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BIOSYNTHESIS OF PTEROCARPAN AND ISOFLAVAN PHYTOALEXINS IN *MEDICAGO SATIVA*: THE BIOCHEMICAL INTERCONVERSION OF PTEROCARPANS AND 2′-HYDROXYISOFLAVANS

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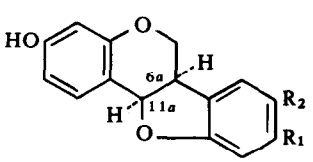
Key Word Index—*Medicago sativa*; *Trifolium pratense*; Leguminosae; lucerne; red clover; biosynthesis; phytoalexin; pterocarpan; isoflavan; demethylhomopterocarpin; sativan; vestitol.

Abstract—Feeding experiments have shown that 2′-7-dihydroxy-4′-methoxy-isoflavone-[Me-^{14}C] and -isoflavanone-[Me-^{14}C] are efficient precursors of the phytoalexins demethylhomopterocarpin, sativan and vesitol in $CuCl_2$-treated lucerne (*Medicago sativa*) seedlings. Demethylhomopterocarpin-[Me-^{14}C] was also incorporated into sativan and vestitol, and vestitol-[Me-^{14}C] was incorporated into demethylhomopterocarpin and sativan. Thus, the pterocarpan demethylhomopterocarpin and the 2′-hydroxy-isoflavan vestitol are interconvertible in *M. sativa*, but incorporation data, and the results of kinetic feeding experiments with L-phenylalanine-[U-^{14}C] suggest that these compounds are synthesized simultaneously from a common intermediate, which could be involved in the interconversion. A carbonium ion, derived from an isoflavanol, a likely intermediate in the biosynthetic reductive sequence from 2′,7-dihydroxy-4′-methoxy-isoflavone and -isoflavanone, is proposed as this common intermediate. 7-Hydroxy-2′,4′-dimethoxyisoflavone-[4′-Me-^{14}C] was a very poor precursor of all three phytoalexins. Sativan, then, is most probably derived by methylation of vestitol. The incorporation of vestitol-[Me-^{14}C] into demethylhomopterocarpin, but not into maackiain, pterocarpan phytoalexins of red clover (*Trifolium pratense*), is also demonstrated.

INTRODUCTION

Pterocarpans on hydrogenolysis are smoothly converted into the corresponding 2′-hydroxyisoflavans [1], and an analogous reaction has been postulated to occur during the biosynthesis of isoflavans in plants [2]. Support for this hypothesis comes from the observation that all of the naturally-occurring isoflavans, except for the animal metabolite equol, have 2′-oxygenation, and where pterocarpans and isoflavans cooccur in a plant, they frequently display the same chirality at the equivalent centre [2]. Biochemically, the pterocarpan → 2′-hydroxyisoflavan conversion has been demonstrated during the fungal detoxification of pterocarpan phytoalexins such as demethylhomopterocarpin (**1**) [3], maackiain (**2**) [4] and phaseollin [5]. However, pterocarpans may chemically be produced by DDQ oxidation of a 2′-hydroxyisoflavan, and this process has also been postulated as a chemical analogy for the biosynthetic relationship [6].

Pterocarpans and isoflavans represent the most abundant classes of isoflavonoid phytoalexins produced by leguminous plants [7, 8], and they probably play an important role in the disease resistance of such plants. Feeding experiments in $CuCl_2$-treated seedlings of red clover (*Trifolium pratense*) [9, 10] have suggested that the biosynthetic pathway to the pterocarpan phytoalexin (6a*R*, 11a*R*)-demethylhomopterocarpin (**1**) proceeds via the isoflavone formononetin (**3**), followed by 2′-hydroxylation to **4** and reduction to the isoflavanone (**6**). This isoflavanone is probably reduced further to the isoflavanol (**7**) which can then cyclize to **1**, perhaps via an intermediate carbonium ion (**8**). The biological reduction sequence is stereospecific, and studies in fenugreek (*Trigonella foenum-graecum*) [11] have demonstrated an overall *E*-addition of hydrogen to the double bond of **3** during the biosynthesis of **1**. To investigate the biosynthesis of isoflavans, and their biochemical relationship to pterocarpans, a number of feeding experiments have been carried out using seedlings of lucerne (*Medicago sativa*), which on fungal infection accumulate three isoflavonoid phytoalexins, (6a*R*, 11a*R*)-demethylhomopterocarpin (**1**) and the two isoflavans (3*R*)-sativan (**9**) and vestitol (**10**) [12, 13]. A preliminary communication of this work has been published [14].



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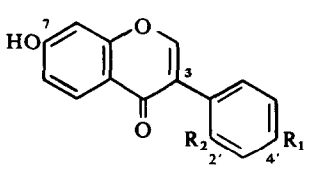

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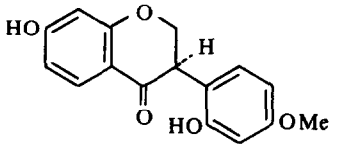

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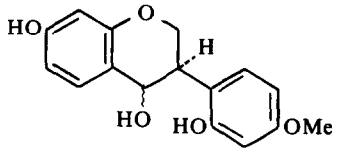

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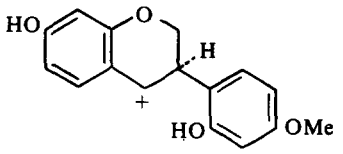

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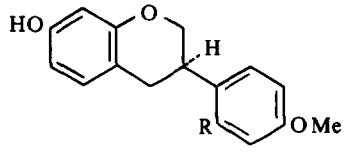

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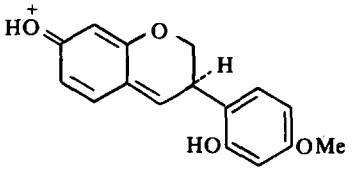

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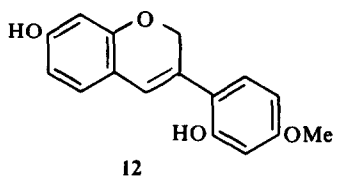

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RESULTS

Phytoalexin synthesis may be stimulated in many plants by abiotic means as well as by fungal or viral infection [7]. The use of chemicals such as heavy metal salts rather than inoculating a plant with fungal spores, greatly facilitates the experimental aspects of biosynthetic research on phytoalexins, and ensures that the results obtained reflect the plant's own metabolic activity rather than fungal modification of a plant product. The use of $CuCl_2$ as a phytoalexin inducer proved particularly useful in the red clover studies [9, 10], and this chemical also induced the production of all three phytoalexins in *M. sativa*, when the roots of 4-day-old seedlings were treated with aqueous solutions. Traces of the phytoalexins were detected in untreated, aseptically-grown seedlings, but treatment with $CuCl_2$ greatly stimulated the synthesis, and typically demethylhomopterocarpin, sativan and vestitol were produced in ratios of *ca* 6:2:1 after an induction period of 24 hr. The individual phytoalexins were separated by TLC of the plant extract (see Experimental) using synthetic racemic isoflavonoids as marker materials. Demethylhomopterocarpin and sativan had virtually identical R_f characteristics in many solvent systems, but could be separated by the multiple use of a benzene–ethyl acetate mixture. Large-scale extractions yielded sufficient demethylhomopterocarpin and sativan for optical activity ($[\alpha]_D$) measurements, and thus confirmed their absolute configurations as (6a*R*, 11a*R*) and (3*R*) respectively. Insufficient vestitol was obtained to confirm its absolute configuration by this measurement, but the isolated vestitol and sativan, and (3*R*)-vestitol obtained by hydrogenolysis of (6a*R*, 11a*R*)-demethylhomopterocarpin, all showed a positive Cotton effect in their ORD curves. Thus, the vestitol isolated also has (3*R*) stereochemistry [15, 16].

A number of labelled isoflavonoids was tested as biosynthetic precursors of the three phytoalexins in comparative feeding experiments. Batches of 4-day-old lucerne seedlings (from 4 g dry seeds) were treated with aq. CuCl for 8 hr, after which time the inducer was removed and replaced with an aq. solution of the radiochemical (*ca* 0.5 mg) in phosphate buffer. After a feeding period of 16 hr, the plant material was worked up, and the phytoalexins were isolated, quantitated by UV spectroscopy and diluted with synthetic (±)-carrier. Typically, *ca* 0.3 mg demethylhomopterocarpin, 0.1 mg sativan and 0.05 mg vestitol were obtained from a batch of seedlings. Demethylhomopterocarpin was converted into its methyl ether, while the isoflavans were acetylated; the products were then recrystallized to constant specific activity and counted.

Labelled compounds tested in the feeding experiments were 2',7-dihydroxy-4'-methoxyisoflavone-[Me-^{14}C] (**4**), 7-hydroxy-2',4'-dimethoxyisoflavone-[4'-Me-^{14}C] (**5**), (±)-2',7-dihydroxy-4'-methoxyisoflavanone-[Me-^{14}C] (**6**), (±)-demethylhomopterocarpin-[Me-^{14}C] (**1**) and (±)-vestitol-[Me-^{14}C] (**10**). Isoflavone (**4**) and isoflavanone (**6**) have already been demonstrated to be excellent precursors of demethylhomopterocarpin in red clover [10], and similar results might be anticipated for the pterocarpan and isoflavan phytoalexins of lucerne. However, sativan could be derived by a reductive sequence from the dimethoxyisoflavone (**5**), and thus labelled **5** was also tested. Labelled demethylhomopterocarpin and vestitol were fed to observe any possible interconversion analogous to the chemical transformations. The syntheses of **4**, **6** and **1** via 7-benzyloxy-2',4'-dimethoxyisoflavone-[4'-Me-^{14}C] have been described [10]. Acid debenzylation of this isoflavone yielded 7-hydroxy-2',4'-dimethoxyisoflavone-[Me-^{14}C], and catalytic hydrogenation of **4** produced (±)-vestitol-[Me-^{14}C].

The results of the feeding experiments are summarized in Table 1. 2',7-Dihydroxy-4'-methoxyisoflavone and (±)-2',7-dihydroxy-4'-methoxyisoflavanone were excellent precursors of demethylhomopterocarpin and both isoflavans. In contrast, 7-hydroxy-2',4'-dimethoxyisoflavone was poorly utilized as a precursor, even for

Table 1. Incorporation of [Me-^{14}C]-labelled isoflavonoids into phytoalexins in *Medicago sativa* seedlings*

Compound fed	Expt	Demethylhomopterocarpin Sp. act. (dpm/mM)	Dilution	Incorp.† (%)	Vestitol Sp. act. (dpm/mM)	Dilution	Incorp.† (%)	Sativan Sp. act. (dpm/mM)	Dilution	Incorp.† (%)
2',7-Dihydroxy-4'-methoxyisoflavone	(i)	5.71×10^7	20	1.7	1.23×10^7	94	0.14	5.59×10^6	210	0.19
	(ii)	1.16×10^8	9.9	10.1	5.53×10^7	21	0.82	3.24×10^7	35	0.63
7-Hydroxy-2',4'-dimethoxyisoflavone	(i)	9.75×10^5	1060	0.073	4.23×10^5	2400	0.011	1.64×10^5	6300	0.004
	(ii)	2.21×10^5	4700	0.024	1.48×10^5	7000	0.0035	6.47×10^4	16000	0.0023
(±)-2',7-Dihydroxy-4'-methoxyisoflavanone	(ii)	6.16×10^7	20	4.1	3.17×10^7	38	0.31	2.41×10^7	50	0.58
(±)-Demethylhomopterocarpin	(i)	2.86×10^8	4.0	14.3	1.55×10^7	74	0.35	8.03×10^6	140	0.17
	(ii)	2.79×10^8	4.1	39.0	2.91×10^7	40	0.31	2.57×10^7	45	0.58
(±)-Vestitol	(i)	1.60×10^7	77	0.74	3.17×10^8	3.9	7.4	2.95×10^7	42	0.58
	(ii)	1.03×10^7	120	0.21	6.28×10^8	2.0	14.9	2.48×10^7	49	0.25

* Four-day-old $CuCl_2$ inducer applied for 8 hr, feeding period 16 hr.
† Incorporation figures are not corrected for possible utilisation of only one enantiomer from racemic mixtures.

sativan. (±)-Demethylhomopterocarpin was significantly incorporated into vestitol and sativan, as was (±)-vestitol into sativan and demethylhomopterocarpin. Thus, in lucerne, demethylhomopterocarpin and vestitol are interconvertible, and there appears to be experimental evidence for the biochemical utilization of both chemical conversions. An essentially similar set of results was obtained from a second series of feeding experiments.

To study the biochemical interconversion of **1** and **10** further, a series of kinetic feeding experiments was performed. L-Phenylalanine-[U-^{14}C] was fed to four batches of lucerne seedlings, pretreated for 8 hr with aq. $CuCl_2$, as above. Batches were then worked up after metabolism periods of 6, 12, 24 and 48 hr. The incorporation of radioactivity into the phytoalexins was measured, and the data are presented in Table 2. These figures show maximum incorporation of activity into demethylhomopterocarpin and vestitol was observed after 6 hr, but into sativan after the 12 hr metabolism period.

To complement these feeding experiments, (±)-vestitol-[Me-^{14}C] was tested as a precursor of demethylhomopterocarpin and maackiain (**2**) in red clover seedlings. (±)-2',7-Dihydroxy-4'-methoxyisoflavanone-[Me-^{14}C] was fed in a comparative experiment (Table 3). Vestitol was incorporated into demethylhomopterocarpin, but not as efficiently as the isoflavanone. Neither compound proved to be a significant precursor of (6a*R*,11a*R*)-maackiain.

Table 2. Incorporation of L-phenylalanine-[U-^{14}C] into phytoalexins in *Medicago sativa* seedlings*

Feeding period (hr)	Demethylhomopterocarpin μM	Sp. act. (dpm/mM × 10^{-8})	Incorp. (%)	Vestitol μM	Sp. act. (dpm/mM × 10^{-7})	Incorp. (%)	Sativan μM	Sp. act. (dpm/mM × 10^{-7})	Incorp. (%)
6	1.00	3.28	1.17	0.24	8.59	0.074	0.27	4.93	0.048
12	1.45	2.11	0.76	0.25	7.30	0.067	0.39	5.55	0.078
24	1.08	1.36	0.53	0.20	6.92	0.051	0.31	2.75	0.031
48	0.77	1.06	0.29	0.18	3.22	0.021	0.31	5.06	0.056

* Four-day-old $CuCl_2$ inducer applied for 8 hr.

Table 3. Incorporation of [Me-^{14}C]-labelled isoflavonoids into phytoalexins in *Trifolium pratense* seedlings*

Compound Fed	Demethylhomopterocarpin Sp. act. (dpm/mM × 10^{-7})	Dilution	Incorp.† (%)	Maackiain Sp. act. (dpm/mM × 10^{-5})	Dilution	Incorp.† (%)
(±)-2',7-Dihydroxy-4'-methoxyisoflavanone	27.0	4.5	2.7	3.80	3200	0.0047
(±)-Vestitol	6.32	19	0.90	1.46	8400	0.0018

* Four-day-old $CuCl_2$ inducer applied for 8 hr, feeding period 16 hr.
† Incorporation figures are not corrected for possible utilisation of only one enantiomer from racemic mixtures.

DISCUSSION

In agreement with the earlier results obtained in red clover seedlings [10], 2',7-dihydroxy-4'-methoxy-isoflavone and -isoflavanone were also excellent precursors of demethylhomopterocarpin in lucerne. These compounds were also well incorporated into the two isoflavans sativan and vestitol, but at a rather lower level than into the pterocarpan. This no doubt reflects the relative proportions of the phytoalexins produced, and perhaps different rates of synthesis during the latter stages of induction when the precursor was applied, together with variation in turnover rates. By comparison, however, incorporation of 7-hydroxy-2',4'-dimethoxyisoflavone was very poor, even into sativan, and a reductive sequence involving this isoflavone thus appears unlikely.

The significant incorporation of demethylhomopterocarpin into sativan and vestitol, and of vestitol into sativan and demethylhomopterocarpin shows that in this plant, demethylhomopterocarpin and vestitol are interconvertible, and that sativan probably arises by methylation of vestitol. However, the incorporation figures for the conversion of demethylhomopterocarpin into the isoflavans, and of vestitol into demethylhomopterocarpin are not particularly large, especially when compared with figures for the isoflavone and isoflavanone, even if values are corrected for the possible utilization of only one enantiomer. This suggests that neither interconversion may represent the normal route to demethylhomopterocarpin and vestitol. The results can however, readily be interpreted if there exists a common intermediate on the pathway to demethylhomopterocarpin and vestitol, which are probably synthesized simultaneously. Reversal of these pathways back to the common intermediate would explain the interconversion. Such an intermediate could be the carbonium ion (**8**) or its mesomeric counterpart (**11**) derived from the isoflavanol (**7**), as postulated in the reductive pathway from isoflavone (**4**) and isoflavanone (**6**) to demethylhomopterocarpin in red clover [10]. This pathway was regarded as analogous to laboratory syntheses of pterocarpans by $NaBH_4$ reduction of 2'-hydroxyisoflavones, followed by treatment with acid [17]. Cyclization and loss of a proton from **8** or **11** would lead to the pterocarpan, whilst reduction/addition of a hydride ion would produce the isoflavan. Structure **11** represents the protonated form of the quinonemethide intermediate postulated by Cornia and Merlini [6] in the chemical conversion of vestitol into demethylhomopterocarpin using DDQ.

The results of the kinetic feeding experiments using phenylalanine offer additional evidence for the existence of a common intermediate and the simultaneous synthesis of demethylhomopterocarpin and vestitol. In the case of these two compounds, maximum incorporation data were observed in the experiment terminated 6 hr after application of the labelled precursor, and these subsequently decreased showing fairly rapid turnover of the phytoalexins. Turnover had also been observed with the $CuCl_2$-induced red clover phytoalexins [9]. However, the figures for sativan indicated maximum incorporation rather later, in the 12 hr experiment, after which time it decreased. The increased incorporation into sativan after 48 hr relative to the 24 hr figure (or alternatively the rather lower 24 hr incorporation) is not readily explained, but may be the result of some biological or experimental variation. The main argument though is unaffected: demethylhomopterocarpin and vestitol show similar trends in their incorporation curves, and differ from sativan in this respect. The curve for sativan is consistent with its biosynthesis by methylation of vestitol. The curves for demethylhomopterocarpin and vestitol suggest simultaneous synthesis rather than a sequential conversion of one compound into the other.

The incorporation of vestitol into demethylhomopterocarpin in red clover shows that similar biosynthetic pathways may also operate in this plant, although isoflavans have not been reported in *T. pratense*. The rather low incorporation compared with that of the isoflavanone similarly indicates that this pathway may not be the normal sequence of events. Neither compound was incorporated to any significant extent into maackiain, in keeping with earlier results [10] and the proposed sequence [18] for the elaboration of the substitution pattern of this pterocarpan.

The proposal of a carbonium ion as a common intermediate in pterocarpan and isoflavan biosynthesis is chemically logical, but biologically, such an entity would have to be suitably stabilized, perhaps by being bound to an appropriate enzyme. More attractive from a biochemical viewpoint, would be the intermediacy of an alternative uncharged species such as an isoflav-3-ene, derived by dehydration of an isoflavanol. Isoflav-3-enes are chemically extremely reactive, and this presumably accounts for lack of reports concerning their natural occurrence until only very recently [19–21]. However, isoflav-3-ene (**12**) has been excluded as a biosynthetic precursor of the lucerne phytoalexins, or as an intermediate in the pterocarpan-2'-hydroxyisoflavan interconversion, although such compounds would appear to play an important role in nature as intermediates in the biosynthesis of 3-arylcoumarins and coumestans [22]. After a number of chemical studies [23], Rall and coworkers have similarly rejected isoflav-3-ene intermediates in favour of carbonium ions during pterocarpan-2'-hydroxyisoflavan interconversions. They also comment on the unlikelihood of detecting 2'-hydroxyisoflavanols as natural products because of their ease of cyclization to pterocarpans via the carbonium ion. The only isoflavanol yet isolated from nature is a 2'-methoxy derivative [24].

The present studies suggest a biosynthetic pathway by 2'-hydroxylation of formononetin (**3**) to **4** (by analogy with the studies in red clover), then a stereospecific reductive sequence via isoflavanone (**6**), isoflavanol (**7**) and carbonium ion (**8**) leading to either demethylhomopterocarpin or vestitol, the latter compound being methylated further to sativan. However, it is possible that the initial 2'-hydroxylation step could equally well occur at the isoflavanone or isoflavanol levels, and that a metabolic grid [25] is involved. Further studies have in fact demonstrated that such a metabolic grid may exist in *M. sativa* [26].

Since both vestitol and sativan are more antifungal than demethylhomopterocarpin [27], it has been suggested [28] that on an evolutionary basis, isoflavans should be derived from pterocarpans. However, plants that produce isoflavan, rather than pterocarpan phytoalexins, could equally well attain this evolutionary distinction by suitable regulation of the enzymes involved in the conversion of the carbonium ion into either class of compound. Red clover synthesizes only pterocarpan

phytoalexins, producing demethylhomopterocarpin and maackiain, and there must be additional enzyme control in this plant for the synthesis of maackiain. This regulation appears to be exerted much earlier on the biosynthetic pathway at or near formononetin [18]. Although no isoflavans appear to be produced on induction, this plant still has the ability to transform vestitol to demethylhomopterocarpin. Further, a major problem in the studies with fungally-infected plants is to be able to separate the metabolic processes of the plant from those of the fungus. Thus, since reductive ring opening of a pterocarpan to a 2'-hydroxyisoflavan [3–5] and methylation of an isoflavonoid [29, 30] are amongst the demonstrated metabolic processes initiated by fungi, and since plant-mediated turnover can also occur, variations in the phytoalexin pattern between fungally-infected and abiotically-induced plants must be expected. This difference is apparent in lucerne where leaves infected with *Helminthosporium carbonum* produce demethylhomopterocarpin and sativan in the leaf tissue in a ratio of *ca* 1:5 (1:2 in diffusion droplets) together with traces of vestitol [13]; this contrasts markedly with the ratio of *ca* 6:2:1 observed in the present studies where the inducer was applied via the roots. Fungal modification of demethylhomopterocarpin could be one contributory factor in these observed differences. The phytoalexin pattern produced by a plant can however vary in a more fundamental manner. Although fenugreek (*Trigonella foenum-graecum*) leaves on fungal infection produce demethylhomopterocarpin and maackiain in roughly equal amounts [31], $CuCl_2$- and UV-treated seedlings from the same batch of seeds, produce only demethylhomopterocarpin, no trace of maackiain being detected [11]. Maackiain is an unlikely fungal metabolite of demethylhomopterocarpin, so there is clearly some important difference in this plant's metabolic response to fungi or abiotic agents.

EXPERIMENTAL

General. Seeds of *Medicago sativa* were purchased (Dickson, Brown and Tait Ltd., Altrincham). TLC was carried out using 0.5 mm layers of Si gel (Merck Kiesel gel GF_{254}) in the solvent systems: A, C_6H_6–EtOAc–MeOH–petrol (60–80°), 6:4:1:3; B, $CHCl_3$–*iso*-PrOH, 10:1; C, C_6H_6–EtOAc, 32:1; D, C_6H_6–EtOH, 94:6, E, C_6H_6–EtOAc–MeOH–petrol (60–80°), 6:4:1:6. Me_2CO was used for elution of TLC zones. Radioactive samples were counted as previously [9].

Radiochemicals. L-Phenylalanine-[U-^{14}C] (sp. act. 10 mCi/mM) was purchased (Radiochemical Centre, Amersham, U.K.). The syntheses of 2',7-dihydroxy-4'-methoxyisoflavone-[Me-^{14}C] (0.518 mCi/mM), (±)-2',7-dihydroxy-4'-methoxyisoflavanone-[Me-^{14}C] (0.546 mCi/mM) and (±)-demethylhomopterocarpin-[Me-^{14}C] (0.514 mCi/mM) have been described [10].

(±)-2',7-*Dihydroxy-4'-methoxyisoflavan* (*vestitol*). 2'-7'-Dihydroxy-4'-methoxyisoflavone [10] (200 mg) was hydrogenated overnight at room temp. and atmos. pres. in HOAc (50 ml) over Pd–C (10%, 100 mg) catalyst. The reaction mixture was filtered, evapd and (±)-vestitol purified by TLC (solvent A), and recrystallization from a small vol. of aq. MeOH. Yield 120 mg, mp 171–4°, lit. [32] 173–5°.

(±)-7-*Hydroxy-2',4'-dimethoxyisoflavan* (*sativan*). 7-Benzyloxy-2',4'-dimethoxyisoflavone [10] (500 mg) was hydrogenated in HOAc (70 ml) over Pd–C catalyst (200 mg) as above. (±)-Sativan was recrystallized from aq. MeOH. Yield 240 mg, mp 153–6°. (Found: C, 70.7; H, 6.65. $C_{17}H_{18}O_4$ requires: C, 71.3; H, 6.30%).

(±)-*Vestitol*-[Me-^{14}C]. 2',7-Dihydroxy-4'-methoxyisoflavone-[Me-^{14}C] [10] (8.0 mg) was hydrogenated in HOAc (10 ml) over Pd–C catalyst (10 mg) as above. (±)-*Vestitol*-[Me-^{14}C] was isolated by TLC (solvent A) and purified to constant sp. act. (0.552 mCi/mM) by TLC (solvents B and C). Yield 6.4 mg.

7- *ydroxy-2',4'-dimethoxyisoflavone*-[4'-Me-^{14}C]. 7-Benzyloxy-2',4'-dimethoxyisoflavone-[4'-Me-^{14}C] [10] (18.4 mg) was treated with HOAc (10 ml) and HCl (5 ml) at 100° for 1.5 hr. The mixture was poured into H_2O, extracted with EtOAc (3 × 25 ml) and the combined extracts washed with 5% aq. $NaHCO_3$ (3 ×), H_2O and evapd. 7-*Hydroxy-2'4'-dimethoxyisoflavone*-[4'-Me-^{14}C] was isolated by TLC (solvent A) and purified to constant sp. act. (0.464 mCi/mM) by TLC (solvents B and D). Yield 11.6 mg.

Plant material, feeding techniques and isolation of metabolites. Seeds of *M. sativa* (1 g) were placed on layers of moist filter paper in a Petri dish (9 cm dia) and germinated in the dark at 25° for 4 days. Batches (4) of seedlings were removed from the paper, combined and placed in a Petri dish (9 cm dia) containing 15 ml aq. $CuCl_2$ (3×10^{-3} M), and grown in the dark for a further 8 hr. The inducer soln was removed from the roots, the seedlings washed with H_2O and the labelled precursor soln added. The labelled phenols (*ca* 0.5 mg) were dissolved in the minimum amount of aq. NaOH (0.5 M), then phosphate buffer (0.1 M, pH 7.0, 2 ml) and H_2O (3.5 ml) were added. Seedlings were grown on in the dark for the feeding period (16 hr), additional H_2O being added as required. The seedlings were homogenized by griding in a mortar with ground glass and H_2O (*ca* 20 ml). The slurry, together with the original feeding soln, was then poured into boiling EtOH (200 ml). After filtration, the tissue was re-extracted with hot EtOH (2 × 100 ml) and the combined extracts were evapd, taken up with H_2O (50 ml) and extracted with Et_2O (100 ml and then 4 × 50 ml). The Et_2O extracts were evapd to dryness and separated by TLC (solvent A). Bands corresponding to demethylhomopterocarpin plus sativan, and vestitol were eluted. The vestitol band was purified further (TLC, solvent B) and its vestitol content assayed by UV absorption (λ_{max} 281 mm, log ε 3.86). The demethylhomopterocarpin/sativan band was applied to TLC plates which were developed twice for a distance of *ca* 2 cm (solvent A), thus concentrating the phytoalexins into a very narrow band. The plates were then developed using solvent C. Bands corresponding to demethylhomopterocarpin and sativan were eluted, then purified further by repeating the above procedure, and combining the appropriate eluates. Phytoalexin content was assayed by UV absorption: demethylhomopterocarpin, λ_{max} 288 mm, log ε 3.94; sativan, λ_{max} 284 mm, log ε 3.80.

Demethylhomopterocarpin was diluted with inactive (±)-carrier (25 mg) and methylated and purified as previously [9]. Vestitol and sativan were separately diluted with (±)-carrier (20 mg), then acetylated with dry Py (5 ml)–Ac_2O (0.5 ml) at room temp. overnight. The reaction mixtures were poured into H_2O and extracted with EtOAc (2 × 20 ml). The extracts were washed with dil. HCl (2 ×), then H_2O, and evapd to dryness. The acetates were purified by TLC (solvent E) then recrystallized to constant sp. act. from aq. MeOH. (±)-*Vestitol diacetate* mp 138–9°. (Found: C, 67.4; H, 5.47. $C_{20}H_{20}O_6$ requires: C, 67.4; H, 5.62%). (±)-*Sativan acetate* mp 106–7°. (Found: C, 69.1; H, 6.57. $C_{19}H_{20}O_5$ requires: C, 69.5; H, 6.10%).

Larger-scale extractions of $CuCl_2$-treated *M. sativa* seedlings yielded (6a*R*,11a*R*)-demethylhomopterocarpin, $[\alpha]_D^{20}$ − 188° (EtOH, *c* = 11.7 mg/2 ml), lit. [33] − 192°, [30] − 214° (MeOH),

and (3*R*)-sativan, $[\alpha]_D^{20} - 18.6°$ (EtOH, $c = 3.77$ mg/ml), lit. [13] −15°, [34] −22°.

Phenylalanine feedings were performed as above, except that the precursor was added as a soln in 3×10^{-3} M aq. $CuCl_2$ (5 ml) and batches were worked up after feeding periods of 6, 12, 24 and 48 hr.

Feeding experiments in *Trifolium pratense* were carried out as described previously [9].

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