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BIOSYNTHESIS OF THE PHYTOALEXIN PHASEOLLIN IN *PHASEOLUS VULGARIS*

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Key Word Index—*Phaseolus vulgaris*; Leguminosae; phytoalexin; pterocarpan; phaseollin; phaseollidin; kievitone; biosynthesis.

Abstract—Feeding experiments in $CuCl_2$-treated French bean (*Phaseolus vulgaris*) seedlings have demonstrated that labelled 2',4',4-trihydroxychalcone, daidzein, 7,2',4'-trihydroxyisoflavone, 3,9-dihydroxypterocarpan and phaseollidin are all good precursors of the pterocarpan phytoalexin phaseollin. These compounds represent a logical sequence in the biosynthetic pathway to phaseollin.

INTRODUCTION

Plants of the tribe Phaseoleae are noteworthy in the family Leguminosae for their synthesis of prenylated isoflavonoids as phytoalexins to counter fungal infection [1]. Several such compounds have been identified in fungus-inoculated tissues of French bean (*Phaseolus vulgaris*), including phaseollin (**8**) [2], phaseollidin (**7**) [3, 4], phaseollinisoflavan (**10**) [4] and kievitone (**9**) [4, 5]. Synthesis of these isoflavonoids may also be stimulated by abiotic treatments [6]. The biosynthetic pathways to simple non-prenylated isoflavonoid phytoalexins such as the pterocarpans medicarpin [7] and maackiain [8] and the isoflavans vestitol [9–11] and sativan [9–11] have now been fairly well established. The pathway delineated by feeding experiments involves 2'-hydroxylation of an isoflavone followed by sequential stereospecific reduction to the isoflavanone and isoflavanol, which is converted into a pterocarpan or isoflavan, probably via an intermediate carbonium ion which serves as the branch-point to pterocarpans or isoflavans. These studies are being extended to include prenylated isoflavonoid phytoalexins, and the results of feeding experiments in *P. vulgaris* seedlings on the biosynthesis of (−)-(6a*R*, 11a*R*)-phaseollin (**8**) are reported here.

RESULTS

Feeding experiments

Phytoalexin synthesis was induced in 12-day-old *P. vulgaris* cv Canadian Wonder seedlings by immersing their roots in 3×10^{-3} M $CuCl_2$ solution for a period of 24 hr, then growing on in distilled water. Work-up of the seedlings after various times and analysis of the extracted tissue showed this treatment to be effective in inducing phaseollin and kievitone synthesis, but phaseollidin could be isolated only very occasionally and other phytoalexins were not detected. The $CuCl_2$ treatment produced significant cellular damage in the leaves of the seedlings. Preliminary feeding experiments with L-[U-^{14}C]phenylalanine (Table 1) demonstrated the satisfactory incorporation of radioactivity into phaseollin and kievitone. Although phaseollin levels continued to increase during the period of the experiment, maximum incorporation of activity was noted *ca* 24 hr after feeding.

In a series of comparative feeding experiments, other labelled compounds were tested as possible precursors of phaseollin. The labelled material (*ca* 0.5 mg) was fed in two ways, either as a sodium salt in phosphate buffer (pH 7.0) or by dissolving in small quantities of ethanol, then diluting with distilled water. The samples were fed to the roots of batches of six bean seedlings which had been pretreated with aq. $CuCl_2$ for 24 hr. After a feeding period of 24–48 hr, the seedlings were worked-up, and phaseollin was isolated, quantified and purified to constant specific activity by repeated TLC. The results are shown in Table 2 and indicate that 2', 4', 4-trihydroxy [carbonyl-^{14}C]chalcone (**1**), [4-^{14}C]daidzein (**2**), 7, 2', 4'-trihydroxy[2-^{3}H]isoflavone (**3**) and (±)-3, 9-dihydroxy[6-^{3}H]pterocarpan (**6**) were all very effective precursors of phaseollin. There was little significant difference in the incorporation data from the two different feeding methods. (−)-(6a*R*, 11a*R*)-[^{14}C]-phaseollidin (**7**) was also tested as a precursor and compared against pterocarpan (**6**) in a further series of experiments (Table 2). This compound was also efficiently incorporated into phaseollin.

Synthesis of labelled precursors

2', 4', 4-Trihydroxy[carbonyl-^{14}C]chalcone and [4-^{14}C]daidzein were available from earlier experiments. 7, 2', 4'-Trihydroxy[2-^{3}H]isoflavone (**3**) was synthesized via base condensation of 4'-benzylresacetophenone with 2,4-dimethoxy[formyl-^{3}H]benzaldehyde to yield 4'-benzyloxy-2'-hydroxy-2, 4-dimethoxy[β-^{3}H]chalcone, which was converted by thallium trinitrate oxidation into 7-benzyloxy-2', 4'-dimethoxy[2-^{3}H]isoflavone. The [2-^{3}H]trihydroxyisoflavone was then obtained by boron tribromide

Table 1. Incorporation of L-[U-^{14}C]phenylalanine into phaseollin and kievitone in $CuCl_2$-induced* *Phaseolus vulgaris* seedlings

Phytoalexin	Feeding period (hr)	μM isolated	Sp. act. (dpm/mM)	Incorporation (%)	Dilution
Phaseollin	12	3.5	1.5×10^8	1.9	147
	24	6.5	1.3×10^8	3.1	168
	36	8.8	9.0×10^7	2.9	244
	48	9.3	4.1×10^7	1.4	537
Kievitone	12	0.47	2.0×10^8	0.3	110
	24	0.59	1.6×10^8	0.3	138
	36	1.3	1.2×10^8	0.6	183
	48	1.2	3.7×10^7	0.2	595

*Induction period 24 hr.

Table 2. Incorporation of labelled precursors into phaseollin in $CuCl_2$-treated* *Phaseolus vulgaris* seedlings

Precursor	Expt	Feeding soln†	Feeding period (hr)	μM isolated	Sp. act. (dpm/mM)	Incorporation (%)	Dilution
2',4',4-Trihydroxychalcone	(ii)	a	48	6.7	1.7×10^5	0.8	424
	(ii)	b	48	5.6	1.7×10^5	0.7	426
Daidzein	(i)	a	24	3.3	4.9×10^5	1.5	153
	(i)	b	24	1.7	5.4×10^5	0.8	138
	(ii)	a	48	4.1	7.8×10^5	2.2	96
	(ii)	b	48	4.3	8.7×10^5	2.6	87
7,2',4'-Trihydroxyisoflavone	(i)	a	24	3.4	2.8×10^6	0.1	1210
	(i)	b	24	2.3	7.9×10^6	0.2	430
	(ii)	a	48	4.3	4.4×10^7	2.1	77
	(ii)	b	48	4.0	5.4×10^7	2.4	63
(±)-3,9-Dihydroxypterocarpan	(i)	a	24	2.7	1.6×10^8	2.5	21
	(i)	b	24	2.4	8.9×10^7	1.2	38
	(ii)	a	48	4.3	2.7×10^7	2.1	126
	(ii)	b	48	4.4	2.3×10^7	1.7	148
	(iii)	b	30	7.3	3.0×10^7	1.7	104
	(iv)	b	30	9.6	1.8×10^7	1.3	180
	(v)	b	30	7.0	2.0×10^7	2.2	159
Phaseollidin	(iii)	b	30	6.0	3.1×10^5	1.2	326
	(iv)	b	30	4.5	4.2×10^5	1.3	238
	(v)	b	30	7.4	9.6×10^4	1.9	1040

*Induction period 24 hr.
†a: As sodium salt in phosphate buffer; b: in EtOH–H_2O–Tween mixture.

dealkylation. Sodium borohydride reduction of [2-^{3}H]**3** smoothly gave good yields of (±)-3, 9-dihydroxy[6-^{3}H]pterocarpan (**6**). 2, 4-Dimethoxybenzaldehyde was labelled with ^{3}H in the formyl group by the method of Bennett *et al.* [12].

(−)-Phaseollidin was synthesized by lithium–liquid ammonia Birch reduction [13] of natural (−)-phaseollin. ^{14}C-labelled phaseollidin was obtained by similar conversion of [^{14}C]phaseollin derived from an L-[U-^{14}C]phenylalanine feeding experiment. Although not proven, such material would be expected to be labelled more or less uniformly in carbons 6, 6a, 11a and the aromatic ring D.

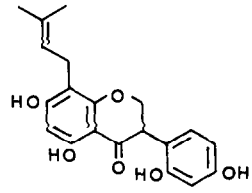
DISCUSSION

The labelled compounds fed to *P. vulgaris* were incorporated sufficiently well into phaseollin to be considered as part of the biosynthetic pathway to this phytoalexin. Ideally, a continuous gradation of incorporation data would be obtained as the precursor fed approached the final product. This is seldom achieved in whole plant systems since solubility, transport and turnover all affect the experimental results. In these studies, incorporation data for (±)-3, 9-dihydroxypterocarpan may be suitably adjusted if only the (−)-(6a*R*, 11a*R*)-isomer is incorporated into (−)-(6a*R*,11a*R*)-phaseollin. Incorporations of phaseollidin were perhaps rather lower than might have been predicted, but this pterocarpan is somewhat labile and does not store well. It was prepared immediately before feeding, but it is highly probable that some decomposition occurred during the feeding period. No identifiable decomposition products of phaseollidin have yet been obtained. Nevertheless,

incorporations of 1.2–1.9% are sufficiently high so that we may assume no random incorporation has occurred.

On the basis of these results it can be postulated that the biosynthetic pathway to phaseollin from 2′, 4′, 4-trihydroxychalcone involves the characteristic aryl ring migration to give daidzein, which is further hydroxylated in the 2′-position (Scheme 1). Stereospecific reduction and cyclization to (6a*R*, 11a*R*)-3, 9-dihydroxypterocarpan follow, almost certainly via the corresponding isoflavanone (**4**) and isoflavanol (**5**), although these compounds were not tested. Prenylation at position 10 to phaseollidin is then presumably followed by ring cyclization to phaseollin. By analogy with postulated pathways for other cyclized hemiterpenoids [14], ring closure may involve an epoxide, but there is as yet no direct evidence for such intermediates.

isoflavans. He has isolated from *P. vulgaris* all of the compounds tested in the present studies, together with 7, 2′, 4′-trihydroxyisoflavanone (**4**). Woodward's studies and our own thus complement each other.



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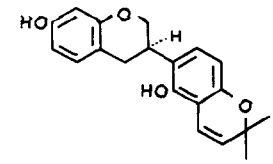

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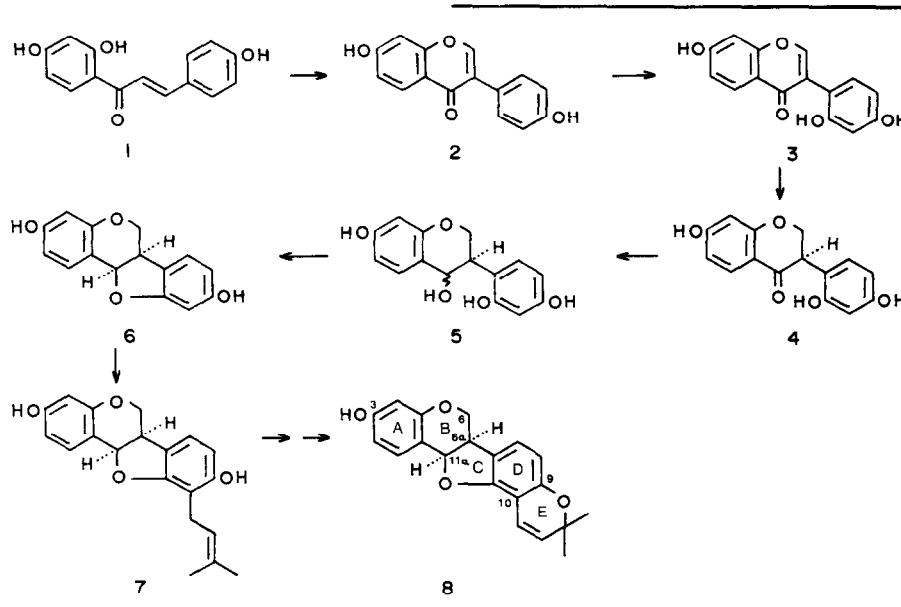
Our inability to detect phaseollinisoflavan (**10**) in Cu^{2+}-treated *P. vulgaris* tissues means that our



Scheme 1.

The compounds chosen for synthesis as possible precursors were selected as those most likely to give positive results from a knowledge of isoflavonoid biosynthesis and *P. vulgaris* metabolites. Thus our earlier studies of rotenoid biosynthesis [15] had clearly demonstrated that prenylation occurred only after the basic rotenoid skeleton had been elaborated and that prenylated isoflavones were not incorporated. An enzyme catalysing the prenylation of 3, 6a, 9-trihydroxypterocarpan has been isolated from soybean [16], and this is probably a key enzyme in the biosynthesis of the glyceollins, the soybean phytoalexins. With the available information on pterocarpan biosynthesis [7–11], the involvement of the isoflavones and 3, 9-dihydroxypterocarpan, together with 10-dimethylallyl-3,9-dihydroxypterocarpan (phaseollidin) became a logical conclusion for phaseollin production. In addition, the elaborate and exhaustive investigation of *Monilinia fructicola*-infected *P. vulgaris* metabolites by Woodward [17–19] demonstrated the absence of prenylated isoflavonoids related to phaseollin other than pterocarpans and

placement of this compound in a general biosynthetic scheme can only be speculative. If an analogy with medicarpin and vestitol biosynthesis holds, this compound should arise by prenylation of 7′, 2′, 4′-trihydroxyisoflavan (demethylvestitol) to phaseollidinisoflavan and subsequent cyclization. 3, 9-Dihydroxypterocarpan and demethylvestitol are probably interrelated by the corresponding carbonium ion [9]. Although Woodward isolated demethylvestitol, he was unable to detect phaseollidinisoflavan. It is possible, therefore, that the prenylated isoflavans are metabolites from the corresponding pterocarpans. Such transformations of phaseollin have been recorded in microbial [20] and plant [21] cells.

The earlier studies of Hess *et al.* [22] on the biosynthesis of phaseollin in $CuCl_2$-treated bean pods have demonstrated the incorporation of daidzein as well as acetate, phenylalanine and cinnamate. Mevalonic acid was poorly utilized in these experiments, however.

Since daidzein was not incorporated into kievitone in accord with expectations, no further investigation

of this metabolite was carried out in these feeding experiments. It is now well demonstrated that the 5-deoxy series of isoflavonoids are the result of a reduction prior to cyclization of the polyketide precursor of chalcones [23]. Virtually all naturally occurring isoflavonoids can be regarded as being derived either from 2', 4', 4-trihydroxychalcone or 2', 4', 6', 4-tetrahydroxychalcone [24]. Kievitone clearly may be expected to be derived from the latter.

EXPERIMENTAL

TLC. TLC was carried out using 0.5 mm layers of Si gel (Merck TLC–Kiesel gel $60GF_{254}$) in the solvent systems: A, toluene–EtOAc–MeOH, 25:8:1; B, hexane–Me_2CO, 2:1; C, $CHCl_3$–MeOH, 50:1; D, $CHCl_3$–MeOH, 10:1; E, $CHCl_3$–propan-2-ol, 9:1; F, $CHCl_3$–propan-2-ol, 10:1; G, hexane–EtOAc–MeOH, 6:4:1. Et_2O was used for elution of TLC zones except where stated.

Growth of plants and feeding techniques. Seeds of *Phaseolus vulgaris* (cv Canadian Wonder) were surface sterilized by washing successively in EtOH, dilute detergent (Teepol) and 0.5% NaClO soln. The seeds were allowed to germinate in running H_2O at 20° and were transferred to sterilized Vermiculite when radicles were *ca* 1 cm long. The seedlings were grown on at 25° in an 18 hr day–6 hr night cycle for 12 days. Phytoalexin synthesis was induced by immersing the roots in aq. $CuCl_2$ (3×10^{-3} M) for 24 hr. Radioactive precursors (*ca* 0.5 mg) dissolved in either EtOH (1 ml) + H_2O (4 ml) + Tween 20 (1 drop) or in aq. NaOH (0.5%, 0.5 ml) + Pi buffer (0.1 M, pH 7.0, 4.5 ml) were then fed to the roots of six induced seedlings for the appropriate period.

Isolation of phaseollin and kievitone. Induced seedlings were macerated in cold EtOH (4 × 50 ml) using a blender, until all green pigment was extracted. The alcoholic extracts were evaporated to dryness, the residue taken up in H_2O (20 ml) and extracted with Et_2O (4 × 50 ml). The evaporated Et_2O extracts were then separated by TLC (solvent A) and phaseollin and kievitone were eluted separately. Phaseollin was further purified by TLC in solvent systems B and C and kievitone in systems E and G. Phytoalexin content of each fraction was assayed by UV spectroscopy (phaseollin: UV λ_{max}^{EtOH} 279 nm (log ϵ 3.96) [25]; kievitone: UV λ_{max}^{EtOH} 293 nm (log ϵ 4.22) [3]).

Radiochemicals. L-[U-^{14}C]Phenylalanine (10 mCi/mM) was purchased (Amersham). The syntheses of 2', 4', 4-trihydroxy[carbonyl-^{14}C]chalcone (0.032 mCi/mM) [15] and [4-^{14}C]daidzein (0.034 mCi/mM) [10] were as described.

7, 2', 4'-*Trihydroxy*[2-^{3}H]*isoflavone.* 2, 4-Dimethoxy [formyl-^{3}H]benzaldehyde, prepared according to the general method of Bennett *et al.*[12] via α-2, 4-dimethoxyphenyl - α - morpholinoacetonitrile, was converted into 7-benzyloxy-2', 4'-dimethoxy[2-^{3}H]isoflavone (1.59 mCi/mM) by our earlier published procedure [7]. This isoflavone (150 mg) was dissolved in dry CH_2Cl_2 (5 ml), and BBr_3 (0.2 ml) was added. The mixture was left at room temp. in a stoppered flask for 3.5 hr, then treated slowly with H_2O (15 ml) and extracted with EtOAc (3 × 20 ml). The combined extracts were washed with H_2O, then evaporated and the residues crystallized from MeOH. Yield 80 mg, identical with authentic material [26]. A portion was further purified by TLC (solvent F), eluting with Me_2CO–MeOH, (1:1). Sp. act. 1.58 mCi/mM. UV λ_{max}^{EtOH} nm (log ϵ): 240 (4.30), 248 (4.32), 261 (4.29), 291 (4.17).

3, 9-Dihydroxy[6-^{3}H]pterocarpan. 7, 2', 4'-Trihydroxy/[2-^{3}H]isoflavone (38 mg) was dissolved in a mixture of THF (5 ml) and EtOH (5 ml). Solid $NaBH_4$ (4 × 50 mg) was added over 2 hr to the stirred soln, which was then stirred at room temp. overnight. After removal of the solvents, the residue was treated with cold dil. HCl, then extracted with EtOAc (× 3). The extracts were washed with H_2O, evaporated and purified by TLC (solvents D, B and F), eluting with Me_2CO. Yield 26 mg, sp. act. 1.58 mCi/mM. UV λ_{max}^{EtOH} nm (log ϵ): 283 sh (3.88), 286.5 (3.93). Other data in agreement with lit. [19].

[^{14}C]*Phaseollidin.* [^{14}C]Phaseollin (4.9 mg, 0.042 mCi/mM) obtained from a L-[U-^{14}C]phenylalanine feeding expt, was dissolved in dry 1, 2-dimethoxyethane (4 ml). Liquid NH_3 (6 ml) was added to the stirred soln followed by freshly-cut Li metal (*ca* 10 mg), which produced an intense blue soln. After 2 min the reaction was terminated by the dropwise addition of H_2O (6 ml). When the temp. had reverted to room temp., conc. HCl was added dropwise to bring the mixture to pH 5–6. The mixture was concd *in vacuo* to remove organic solvent, then diluted further with H_2O and extracted with Et_2O (× 3). The Et_2O extracts were evaporated, then purified by TLC (solvent system A) to yield [^{14}C]phaseollidin (2.0 mg), sp. act. 0.045 mCi/mM. (Note: to minimize phaseollidin decomposition, all chromatography and eluting solvents were redistilled before use, and the product was stored under N_2.) UV λ_{max}^{EtOH} 286.5 nm (log ϵ 3.78) [27].

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REFERENCES

1. Ingham, J. L. (1981) in *Advances in Legume Systematics* (Polhill, R. M. and Raven, P. H., eds.) p. 599. Royal Botanic Gardens, Kew.
2. Perrin, D. R. (1964) *Tetrahedron Letters* 29.
3. Burden, R. S., Bailey, J. A. and Dawson, G. W. (1972) *Tetrahedron Letters* 4175.
4. Perrin, D. R., Whittle, C. P. and Batterham, T. J. (1972) *Tetrahedron Letters* 1673.
5. Smith, D. A., VanEtten, H. D., Serum, J. W., Jones, T. M., Bateman, D. F., Williams, T. H. and Coffen, D. L. (1973) *Physiol. Plant. Pathol.* **3**, 293.
6. VanEtten, H. D. and Pueppke, S. G. (1976) in *Biochemical Aspects of Plant-Parasite Relationships* (Friend, J. and Threlfall, D. R., eds.) p. 239. Academic Press, New York.
7. Dewick, P. M. (1977) *Phytochemistry* **16**, 93.
8. Dewick, P. M. and Ward, D. (1978) *Phytochemistry* **17**, 1751.
9. Dewick, P. M. and Martin, M. (1979) *Phytochemistry* **18**, 591.
10. Dewick, P. M. and Martin, M. (1979) *Phytochemistry* **18**, 597.
11. Martin, M. and Dewick, P. M. (1980) *Phytochemistry* **19**, 2341.
12. Bennett, D. J., Kirby, G. W. and Moss, V. A. (1970) *J. Chem. Soc. C* 2049.
13. Brink, A. J., Rall, G. J. H. and Engelbrecht, J. P. (1974) *Phytochemistry* **13**, 1581.
14. Grundon, M. F. (1978) *Tetrahedron* **34**, 143.
15. Crombie, L., Dewick, P. M. and Whiting, D. A. (1973) *J. Chem. Soc. Perkin Trans.* 1, 1285.

16. Zähringer, U., Ebel, J., Mulheirn, L. J., Lyne, R. L. and Grisebach, H. (1979) *FEBS Letters* **101**, 90.
17. Woodward, M. D. (1979) *Phytochemistry* **18**, 365.
18. Woodward, M. D. (1979) *Phytochemistry* **18**, 2007.
19. Woodward, M. D. (1980) *Phytochemistry* **19**, 921.
20. Higgins, V. J., Stoessl, A. and Heath, M. C. (1974) *Phytopathology* **64**, 105.
21. Hargreaves, J. A. and Selby, C. (1978) *Phytochemistry* **17**, 1099.
22. Hess, S. L., Hadwiger, L. A. and Schwochau, M. E. (1971) *Phytopathology* **61**, 79.
23. Stoessl, A. and Stothers, J. B. (1979) *Z. Naturforsch. Teil C* **34**, 87.
24. Dewick, P. M. (1976) *Phytochemistry* **15**, 249.
25. VanEtten, H. D. and Bateman, D. F. (1970) *Phytopathology* **60**, 385.
26. Berlin, J., Dewick, P. M., Barz, W. and Grisebach, H. (1972) *Phytochemistry* **11**, 1689.
27. Perrin, D. R., Biggs, D. R. and Cruickshank, I. A. M. (1974) *Aust. J. Chem.* **27**, 1607.