[Chem. Pharm. Bull. 33(9)3811—3816(1985)]

Formation of Chalcones and Isoflavones by Callus Culture of *Glycyrrhiza uralensis* with Different Production Patterns

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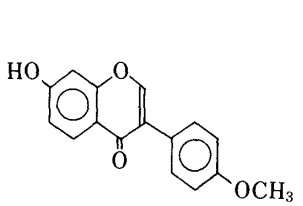
(Received December 10, 1984)

Formononetin (**1**), isoliquiritigenin (**2**), echinatin (**3**), liquiritigenin (**4**), *p*-hydroxybenzoic acid (**5**), 3′-hydroxyformononetin (**6**) and isobavachalcone (**7**) were isolated from callus culture of *Glycyrrhiza uralensis* which was established on Murashige-Skoog's medium containing naphthaleneacetic acid (NAA, 2 ppm), (2,4-dichlorophenoxy)acetic acid (2,4-D, 1 ppm) and benzyladenine (0.1 ppm). Formononetin (**1**), 3′-hydroxyformononetin (**6**) and isobavachalcone (**7**) showed different patterns of production.

Keywords——*Glycyrrhiza uralensis*; Leguminosae; tissue culture; isoflavonoids; chalcone

Introduction

Glycyrrhizae Radix, the root of *Glycyrrhiza* plants (called "licorice"), has been used as an important drug in oriental medicine, while in Europe, it has been a popular sweetener for a long time. Flavonoid constituents of licorice have been extensively studied, since the flavonoid-rich fractions showed an antigastric ulcer effect.[1,2] *Glycyrrhiza uralensis* has been shown to contain a number of flavonoids.[3,4] Among these, formononetin (**1**) has been reported to be formed in cell suspension cultures of Leguminosae plants,[5] although certain tissue cultures do not produce species-specific flavonoids. Ayabe *et al.* investigated the constituents of callus culture of *Glycyrrhiza echinata* which produced no sweet constituents, and isolated a biosynthetically unique chalcone, echinatin (**3**), along with formononetin (**1**).[6,7] Thus, it was expected that cell cultures of *G. uralensis* might accumulate isoflavonoids. Our current interest in flavonoid biosynthesis prompted us to investigate the constituents of the cell culture of *G. uralensis*. Although the kinetics of isoflavonoid production have been briefly reported by Takeya and Itokawa in tissue culture of *Puerallia lobata*,[8] it is necessary to study the time course of flavonoid production to extend our knowledge of metabolism kinetics



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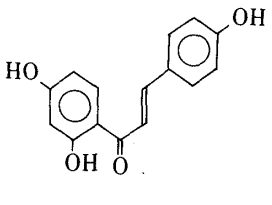

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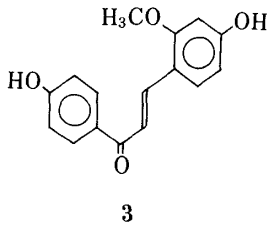

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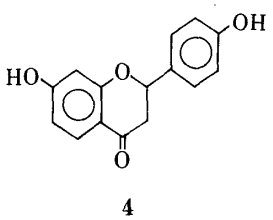

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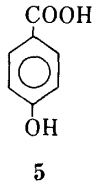

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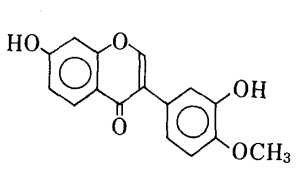

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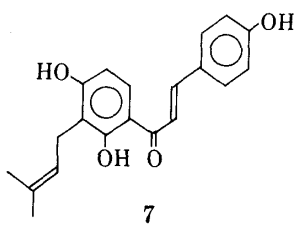

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in *Glycyrrhiza* species. This paper deals with the isolation and identification of chalcones and isoflavones produced by callus culture derived from the seedlings of *Glycyrrhiza uralensis*, as well as the time course of flavonoid production. The effect of auxins and cytokinins on the growth and production of flavonoids is also discussed.

Results

Callus tissue was initiated[9] from aseptically grown seedlings of *G. uralensis* on Murashige-Skoog's (M-S) medium containing (2,4-dichlorophenoxy)acetic acid (2,4-D, 3 ppm) and kinetin (0.1 ppm), and was maintained on the M-S medium containing 2,4-D (2.5 ppm) and benzyladenine (0.1 ppm). It showed a pale yellow colour throughout all stages of growth. However, subcultures tended to become white when they were maintained on the medium containing 2,4-D. Preliminary investigation revealed that the main metabolite of this culture was formononetin (**1**). Therefore the content of formononetin (**1**) was used as an index of flavonoid production by the callus culture to investigate the effects of culture media, auxins and cytokinins on the growth and production of the culture. The results shown in Tables I and II were obtained by using the second subcultures of callus grown on media containing different hormones. Callus tissue transferred onto the M-S medium containing naphthaleneacetic acid (NAA, 2.5 ppm) or indoleacetic acid (IAA, 3.0 ppm) produced nine to ten times larger amounts of formononetin (**1**) than the callus culture grown on the medium with 2,4-D as an auxin. However, the third transfer generation without 2,4-D stopped growing. Since the considerable growth rate of callus culture in the first and second subcultures on the medium containing NAA or IAA seemed to be caused by 2,4-D carried over to the subcultures, a combination of NAA (2 ppm) and 2,4-D (1 ppm) was tested to obtain higher growth and production. As shown in Table I, subcultures had the highest growth rate when the combination of NAA and 2,4-D was used as auxins. Benzyladenine and kinetin were tested to investigate their effect on the growth and production of the callus culture. The results indicate that benzyladenine is generally more effective for growth and formononetin (**1**) production than kinetin. The Nitsch and Nitsch ('69)[10] medium with any combination of auxins and cytokinins tested showed a lower growth rate and a higher production of formononetin (**1**) (Table II) than the corresponding combination in the M-S medium. The best medium to maintain the callus culture was M-S medium containing NAA (2 ppm), 2,4-D (1 ppm) and benzyladenine (0.1 ppm), since the callus culture grown on this medium showed reproducible growth rate and flavonoid production. The callus culture was then maintained by subculturing at a transfer interval of 28 d at 25 °C on the M-S medium for more than two years.

TABLE I. Effect of Auxins and Cytokinins on Growth and Formononetin (**1**) Formation in a 25-Day Callus Culture of *Glycyrrhiza uralensis*

Auxin (ppm)	Cytokinin (ppm)	Callus fresh weight (g/flask)	Formononetin (**1**)	
			(mg/100 g fr. wt.)	(mg/flask)
2,4-D (2.5)	Benzyladenine (0.1)	16.3	1.04	0.17
	Kinetin (0.1)	13.9	1.41	0.20
NAA (2.5)	Benzyladenine (0.1)	12.5	10.76	1.35
	Kinetin (0.1)	13.9	4.04	0.56
IAA (3.0)	Benzyladenine (0.1)	13.7	8.98	1.23
	Kinetin (0.1)	12.7	5.24	0.67
NAA (2.0)+ 2,4-D (1.0)	Benzyladenine (0.1)	17.1	6.35	1.08
	Kinetin (0.1)	15.9	4.40	0.71

At 25 °C in the dark. Culture medium: Murashige-Skoog's (1962).

TABLE II. Effect of Auxins and Cytokinins on Growth and Formononetin Formation in a 25-Day Callus Culture of *Glycyrrhiza uralensis*

Auxin (ppm)	Cytokinin (ppm)	Callus fresh weight (g/flask)	Formononetin (1)	
			(mg/100 g fr. wt.)	(mg/flask)
NAA (2.5)	Benzyladenine (0.1)	7.7	13.6	1.01
	Kinetin (0.1)	5.4	8.78	0.47
2,4-D (2.5)	Kinetin (0.1)	10.6	3.72	0.39
NAA (2.0)+ 2,4-D (1.0)	Benzyladenine (0.1)	13.7	9.24	1.27

At 25 °C in the dark. Culture medium: Nitsch and Nitsch (1969).

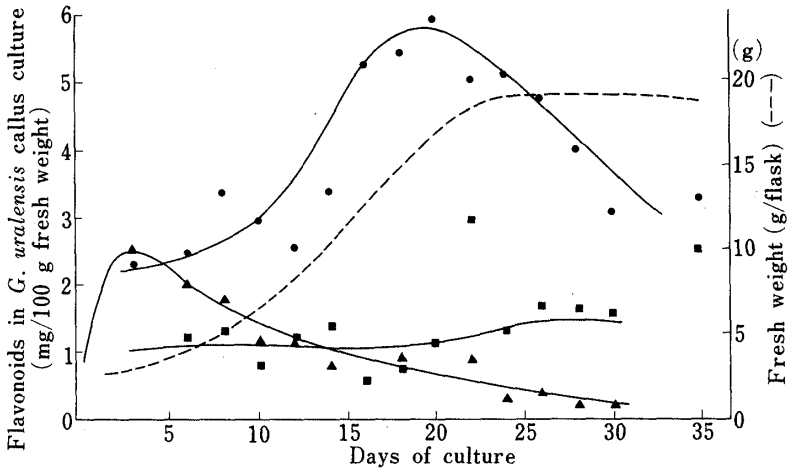

Fig. 1. Growth Kinetics and Production of Flavonoids in *G. uralensis*

Time courses of changes in fresh weight (g) of callus culture (----) and in contents (mg/100 g fr. wt.) of formononetin (●), 3′-hydroxyformononetin (■) and isobavachalcone (▲).

In order to identify the constituents of the culture, 20- to 25-day-old callus tissues grown on the above medium were collected and extracted with ethyl acetate followed by acetone. Formononetin (**1**), isoliquiritigenin (**2**) and echinatin (**3**), flavonoids already isolated from *G. uralensis*, were mainly contained in the ethyl acetate extract and liquiritigenin (**4**) and *p*-hydroxybenzoic acid (**5**) in the acetone extract. An isoflavone obtained from the ethyl acetate-soluble fraction was not identical to any isoflavone hitherto obtained from *Glycyrrhiza* plants. The spectral and chemical data of this isoflavone corresponded to those of 3′-hydroxyformononetin (**6**), which has been isolated from the heartwood of *Pterocarpus dalbergioides*[11] and the root of *Thermopsis fabacea*.[12] It was identified as 3′-hydroxyformononetin (**6**) by direct comparison with an authentic sample. Another yellow compound isolated was also not identical to any flavonoid previously obtained from *Glycyrrhiza* plants. The high-resolution mass spectrum suggested the molecular formula $C_{20}H_{20}O_4$. The proton nuclear magnetic resonance (^{1}H-NMR) spectrum revealed the presence of a dimethylallyl group, six aromatic protons (two of which showed *ortho* couplings), and two protons on a *trans* double bond. All the above data are in accord with structure **7**, which has already been assigned to isobavachalcone (**7**) isolated from the seeds of *Psoralea corylifolia* (Leguminosae).[13]

In order to investigate the possible relationship between product accumulation and growth, the time courses of the accumulation of formononetin (**1**), 3′-hydroxyformononetin (**6**) and isobavachalcone (**7**) were followed over a period of 30 d after transfer (Fig. 1). The

maximum cell mass was attained about 22—24 d after the transfer. The content of formononetin (**1**) in cells was parallel to the growth rate during the lag phase (0—4 d) and the logarithmic phase (4—17 d), and then showed a maximum (5.8 mg/100 g fresh weight) at the end of logarithmic phase (17—22 d). After reaching the maximum, the content declined in the stationary phase. On the other hand, the content of 3′-hydroxyformononetin (**6**) remained in the range of 1—1.5 mg/100 g fresh weight throughout all the stages of growth. These results suggested that 3′-hydroxylation of formononetin (**1**) is not the major metabolic pathway of formononetin (**1**). Soon after the inoculation, the isobavachalcone (**7**) content reached a maximum (2.5 mg/100 g fresh weight) at the lag phase (0—4 d), and then declined throughout the logarithmic and stationary phases.

Discussion

In contrast to the production and accumulation of formononetin (**1**), isoliquiritigenin (**2**) and liquiritigenin (**4**), licoricone and licoricidin, which are the normal constituents of intact *G. uralensis* plant, are not detected in the callus culture.[3,4] Licoricone has the same substitution pattern of oxygen functionality as retrochalcones, in which the origin of the two aromatic rings is different from that of normal chalcones.[5] However, the isolation of echinatin (**3**) indicates that the biosynthetic pathway leading to retrochalcones from normal chalcones is not blocked in the callus culture. The occurrence of *p*-hydroxybenzoic acid (**5**) was reported along with that of formononetin (**1**) in the cell culture of *Cicer arietinum*.[14] Isobavachalcone (**7**) (a prenyl derivative of isoliquiritigenin (**2**)) and 3′-hydroxyformononetin (**6**), which had not been obtained from intact *Glycyrrhiza* plants, were isolated for the first time from the tissue culture of *Glycyrrhiza* plant. It is well known that cell cultures often produce a certain group of compounds not found in the intact plant.[15]

The production of formononetin (**1**) and the growth of the callus culture depend on the kinds and concentrations of auxins and cytokinins that are present. Although the callus culture of *G. uralensis* requires 2,4-D to be maintained, 2,4-D suppressed the production of formononetin (**1**).[14] The suppressing effect of 2,4-D was previously observed in the production of various secondary metabolites in callus and cell suspension cultures, for example anthraquinones in *Morinda* and *Digitalis lanata*,[16,17] and anthocyanins in *Daucus carota*.[18] Growth and production patterns of tissue cultures can be classified into several types.[19] The callus culture of *G. uralensis* showed three different patterns of product formation. The production of formononetin (**1**) proceeds almost in parallel with cell growth and then decreases gradually, as was observed in the production of daidzein in callus culture of *Pueraria lobata*.[8] Apigenin and kaempferol are also accumulated in parallel with growth in parsley cell suspension culture.[20] The content of isobavachalcone (**7**) was found to reach its maximum just after the inoculation and gradually decreased up to 30 d. In contrast the content of 3′-hydroxyformononetin (**6**) remained more or less constant over the entire period after the transfer.

It is generally recognized that the metabolic activities in cultured cells increase immediately after inoculation.[21] Enzyme activities relating to primary metabolism, for example, carbohydrate metabolism and oxygen consumption, increase immediately after inoculation. The induction of biosynthesis and accumulation of secondary products in the lag phase were observed in germichrysone production by *Cassia torosa* culture.[22] Hahlbrock reported that the enzymes leading to the formation of flavonoids can be classified into two groups.[23] The first group consists of three enzymes, phenylalanine ammonialyase, cinnamate-4-hydroxylase, and *p*-coumarate coenzyme A (CoA) ligase, which are recognized to be activated or induced by the inoculation as well as by the onset of light irradiation. The second group of enzymes (*e.g.* chalcone synthase in parsley (*Pteroselinum hortense*) cell culture)

require light irradiation for their induction and are not induced by the inoculation. However, Ebel *et al.* reported that chalcone synthase could be induced by elicitor treatment in soybean (*Glycine max*) cell culture without light irradiation, in contrast to the case of parsley cell culture.[24] Even without elicitor treatment, the chalcone synthase activity showed two maxima during the culture; the first maximum was at 24 h after inoculation and the second one was on the 7th day. The characteristic increase in the production of isobavachalcone (**7**) in the *Glycyrrhiza* culture is presumably caused by the inoculation. The results suggest that the pattern of isobavachalcone (**7**) biosynthesis resembles that in soybean rather than parsley. However the regulatory mechanism of the flavonoid biosynthesis in *G. uralensis* callus culture still requires further investigation.

Experimental

All melting points are uncorrected. Spectral data were recorded on the following instruments: infrared (IR) spectra on a JASCO DS 701G spectrometer, ultraviolet (UV) spectra on a Hitachi 100-60 spectrometer, and mass spectra (MS) with a JEOL DX-200 spectrometer. ^{1}H- and ^{13}C-NMR spectra were obtained with JEOL FX-100 and JEOL FX-400 spectrometers.

Plant Materials——Seeds of *Glycyrrhiza uralensis* were a kind gift of Prof. H. Mitsuhashi of Hokkaido University. Callus tissues were induced from the seedlings of *G. uralensis* on 40 ml of M-S medium supplemented with 2,4-D (3 ppm), kinetin (0.1 ppm), 3% sucrose and 0.9% agar in 100 ml flasks at 25 °C in the dark. The callus tissues used for the preliminary investigation were maintained on the M-S medium containing 2,4-D (2.5 ppm) and benzyladenine (0.1 ppm). The callus tissues were subcultured every 28 d on the M-S medium supplemented with NAA (2 ppm), 2,4-D (1 ppm) and benzyladenine (0.1 ppm) at 25 °C in the dark for two years. Nitsch and Nitsch ('69) medium was also tested as a basal medium. In experiments to test the influence of auxins and cytokinins, the values shown in Tables I and II are the means for 5 flasks in two separate experiments.

Quantitative Analysis of Phenolic Compounds——Fresh callus tissues was extracted with ethyl acetate under reflux and the extracts were evaporated to dryness. The residue was dissolved in a known volume of acetone and an appropriate volume of the solution was subjected to thin layer chromatography (TLC) (Merck GF_{254}), developed with benzene–acetone (3 : 1). The quantities of formononetin (**1**), 3′-hydroxyformononetin (**6**) and isobavachalcone (**7**) were measured with a Shimadzu dual-wavelength TLC zig-zag scanner (model CS 900) with λ 280/370 nm (sample) and λ 420/700 nm (reference). The calibration curves of authentic samples were prepared in the manner described previously.[22]

Extraction and Isolation of Phenolic Compounds——The callus tissues (6 kg fresh wt.) were refluxed with ethyl acetate. The residue was refluxed with acetone and the acetone extract was extracted again with ethyl acetate. Evaporation of the combined ethyl acetate layer gave 3.4 g of a gum, which was chromatographed on silica gel using benzene–acetone mixture as an eluent, and the resulting fractions were further purified on Sephadex LH 20, Lobar column and Lichroprep Si 60 (Merck) to give formononetin (**1**) (150 mg), isoliquiritigenin (**2**), isobavachalcone (**7**) (5 mg) echinatin (**3**), liquiritigenin (**4**), 3′-hydroxyformononetin (**6**) (3 mg) and *p*-hydroxybenzoic acid (**5**) (3 mg). The yields of isoliquiritigenin (**2**), ethinatin (**3**) and liquiritigenin (**4**) were less than 1 mg. Formononetin (**1**), isoliquiritigenin (**2**), echinatin (**3**), liquiritigenin (**4**) and *p*-hydroxybenzoic acid (**5**) were identified by direct comparison with authentic specimens.

3′-Hydroxyformononetin (Clycosin) (6)——mp 250—251 °C (colourless needles from MeOH). Positive Gibbs test.[25] UV λ_{max}^{MeOH} nm (log ε): 221 (4.44), 248 (4.36), 260 (sh, 4.32), 292 (4.15). IR ν_{max}^{KBr} cm^{-1}: 3420, 1615, 1570, 1380, 1280, 1240, 1130. MS *m/z*: 284 (M^+), 269 (M^+-15), 241 (M^+-43), 213, 148, 137, 133. *Anal.* Calcd for $C_{16}H_{12}O_5$: C, 67.60; H, 4.24. Found: C, 67.36; H, 4.12. ^{1}H-NMR (d_6-acetone) δ: 3.90 (3H, s), 6.90 (1H, d, J=2.3 Hz, C-8), 6.98 (1H, d, J=8.2 Hz, C-5′), 7.00 (1H, dd, J=8.8 and 2.3 Hz, C-6), 7.07 (1H, dd, J=8.2 and 2.1 Hz, C-6′), 7.16 (1H, d, J=2.1 Hz, C-2′), 8.06 (1H, d, J=8.8 Hz, C-5′), 8.16 (1H, s, C-2). ^{13}C-NMR (d_4-MeOH) δ: 56.5 (q), 103.3 (d), 112.7 (d), 116.5 (d), 117.4 (d), 118.2 (s), 121.7 (d), 125.2 (s), 125.8 (s), 128.5 (d), 147.4 (s), 149. 2 (s), 154.8 (d), 159.8 (s), 164.6 (s), 178.0 (s).

Isobavachalcone (7)——mp 155—157 °C (yellow powder recryst. from MeOH–H_2O). UV λ_{max}^{MeOH} nm: 209, 299 (sh), 369. IR ν_{max}^{KBr} cm^{-1}: 3360, 1618, 1600, 1545, 1509, 1438, 1288, 1235, 1162. MS *m/z*: 324 (M^+), 281 (M^+-43), 269 (M^+-55), 149, 147. High-resolution MS Calcd for $C_{20}H_{20}O_4$: *m/z* 324.1359. Found: 324.1344. ^{1}H-NMR (d_6-acetone) δ: 1.65 (3H, s), 1.73 (3H, s), 3.38 (2H, d, J=7.6 Hz, allyl H), 5.27 (1H, t, J=7.6 Hz, =CH–), 6.53 (1H, d, J=9.1 Hz, C-5′), 6.92 (2H, d, J=9.1 Hz, C-3, 5), 7.64 (2H, d, J=9.1 Hz, C-2, 6), 7.73 (1H, d, J=16 Hz, α-H), 7.87 (1H, d, J=16 Hz, β-H), 7.98 (1H, d, J=9.1 Hz, C-2′).

Acknowledgement The authors are indebted to Prof. N. Morita and Dr. M. Shimizu, Toyama University, for

supplying authentic samples and spectral data.

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