Chemistry of Natural Compounds, Vol. 41, No. 5, 2005

FLAVONES FROM CALLUS TISSUE OF *Iris ensata*

E. V. Boltenkov,[1] V. G. Rybin,[2] and E. V. Zarembo[3]

UDC 547.972:581.143.6:582.579.2

Flavonoids uncharacteristic of intact plants were isolated from callus tissue of Iris ensata *and were identified as 5-hydroxy-4′-methoxyflavone, 5-hydroxy-3′-methoxyflavone, and 5-hydroxy-2′-methoxyflavone using PMR and mass spectrometry. It was proposed that the lack of growth of callus tissue after changing cultivation conditions was related to the inhibiting effect of these flavones on cell proliferation.*

Key words: *Iris ensata*, Iridaceae, callus tissue, 5-hydroxy-4′-methoxyflavone, 5-hydroxy-3′-methoxyflavone, 5-hydroxy-2′-methoxyflavone.

Flavonoids are the most studied group of secondary plant metabolites from the Iridaceae family [1]. Information on the contents of these compounds is used in chemotaxonomic studies of representatives of this family. The flavonoid composition is known for almost 60% of *Iris* species [2]. However, little attention has been paid to the secondary metabolites of in vitro tissue from cultivated species of this genus. It has been demonstrated that *Iris* cell cultures contain terpenoids [3, 4]. Earlier we studied the qualitative pigment composition of *I. ensata* Thunb. heterotrophic callus culture [5]. A determination of the nature of pigments from callus tissue of this plant indicated an acculumation of flavonoids. The goal of the present work was to isolate and identify red pigments from callus tissue of *I. ensata*.

Extract of *I. ensata* callus tissue contained three isomers of 5-hydroxy-monomethoxyflavone. The isolated compounds were identified as 5-hydroxy-2-(4-methoxyphenyl)-chromen-4-one (5-hydroxy-4′-methoxyflavone) (**1**), 5-hydroxy-2-(3-methoxyphenyl)-chromen-4-one (5-hydroxy-3′-methoxyflavone) (**2**), and 5-hydroxy-2-(2-methoxyphenyl)-chromen-4-one (5-hydroxy-2′-methoxyflavone) (**3**).

Compound **3** and other lipophilic flavones unsubstituted at the 7-position were observed in secretory emissions of aerial organs of plants from the genus *Primula* [6, 7]. It has been found that flavones found in leaves of *P. veris* are synthesized in in vitro cultivated tissues of this plant [8].

The results suggest that the spectrum of synthesized secondary-exchange compounds changed in callus culture of *I. ensata* when compared with the intact plants. According to the literature, intact plants of this species contain flavonoid pigments of the anthocyan [9, 10], flavone, and xanthone [11] types. However, the dominant pigments in callus tissue were lipophilic flavones **1**-**3** uncharacteristic of this plant.

Our results for the pigment composition of *I. ensata* callus tissue showed that flavone formation in tissue was suppressed in the subcultivation medium [5]. However, the change of cultivation conditions (reduced content of phytohormones, increased concentration of saccharose, change of pH) favored flavonoid accumulation and inhibited callus growth.

It was noted that the synthesis of secondary compounds in isolated instances was related to a slowing or cessation of cell proliferation [12, 13]. Apparently the flavonoids identified by us and compounds similar to them in biogenetic origin were responsible for the suppression of cell growth. An analogous conclusion was reached in an investigation of flavones isolated from *P. denticulata* that were similar in nature to **1**-**3** [14]. It was demonstrated that the flavonoids exhibited distinct cytostatic properties in an experiment with human tumor cells.

The conditions favoring formation of primarily lipophilic flavones in auxin-dependent *I. ensata* callus tissue can be considered stress factors. Such factors act as elicitors, which facilitate biosynthesis of phytoalexins [15, 16]. It was

1) Botanical Garden-Institute, Far-East Division, Russian Academy of Sciences, 690024, Vladivostok, ul. Makovskogo, 142; 2) Pacific Scientific-Research Fisheries Center, 690950, Vladivostok, per. Shevchenko, 4, fax (4232) 30 07 51, e-mail: vgrybin@tinro.ru; 3) Pacific Institute of Bioorganic Chemistry, Far-East Division, Russian Academy of Sciences, 690022, Vladivostok, pr. 100-letiya Vladivostoka, 159. Translated from Khimiya Prirodnykh Soedinenii, No. 5, pp. 440-442, September-October, 2005. Original article submitted August 3, 2005.

0009-3130/05/4105-0539

demonstrated [17-19] that flavonoid formation in cultivated in vitro plant cells and tissues changes under stressful conditions. The increased content of pigments in response to the effect of abiotic elicitors in *I. ensata* callus tissue indicates that the compounds studied by us may be phytoalexins.

EXPERIMENTAL

UV spectra of flavones were recorded in CH_3CN on a Hitachi-330 (Japan) spectrophotometer; PMR spectra, in $CDCl_3$ on an AMX spectrometer (Bruker, Germany) at working frequency 500.12 MHz relative to TMS as an internal standard ($\delta = 0$); mass spectra, in a 9000S instrument (LKB, Sweden) with electron-impact ionization (EI, 70 eV) and direct sample introduction into the source (ion-source temperature 210°C). HPLC—MS was carried out in an Agilent 1100 Series LC/MSD GC-MS (Hewlett—Packard, USA) equipped with a mass-selective detector (chemical ionization at atmospheric pressure, CIAP) and a diode-array detector and separation over a Shim-Pack FLC-ODS column (4.6×50 mm, 3 μm, Shimadzu, Japan) at 55°C. The eluent was $CH_3CN:H_2O$ (40:60) at flow rate 0.5 mL/min. The range of recorded masses was 150-1000 Da (positive-ion recording). The fragmenting potential was 75 V; ionization chamber potential, 2.5 kV; carrier-gas (N_2) flow rate, 6 L/min; and gas-sprayer (N_2) pressure, 50 kgs/cm^2. TLC was performed on Silufol plates (Kavalier, Czech Rep.) using benzene and ethylacetate. Uncolored compounds were developed on chromatograms using phosphomolybdic acid in ethanol (10%) with subsequent slow heating to 100°C.

Auxin-dependent *I. ensata* callus culture was obtained from embryos and cultivated on modified Murashige—Scoog agar nutrient medium [20] supplemented with phytohormones: 2,4-dichlorophenoxyacetic acid (1 mg/L) and 6-benzylaminopurine (0.5 mg/L) [5]. Tissues were incubated in the dark at 22-24°C and relative humidity 70%. Pigments were isolated from dark-red callus tissue that was produced by changing the composition of the subcultivation medium.

Flavone Isolation. Pigments were extracted from air-dried tissue by ethanol (96%) at 25°C. Supernatant was separated, filtered, and evaporated in vacuo at 35°C to constant weight to give a dry mixture (0.5 mg) from callus tissue (4-5 g). The mixture was placed on a silica-gel (20 g, Porokvarts PKN-200, 100-200 μm, APO, Russia) column and eluted in the following sequence: hexane (600 mL), benzene (500 mL), ethylacetate (900 mL), and ethanol (50 mL). Fractions containing mixtures with similar TLC behavior were combined and evaporated in vacuo at 35°C to constant weight. Pure flavones were isolated by preparative HPLC over a column (Zorbax ODS, 9.4×250 mm, 5 μm, Shimadzu, Japan) using $CH_3CN:H_2O:CH_3CO_2H$ (30:70:0.05) to produce chromatographically pure red compounds **1**-**3**.

Methoximes of hydroxyflavones were prepared as follows. Carbonyl compound (3-10 mg) was dissolved in a saturated solution (100 μL) of methoxyamine hydrochloride in pyridine. The reaction mixture was incubated at 40°C for 12 h, cooled to 25°C, and evaporated under Ar. The dry solid was dissolved in hexane (2 mL).

Trimethylsilyl derivatives of hydroxyflavone methoximes were prepared as follows. Compounds (3-10 mg) were dissolved in a mixture (100 mL) of N,O-bis(trimethylsilyl)trifluoroacetamide and pyridine (1:1 v/v). The reaction mixture was held for 1 h at 60°C, cooled to 25°C, treated with hexane (1 mL) and saturated aqueous NaCl (1 mL), vigorously shaken, dried over Na_2SO_4 (anhydr.), and evaporated under Ar. The dry solid was dissolved in hexane (2 mL).

Compound 1. UV spectrum (CH_3CN, λ_{max}, nm, log ε): 355 (3.9).

PMR spectrum (500 MHz, $CDCl_3$, δ, ppm, J/Hz): 3.73 (3H, s, OCH_3-7′), 6.48 (2H, dd, J = 8, J = 1.5, H-6, H-8), 6.71 (1H, s, H-3), 6.72 (2H, d, J = 8.7, H-3′, H-5′), 7.19 (2H, d, J = 8.7, H-2′, H-6′), 7.2 (1H, dd, $J_1 = J_2 = 8$, J = 8, H-7), 12.74 (1H, s, OH-5).

Mass spectra (principal ions) (*m*/*z*, I_{rel}, %): EI 268 (100) $[M]^+$, 253 (5), 240 (15), 225 (10), 197 (4), 132 (60), 117 (17), 108 (4); EI (trimethylsilyl methoxime derivative) 369 (3) $[M]^+$, 255 (25), 221 (90), 205 (50), 89 (100); CIAP 269 (100) $[M + H]^+$.

Compound 2. UV spectrum (CH_3CN, λ_{max}, nm, log ε): 355 (3.9).

PMR spectrum (500 MHz, $CDCl_3$, δ, ppm, J/Hz): 3.73 (3H, s, OCH_3-3′), 6.48 (2H, dd, J = 8, J = 1.5, H-6, H-8), 6.65 (1H, dd, $J_{4'5'} = 8$, $J_{4'6'} = 1.5$, H-4′), 6.71 (1H, s, H-3), 6.81 (1H, s, H-2′), 6.86 (1H, dd, J = 8.7, J = 1.5, H-6′), 7.1 (1H, dd, $J_{5'4'} = 8$, $J_{5'6'} = 8.7$, H-5′), 7.2 (1H, dd, $J_1 = J_2 = 8$, H-7), 12.74 (1H, s, OH-5).

Mass spectra (principal ions) (*m*/*z*, I_{rel}, %): EI 268 (100) $[M]^+$, 253 (6), 240 (12), 225 (10), 197 (5), 132 (63), 117 (15), 108 (4); EI (trimethylsilyl methoxime derivative) 369 (3) $[M]^+$, 255 (22), 221 (92), 205 (47), 89 (100); CIAP 269 (100) $[M + H]^+$.

Compound 3. UV spectrum (CH_3CN, λ_{max}, nm, log ε): 355 (3.9).

PMR spectrum (500 MHz, $CDCl_3$, δ, ppm, J/Hz): 3.73 (3H, s, OCH_3-2′), 6.48 (2H, dd, J = 8, J = 1.5, H-6, H-8), 6.54 (1H, s, H-3), 6.72 (1H, ddd, $J_{3'4'} = 8$, $J_{3'5} = 1.5$, $J_{3'6} = 0.41$, H-3′), 6.77 (1H, ddd, $J_{5'4} = 8.7$, $J_{5'6'} = 8.7$, $J_{5'3'} = 1.5$, H-5′), 7.03 (1H, ddd, $J_{4'3'} = 8.7$, $J_{4'5'} = 8.7$, $J_{4'6'} = 1.5$, H-4′), 7.19 (1H, ddd, $J_{6'5'} = 8.7$, $J_{6'4'} = 1.5$, $J_{6'3'} = 0.41$, H-6′), 7.2 (1H, dd, $J_1 = J_2 = 8$, H-7), 12.74 (1H, s, OH-5).

Mass spectra (principal ions) (*m*/*z*, I_{rel}, %): EI 268 (100) $[M]^+$, 253 (5), 240 (17), 225 (10), 197 (4), 132 (64), 117 (12), 108 (3); EI (trimethylsilyl methoxime derivative) 369 (3) $[M]^+$, 255 (27), 221 (90), 205 (46), 89 (100); CIAP 269 (100) $[M + H]^+$.

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