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STRESS-INDUCED FORMATION OF ECHINATIN AND A METABOLITE, 5′-PRENYL-LICODIONE, IN CULTURED *GLYCYRRHIZA ECHINATA* CELLS*

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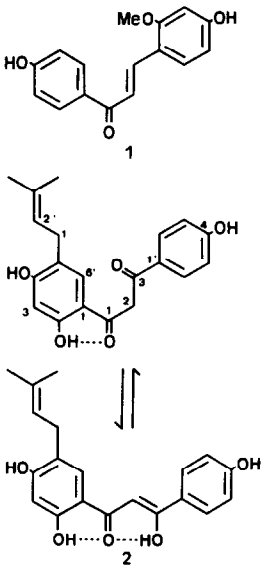
Key Word Index—*Glycyrrhiza echinata*; Leguminosae; cell culture; induction; stress metabolites; yeast extract; flavonoids; retrochalcone; echinatin; 5′-prenyl-licodione.

Abstract—The production of a retrochalcone, echinatin, by isoflavone-rich *Glycyrrhiza echinata* (M-2) cultured cells was stimulated by the addition of yeast extract or calcium alginate beads to the culture medium. Combined addition of yeast extract and cycloheximide suppressed the formation of retrochalcone, suggesting *de novo* synthesis. A new metabolite was isolated from the induced cells and its structure was determined to be 1-[2,4-dihydroxy-5-(3-methyl-2-butenyl)phenyl]-3-(4-hydroxyphenyl)-1,3-propanedione (5′-prenyl-licodione).

INTRODUCTION

Physiologically stressed plant cells show a variety of biochemical changes including an altered secondary metabolism [1]. Several biological and environmental factors, such as infection with micro-organisms, mechanical wounding, UV irradiation and dehydration, can stimulate the synthesis of stress metabolites [2]. The induction mechanism of the synthesis of phytoalexins, antimicrobial stress compounds, and also of the UV-mediated formation of flavonoids which may be defensive to the injury caused by light, has been extensively studied [3, 4]. Also, new compounds or metabolites which have previously not been categorized as stress metabolites have been characterized in cultured plant cells after treatment with fungal elicitors or antibiotics, or inoculation with micro-organisms [5–8].

Recently, we reported that immobilization in calcium alginate gels of an isoflavonoid-rich cell line of cultured *Glycyrrhiza echinata* cells (M-2 strain) causes a rapid and transient accumulation of a retrochalcone, echinatin (**1**), and a hitherto unknown metabolite in both alginate beads containing the cells and the culture medium [9]. Whereas the retrochalcone level in normal culture is very low (< 0.03 mg/g fr. wt of the cells), the immobilized system shows much higher production (*ca* 0.9 mg/g fr. wt) in a 2–3 day culture period. Induction of echinatin synthesis was also observed when sodium alginate, a component involved in the immobilization procedure, was added to the free suspension culture, and the activities of the enzymes involved in echinatin biosynthesis have been shown to increase rapidly by immobilization. These facts led us to suggest that the retrochalcone might be a novel type of stress metabolite of the Leguminosae. Herein we report further studies on the induction of echinatin synthesis in the M-2 cells, and describe the structure of another metabolite isolated from the induced cells.



RESULTS AND DISCUSSION

Induction of echinatin synthesis by yeast extract

Yeast extract has been used as a component of the medium for *G. echinata* M-1 cells which contain echinatin as a major constituent [10]. Formononetin (an isoflavone)-rich cell line (M-2) grows on Murashige and Skoog's medium supplemented with IAA and kinetin [10]. When a 7-day-old suspension culture of M-2 cells accepted 0.1% (w/v) yeast extract, echinatin production increased in 24–48 hr, and *ca* 70% of the product was released into the medium (Table 1). We also observed that the addition of calcium alginate beads to the same suspension culture caused increased echinatin production in 48 hr. This latter result is complementary to our

*Part 46 in the series "Studies on Plant Tissue Culture". For Part 45, see ref. [9].

Table 1. Echinatin content in *G. echinata* M-2 cultured cells after treatment with yeast extract or Ca alginate beads*

Additive	Time after addition					
	24 hr			48 hr		
	Cells	Medium (mg/flask)	Total	Cells	Medium (mg/flask)	Total
Control†	0.88	2.34	3.22	0.40	0.90	1.30
Yeast extract‡	1.44	3.96	5.40	1.90	4.32	6.32
Beads§	0.84	0.48	1.32	0.77	3.62	4.39

*1 l. Erlenmeyer flasks containing *ca* 18 g of cells suspended in 250 ml of medium were used.

†10 ml of water.

‡0.25 g/10 ml.

§4 g of Na alginate equivalent.

previous observations [9], and confirms that echinatin production is stimulated by alginate (either Ca or Na salt) and not necessarily by immobilization of the cells. The induction of acridone alkaloid epoxides by contact of cultured *Ruta graveolens* cells with Ca alginate beads has been reported [5].

The effects of the combined addition of yeast extract and cycloheximide to the culture medium on retrochalcone production by the M-2 cells were also examined. The results (Fig. 1) indicate that yeast extract brings about a rapid and transient increase of echinatin, and that cycloheximide, when added simultaneously with yeast extract, suppresses its induction. Addition of cycloheximide alone did not cause echinatin production to alter compared to the control.

Yeast extract (the water-soluble portion of autolysed *Saccharomyces* spp.) has been demonstrated to contain an elicitor (or elicitors) which stimulates the synthesis of phytoalexins in soybean cotyledons and hypocotyls [11], and has also been shown to be an effective medium component for the production of phytoalexins (*l*-maackiain and ipomeamarone) by the cultured cells of *Sophora angustifolia* and sweet potato [12, 13]. It is thus likely that the *de novo* synthesis of echinatin (indicated by the experiment involving a translation inhibitor) is regulated by a similar mechanism operating in the synthesis of phytoalexins [14]. Of course, it remains to be determined whether echinatin shows antimicrobial activity and whether the original plant produces it in response to microbial attack before the retrochalcone is claimed to be a phytoalexin of *Glycyrrhiza*.

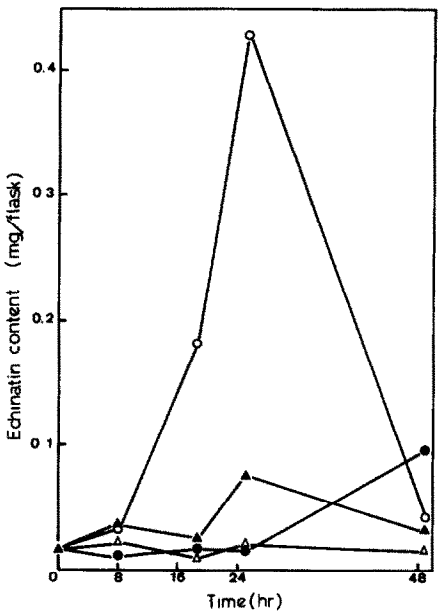


Fig. 1. Effect of the addition of yeast extract (0.1%) and/or cycloheximide (0.001%) to the culture medium on echinatin production by *G. echinata* M-2 cells. The total echinatin content in each 100 ml Erlenmeyer flask (containing 3–4 g of cells and 20 ml of medium) after the addition of yeast extract alone (○), yeast extract and cycloheximide (●), or cycloheximide alone (△) was measured. ▲, Control (no addition).

Structure of IM-1

We observed the appearance of a new spot, in addition to echinatin, on TLC of the ethyl acetate extracts prepared from the cells and media of the beads and yeast extract-treated cultures. This compound, temporarily named IM-1, has been shown to be formed in Ca alginate-entrapped M-2 cells, but absent in the cells under normal culture conditions [9].

Compound IM-1 (**2**) was then isolated as yellow needles from the induced cells (either immobilized or yeast extract-treated) and also from the culture media (see Experimental). The molecular formula of IM-1 was deduced to be $C_{20}H_{20}O_5$ from high-resolution mass spectrometry ($[M]^+$ at m/z 340.1303). The orange colour on TLC under UV illumination and its change to light blue after spraying with sulphuric acid and subsequent slight heating resemble the colour reaction of licodione (**3**), a dibenzoylmethane demonstrated to be a key intermediate in echinatin biosynthesis [10]. The UV and IR

spectra of **2** are also consistent with a β-hydroxychalcone (a tautomer of dibenzoylmethane) moiety in the molecule. The characteristic fragment ions in the mass spectrum at *m/z* 205.0846 ($C_{12}H_{13}O_3$) and 121.0279 ($C_7H_5O_2$) in addition to those at 323 $[M-OH]^+$ and 285 $[M-C_4H_7]^+$ suggest two benzoyl groups, one of which is monohydroxylated and the other bearing two hydroxyls and an alkyl (C_5H_9) substituent.

The ^{1}H NMR spectrum (acetone-d_6) of IM-1 shows singlet signals at δ4.67 (0.8H) and 7.08 (overlapping on the aromatic signals at δ7.10 and 7.08) which are attributed to the α-proton of a dibenzoylmethane existing in an equilibrium mixture of keto and enol forms [15, 16]. All the other proton signals appear as pairs arising from the two tautomeric structures. Analysis of the higher field region of the spectrum revealed a 3-methyl-2-butenyl (prenyl) group in the structure (see Table 2). The appearance of singlet aromatic signals attributed to C-3' and C-6' suggested that the position of alkyl attachment is C-5'.

From the above evidence the structure of IM-1 was proposed to be 1-[2,4-dihydroxy-5-(3-methyl-2-butenyl)phenyl]-3-(4-hydroxyphenyl)-1, 3-propanedione (5'-prenyl-licodione; **2**). Integration of the signal areas in the ^{1}H NMR spectrum of each tautomer suggests that **2** exists in *ca* 35% keto and *ca* 65% enol forms in acetone-d_6. A notable feature in the ^{1}H NMR spectrum is the large higher field shift (0.25 ppm) of the signal assignable to 6'-H, and also a small but substantial shift (0.08 ppm) of those attributable to H-1''' and H-2''', of the keto form, compared to the corresponding signals arising from the enol counterpart. This can be explained by the fact that, in the keto tautomer, two benzoyl groups are twisted, and thus an anisotropic effect of the 3-keto group to these protons is prominent, whereas in the enol tautomer the whole molecular conformation is extended because of the two hydrogen bonds (see the formulae).

Further confirmation of the structure was afforded by chemical conversion of IM-1 (**2**) to licoflavone A (**4**), which has been isolated from *G. echinata* callus culture and from Sinkiang licorice [15, 17], by acid treatment (see Experimental).

The facile chemical conversion of 5'-prenyl-licodione (**2**) to licoflavone A (**4**) and the co-occurrence of both of these compounds in *G. echinata* cell culture may suggest that **2** is a direct precursor in the biosynthesis of **4**. However, our previous feeding experiments have shown that the incorporation of ^{14}C-labelled licodione (**3**) into 7,4'-dihydroxyflavone (**5**) is poor in *G. echinata* cells [10]. On the other hand, a 2-hydroxyflavanone-type intermediate, one of the possible tautomers of *o*-hydroxydibenzoylmethane, in flavone biosynthesis has been suggested [18, 19]. Therefore, by analogy, 5'-prenyl-licodione (**2**) is not likely to be a precursor of licoflavone A (**4**). Instead, the occurrence of another presumed retrochalcone, licochalcone A (**6**), in commercial Sinkiang licorice [17], the original plant of which is a cultivar of *Glycyrrhiza glabra* [20], may indicate a biosynthetic relationship between **2** and **6**.

Grisebach and co-workers [21, 22] have reported the

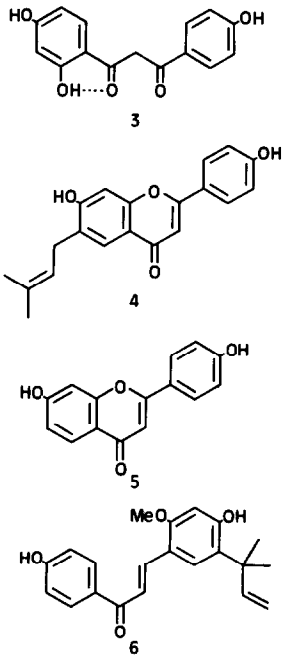


Table 2. ^{1}H NMR spectral data of 5'-prenyl-licodione (**2**) (acetone-d_6, 400 MHz)*

Proton No.	Enol	Keto	$\delta_{enol}-\delta_{keto}$
2	7.08 *s* (*ca* 0.7H)	4.67 *s* (0.8H)	2.41
3'	6.52 *s* (0.6H)	6.49 *s* (0.4H)	0.03
6'	7.96 *s* (0.6H)	7.71 *s* (*ca* 0.4H)	0.25
2'', 6''	8.07 *d* (*ca* 1.2H)	8.09 *d* (*ca* 0.8H)	−0.02
	J = 8.5	*J* = 8.5	
3'', 5''	7.10 *d* (*ca* 1.2H)	7.08 *d* (*ca* 0.8H)	0.02
	J = 8.5	*J* = 8.5	
1'''	3.40 *d* (1.3H)	3.32 *br d* (0.7H)	0.08
	J = 8	*J* = 7.5	
2'''	5.45 *m* (0.6H)	5.37 *m* (0.4H)	0.08
Me	1.85 *d* (4.2H)	1.83 *d* (1.8H)	0.02
	J = 1	*J* = 1	

*Chemical shift, δ (ppm); TMS as internal standard; coupling constant (*J*) in Hz.

induction of dimethylallyl pyrophosphate: pterocarpan dimethylallyl-transferase in elicitor-treated soybean cells. In *Glycyrrhiza* cells, too, similar inducible enzyme(s) but with different substrate specificities would be involved in the biosynthesis of 5'-prenyl-licodione (**2**).

EXPERIMENTAL

Tissue cultures and induction methods. Isoflavonoid-rich *Glycyrrhiza echinata* M-2 cells were cultured on Murashige–Skoog's agar medium supplemented with 1 ppm IAA and 0.1 ppm kinetin [10]. A portion (5 g) of suspension cultured cells in the same medium but without agar was either immobilized by entrapment in Ca alginate gels [9] or transferred to a fresh medium, and cultured further in the dark at 26°. This second passage of cell suspension was used for the induction expts. At day 7, aq. yeast extract (Difco Laboratories, Detroit; 0.25 g/10 ml), Ca alginate beads (see below) or H_2O (10 ml; as the control) was added to the culture (250 ml medium). The Ca alginate beads without cells were prepared by dropwise pouring of 100 ml 4% aq. Na alginate into 0.1 M aq. $CaCl_2$ and thorough washing of the resultant beads. Extraction of echinatin from the induced cells, and from the culture media, and determination of echinatin content with a TLC scanner were carried out by the method in ref. [9].

Effects of cycloheximide on yeast extract-induced G. echinata *cells.* Four-week-old M-2 callus (*ca* 30 g) was transferred to liquid medium (250 ml in 1 l. flasks) and suspension-cultured for 1 week. The medium containing cells was divided equally into 20 ml portions, then yeast extract (final concn 1 mg/ml) and/or cycloheximide (final concn 0.01 mg/ml) were added and cultured as before. To determine the echinatin content, EtOAc (15 ml) was added to the culture, and the mixture was homogenized for *ca* 30 sec by the use of a Polytron homogenizer. After centrifugation (2000 *g*, 10 min) of the homogenate, the EtOAc layer of the supernatant was separated, dried (Na_2SO_4) and evaporated. An aliquot of this EtOAc extract was applied to silica gel TLC (toluene–EtOAc–MeOH–petrol, 6:4:1:3), the echinatin spot was scraped off, and echinatin was redissolved in EtOAc. After concn, a portion of this extract was analyzed by high-performance liquid chromatography: column, Nucleosil C_{18} 30 cm × 10 mm i.d.; eluant, 80% MeOH; detector, UV 370 nm; flow rate, 2 ml/min; R_t of echinatin, 12.2 min.

Isolation of 5'-prenyl-licodione (**2**). *G. echinata* M-2 cultured cells were entrapped in Ca alginate gels and shaken in the same medium as above. After 48 hr, the beads containing the cells and the medium were separated by filtration through a Nylon screen. The beads from 15 flasks (original cell wt 75 g) were homogenized using a Waring blender with MeOH, filtered and successively extracted with hot MeOH. The MeOH extract was partitioned between H_2O and EtOAc. The EtOAc layer (0.66 g) after evaporation *in vacuo* was submitted to silica gel CC with the eluting solvent C_6H_6–EtOAc (from 3:1 to 1:1). Fractions containing **2** were combined and applied to repeated prep. TLC (silica gel; toluene–EtOAc–MeOH–petrol, 6:4:1:3 and *n*-hexane–Me_2CO, 4:1). The combined medium (1250 ml) was directly extracted with EtOAc, and the EtOAc extract was subjected to CC and prep. TLC as above. Isolation yield of **2** was 4.05 mg from the beads and 13.5 mg from the medium (recrystallized from EtOH–H_2O). In another expt, the cells and the medium from the suspension culture, which had accepted yeast extract (0.1% w/v) and had been shaken for 48 hr, were extracted separately in the same manner as in the immobilized system. The yield of **2** was 20.05 mg from 250 × 16 ml medium and 18.0 mg from 223.1 g of the cells. **2**: yellow needles; mp 130–135°; $C_{20}H_{20}O_5$ ([M]$^+$: 340.1303; calc.: 340.1311); MS (rel. int.): *m/z* 340 [M]$^+$ (28), 323 (4), 285 (2), 205 (17), 121 (100); IR ν_{max}^{KBr} cm^{-1}: 3375, 2925, 1630, 1580, 1510, 1350, 1170; UV λ_{max}^{MeOH} (log ε) nm: 283 (4.13), 382 (4.27); ^{1}H NMR: see Table 2.

Licoflavone A (**4**). 5'-Prenyl-licodione (**2**; 5.10 mg) was added to 0.5 ml 10% HCl–HOAc (v/v) and stirred for 5 min at room temp. H_2O was added to the reaction mixture and extracted with EtOAc repeatedly. The EtOAc layer was washed with 5% aq. Na_2CO_3 and dried (Na_2SO_4). The evaporated EtOAc extract was applied to prep. TLC (silica gel; $CHCl_3$–MeOH, 9:1) and the major band was collected. Recrystallization from H_2O–EtOH gave 2.25 mg licoflavone A (**4**); mp, MS, IR and TLC data identical to a standard specimen [15].

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