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BIOSYNTHESIS OF GLYCEOLLINS I, II AND III IN SOYBEAN

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Key Word Index—*Glycine max*; Leguminosae; soybean; phytoalexins; pterocarpan; isoflavonoid; glyceollin; biosynthesis.

Abstract—In a series of feeding experiments using $CuCl_2$-treated soybean (*Glycine max*) seedlings and pods, ^{14}C-labelled phenylalanine, daidzein, 7,2′,4′-trihydroxyisoflavone, 3,9-dihydroxypterocarpan and glycinol (3,6a,9-trihydroxypterocarpan) were all good biosynthetic precursors of the phytoalexins glyceollins I, II and III. The results confirm that the prenylation step occurs after the 6a-hydroxypterocarpan skeleton of glycinol has been synthesized. The proportions of the individual glyceollins isolated from seedlings or pods differed markedly, being *ca* 2:1:2 for glyceollins I, II and III, respectively, in seedlings, and 5:3:1, respectively, in pods.

INTRODUCTION

Several 6a-hydroxypterocarpan derivatives are synthesized as phytoalexins by soybean (*Glycine max*) tissues upon treatment with a variety of biotic or abiotic agents. These are mainly prenylated (6a*S*, 11a*S*)-6a-hydroxypterocarpans and include the major phytoalexins glyceollin I (**7**) [1, 2], glyceollin II (**8**) [2] and glyceollin III (**9**) [2] together with smaller amounts of glyceollin IV [3], glyceollidin I (**5**) [4] and glyceollidin II (glyceocarpin) (**6**) [4, 5]. The parent non-prenylated 6a-hydroxypterocarpan glycinol (3,6a,9-trihydroxypterocarpan) (**4**) [3, 6] also accumulates in treated tissue. Other isoflavonoids, not strictly regarded as phytoalexins, have been reported to be inducibly formed, or synthesized in greater amounts by the stressed plant, including daidzein (**1**) [7], coumestrol [7], isoformononetin [5], glyceofuran [5] and 9-*O*-methylglyceofuran [5].

By analogy with biosynthetic studies on other prenylated isoflavonoids [8, 9], it is logical to predict that the prenylated derivatives above are derived from glycinol via glyceollidin I or II. Glyceollins I–III would then be produced by cyclization of the 3-hydroxyl group onto the dimethylallyl, perhaps via an intermediate epoxide. The isolation of dimethylallyl transferase enzymes from glucan elicitor-treated soybean cotyledons catalysing the synthesis of glyceollidins I and II from glycinol and dimethylallylpyrophosphate [4] strongly supports this hypothesis. To help define the biosynthetic pathway in more detail, we have conducted a series of feeding experiments in $CuCl_2$-treated *G. max* tissues using a number of non-prenylated isoflavonoid and pterocarpan precursors, and have quantified the incorporations into the individual phytoalexins glyceollins I, II and III.

RESULTS

Feeding experiments

Phytoalexin synthesis was induced in 7-day-old *Glycine max* cv Wayne seedlings by immersing their roots in $CuCl_2$ solution for a period of 12 hr. After this period, the cotyledons were excised, and the $CuCl_2$ solution was replaced with a solution of the ^{14}C-labelled precursor (*ca* 0.4 mg per batch of 25 seedlings). After a feeding period of 36 hr, the seedlings were worked up, and a crude glyceollin mixture was isolated using TLC. This mixture was quantified by UV spectroscopy and then the individual glyceollins were separated using HPLC. The glyceollin mixture was firstly separated from pigments and other minor impurities, and was then rechromatographed to achieve complete separation of glyceollins I, II and III. The individual bands were rechromatographed in the second solvent system to ensure complete purification. Samples were then assayed for radioactivity, and total incorporation values were calculated using UV ε values for the individual glyceollins, peak areas from the HPLC traces, and the previously measured total glyceollin content. The identities of glyceollins I, II and III were confirmed by their NMR spectra [2].

A further series of feeding experiments was conducted using $CuCl_2$-treated pod tissue. $CuCl_2$ solution was injected into the seed cavities of young, immature pods from garden-grown *G. max* plants, left for 12 hr, then removed and replaced with precursor solution (*ca* 0.2 mg precursor per batch of 25 pods). Phytoalexins were extracted and purified as from seedlings. This sytem of feeding was particularly convenient, it produced higher amounts of glyceollins, and very efficient incorporations were observed. However, the availability of pod tissue was strictly seasonal and the slightly less efficient seedling system was mainly employed. A significant variation in proportions of the individual glyceollins was noticed, however. Thus for seedlings, glyceollins I, II and III were produced in proportions of *ca* 2:1:2, whereas in pods the proportions were *ca* 5:3:1 (Table 1).

The results of the feeding experiments are summarized in Table 1. D,L-Phenyl[2-^{14}C]alanine, [4-^{14}C]daidzein (**1**), [2-^{14}C]-7,2′,4′-trihydroxyisoflavone (**2**), (±)-[6-^{14}C]-3,9-dihydroxypterocarpan (**3**) and (6a*S*, 11a*S*)-[^{14}C]glycinol (**4**) all proved to be efficient precursors of each of the three glyceollins in seedlings and pods.

Table 1. Incorporation of labelled precursors into glyceollins I, II and III in $CuCl_2$-treated *Glycine max*

Precursor	Expt	Glyceollin I			Glyceollin II			Glyceollin III		
		μg/g fr. wt.	Dilution	Incorp. (%)	μg/g fr. wt.	Dilution	Incorp. (%)	μg/g fr. wt.	Dilution	Incorp. (%)
Seedlings										
Daidzein*	(i)	5.3	416	0.13	3.9	1610	0.025	11.9	4690	0.023
7,2′,4′-Trihydroxyisoflavone†	(i)	8.1	244	0.35	4.1	600	0.075	7.7	1730	0.049
(±)-3,9-Dihydroxypterocarpan‡	(i)	9.3	63	1.20	5.0	305	0.13	9.7	776	0.17
	(ii)	5.5	22	1.78	3.6	38	0.68	7.8	42	1.35
Glycinol§	(ii)	5.2	16	3.08	2.8	26	0.97	5.9	35	1.54
	(ii)	5.4	20	2.67	3.5	37	0.92	6.4	27	2.30
Pods										
D,L-Phenylalanine†	(iii)	21.9	1030	0.18	12.6	1730	0.061	4.5	1500	0.025
Daidzein*	(iv)	27.0	398	0.79	12.0	604	0.22	1.7	403	0.049
7,2′,4′-Trihydroxyisoflavone†	(iv)	22.0	372	0.56	13.7	315	0.46	4.1	233	0.16
(±)-3,9-Dihydroxypterocarpan‡	(iv)	26.1	244	0.89	18.0	206	0.66	4.7	160	0.25

*[4-^{14}C].
†[2-^{14}C].
‡[6-^{14}C].
§[^{14}C].

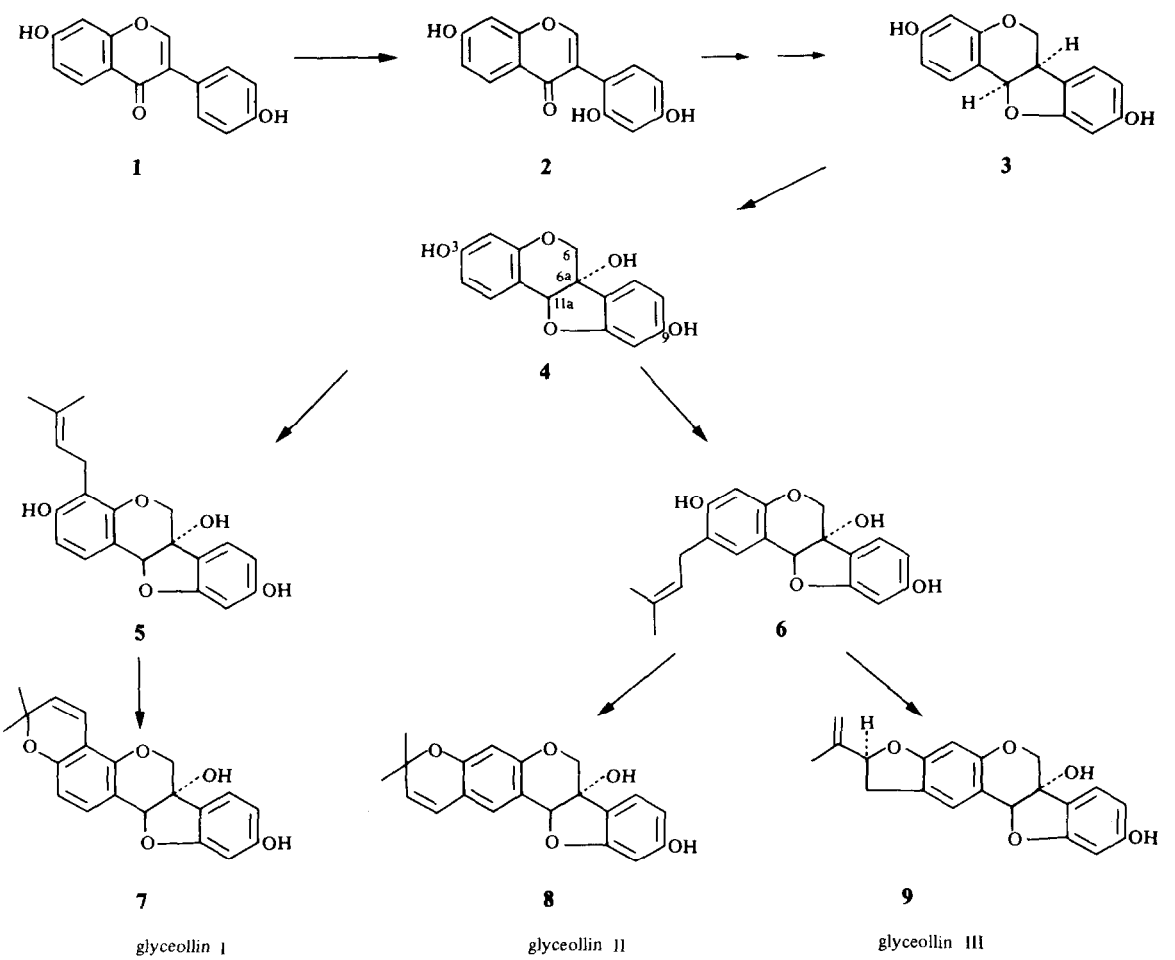
Synthesis of labelled precursors

[4-^{14}C]Daidzein was available from earlier experiments [10]. [2-^{14}C]-7,2′,4′-Trihydroxyisoflavone was synthesized by BBr_3 dealkylation [8] of [2-^{14}C]-7-benzyloxy-2′,4′-dimethoxyisoflavone obtained by reacting the deoxybenzoin 2,4-dimethoxybenzyl-4-benzyloxy-2-hydroxyphenylketone with triethyl-[^{14}C]orthoformate in pyridine/piperidine [11]. Sodium borohydride reduction of this labelled trihydroxyisoflavone gave (±)-[6-^{14}C]-3,9-dihydroxypterocarpan [8]. (6a*S*,11a*S*)-[^{14}C] Glycinol was obtained biosynthetically by feeding D,L-phenyl[2-^{14}C]alanine to UV-irradiated *G. max* cotyledons [6]. Under these conditions, glycinol tends to be formed as the major phytoalexin, in contrast to the $CuCl_2$-induction process employed in these studies, where glycinol could not normally be detected in significant quantities. Though not confirmed, it may be assumed that glycinol produced from this feeding was labelled at position 6a.

DISCUSSION

The incorporations of daidzein, 7,2′,4′-trihydroxyisoflavone, 3,9-dihydroxypterocarpan and glycinol into glyceollins I, II and III are all sufficiently large that it is reasonable to assume they may be natural precursors of these phytoalexins. The two pterocarpans were particularly well incorporated. The ready incorporation of all precursors tested in these experiments suggests that the basic 6a-hydroxypterocarpan skeleton, i.e. glycinol is synthesized before any prenylation occurs (Scheme 1). This parallels data obtained with the pterocarpan phytoalexin phaseollin [8] and the rotenoid amorphigenin [9] where prenylation is also delayed until the basic isoflavonoid skeleton (pterocarpan or rotenoid, respectively) has been constructed. The results thus support the suggestion that dimethylallylpyrophosphate:glycinol dimethylallyl transferases isolated from elicitor-treated soya bean are key enzymes in the biosynthesis of the glyceollins [4].

By analogy with the biosynthetic pathways to simple non-prenylated isoflavonoid phytoalexins such as medicarpin [10, 12], maackiain [13] and pisatin [14], it can be postulated that glycinol arises from daidzein, which is further hydroxylated in the 2′-position, then successively reduced in a stereospecific manner via the corresponding isoflavanone and isoflavanol to give 3,9-dihydroxypterocarpan. 6a-Hydroxylation of pterocarpans occurs with retention of configuration at C-6a [15], so the production



Scheme 1. Biosynthesis of glyceollins I, II and III in *Glycine max*.

of (6a*S*, 11a*S*)-glycinol presumably involves the (6a*R*,11a*R*)-isomer of 3,9-dihydroxypterocarpan (Scheme 1). Although racemic **3** was fed, no correction has been made for the possible utilization of only the (6a*R*,11a*R*)-isomer in view of the observed aberrant synthesis of some (−)-(6a*S*,11a*S*)-pisatin instead of (+)-(6a*R*,11a*R*)-pisatin when (−)-(6a*R*,11a*R*)-maackiain was tested as a precursor in $CuCl_2$-induced pea tissue [16].

4,2',4'-Trihydroxychalcone, a known biosynthetic precursor of daidzein [17], has been shown to be incorporated into the mixed glyceollin fraction in earlier feeding experiments [7]. Mevalonic acid, the precursor of dimethylallylpyrophosphate, has also been incorporated into the mixed glyceollins [18].

The site of dimethylallylation of glycinol is presumably controlled in some way in the induced plant tissue, and this may depend on the tissue, or the method of induction. Analysis of the proportions of glyceollins I, II and III produced in these experiments (Table 1) shows that the seedling tissue produced more material as 2-prenylated structures (glyceollins II and III) than as 4-prenylated derivatives (glyceollin I), whereas the reverse was true in the pod tissue. Proportions reported in $CuCl_2$-treated cotyledons [2] were almost identical to our seedling figures, but in iodoacetate-induced leaves, ratios of 1:3:6 for glyceollins I, II and III were noted [5]. In glucan elicitor-treated cotyledons, a system that produces glyceollin I (4-prenylated) as the major glyceollin, the internal pools of glyceollidins I and II were very heavily weighted in favour of the 2-prenylated isomer, glyceollidin II [4], although this may partly be due to increased turnover. In UV-treated cotyledons, however, the prenylation process becomes severely inhibited, resulting in accumulation of glycinol itself [6].

A further unusual feature of soya phytoalexins is the mode of cyclization of the two glyceollidins. Thus, whereas pyrano- and several furano-derivatives from glyceollidin II have been isolated, only the pyrano-derivative glyceollin I has been reported from the glyceollidin I series in *G. max*. However, one example of a furano-analogue, clandestacarpin, has been found in other *Glycine* species [19].

EXPERIMENTAL

General. G. max cv Wayne seeds were purchased from Wilken Seed Co., Pontiac, Illinois, U.S.A. TLC was carried out using 0.5 mm layers of silica gel $60GF_{254}$. TLC zones were eluted with Me_2CO–MeOH (1:1). HPLC was carried out on a Spherisorb 5 silica column (250 × 5 mm) with a solvent flow rate of 2 ml/min using UV detection (285 nm).

Glyceollin production in seedlings. Soybean seeds were surface-sterilized by immersing successively in EtOH (10 min), dilute detergent (Teepol, 10 min) and NaOCl soln (5%, 5 min). Seeds were germinated in running H_2O (30°) for 24 hr, then placed in trays containing moist sterilized vermiculite and covered with a 1 cm layer of dry vermiculite. The trays were placed in a growth cabinet at 25° in a 12 hr light (4000 l×)–12 hr dark cycle for 6–7 days. The seedlings were removed and placed in batches of 25 in glass dishes (10 cm diameter × 5 cm deep) containing sufficient aq. $CuCl_2$ (0.003 M) to cover the roots. After 12 hr, the $CuCl_2$ soln was removed, the roots were washed with distilled H_2O and the cotyledons excised. Radioactive precursors (*ca* 0.4 mg) were administered to the roots over 36 hr in 2-methoxyethanol (0.5 ml), Pi buffer (0.1 M, pH 7.0, 2 ml), Tween 20 (1 drop) and H_2O (sufficient to cover the roots, *ca* 40 ml). The seedlings and residual feeding soln were homogenized in EtOH (100 ml) at room temp., filtered, and the solids re-extracted with EtOH (3 × 100 ml). The combined extracts were evapd almost to dryness, treated with H_2O (50 ml), then extracted with Et_2O (3 × 100 ml). The evapd Et_2O extract was separated by TLC ($CHCl_3$–Me_2CO–38% aq. NH_4OH, 50:50:1) and the glyceollin mixture eluted from a band at R_f *ca* 0.66. The mixed glyceollins were quantified by UV using log ε_{mean} 3.94 at 285 nm (EtOH soln). Further purification was achieved by HPLC in hexane–2-propanol (97:5) and the combined glyceollin fraction (R_fs for glyceollins I, II and III: 10.5–11, 10–11.5 and 11–13 min, respectively) was rechromatographed in hexane–2-propanol (97:3). Individual glyceollins were collected (R_fs for glyceollins I, II and III: 17–19, 19–21, 21–22 min, respectively) and rechromatographed in this system, combining fractions as appropriate. Quantification was by UV absorption (EtOH) at 285 nm; glyceollin I, log ε 3.92 [7]; glyceollin II, log ε 3.94 [2]; glyceollin III, log ε 3.97 [2].

Glyceollin production in pods. Immature pods (25) from garden-grown soybean plants were slit longitudinally along the edge opposite the seeds and the seeds removed with forceps. Aq. $CuCl_2$ soln was injected into the seed cavities, the pods were placed on edge in trays lined with moist filter paper and the trays covered with a glass sheet. The trays were placed in a growth cabinet as before for 12 hr, when the $CuCl_2$ soln was replaced with precursor soln (*ca* 0.2 mg precursor/5 ml soln) and incubation was continued for 36 hr. Extraction and purification of glyceollins were as above.

Radiochemicals. D,L-Phenyl[2-^{14}C]alanine (25 mCi/mM) and triethyl-[^{14}C]orthoformate (59 mCi/mM) were purchased (Amersham). [4-^{14}C]Daidzein (0.0338 mCi/mM) was available from earlier expts [10].

[^{14}C]*Glycinol.* Cotyledons (*ca* 500) were excised from 8-day-old soybean seedlings and a broad, hollow groove was cut from the convex surface. The cotyledons were placed, cut surface uppermost, in a tray lined with moist filter paper and exposed to UV light (254 nm, 5 cm distance from cotyledons) for 4 hr. D,L-Phenyl[2-^{14}C]alanine (50 μCi) in H_2O (25 ml) was administered to the cut surfaces (50 μl per cotyledon) and a glass cover placed over the tray. The cotyledons were then incubated at 25° for 24 hr (12 hr light–12 hr dark), then placed in a 250 ml Buchner flask with EtOH (100 ml) and subjected to vacuum infiltration (water pump) for 10 min and the EtOH was decanted off. This procedure was repeated × 4. The combined ethanolic extracts were evapd to dryness, treated with H_2O and extracted with Et_2O (100 ml, then 3 × 50 ml). The evapd Et_2O extracts were purified by TLC (toluene–$CHCl_3$–Me_2CO, 8:5:10) and glycinol eluted from a band at R_f *ca* 0.25. Glycinol was purified further by HPLC in hexane–2-propanol (97:7), then hexane–2-propanol (97:5). R_ts were 16 and 30 min, respectively. Yield 1.0 mg, sp. act. 0.00181 mCi/mM, quantified via UV absorption at 287 mm, log ε 3.76 [6].

[2-^{14}C]-*7,2',4'-Trihydroxyisoflavone.* 7-Benzyloxy-2',4'-dimethoxyisoflavone [12] (100 mg) was heated under reflux for 1 hr with KOH (5 g) in EtOH (50 ml) and H_2O (25 ml). The mixture was cooled, coned, diluted with H_2O and acidified with 10% HCl. The product was extracted with EtOAc (3 × 100 ml), the combined extracts were washed with H_2O and evapd to dryness. The residue was recrystallized from aq. MeOH to give 2,4-dimethoxybenzyl-4-benzyloxy-2-hydroxyphenylketone, 80 mg, mp 103–104°. ^{1}H NMR (60 MHz, $CDCl_3$, TMS): δ 12.70 (1H, *s*, OH), 7.83 (1H, *d*, J = 9 Hz, H-6), 7.38 (5H, *s*, $OCH_2\underline{Ph}$), 7.07 (1H, *d*, J = 9 Hz, H-6'), 6.3–6.6 (4H, *m*, H-3,3',5,5'), 5.08 (2H, *s*, $OC\underline{H}_2Ph$), 4.15 (2H, *s*, $COC\underline{H}_2Ar$), 3.78 (3H, *s*, OMe), 3.75 (3H, *s*, OMe). The above deoxybenzoin (50 mg) was stirred and heated at 110° for 3 days in a Reactivial with dry pyridine (800 μl), dry piperidine (10 μl), triethylorthoformate (108 μl) and triethyl-[^{14}C]orthoformate (0.5 μl, 200 μCi). The mixture was then

transferred into 5% HCl (70 ml) at 0° and extracted with EtOAc (4 × 100 ml). The combined extracts were washed with H_2O, evapd to dryness and separated by TLC [hexane–EtOAc (3:2); hexane–Me_2CO (2:1)] to give [2-^{14}C]-7-benzyloxy-2',4'-dimethoxyisoflavone. This was dried and treated with BBr_3 in CH_2Cl_2 as described earlier [8], giving, after TLC purification, [2-^{14}C]-7,2',4'-trihydroxyisoflavone, 6.6 mg, sp. act. 0.0764 mCi/mM, identical with authentic material [8].

(±)-[6-^{14}C]-3,9-*Dihydroxypterocarpan*. $NaBH_4$ reduction of [2-^{14}C]-7,2',4'-trihydroxyisoflavone (3 mg) as described earlier [8], gave, after TLC purification, (±)-[6-^{14}C]-3,9-dihydroxypterocarpan, 1.1 mg, sp. act. 0.0770 mCi/mM, identical with authentic material [8].

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