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BIOSYNTHESIS OF THE 6a-HYDROXYPTEROCARPAN PHYTOALEXIN PISATIN IN *PISUM SATIVUM*

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Key Word Index—*Pisum sativum*; Leguminosae; phytoalexin; pterocarpan; isoflavonoid; pisatin; biosynthesis.

Abstract—Comparative feeding experiments in cupric chloride-treated *Pisum sativum* pods and seedlings have demonstrated excellent incorporation into the 6a-hydroxypterocarpan phytoalexin (+)-pisatin of [^{14}C]phenylalanine, 2′,4′,4-trihydroxychalcone, 7-hydroxy-4′-methoxyisoflavone, 7,3′-dihydroxy-4′-methoxyisoflavone, 7-hydroxy-3′,4′-methylenedioxyisoflavone, 7,2′-dihydroxy-4′,5′-methylenedioxyisoflavone, (±)-maackiain and (+)-6a-hydroxymaackiain. 2′,4′-Dihydroxy-4-methoxychalcone, 7,4′-dihydroxyisoflavone, 7,4′-dimethoxyisoflavone, (±)-pterocarpin, 3-hydroxy-8,9-methylenedioxypterocarp-6a-ene and 3-methoxy-8,9-methylenedioxypterocarp-6a-ene were poor precursors however. The incorporation of these precursors thus indicates that pisatin is produced in pea tissues via 6a-hydroxylation of maackiain, followed by O-methylation.

INTRODUCTION

(+)-Pisatin (**1**) is a 6a-hydroxypterocarpan produced by pea (*Pisum sativum*) tissues on fungal infection or abiotic treatments [1], and was the first phytoalexin to be characterized chemically [2]. Its biosynthesis in cupric chloride-treated pea pods was briefly investigated some time ago [3] and good incorporations of simple precursors phenylalanine, cinnamic acid and methionine were recorded. Acetate and glucose were also incorporated, but tyrosine proved a poor precursor. The mode of incorporation of acetate has since been established using ^{13}C-labelling techniques [4], demonstrating a specific folding of the polyketide chain, and reduction of one carbonyl function prior to aromatization of this portion. Although there is now considerable data concerning the biosynthetic origins of pterocarpans [5–10], there has been no experimental information inter-relating 6a-hydroxypterocarpans with pterocarpans. This important class of phytoalexin has been considered to arise by hydration of a pterocarp-6a-ene intermediate [11], but the non-involvement of pterocarpenes in the biosynthesis of pterocarpans [6] and coumestans [10] makes this transformation now less likely. The direct 6a-hydroxylation of pterocarpans though has analogies in the microbial metabolism of a number of pterocarpan phytoalexins [12–15]. We now present evidence from feeding experiments supporting the 6a-hydroxylation mechanism for the biosynthesis of pisatin in pea seedlings and pods.

RESULTS AND DISCUSSION

Feeding experiments

Feeding experiments with radioactive labelled precursors were carried out in 7-day-old *P. sativum* seedlings or young unexpanded pods from garden-grown plants. Phytoalexin synthesis was initiated by treating the roots of seedlings with dilute aqueous cupric chloride, or by injecting a similar solution into the pods. After 12 hr, the inducing solution was replaced by a solution of the appropriate labelled compound, and the plant tissue was worked-up after a 36–48 hr metabolism period. Pisatin was isolated by TLC, then purified rigorously to constant specific activity by further TLC in a number of other solvent systems.

Because mercuric chloride is reported to be a more efficient inducer of pisatin production in pea tissue than cupric chloride [16], preliminary experiments were conducted using this abiotic inducer. Seedlings readily produced pisatin after their roots were immersed in aqueous mercuric chloride (10^{-2} M), and L-[U-^{14}C]phenylalanine was satisfactorily incorporated into the phytoalexin (Table 1). Maximum incorporation of precursor in these experiments was observed after a 48 hr metabolism period, although no experiments were conducted beyond this time. Pisatin production was still increasing at the 48 hr period. Preliminary experiments with more advanced precursors were then undertaken using the 12 hr induction–48 hr metabolism conditions established.

Pisatin is structurally similar to the widely-distributed pterocarpan phytoalexin maackiain (**3**), and indeed, maackiain is a minor phytoalexin of *P. sativum* [17, 18]. [carbonyl-^{14}C]2′,4′,4-Trihydroxychalcone (**5**) and [Me-^{14}C]formononetin (**8**) are both good precursors of maackiain in cupric chloride-treated red clover (*Trifolium pratense*) seedlings [5], so these labelled materials were also tested as biosynthetic precursors of pisatin. [4′-Me-^{14}C]7,4′-

Dimethoxyisoflavone (methylformononetin) (**9**) was also tested to establish if introduction of the 3-*O*-methyl group (7-*O*-methyl for isoflavonoid numbering) was a fairly early step in the pathway. The results are shown in Table 2. Although a 0.11% incorporation (dilution 8300) of formononetin into pisatin was recorded, this appeared a very low figure compared with the incorporation of this material into, for example maackiain in red clover (1.8%, dilution 43) [7], and all figures were surprisingly low compared with the incorporation of phenylalanine (Table 1). Repeat feedings with increased metabolism times up to 96 hr produced no better incorporations. At these activity levels, the role of methylformononetin could not be accurately assessed.

Previous feeding experiments in *Trifolium pratense* [5–7], *Medicago sativa* [8–10] and *Trigonella foenum-graecum* [19] had all employed cupric chloride (3×10^{-3} M) rather than mercuric chloride as abiotic inducer of phytoalexins. Thus, some further experiments using [Me-^{14}C]formononetin were conducted on cupric chloride-treated *P. sativum* tissue (Table 3). In these cases, extremely good incorporations were observed. All three tissues tested, viz. excised hypocotyls, seedlings deprived of endosperm tissue, and intact pods, very efficiently metabolized formononetin to pisatin with incorporations of 1–5%. The significantly lower incorporations in mercuric chloride-induced seedlings (Table 2) are probably a result of poorer transport of the isoflavone precursor caused by cellular damage, since mercury is generally accepted to be more phytotoxic than copper. Subsequent experiments thus utilized cupric chloride-induced tissue, and for experimental convenience, pods or seedlings were used according to availability.

With the establishment of formononetin as a good precursor of pisatin, comparative feeding experiments using [carbonyl-^{14}C]2′, 4′, 4-trihydroxychalcone (**5**), [Me-^{14}C]-formononetin and [4′-Me-^{14}C]methylformononetin (**9**) were conducted in cupric chloride-

Table 1. Incorporation of L-[U-^{14}C]phenylalanine into pisatin in mercuric chloride-treated *P. sativum* seedlings*

Feeding period (hr)	Pisatin produced (μg/g)	Specific activity (dpm/mM)	Dilution	Incorporation (%)
6	23	7.34×10^5	30200	0.008
12	37	5.78×10^6	3840	0.11
24	59	7.24×10^6	3070	0.19
36	70	8.08×10^6	2750	0.24
48	124	2.07×10^7	1070	0.93

*7-day-old seedlings, induction period 12 hr.

Table 2. Incorporation of ^{14}C-labelled compounds into pisatin in mercuric chloride-treated *P. sativum* seedlings*

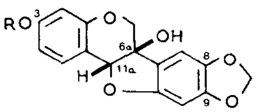
Compound	Pisatin produced (μg/g)	Specific activity (dpm/mM)	Dilution	Incorporation (%)
2′, 4′, 4-Trihydroxychalcone†	65	8.54×10^4	5800	0.08
Formononetin‡	76	1.89×10^5	8300	0.11
Methylformononetin‡	37	1.28×10^5	12500	0.04

*7-day-old seedlings, 12 hr induction, 48 hr feeding period.
†[carbonyl-^{14}C].
‡[4′-methyl-^{14}C].

Table 3. Incorporation of [Me-^{14}C]formononetin into pisatin in cupric chloride-treated *P. sativum* tissue*

Tissue	Pisatin produced (μg/g)	Specific activity (dpm/mM)	Dilution	Incorporation (%)
Pods	160	7.00×10^6	214	4.9
Hypocotyls	42	1.42×10^7	105	1.0
Seedlings	70	1.08×10^7	136	2.2

*Induction period 12 hr, feeding period 36 hr.



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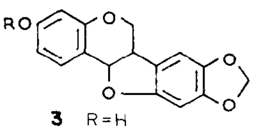
2 R = H



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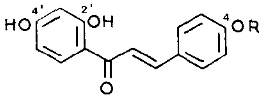

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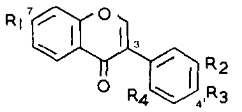
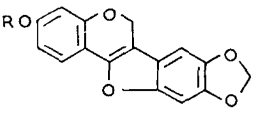
6 R = Me



	R_1	R_2	R_3	R_4
7	OH	H	OH	H
8	OH	H	OMe	H
9	OMe	H	OMe	H
10	OH	OH	OMe	H
11	OH	OCH_2O		H
12	OH	OCH_2O		OH



13 R = Me
14 R = H

treated pods. In the same series of experiments, [4-^{14}C]-labelled isoflavone precursors of maackiain [7], 7, 3′-dihydroxy-4′-methoxyisoflavone (calycosin) (**10**), 7-hydroxy-3′,4′-methylenedioxyisoflavone (ψ-baptigenin) (**11**) and 7, 2′-dihydroxy-4′, 5′-methylenedioxyisoflavone (hydroxy-ψ-baptigenin) (**12**) were also tested. The results are presented in Table 4, and demonstrate excellent incorporations of all compounds except for methylformononetin. This suggests that the 3-*O*-methyl group of pisatin is introduced rather late in the pathway. The excellent incorporations of the other isoflavones, all good precursors of maackiain in red clover [7], are strongly indicative of maackiain itself being on the pathway to pisatin. Further experiments with the [carbonyl-^{14}C]-labelled chalcones (**5**) and (**6**), [Me-^{14}C]formononetin and [4-^{14}C]daidzein (**7**) are also included in Table 4. Of these four compounds, only the trihydroxychalcone and formononetin can be regarded as well incorporated, and the results parallel those of similar feeding experiments in *Trifolium pratense* [5], *Medicago sativa* [9], *Onobrychis viciifolia* [20] and *Amorpha fruticosa* [21] for other isoflavonoids. They again indicate that the 4-hydroxy group of (**5**) is necessary for the aryl migration process associated with isoflavonoid biosynthesis, and the poor incorporation of daidzein (**7**) has been interpreted in terms of methylation of this hydroxyl group occurring during the aryl migration process [21]. Although daidzein may be enzymically methylated to formononetin [22], incorporation data as above suggest this process may not account for the major portion of formononetin production. The incorporation of chalcone (**5**) containing a resorcinol ring is entirely in accord with the ^{13}C NMR data [4] for the acetate-derived portion of pisatin. Further, the isolation of a number of isoflavonoids from cupric chloride-treated *P. sativum* [23], including formononetin and ψ-baptigenin offers circumstantial evidence for the involvement of these compounds in pisatin biosynthesis.

The later stages of the biosynthetic pathway to pisatin were investigated by feeding labelled maackiain and a number of related pterocarpanoid compounds in a series of comparative experiments to either induced pods or seedlings (Table 5). (±)-[11a-^{14}C]Maackiain was indeed a good precursor, significantly better than hydroxy-ψ-baptigenin (**12**), which was also included as a standard. The methyl ether of maackiain, (±)-[11a-^{14}C]pterocarpin (**4**) was in contrast poorly utilized, again indicating 3-*O*-methylation to be even later in the pathway. This is probably the last reaction of the route, because (+)-[^{14}C]6a-hydroxymaackiain (**2**) was incorporated into pisatin at extremely high levels (18–28%). These feedings indicate maackiain → 6a-hydroxymaackiain → pisatin as the most likely route. The two pterocarpenes, [^{14}C]3-methoxy-8, 9-methylenedioxypterocarp-6a-ene (anhydropisatin) (**13**) and [^{14}C]3-hydroxy-8, 9-methylenedioxypterocarp-6a-ene (dehydromaackiain) (**14**) were much poorer precursors. Although the incorporation levels of **13** and **14**, and also pterocarpin (**4**) were insignificant compared with those of maackiain and 6a-hydroxymaackiain, they are sufficiently large (0.1–0.7%) to indicate less direct methods for the metabolism of these compounds to pisatin. Demethylation and/or hydrolysis of a pterocarpene to an isoflavanone could make these compounds acceptable precursors.

The biosynthetic pathway

The pathway from trihydroxychalcone (**5**) to pisatin may thus be formualted as in Scheme 1. Conversion of the chalcone to formononetin (**8**) is followed by a gradual elaboration of the ring B substitution pattern, via 3′-hydroxylation to calycosin (**10**), oxidative ring closure to give the methylenedioxy derivative ψ-baptigenin (**11**), and further hydroxylation to yield hydroxy-ψ-baptigenin (**12**). By analogy with the pathway to medicarpin [6, 8, 9], this isoflavone is then presumably reduced sequentially to the isoflavanone (**15**) and isoflavanol (**16**) which can then cyclize to the pterocarpan maackiain (**18**) via the corresponding carbonium ion intermediate (**17**). It is, of course, quite likely that a metabolic grid of isoflavones, isoflavanones and isoflavanols may exist, and no unique route from formononetin to maackiain is operative. A less complex metabolic grid appears to function in *Medicago sativa* for the biosynthesis of medicarpin and the isoflavans vestitol and sativan [9]. Maackiain is then 6a-hydroxylated to (**2**) and *O*-methylated to yield pisatin. The 6a-hydroxylation step is thus analogous to fungal metabolism of maackiain [15] and other pterocarpans [12–14]. Hydration of pterocarp-6a-ene structures can be excluded.

The reduction sequence from a 2′-hydroxyisoflavone to a pterocarpan is stereospecific [19], and for phytoalexin synthesis, a (−)-(6a*R*,11a*R*)-pterocarpan is usually produced. In the biosynthesis of (−)-medicarpin in *Trigonella foenum-graecum*, an overall E addition of hydrogen to the isoflavone double bond was observed [19]. Pisatin is unusual amongst pterocarpan phytoalexins in having the

Table 4. Incorporation of ^{14}C-labelled chalcones and isoflavones into pisatin in cupric chloride-treated *P. sativum* pods*

Compound	Experiment	Pisatin produced (μg/g)	Specific activity (dpm/mM)	Dilution	Incorporation (%)
2′, 4′, 4-Trihydroxychalcone†	(i)	111	4.99×10^5	990	0.55
	(ii)	199	3.33×10^5	1490	0.86
2′, 4′-Dihydroxy-4-methoxychalcone†	(ii)	121	3.58×10^4	12600	0.06
Daidzein†	(ii)	159	2.52×10^4	2970	0.30
Formononetin‡	(i)	139	1.31×10^7	115	4.7
	(ii)	181	6.99×10^6	215	5.4
Methylformononetin‡	(i)	54	3.97×10^5	3780	0.05
Calycosin†	(i)	132	4.57×10^5	130	5.1
ψ-Baptigenin†	(i)	179	2.73×10^5	220	3.6
7, 2′-Dihydroxy-4′, 5′-methylenedioxyisoflavone†	(i)	151	2.36×10^5	250	4.9

*Induction period 12 hr, feeding period 36 hr.

†[carbonyl-^{14}C].

‡[4′-methyl-^{14}C].

Table 5. Incorporation of ^{14}C-labelled pterocarpanoids into pisatin in cupric chloride-treated *P. sativum**

Compound	Experiment	Tissue†	Pisatin produced (μg/g)	Specific activity (dpm/mM)	Dilution	Incorporation‡ (%)
7, 2′-Dihydroxy-4′, 5′-methylenedioxyisoflavone§	(i)	S	44	3.38×10^5	180	3.0
(±)-Maackiain‖	(i)	S	42	1.53×10^6	38	16.0
	(ii)	P	91	6.86×10^5	86	5.2
	(iii)	P	200	1.69×10^5	350	5.0
(±)-Pterocarpin‖	(iii)	P	178	4.66×10^4	1300	0.71
(±)-6a-Hydroxymaackiain	(i)	S	72	1.79×10^7	43	18.1
	(iv)	S	68	1.18×10^6	41	27.7
	(v)	P	151	4.79×10^5	101	27.1
Anhydropisatin	(ii)	P	102	9.91×10^4	7600	0.12
	(iii)	P	185	2.36×10^4	2100	0.51
	(iv)	S	49	5.60×10^4	860	0.73
3-Hydroxy-8, 9-methylenedioxypterocarp-6a-ene	(iv)	S	61	1.99×10^4	2440	0.32

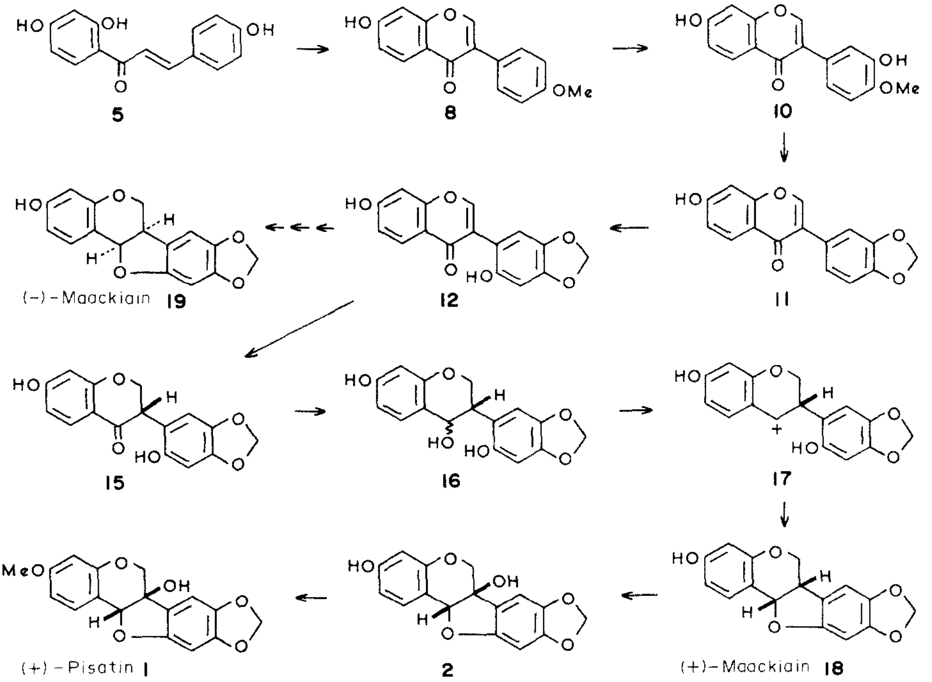
*Induction period 12 hr, feeding period 36 hr.

†S = seedling, P = pod.

‡Uncorrected for possible utilization of one enantiomer from racemic.

§[4-^{14}C].

‖[11a-^{14}C].



Scheme 1. Biosynthesis of (+)-pisatin in *Pisum sativum*.

opposite (+)-(6a*R*,11a*R*) configuration* to other reported examples which are all laevorotatory. Indeed, the minor phytoalexin maackiain produced in induced pea tissue is also laevorotatory [17, 18], and has the opposite absolute configuration (**19**) to the (+)-pisatin produced simultaneously. An overall Z addition of hydrogen to the isoflavone double bond, followed by a stereospecific 6a-hydroxylation of (+)-maackiain with retention of configuration is believed to be involved during biosynthesis of (+)-pisatin [18, 26]. Pea plants thus appear to have reductive enzymes capable of producing (3*R*)- and (3*S*)-isoflavanones via E and Z addition of hydrogen respectively [18]. Several other plants are known to constitutively accumulate enantiomeric pterocarpans [11, 27]. For the production of (+)-pisatin, the (3*S*)-isoflavanone (**15**) and -isoflavanol (**16**), and (+)-(6a*S*,11a*S*)-maackiain (**18**) are presumably intermediates and Scheme 1 has been drawn accordingly.

Feeding experiments [18] have shown that in responsive cupric chloride-treated tissue, the addition of exogenous (−)-maackiain can result in the synthesis of the abnormal phytoalexin (−)-pisatin, probably because new induced enzymes capable of metabolizing this compound are produced. Since the maackiain precursors used here were racemic, it is not possible to assign the reported incorporation data (Table 5) to (+)-pisatin only, but these will also include any incorporation of (−)-maackiain into (−)-pisatin (if produced). Observations do indicate that supply of (±)-maackiain can result in a significant proportion of (−)-pisatin (up to *ca* 10%) in the pisatin produced.

Synthesis of labelled compounds

[carbonyl-^{14}C]2′,4′,4-Trihydroxychalcone [21], [carbonyl-^{14}C]2′,4′-dihydroxy-4-methoxychalcone [21], [Me-^{14}C]formononetin [9] and [4-^{14}C]-labelled isoflavones daidzein [9], calycosin [7], *ψ*-baptigenin [7] and hydroxy-*ψ*-baptigenin [7] had been synthesized for earlier experiments. [4′-Me-^{14}C]Methylformononetin was synthesized by methyl iodide methylation of [Me-^{14}C]formononetin. (±)-[11a-^{14}C]Maackiain was obtained by sodium borohydride reduction of the labelled hydroxy-*ψ*-baptigenin; methylation with methyl iodide yielded (±)-[11a-^{14}C]pterocarpin. (+)-[^{14}C]6a-Hydroxymaackiain was produced by fungal demethylation of ^{14}C-labelled (+)-pisatin using cultures of *Fusarium avenaceum* as reported by Lappe and Barz [28], labelled pisatin being obtained from L-[U-^{14}C]phenylalanine or [3-^{14}C]cinnamic acid feedings of *P. sativum*. Acid-catalysed dehydration in ethanolic solution then readily produced [^{14}C]dehydromaackiain, whereas a similar reaction using [^{14}C]pisatin gave [^{14}C]anhydropisatin.

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, C_6H_6-EtOAc-propan-2-ol (90:10:1); B, $CHCl_3$-MeOH (25:1); C, $CHCl_3$-MeOH (97:3); D, hexane-Me_2CO (2:1); E, hexane-Me_2CO (3:1); F, hexane-Me_2CO (7:3); G, hexane-EtOAc-MeOH (60:40:1); H, C_6H_6-EtOAc-MeOH-petrol (bp 60–80°) (6:4:1:8); I, C_6H_6-EtOAc-MeOH-petrol (60–

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*Pterocarpans having a large negative $[\alpha]_D$ are assigned the (6a*R*,11a*R*) configuration [24]; (−)-6a-hydroxypterocarpans are regarded as having the same absolute configuration [25], but the priority rules give a (6a*S*,11a*S*) nomenclature.

80°) (6:4:1:6); J, C_6H_6–EtOAc–MeOH–petrol (bp 60–80°) (6:4:1:3); K. C_6H_6–EtOAc (32:1); L, C_6H_6–EtOH (9:1). Me_2CO (Analar) was used for elution of TLC zones.

Plant material, feeding techniques and isolation of pisatin. Seeds of *Pisum sativum* (cv. Kelvedon Wonder) were surface sterilized by washing successively in EtOH, dilute detergent (Teepol) and 2% NaOCl soln. The seeds were allowed to germinate in running H_2O at 20°, and were transferred to sterilized Vermiculite after 3 days. The seedlings were grown on at 25° in a 12 hr light–12 hr dark cycle for 4 days. Phytoalexin synthesis was induced by immersing the roots in aq. $CuCl_2$ (3×10^{-3} M) for 12 hr. The inducer soln was removed, the roots washed with H_2O, and the endosperm was then excised. Radioactive precursors (*ca* 0.2 mg) were administered to the roots of *ca* 20 seedlings over 36 hr in either aq. NaOH (0.5%, 0.5 ml) + Pi buffer (0.1 M, pH 7.0, 2 ml) + H_2O (4 ml), or in 2-methoxyethanol (0.5 ml) + Pi buffer (2 ml) + Tween 20 (1 drop) + H_2O (4 ml).

Pea pods were obtained from plants (cvs. Kelvedon Wonder, Little Marvel and Hurst Green Shaft) grown under normal garden conditions. Pods were chosen when beginning to expand, though containing very immature peas. Pods (*ca* 6) were induced by injecting and filling with aq. $CuCl_2$, then leaving for 12 hr. Any unabsorbed $CuCl_2$ was removed by syringe, then radioactive precursors as above were injected into the pods. The total vol. of feeding soln utilized was equivalent to the amount of $CuCl_2$ soln injected for induction. The pods were left for 36 hr in a 12 hr light–12 hr dark regimen under conditions of high humidity.

Etiolated hypocotyls from 7-day-old dark-grown seedlings were excised, exposed to UV light (254 nm) for 30 min, then placed in a small flask with their ends dipping into a 0.5 cm depth of aq. $CuCl_2$ for 12 hr in the light. The $CuCl_2$ was replaced by the feeding soln, and metabolism was allowed to continue for 36 hr in continuous light.

The plant tissue was homogenized in a mortar with powdered glass and a little H_2O, then poured into cold EtOH (100 ml), stirred and left at room temp. for 10 min. The mixture was filtered, and the solids re-extracted with cold EtOH (2×100 ml). The combined extracts were evaporated to dryness, treated with H_2O (50 ml) and extracted with Et_2O (100 ml, then 3×50 ml). The evaporated Et_2O extract was separated by TLC (solvent A), and the pisatin band purified further by TLC using the solvent systems B, D, G and A. Pisatin content was assayed by its UV absorption at 307 nm, lit. [2] log ϵ 3.86.

Radiochemicals. L-[U-^{14}C]Phenylalanine (10 mCi/mM) and [3-^{14}C]cinnamic acid (57 mCi/mM) were purchased (Amersham). The syntheses of [carbonyl-^{14}C]2′,4′,4-trihydroxychalcone (0.223 mCi/mM) [21], [carbonyl-^{14}C]2′,4′-dihydroxy-4-methoxychalcone (0.203 mCi/mM) [21], [Me-^{14}C]formononetin (0.676 mCi/mM) [9], [4-^{14}C]daidzein (0.0338 mCi/mM) [9], [4-^{14}C]calycosin (0.0271 mCi/mM) [7], [4-^{14}C]ψ-baptigenin (0.0274 mCi/mM) [7] and [4-^{14}C]7,2′-dihydroxy-4′, 5′-methylenedioxyisoflavone (0.0267 mCi/mM) [7] have been described.

[4′-Me-^{14}C]4′, *7-Dimethoxyisoflavone* (*methylformononetin*). This was obtained by methylation [5] of [Me-^{14}C]formononetin. The product was purified by TLC (solvent systems H, K, I). Sp. act. 0.676 mCi/mM. UV $\lambda_{\max}^{\text{EtOH}}$ nm: 261 (log ϵ 4.44), 300 sh.

(±)-[11a-^{14}C]*Maackiain.* $NaBH_4$ reduction of [4-^{14}C]7,2′-dihydroxy-4′, 5′-methylenedioxyisoflavone according to earlier procedures [5, 6] yielded (±)-[11a-^{14}C]maackiain. This was purified by TLC (solvent systems D, B, A). Sp. act. 0.0267 mCi/mM.

(±)-[11a-^{14}C]*Pterocarpin.* (±)-[11a-^{14}C]Maackiain was methylated [5], then purified by TLC (solvent systems E, A, D). Sp. act. 0.0313 mCi/mM. UV $\lambda_{\max}^{\text{EtOH}}$ nm: 280, 286, 309 (log ϵ 3.87).

(+)-[^{14}C]6a-*Hydroxymaackiain.* [^{14}C]Pisatin (5 mg) in 2-methoxyethanol (0.5 ml) was added to an actively growing culture of *Fusarium avenaceum* [28–30] in 0.05 M Pi buffer (pH 7.4, 50 ml). After 24 hr, the mixture was extracted with Et_2O (3×25 ml), and the extracts evaporated to dryness. The product was isolated and purified by TLC (solvents C, B, D). Yield 3.5 mg. Sp. act. 0.343 and 0.0219 mCi/mM. $[\alpha]_D + 340°$ (EtOH; c 1.10) lit. [31] + 337°. UV $\lambda_{\max}^{\text{EtOH}}$ nm: 279, 284, 307 (log ϵ 3.88 [31]). ^{1}H NMR (250 MHz, $CHCl_3$, TMS): δ 7.35 (1H, *d*, $J = 8.4$ Hz, H-1), 6.80 (1H, *s*, H-7), 6.57 (1H, *dd*, $J = 8.3$, 2.4 Hz, H-2), 6.41 (1H, *d*, $J = 2.4$ Hz, H-4), 6.40 (1H, *s*, H-10), 5.95 (1H, *d*, $J = 1.2$ Hz, O–CH_2–O), 5.91 (1H, *d*, $J = 1.2$ Hz, O–CH_2–O), 5.27 (1H, *br s*, H-11a), 4.18 (1H, *dd*, $J = 11.3$, 0.9 Hz, H-6_S), 3.99 (1H, *d*, $J = 11.3$ Hz, H-6_R).

[^{14}C]-*Methoxy*-8, 9-*methylenedioxypterocarp*-6a-*ene* (*anhydropisatin*). [^{4}C]Pisatin (1 mg) in EtOH (20 ml) was treated with conc. HCl (0.1 ml) at 4° in the dark for 5 hr. The mixture was concd under red. pres., diluted with H_2O (20 ml) and extracted with EtOAc (50 ml, 2×30 ml). The combined extracts were washed with H_2O (2×40 ml), evaporated to dryness and purified by TLC (solvents F, L, J, G). This material was purified immediately before feeding, and feeding solns were made up using deoxygenated H_2O. Yield 0.5 mg. Sp. act. 0.343 and 0.0219 mCi/mM. UV $\lambda_{\max}^{\text{EtOH}}$ nm: 291, 339 (log ϵ 4.58 [2]), 358. ^{1}H NMR (250 MHz, $CDCl_3$, TMS): δ 7.37 (1H, *d*, $J = 8.3$ Hz, H-1), 7.02 (1H, *s*, H-7), 6.74 (1H, *s*, H-10), 6.54 (1H, *dd*, $J = 8.2$, 2.4 Hz, H-2), 6.50 (1H, *d*, $J = 2.4$ Hz, H-4), 6.00 (2H, *s*, O–CH_2–O), 5.52 (2H, *s*, C-6), 3.81 (3H, *s*, OMe).

[^{14}C]3-*Hydroxy*-8, 9-*methylenedioxypterocarp*-6a-*ene* (*dehydromaackiain*). This was prepared from [^{14}C]6a-hydroxymaackiain by a procedure analogous to that above. Sp. act. 0.0219 mCi/mM. UV $\lambda_{\max}^{\text{EtOH}}$ nm: 291, 339, 358 (for quantification, log ϵ values assumed to be as for anhydropisatin).

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