 25 for UV spectra and Merck silica plates for analytical (cat. 1.05715) and preparative (1.05717) TLC. HPLC analysis of isolated compounds was performed on a Shimadzu system with a UV detector. A Spherisorb C18 (5 μm, 4.6×250 mm i.d.) column was used for analysis. Acetonitrile : water/aqueous acetic acid 5% (1 : 1) was used as the mobile phase (1 ml/min).

Plant Materials The plant materials were collected in June 2001, at Follina (TV) (Italy) (a voucher is deposited at the Botanical Garden of the University of Padova, no. 42—73).

Cytotoxic Activity The extracted residues, after solvent removal under a vacuum, were dissolved in DMSO at a concentration of 20 mg/ml. Stock solutions were used to prepare diluted solutions used in tests.

Activity against the cell lines was evaluated in experimentally growing cultures seeded at 5×10^4 cell/ml which were allowed to adhere to culture plates for 18 h before adding the compounds.

Cell viability was determined using the MTT method[21] 72 h later. Tumor cell growth at each drug concentration was expressed as a percentage of that of untreated controls, and the concentration resulting in 50% growth inhibition (IC_{50}) was determined by linear regression analysis.

Extraction Procedure The air dried-roots (9.17 g) and aerial parts (63.95 g) were separated and ground. The plant materials were exhaustively extracted in a Soxhlet apparatus with solvents (petroleum ether, chloroform, and methanol) of increasing polarity. The solvents were removed under a vacuum. The yields, in percentage of weight residue of the weight of dry material extracted, were: petroleum ether 0.83% (roots) and 1.29% (aerial parts); chloroform 3.56% (roots) and 3.49% (aerial part); and methanol 30.7% (roots) and 18.9% (aerial parts).

Fractionation of Chloroform Extract from Roots The residue (0.8 g) was repeatedly chromatographed on silica gel plates using chloroform, chloroform–methanol 2%, and ethyl acetate–cyclohexane in different ratios (1 : 2

and 1 : 1; v/v) as eluents. Compounds **1** and **2** were isolated and purified. The purity of isolated compounds was further checked by HPLC as described.

Fractionation of Chloroform Extract from Aerial Parts Chloroform extract (2 g) was suspended in aqueous methanol (10%) and extracted with *n*-hexane, chloroform, and ethyl acetate. The hexane fraction contained mainly an oily residue made up of chlorophylls. The chloroform and ethyl acetate fractions were collected on the basis of their chromatographic behavior. The residue obtained was then repeatedly chromatographed on silica gel plates and yielded compounds **3** and **4**. The purity of isolated compounds was further examined by HPLC as described.

Compound **1**, 3′,5′-Dimethoxy-biphenyl-4-ol, white powder (7.6 mg), $C_{14}H_{14}O_3$ UV (EtOH) λ_{max} [210, 227, 265 nm; (NaOH) 211, 293 nm; HR-MS API-TOF *m/z* 231,1077 $M+H]^+$. ^{1}H-NMR ($CDCl_3$) δ: 7.42 (2H, d, J=8.4 Hz, 2-H, 6-H), 6.88 (2H, d, J=8.4 Hz, 3-H, 5-H), 6.67 (2H, d, J=1 Hz, 2′-H, 6′-H), 6.42, (1H, d, J=1 Hz, 4′-H), 3.90 (6H, s); ^{13}C-NMR δ: 161.4 (C-3′-5′); 155.8 (C-4), 143.4 (C-1′), 134.3 (C-1), 128.8 (C-2-6), 115.9 (C-3-5), 99.1 (C-4′), 55.8 (OCH_3). t_R: 9.46 min.

Compound **2**, 3-(4-Hydroxy-3,5-dimethoxy-phenyl)-acrylic acid methyl ester (methylsinapate), slightly yellow powder (5.4 mg), $C_{12}H_{14}O_5$. HR-MS API-TOF *m/z* 239,0967 $[MH]^+$. ^{1}H-NMR ($CDCl_3$) δ: 7.61 (1H, d, J= 15.95 Hz), 6.80 (2H, s), 6.35 (1H, d, J=15.95 Hz); 3.90 (6H, s), 3.84 (3H, s). ^{13}C-NMR (168.0 (C-9), 148.1 (C-3, C-5), 145.0 (C-8), 138.4 (C-4), 126.1 (C-1), 116.0 (C-7), 106.0 (C-2, C-6), 56.1 (OCH_3-5) 51.0 (OCH_3 ester) identical to lit.[15] t_R: 5.27 min.

Compound **3**, 1,3-Dihydroxy-2,4,7-trimethoxyxanthone was obtained as a yellow solid (4.5 mg), $C_{16}H_{14}O_7$. UV (EtOH) λ_{max} 236, 264, 301, 386 nm, (NaOH) 236, 422 nm. HR-MS API-TOF *m/z* 341.0667 $[M+Na]^+$; MS EI (70 eV) *m/z* 318 $[M]^+$; ^{1}H- and ^{13}C-NMR data are shown in Table 2. t_R: 9.87 min.

Compound **4**, 7-Chloro-1,2,3-trihydroxy-6-methoxyxanthone was obtained as a yellow solid (4.2 mg), $C_{14}H_9O_6Cl$. HR-MS API-TOF *m/z* 308.0103 $[C_{14}H_9O_6\ ^{35}Cl]^+$; ^{1}H- and ^{13}C-NMR data are shown in Table 2. t_R: 6.06 min.

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