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Halina Wysoki#ska, Zuzanna Skrzypek, and Jolanta Kunert-Radek

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STUDIES ON IRIDOIDS OF TISSUE CULTURES OF *PENSTEMON SERRULATUS*: ISOLATION AND THEIR ANTIPROLIFERATIVE PROPERTIES

HALINA WYSOKIŃSKA,* ZUZANNA SKRZYPEK,

Department of Botany, Institute of Environmental Research and Bioanalysis, Medical Academy, Muszyńskiego 1, PL 90-151 Łódź, Poland

and JOLANTA KUNERT-RADEK

Institute of Endocrinology, Medical Academy, Dr Sterling 3, PL 91-425 Łódź, Poland

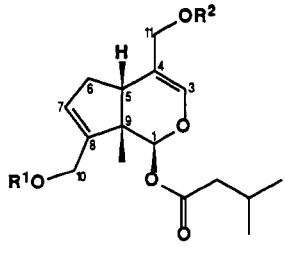
ABSTRACT.—Penstemide [**1**] and serrulatoloside [**2**] as well as penstemide aglycone [**3**] and serrulatoloside aglycone [**4**] were isolated and identified in the calli and suspension cultures of *Penstemon serrulatus*. The influence of serrulatoloside, penstemide, and its aglycone on the spontaneous proliferation of mouse spleen lymphocytes or hepatoma cells in the Syrian hamster has been estimated in vitro. It has been found that these compounds produce a dose-dependent inhibition of [^{3}H]-thymidine incorporation into the DNA of the examined cells.

The genus *Penstemon* Mitch belongs to the Scrophulariaceae and comprises about 300 species (1) widespread in North America. From *Penstemon* several valeriana-type ester iridoids have been isolated (2–7). Some of them have been found to be biologically active (8,9). For example, penstemide [**1**] first reported by Jensen *et al.* (10) from *Penstemon deustus* Dougl. has been described as having cytotoxic properties (8). In order to obtain an alternative source of valeriana-type iridoids we initiated tissue cultures of *Penstemon serrulatus* Menz. We reported previously on the growth and iridoid production by calli and suspended-cells of *P. serrulatus* (11–13).

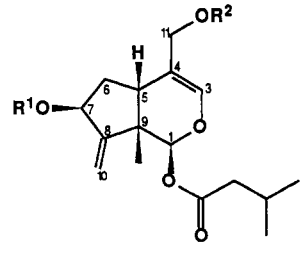
Continuing our investigations on *P. serrulatus* tissue culture, we have now described the isolation and identification of iridoids from static and liquid cultures of this species. We have also tested the influence of penstemide [**1**], serrualatoloside [**2**], and penstemide aglycone [**3**] on the spontaneous proliferation of mouse spleen lymphocytes or hepatoma cells in the Syrian hamster in vitro. [^{3}H]-thymidine incorporation into DNA was used as a sensitive index of cell proliferation.

RESULTS AND DISCUSSION

Tlc investigation revealed the presence of two major and three minor iridoid components in the aqueous-alcoholic extract of calli and suspension-cultured cells of *P. serrulatus*. In order to isolate them, the aqueous-alcoholic extract was successively extracted with petroleum ether and EtOAc. The residue was then chromatographed over a Si gel column. Two main fractions (polar and nonpolar) were obtained. From the polar



1 R^1=H, R^2=glc
3 R^1=R^2=H
5 R^1=R^2=Ac



2 R^1=H, R^2=glc
4 R^1=R^2=H
6 R^1=R^2=Ac

fraction two products were isolated and identified as penstemide [**1**] and serrulatoloside [**2**]. The identification of these iridoids was made on the basis of physical (optical rotations) and spectroscopic (ir, ^{1}H nmr, ms) properties and by comparison of their data with published values (6,7). The third component of the polar fraction was obtained in quantities too small for identification. Results from our previous paper (12) showed that cultured cells growing under optimized conditions produced higher amounts of penstemide and serrulatoloside (up to ca. 4% and 2% on a dry wt basis, respectively, after 2 years of periodic subculture) than the leaves of intact *P. serrulatus* plants. The latter accumulated ca. 3% of penstemide and 1.4% (dry wt) of serrulatoloside (11).

The residue of the nonpolar fraction contained two compounds, **3** and **4**, which showed on tlc, after detection with phloroglucinol-concentrated HCl, spots of the same colors (brown and blue) as the mixture of **1** and **2** did. Enzymatic cleavage with β-glucosidase of **1** and **2** resulted in glucose and the mixture of the corresponding aglycones with identical R_f values and colors to **3** and **4** after detection on tlc. Also ir spectra of **3** and **4** were identical to those of the penstemide and serrulatoloside aglycones obtained after enzymatic hydrolysis. Further confirmation was afforded by acetylation of **3** and **4**. Acetylation gave the more stable acetyl derivatives **5** and **6**. As ms fragmentations and ir spectral data of **5** and **6** were similar, their structural assignments were based mainly on ^{1}H-nmr spectral data (see Experimental). The ^{1}H-nmr spectra of **5** and **6** revealed that compounds **3** and **4** were penstemide and serrulatoloside aglycones, respectively. This is the first report of occurrence of these compounds in cultures of *P. serrulatus*. Also, the chemical investigation carried out by Junior has not led to the isolation of **3** and **4** from the leaves of this species (5–7). Our studies of intact plants (leaves and flowers) of *P. serrulatus* for aglycones of penstemide and serrulatoloside showed that these compounds were found only in flowers of this plant (unpublished data). Because all materials have been extracted under identical conditions this suggests that the aglycones isolated are not derived by hydrolysis of **1** and **2** during the extraction procedure but occur as natural substances. This is in accordance with the observation of Gering and co-workers (2,4), who have been able to isolate penstemide and serrulatoloside aglycones as genuine compounds from leaves of *Penstemon richardsonii* Dougl.

The compounds isolated from tissue cultures of *P. serrulatus* (serrulatoloside, penstemide, and its aglycone) were tested for their antiproliferative activity as described in Experimental. There was no noticeable difference in this activity between penstemide and serrulatoloside. Both glucosides suppressed [^{3}H]-thymidine uptake incorporation into DNA of the examined cell lines (mouse spleen lymphocytes and hamster hepatoma cells) in a dose-dependent manner at concentrations of 10^{-4} and 10^{-5}M (Tables 1 and 2). This is the first time that serrulatoloside has been reported to

TABLE 1. The Effect of Penstemide [**1**] and Serrulatoloside [**2**] on [^{3}H]-thymidine Incorporation into DNA of Mouse Spleen Lymphocytes in vitro.

Compound	n[a]	cpm/10^6 cells ± SD	% Inhibition	Significance
Control	6	63.516 ± 6.455		
Penstemide [**1**]				
10^{-6} M	3	61.360 ± 6.795	4	n.s.
10^{-5} M	3	48.503 ± 1.923	24	$P<0.05$
10^{-4} M	3	26.310 ± 832	59	$P<0.01$
Serrulatoloside [**2**]				
10^{-6} M	3	58.282 ± 3.466	9	n.s.
10^{-5} M	3	53.714 ± 7.507	16	n.s.
10^{-4} M	3	35.400 ± 2.649	45	$P<0.01$

[a]n = number of determinations.

TABLE 2. The Effect of Penstemide [**1**] and Serrulatoloside [**2**] on [^{3}H]-thymidine Incorporation into DNA of Hepatoma Cells in the Syrian Hamster.

Compound	n[a]	cpm/0.5 × 10^6 cells ± SD	% Inhibition	Significance
Control	3	37.738 ± 1.922		
Penstemide [**1**]				
10^{-5} M	3	33.572 ± 529	12	$P<0.05$
10^{-4} M	3	11.936 ± 2.204	69	$P<0.001$
Serrulatoloside [**2**]				
10^{-5} M	3	31.310 ± 1.378	18	$P<0.05$
10^{-4} M	3	9.787 ± 1.742	75	$P<0.001$

[a]n = number of determinations.

reveal antiproliferative activity. Penstemide was previously found to have cytostatic activity against the P388 lymphocytic leukemia system (8). This report taken along with the present data suggests that the effect of penstemide is due to interference with DNA synthesis. Our experiments were also performed on penstemide aglycone. Its antiproliferative action was studied on the mouse spleen lymphocytes cell line. The inhibitory effect of aglycone was slightly more potent than that of the parent glucoside: the data presented in Table 3 indicate a significant inhibition of incorporation of [^{3}H]-thymidine into DNA at the concentrations 10^{-4}–10^{-6}M.

TABLE 3. The Effect of Penstemide Aglycone [**3**] on [^{3}H]-thymidine Incorporation into DNA of Mouse Spleen Lymphocytes In Vitro.

Compound concentration (M)	n[a]	cpm/10^6 cells ± SD	% Inhibition	Significance
0	3	3.621 ± 199		
10^{-6}	3	3.090 ± 177	15	$P<0.05$
10^{-5}	3	2.704 ± 268	25	$P<0.02$
10^{-4}	3	1.430 ± 121	60	$P<0.001$

[a]n = number of determinations.

The present experiments suggest that valeriana-type ester iridoids show activity not only in the form of aglycones, like iridoids studied by Isiguro *et al.* (14), but also in the form of glucosides. The inhibitory activity of the valeriana-type iridoids may be partially connected with esterification with isovaleric acid on the hydroxy group at C-1 position. Some authors suggest that even the esterification of one group increases liposolubility of the molecules, which allows them to penetrate into the cells and reach their activity site (15,16). A similar mechanism has been postulated for the cytotoxic action of other iridoid substances, valepotriates. The tests of proliferation capacity and viability of HTC-cultured hepatoma cells showed that these monoterpenic esters of *Valeriana* sp. having structural similarities to valeriana-type iridoids were good inhibitors of cellular growth (17,18). On the other hand, as investigated by Berger *et al.* (19), the growth of transplanted and chemically induced autochthonous rat tumors was not influenced significantly by intravenous or peroral administration of a standardized mixture of valepotriates. It is now of interest to test penstemide and serrulatoloside on different tumor cell lines in culture and on tumors in vivo. This is under current study.

EXPERIMENTAL

PLANT MATERIAL AND METHOD OF CULTURE.—Callus and suspension cultures (established from anthers of *P. serrulatus* plants grown in Botanical Garden, Warsaw) were cultivated as described earlier (11,12), and voucher specimens are deposited in the Botany Department of the Medical Academy, Lódż.

GENERAL EXPERIMENTAL PROCEDURES.—Si gel 60 (70–230 mesh, Merck) was used for cc, and Si gel F_{254} (Merck) plates were used for tlc. Solvent systems: A, Me_2CO-$CHCl_3$-H_2O (16:4:1); B, EtOAc-cyclohexane (4:1); C, Me_2CO–*n*-BuOH–H_2O (8:1:1). Spots were detected by spraying with 2% solution of phloroglucinol in EtOH followed (after heating at 100° for 2–3 min) by concentrated HCl. Hplc was carried out using a model LC-6A Shimadzu, injector Rheodyne model 7125, detector uv-vis LC-SPD 6A, column TSK C_{18} 10 μm (300 × 4.6 mm), eluent MeOH-H_2O (28:72), flow rate 2.5 ml/min, detection 220 nm. Optical rotations were measured with Perkin-Elmer 241 MC photopolarimeter. Ir spectra were recorded on a SP 200 G spectrometer. ^{1}H-nmr spectra were obtained with a Tesla (100 MHz) (for **1** and **2**) and an EM 360 NMR (60 MHz) (for **5** and **6**) spectrometer, using TMS (δ=0) as an internal standard, *J* values in Hz. Ms spectra were run on a LKB 2091 spectrometer at 70 eV ionization potential.

EXTRACTION AND ISOLATION OF IRIDOIDS.—Dried and powdered material (540 g) was extracted for two 3-h periods with boiling 80% EtOH. The combined extracts were concentrated under reduced pressure and diluted with hot H_2O. The aqueous solution was left for 24 h and then filtered. The aqueous solution was successively extracted with petroleum ether and EtOAc. The concentrated EtOAc extract (11.5 g) was chromatographed over Si gel 60 (300 g) column (100 × 4.5 cm) with EtOAc-MeOH (9:1). The elution was monitored by tlc (system A), and eluates were combined into two main fractions (polar and nonpolar).

Isolation of **1** *and* **2**.—The residue of the polar fraction (9.77 g) was further separated by cc of Si gel 60 eluting with EtOAc-cyclohexane-MeOH (75:15:1 to 15:3:2), and 164 fractions (10 ml each) were collected and combined on the basis of tlc (system A). After concentration in vacuo of combined fractions 50–94 having R_f 0.42, they yielded 480 mg of **1**. Fractions 104–164 (R_f 0.36) gave **2** (232 mg). Compounds **1** and **2** were purified by semi-preparative hplc. Spectroscopic data (od, ir, ^{1}H nmr, ms) were in agreement with literature data (6,7,12).

Isolation of **3** *and* **4**.—The residue of a nonpolar fraction (0.41 g) was chromatographed on Si gel 60 (50 g) column (60 × 2.5 cm), using EtOAc-cyclohexane (3:2 to 7:3). Fractions in 10-ml portions were collected and analyzed by tlc (system B). Fractions 6–13, R_f 0.41, were combined, and after concentration in vacuo 37 mg of **3** was obtained. Fractions 16–22, R_f 0.27, were collected and evaporated to give 10 mg of **4**. R_f values on tlc and ir spectra of **3** and **4** were compared with authentic samples of aglycones **1** and **2** obtained after enzymatic cleavage.

ENZYMATIC HYDROLYSIS OF **1** AND **2**.—A solution of **1** (25 mg) and β-glucosidase (Fluka AG) (15 mg) was kept at 36° for 2 h and then extracted with EtOAc. The EtOAc extract evaporated to dryness gave a residue which, when chromatographed on Si gel 60 (6 g) and eluted with EtOAc-MeOH (9:1), afforded pure **3** (14 mg) as a colorless oil. Similar hydrolysis with **2** (25 mg) was carried out, giving **4** (11 mg). The aqueous layers obtained from hydrolysis of **1** and **2** were evaporated to dryness, and glucose in residue was identified by co-tlc with an authentic sample (system C).

ACETYLATION OF **3** AND **4**.—Compounds **3** (14 mg) and **4** (11 mg) were treated separately with anhydrous pyridine (1 ml) and Ac_2O (1 ml) for 2 h at room temperature. After the addition of MeOH (3 ml) the solution was left for 20 min, then evaporated in vacuo to give crude **5** or **6**, which chromatographed on Si gel 60 (6 g) in cyclohexane-EtOAc (4:1) gave pure **5** (17 mg) or **6** (12.5 mg) as colorless oils. The identification of **5** and **6** was based on spectral data (ir, ms, and ^{1}H nmr).

10,11-Di-acetyl derivative of penstemide aglycone [**5**].—^{1}H nmr (60 MHz, $CDCl_3$) δ 5.75 (d, H-1, $J_{1,9}$=7), 6.40 (bs, H-3), 2.15–3.10 (m, H-5, H-6), 5.70 (bs, H-7), 2.15–3.10 (m, H-9), 4.40–4.70 (m, H-10, H-11), isovaleric acid protons 0.98 (d, J=6.5, 2 × Me), 2.06 (m, -CH_2-,-CH<), 2.10 (s, Me_3CO).

7,11-Di-acetyl derivative of serrulatoloside aglycone [**6**].—^{1}H nmr (60 MHz, $CDCl_3$) δ 5.83 (d, H-1, $J_{1,9}$=5.8), 6.36 (bs, H-3), 2.06–3.30 (m, H-5, H-6), 4.46 (m, H-7), 2.06–3.30 (m, H-9), 5.45 and 5.37 (s, H-10, =CH_2), 4.46 (m, H-11), isovaleric acid protons 1.03 (d, J=6.5, 2 × Me), 2.06 (m, -CH_2-, -CH<), 2.10 (s, CH_3CO).

ANTIPROLIFERATIVE ACTIVITY TESTS.—Influence of iridoids on [^{3}H]-thymidine incorporation into DNA of cells was investigated using the culture of mouse spleen lymphocytes or hamster hepatoma cells.

Mouse spleen lymphocyte cultures.—Male intact BALB/c mice, approximately 5–6 weeks old, housed in a room with controlled illumination (LD 12:12) and temperature (23±2°) were used as spleen donors. Spleens were aseptically removed and transferred to RPMI-1640 medium (Gibco) with 20 mM Hepes buffer (pH 7.35), penicillin (100 U/ml), and streptomycin (100 μg/ml). Single-cell suspensions from the spleens were prepared at room temperature by gentle teasing of the tissues in RPMI-1640 medium, according to Le Boeuf *et al.* (20) with our minor modification. The isolated cells were checked by

trypan blue. After 30 min of preincubation the cells were counted and resuspended in the same medium supplemented with 15% fetal calf serum (Gibco). One-ml aliquots of cell suspension, each containing 10^6 cells, were distributed into plastic tubes (12 × 75 mm, Kimble Products, Houston, Texas).

Hepatoma cell culture preparation.—Hepatoma was induced in Syrian hamsters by subcutaneous injection of 0.2 ml of Kirkman-Robbins hepatoma pulp. The animal with induced hepatoma was killed by decapitation, and the tumor was aseptically removed. The suspension of tumor cells was obtained by mechanical dispersion in RPMI-1640 medium and by enzymatic digestion with 0.4% collagenase (537 U/mg *Clostridium histolyticum* type I, Sigma) and 0.2% trypsine (Koch-Light Lab., Ltd.) according to Kedar *et al.* (21) with our slight modification. This procedure yielded a population containing more than 95% viable cells. Following 30 min of preincubation the cells were counted and resuspended in the same medium with 15% fetal calf serum (Gibco). One-ml aliquots of the cell suspension, each containing 0.5×10^6 cells, were distributed into plastic tubes.

Incubation procedure.—The investigated compounds dissolved in the culture medium at the concentrations 10^{-4}–10^{-6}M were added to the appropriate tubes. Penstemide and serrulatoloside were isolated from tissue cultures and purified as described above until the isolated products gave a single peak each on hplc. Penstemide aglycone was obtained by enzymatic hydrolysis of parent glucoside. All the tubes were incubated at 37° in humidified atmosphere of air-CO_2 (95:5). After 1 h of incubation, 2 μCi/ml of [^{3}H]-thymidine (sp. act. 28.9 Ci/mM, Amersham Centre, England) in 50 μl of RPMI-1640 medium was added. The incubation was terminated 24 h later, and the cells were washed with 2 ml cold 0.9% NaCl. The replication of DNA and the determination of radioactivity ([^{3}H] cpm/10^6 cells) were conducted according to previously published methods (22). Incorporation of [^{3}H]-thymidine into lymphocytes or tumor cells was shown to be correlated with cell growth (23). The inhibition of proliferation was determined by the formula:

$$\% \text{ inhibition} = \left[1 - \frac{\text{cpm in cultures with tested drug}}{\text{cpm in control cultures}} \right] \times 100\%$$

Student's *t*-test was used to determine the statistical significance.

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Nominations and inquiries should be received no later than March 1, 1992, and should be directed to:

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