Xanthones from *Polygala alpestris* (Rchb.)

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Bioactivity-guided fractionation of *Polygala alpestris* L. (Rchb.) extracts led to the identification of two new xanthones, 1,3,7-trihydroxy-2,6-dimethoxyxanthone (**1**) and 2,3-methylenedioxy-4,7-dihydroxyxanthone (**2**). In addition five known compounds 3,4-dimethoxy-1,7-dihydroxyxanthone (**3**), 1,3-dihydroxy-7-methoxyxanthone (**4**), 1,7-dihydroxy-2,3-dimethoxyxanthone (**5**), 3′,6-*O*-disinapoyl sucrose (**6**) and 3′,5′-dimethoxybiphenyl-4-olo (**7**) were isolated. The structures of the isolated compounds were established by means of high resolution mass spectrometry, mono- and bi-dimensional NMR spectroscopy. All isolated compounds were tested for cytotoxic activity against three tumor cell lines (LoVo, HL-60, K 562).

Key words: Polygala alpestris, Xanthones, Cytotoxicity

Introduction

The genus *Polygala* is widely distributed in almost all parts of the world, and many of the species are used in folk medicine because of their content of triterpenic saponins. In addition many *Polygala* species are well known for producing several classes of secondary metabolites having biological activities like xanthones (Dreyer, 1969; Pinheiro *et al.*, 1998; Dall'Acqua *et al.*, 2002; Rodrigo *et al.*, 2003) and aryltetrahydronaphthalene lignans (Hoffmann, *et al.*, 1977; Hokanson, 1978). In a previous paper we described the isolation and characterization of cytotoxic xanthones from *Polygala vulgaris* L. (Dall'Acqua *et al.*, 2002).

Here, we report on the isolation and structure elucidation of *Polygala alpestris* Rchb. (Polygalaceae) constituents, a species not previously investigated from this point of view. Sequential extracts were prepared from both dry roots and aerial parts using solvents with increasing polarity, in particular petroleum ether, chloroform and methanol. The extracts obtained were used to perform preliminary cytotoxicity tests against a solid tumour human intestinal adenocarcinoma cell line (LoVo). The chloroform extracts from both roots and aerial parts were active as shown in Table I.

The phytochemical investigations were carried out on active extracts and yielded to the two xanthones **1** and **2** and the five known compounds **3**, **4**, **5**, **6** and **7**.

Table I. IC_{50} values for *P. alpestris* extracts against LoVo cell line.

Extract	IC_{50} [μg/ml]	
	Aerial parts	Roots
Petroleum ether	50.0 ± 3. 5	150.0 ± 3.50
Chloroform	36.1 ± 2.18	100.1 ± 0.99
Methanol	> 200	> 200

The cytotoxic activity of the isolated compounds **1**–**7** was measured against three cell lines, HL-60 human promyelocytic leukaemia, K 562 human erythro leukaemia, and LoVo human adenocarcinoma cells. The most active compound **2** was also tested against LoVo/Doxo cell line (human adenocarcinoma expressing the *mdr* factor).

Materials and Methods

General

The UV spectra were recorded on a Perkin Elmer lambda 25 spectrometer. NMR spectra were recorded in CD_3OD on a Bruker AMX 300. The mass spectra (HR-MS) were recorded on a Mariner Biosystem (API-TOF) mass spectrometer. Column chromatography was performed using Merck Silica Gel 60 (0.040–0.063 mm, 230–400 mesh) and Sephadex® LH 20. TLC was performed with silica gel 60 F_{254} (Merck , cat. 5715 and 5717).

Plant material

Polygala alpestris (Rchb.) was collected in June 2002, at Monte Bondone, Trento (Italy); a voucher have been deposited at the Botanical Garden of the University of Padova (n° 48–72).

Extraction and isolation

Air-dried roots (10.6 g) and aerial parts (78.2 g) were separated and ground. The vegetal materials were extracted at room temperature using solvents of increasing polarity, namely petroleum ether, chloroform and methanol for one week each. The solvents were removed under vacuum. The yields in weight percent of residue referring to the weight of dry material extracted were as follows: petroleum ether 1.4 (aerial parts) and 0.9 (roots), chloroform 3.5 (aerial parts) and 2.0 (roots), and methanol 35 (aerial parts) and 20 (roots).

The chloroform extract obtained from aerial parts (1.5 g) was chromatographed on a silica gel column (300 ml) using $CHCl_3$ (300 ml) and mixtures of $CHCl_3$/MeOH in increasing ratios (from 90:10 to 50:50 v/v) as eluents. Fractions collected were pooled on the basis of their chromatographic behaviour in five groups. Further chromatographic steps on silica gel plates yielded compounds **1** (6.5 mg), **2** (3.6 mg), **3** (4.2 mg) and **4** (4.3 mg).

The chloroform extract obtained from roots (370 mg) was fractionated on a Sephadex column (100 ml) and eluted with MeOH. Fractions were collected on the basis of their chromatographic behaviour and the residues repeatedly chromatographed on silica gel plates using $CHCl_3$ and mixtures of toluene/EtOEt, toluene/$(CH_3)_2CO$, EtOAc/cyclohexane and $CHCl_3$/MeOH in different ratios as eluents. Compounds **2** (3.3 mg), **3** (3.1 mg), **4** (8.6 mg), **5** (7.6 mg), **6** (8.2 mg) and **7** (9.2 mg) were isolated.

Spectroscopic data

1,3,7-Trihydroxy-2,6-dimethoxyxanthone (**1**): Yellow powder. – UV(EtOH): λ_{max} = 236, 255, 321, 364 nm. – HR-MS API-TOF: *m/z* = 305.0664 $[M+H]^+$ (calcd. for $C_{15}H_{12}O_7$ + H 305.0656). – ^{1}H NMR and ^{13}C NMR: data are shown in Table II.

2,3-Methylenedioxy-4,7-dihydroxyxanthone (**2**): Light yellow powder. – UV(EtOH): λ_{max} = 249, 297, 327, 379 nm. – HR-MS API-TOF: *m/z* = 273.0402 $[M+H]^+$ (calcd. for $C_{14}H_8O_6$ + H 273.0399). – ^{1}H NMR and ^{13}C NMR: data are shown in Table II.

Compounds **3**–**7**, were characterized by comparison of their spectral data (MS, NMR) with those reported in literature (Yang *et al.*, 2001; Pinheiro *et al.*, 1998; Miyase *et al.*, 1999; Dall'Acqua *et al.*, 2002).

Measurement of cytotoxic activity

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

Activity against cell lines was evaluated as we previously described (Dall'Acqua *et al.*, 2002) in experimentally growing cultures seeded at 5×10^4 cells/ml which were allowed to adhere for 18 h to culture plates before adding the compounds.

Cell viability was determined by the tetrazolium salts assay (MTT) (Mosmann, 1983) 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.

Results and Discussion

Fractionation of the chloroform extracts resulted in the isolation of seven compounds, two new xanthones **1** and **2**, and five already known compounds **3**–**7** (Fig. 1).

HR-MS analysis of **1** indicated a molecular formula of $C_{15}H_{12}O_7$. ^{1}H NMR data showed three aromatic singlets at δ 7.39, 6.62 and 6.30 integrating for one protons at each; further signals at δ 3.89 and 3.83 integrating for three protons each, were also present (Table II). Protonated carbon atoms were assigned by a HMQC spectrum while the complete structure assignment was performed by a HMBC spectrum. Diagnostic long-range correlations were observed between an aromatic singlet at δ 7.39 and signals at δ 154.3 (C-4b) and 148.4 (C-6) and a ketone carbon at δ 179.6. Further long-range correlations were observed between a singlet at δ 6.62 and a carbon signal at δ 109.6 (C-8a), 161.0 (C-7), and between a signal at δ 6.30 and carbon signals at δ 131.3 (C-2) and 101.7 (C-8b). Thus compound **1** was characterized as 1,3,7-trihydroxy-2,6-dimethoxyxanthone, a new xanthone.

The mass spectrum of **2** indicated a molecular formula of $C_{14}H_8O_6$. The ^{1}H NMR spectrum showed two doublets at δ 7.50 (J = 2.0 Hz) and at

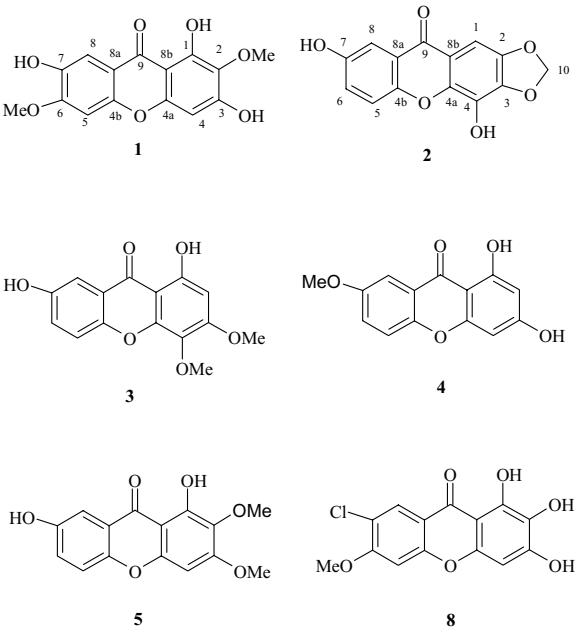

Fig. 1. Structures of isolated xanthones **1**–**5** and reference compound **8**; 1,3,7-trihydroxy-2,6-dimethoxyxanthone (**1**), 2,3-methylenedioxy-4,7-dihydroxyxanthone (**2**), 3,4-dimethoxy-1,7-dihydroxyxanthone (**3**), 1,3-dihydroxy-7-methoxyxanthone (**4**), 1,7-dihydroxy-2,3-dimethoxyxanthone (**5**) and 1-chloro-2-methoxy-6,7,8-trihydroxyxanthone (**8**).

7.46 (J = 9.0 Hz) and a doublet of doublets at δ 7.27 (J = 9.0, 2.0 Hz); in addition two singlets were observed at δ 6.58, integrating for one proton, and 6.0 integrating for two protons, the latter suggesting the presence of a dioxymethylene group. The group is linked to carbons C-2 and C-3 which was established by the long-range correlation between the singlet at δ 6.0 and carbon signals at δ 153.0 (C-2) and 129.0 (C-3) and the signal at δ 6.58 and a carbon signal at δ 129.0 (C-3). Thus compound **2** was identified as a new natural xanthone, 2,3-methylenedioxy-4,7-dihydroxyxanthone.

Compounds **3**–**5** were identified by NMR experiments as 3,4-dimethoxy-1,7-dihydroxyxanthone (**3**), 1,3-dihydroxy-7-methoxyxanthone (**4**) and 1,7-dihydroxy-2,3-dimethoxyxanthone (**5**), respectively. Their proton and carbon assignments are in accordance with published data (Yang *et al.*, 2001; Pinheiro *et al.*, 1998; Dall'Acqua *et al.*, 2002).

Structures of the isolated 3′,6-*O*-disinapoyl sucrose (**6**) and 3′,5′-dimethoxybiphenyl-4-olo (**7**) were elucidated by comparing the results of the NMR experiments with literature data (Miyase *et al.*, 1999; Dall'Acqua *et al.*, 2002).

Table II. ^{1}H NMR and ^{13}C NMR data of compounds **1** and **2** in CD_3OD; numbers in parenthesis are coupling constants in Hz.

Position	Compound **1**		Compound **2**	
	δ ^{1}H	δ ^{13}C	δ ^{1}H	δ ^{13}C
1	–	154.0	6.58 s	90.2
2	–	131.3	–	153.0
3	–	160.2	–	129.0
4	6.30 s	95.9	–	150.1
4a	–	153.9	–	155.2
4b	–	154.3	–	151.1
5	6.62 s	104.5	7.46 d (9.0)	120.8
6	–	148.4	7.27 dd (9.0, 2.0)	126.6
7	–	161.0	–	155.4
8	7.39 s	104.9	7.50 d (2.0)	109.0
8a	–	109.6		110.7
8b	–	101.7		106.3
9	–	179.6		174.6
2-OCH_3	3.83 s	61.1		
6-OCH_3	3.89 s	56.5		
3-OCH_3	–	–		
O-CH_2-O	–	–	6.0 s	104.8

The antiproliferative activity of the isolated compounds was evaluated against leukaemic line. In addition the human intestinal adenocarcinoma cell line LoVo and the drug-resistant subclone LoVo/Doxo (Grandi *et al.*, 1986) were used (this latter only for compound **2**). Doxorubicin hydrochloride was used as a reference. Results are documented in Table III. The cytotoxic activity of the isolated compounds was low compared to doxorubicin hydrochloride. The xanthone **2** resulted as the most active isolated compound, and its cytotoxic effect was also measured against the LoVo/Doxo cell line. This compound was found less active than 1-chloro-2-methoxy-6,7,8-trihydroxyxanthone (**8**) (Dall'Acqua *et al.*, 2002). However, the residual activity against the LoVo/Doxo cell line suggested that activity of **2** does not interfere with DNA synthesis *via* the Topo II catalytic step.

The resistance index calculated for doxorubicin was 66.0 whereas for compound **2** resulted 1.3 indicating that this compound is in part able to overcome the mechanism of resistance.

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Table III. IC_{50} values for compounds **1**-**7** against HL-60, LoVo, K 562, and LoVo/Doxo cell lines; doxorubicin hydrochloride and 1-chloro-2-methoxy-6,7,8-trihydroxyxanthone (**8**) were used as reference compounds.

Compound	IC_{50} [μM] K 562	IC_{50} [μM] HL-60	IC_{50} [μM] LoVo	IC_{50} [μM] LoVo/Doxo
1	35.9 ± 3.1	36.5 ± 2.5	> 40	–
2	27.0 ± 2.6	> 40	15.5 ± 0.11	19.5 ± 0.14
3	> 40	31.9 ± 3.0	31.0 ± 4.2	–
4	> 40	21.6 ± 3.1	> 40	–
5	20.7 ± 21	24.1 ± 2.3	> 40	–
6	> 40	> 40	29.7 ± 2.4	–
7	20.5 ± 4.0	22.7 ± 1.9	35.6 ± 1.8	–
8	–	–	8.30 ± 0.09	6.7 ± 0.14
Doxorubicin hydrochloride	0.01 ± 0.005	0.01 ± 0.007	0.20 ± 0.06	13.2 ± 0.01

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