Phytochemistry, 1979, Vol. 18, pp. 597–602. © Pergamon Press Ltd. Printed in England. 0031-9422/79/0401-0597 $02.00/0

BIOSYNTHESIS OF PTEROCARPAN, ISOFLAVAN AND COUMESTAN METABOLITES OF *MEDICAGO SATIVA*: CHALCONE, ISOFLAVONE AND ISOFLAVANONE PRECURSORS

PAUL M. DEWICK and MARIA MARTIN

Department of Pharmacy, University of Nottingham, Nottingham, NG7 2RD, U.K.

(*Revised received* 24 *October* 1978)

Key Word Index—*Medicago sativa*; Leguminosae; lucerne; biosynthesis; phytoalexin; pterocarpan; isoflavan; coumestan; demethylhomopterocarpin; sativan; vestitol; coumestrol; 9-*O*-methylcoumestrol.

Abstract—Comparative feeding experiments in $CuCl_2$- and UV-treated lucerne (*Medicago sativa*) seedlings have shown that 2',4,4'-trihydroxychalcone-[carbonyl-^{14}C] and formononetin-[Me-^{14}C] but not 2',4'-dihydroxy-4-methoxychalcone-[carbonyl-^{14}C] or daidzein-[4-^{14}C] were incorporated into the phytoalexins demethylhomopterocarpin, sativan and vestitol, and also into 9-*O*-methylcoumestrol. The synthesis of 9-*O*-methylcoumestrol is greatly stimulated by this abiotic treatment, but coumestrol production is not noticeably affected. Daidzein and the trihydroxychalcone were precursors of coumestrol. The results are interpreted in favour of a mechanism in which methylation is an integral part of the aryl migration process associated with the biosynthesis of 4'-methoxyisoflavonoids. Formononetin, 2',7-dihydroxy-4'-methoxyisoflavone-[Me-^{14}C], 7-hydroxy-4'-methoxyisoflavanone-[Me-^{14}C] and 2',7-dihydroxy-4'-methoxyisoflavanone-[Me-^{14}C] were all excellent precursors of demethylhomopterocarpin, sativan, vestitol and 9-*O*-methylcoumestrol, and thus a metabolic grid may be involved in their biosynthetic origin.

INTRODUCTION

Lucerne (*Medicago sativa*), on fungal infection, produces three isoflavonoid phytoalexins, the pterocarpan (6a*R*, 11a*R*)-demethylhomopterocarpin(**1**), and two isoflavans (3*R*)-sativan (**3**) and (3*R*)-vestitol (**4**) [1, 2], compounds which may well play an important role in the natural disease resistance of this plant [3, 4]. Synthesis of the same three compounds may also be stimulated by treating the roots of lucerne seedlings with aq. $CuCl_2$, and the biosynthetic pathway to demethylhomopterocarpin in $CuCl_2$-treated lucerne parallels that observed [5] in red clover (*Trifolium pratense*) seedlings [6]. Isoflavone (**7**) is sequentially reduced to isoflavanone (**9**) and then most probably to the isoflavanol (**11**), which is converted into the pterocarpan (**1**) via the carbonium ion (**13**). The biological reduction sequence appears to be stereospecific and studies in fenugreek (*Trigonella foenum-graecum*) [7] have demonstrated an overall *E*-addition of hydrogen to the double bond of **7**. In lucerne, there is evidence that demethylhomopterocarpin and vestitol are interconvertible, but appear to be synthesized simultaneously from a common intermediate, and the carbonium ion has been postulated as this branchpoint [6]. Reversal of the pathways back to this common intermediate would explain the interconversion. Sativan (**3**) is most probably derived by methylation of vestitol.

The present paper reports the results of feeding experiment using labelled precursors to investigate some of the earlier parts of the biosynthetic pathways to demethylhomopterocarpin, sativan and vestitol, and to establish whether a metabolic grid [8] may be involved in the elaboration of the substitution patterns of these compounds. Also studied in these experiments were the biosynthetic pathways to the coumestans coumestrol (**14**) and 9-*O*-methylcoumestrol (**15**). Although coumestans are not usually fungitoxic, they are frequently synthesized in increased amounts in fungally-infected leguminous plants [9–15], and there is presumably a very close relationship between the biosynthesis of these compounds and structurally-related isoflavonoid phytoalexins.

RESULTS

Feeding experiments in $CuCl_2$-treated red clover seedlings have demonstrated that 2',4,4'-trihydroxychalcone (**17**) and 7-hydroxy-4'-methoxyisoflavone (formononetin (**6**) were readily incorporated into the pterocarpan phytoalexins (6a*R*,11a*R*)-demethylhomopterocarpin (**1**) and (6a*R*,11a*R*)-maackiain (**2**), but 2',4'-dihydroxy-4-methoxychalcone (**18**) and 4',7'-dihydroxyisoflavone (daidzein) (**5**) were poor precursors [16]. These results may be rationalized if methylation is an integral part of the aryl migration process involved in the biosynthesis of 4'-methoxyisoflavones [17, 18]. The same four compounds were also tested as precursors of demethylhomopterocarpin and the two isoflavans sativan and vestitol in seedlings of lucerne. Batches of 4-day-old lucerne seedlings (from 4 g dry seeds) were treated with aq. $CuCl_2$ for a period of 8 hr, and also exposed to UV radiation for the first 30 min of this time. Both forms of abiotic treatment may stimulate phytoalexin production [3]; in the present experiments, the combination was found to produce increased (10–20%) amounts of all three phytoalexins than did either treatment alone. After 8 hr, the inducer solution 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, the phytoalexins were isolated by TLC, quantitated by UV spectroscopy and

Table 1. Incorporation of labelled chalcones and isoflavones

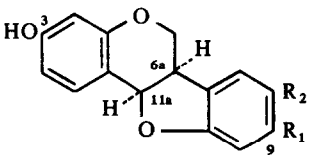
Compound fed	Expt	Demethylhomopterocarpin Sp. act. (dpm/mM)	Dilution	Incorp. (%)	Vestitol Sp. act. (dpm/mM)	Dilution	Incorp. (%)
2',4,4'-Trihydroxychalcone†	(i)	8.52×10^5	580	0.14	6.51×10^5	770	0.021
	(ii)	1.47×10^6	340	0.29	1.30×10^6	380	0.041
2',4'-Dihydroxy-4-methoxychalcone†	(i)	7.00×10^4	6400	0.010	8.74×10^4	5200	0.003
Daidzein†	(ii)	2.20×10^4	3400	0.02		n.d.	
Formononetin‡	(i)	9.70×10^7	16	6.6		§	

* Four-day-old $CuCl_2$ inducer applied for 8 hr, UV for 0.5 hr., feeding period 16 hr.
† [carbonyl-^{14}C].
‡ [Me-^{14}C]. n.d. No activity detected.
§ Due to experimental difficulties, these figures are not available.

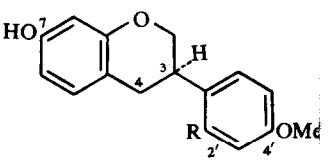
diluted with synthetic racemic carrier. Demethylhomopterocarpin was then converted into its methyl ether, and sativan and vestitol were acetylated. The derivatives were then purified to constant specific activity and counted.

Coumestrol (**14**) and 9-*O*-methylcoumestrol (**15**) were also isolated from the plant extract and studied in the feeding experiments. The identity of the compounds was confirmed by comparison with authentic specimens. A number of other coumestans have been reported [19] in *M. sativa*, including medicagol (**16**). In the TLC systems employed, medicagol had virtually identical R_f characteristics to 9-*O*-methylcoumestrol, but none of this compound was detected in the 9-*O*-methylcoumestrol extracts. A mixture of the two compounds may be partially separated via their acetates [20]; no medicagol acetate was observed in the acetylated coumestan band from the present studies. The amount of 9-*O*-methylcoumestrol isolated from the seedlings was significantly increased (*ca* 3 times) as a result of the phytoalexin induction treatment. Coumestrol production was not noticeably affected, and amounts isolated were much smaller than for the methyl derivative. Typically *ca* 0.06 mg of **15** and 0.02 mg of **14** were isolated from a batch of seedlings. Both coumestans were methylated prior to purification to constant specific activity and counting.

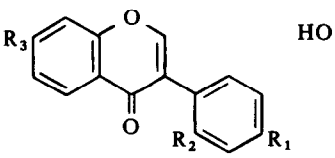
The labelled compounds tested as precursors in the feeding experiments were 2',4,4'-trihydroxychalcone-[carbonyl-^{14}C], 2',4'-dihydroxy-4-methoxychalcone-[carbonyl-^{14}C], formononetin-[Me-^{14}C] and daidzein-[4-^{14}C], and the results are summarized in Table 1. For experimental reasons, two sets of feedings were performed; 2',4,4'-trihydroxychalcone was fed in both sets. The results indicate the significant incorporation of 2',4,4'-trihydroxychalcone and formononetin into demethylhomopterocarpin, sativan, vestitol and 9-*O*-methylcoumestrol. (For incorporation of formononetin into vesitol, see Table 2.) By comparison, 2',4'-dihydroxy-4-methoxychalcone was a poor precursor. Incorporation of daidzein was similarly very poor, except into coumestrol; because of the small quantity of coumestrol isolated, the absolute (percentage) incorporation was low, and the dilution value is thus a more meaningful measure of this incorporation. Similar comments apply to the incorporation of 2',4,4'-trihydroxychalcone into coumestrol. Both compounds are proven precursors of coumestrol in *M. sativa* [21, 22] and *Phaseolus aureus* [23, 24].



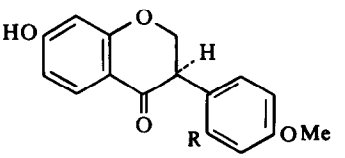
1 R_1 = OMe; R_2 = H
2 R_1R_2 = OCH_2O



3 R = OMe
4 R = OH



5 R_1 = R_3 = OH; R_2 = H
6 R_1 = OMe; R_3 = OH; R_2 = H
7 R_1 = OMe; R_2 = R_3 = OH
8 R_1 = OMe; R_3 = OCH_2Ph; R_2 = H



9 R = OH
10 R = H

Table 2. Incorporation of [Me-^{14}C]-labelled isoflavones and

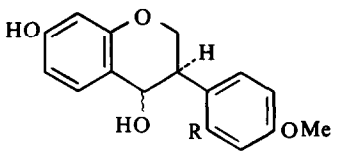
Compound fed	Demethylhomopterocarpin Sp. act. (dpm/mM × 10^{-7})	Dilution	Incorp.† (%)	Vestitol Sp. act. (dpm/mM × 10^{-7})	Dilution
Formononetin	8.69	17	6.4	3.75	40
(±)-7-Hydroxy-4'-methoxyisoflavanone	2.41	56	2.1	1.31	103
2',7-Dihydroxy-4'-methoxyisoflavone	5.78	20	6.6	5.11	23
(±)-2',7-Dihydroxy-4'-methoxyisoflavanone	5.33	23	5.6	3.44	35

* Four-day-old, $CuCl_2$ inducer applied for 8 hr, UV for 0.5 hr, feeding period 16 hr.
† Incorporation figures are not corrected for possible utilization of only one enantiomer from racemic mixtures.

into isoflavonoids in *Medicago sativa* seedlings*

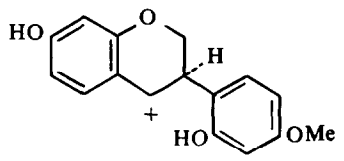
Sativan			9-*O*-Methylcoumestrol			Coumestrol		
Sp. act (dpm/mM)	Dilution	Incorp. (%)	Sp. act. (dpm/mM)	Dilution	Incorp. (%)	Sp. act. (dpm/mM)	Dilution	Incorp. (%)
3.28×10^5	1500	0.017	1.76×10^6	280	0.013		n.d.	
6.82×10^5	730	0.031	6.73×10^6	74	0.039	7.15×10^5	690	0.007
4.79×10^4	9400	0.002		§				
	n.d.			n.d.		6.79×10^5	110	0.023
1.65×10^7	91	0.26	9.31×10^7	16	0.18			

The further substitution, and reduction, necessary to transform formononetin to 2',7-dihydroxy-4'-methoxyisoflavanol (**12**), the proposed [6] precursor of demethylhomopterocarpin, sativan and vestitol may be effected not by a unique route, but via a metabolic grid [8] of isoflavones, isoflavanones and isoflavanols in *M. sativa*. To test this hypothesis, further comparative feeding experiments were conducted, and incorporations into demethylhomopterocarpin, sativan, vestitol and 9-*O*-methylcoumestrol were measured. In addition to the demonstrated precursors of demethylhomopterocarpin, sativan and vestitol, namely formononetin-[Me-^{14}C] (**6**), 2',7-dihydroxy-4'-methoxyisoflavone-[Me-^{14}C] (**7**) and (±)-2',7-dihydroxy-4'-methoxyisoflavanone-[Me-^{14}C] (**9**), (±)-7-hydroxy-4'-methoxyisoflavanone-[Me-^{14}C] (**10**) was also tested. The results of these experiments are listed in Table 2. All four compounds were excellent precursors of the phytoalexins and 9-*O*-methylcoumestrol.



11 R = OH
12 R = H

The syntheses of 2',4,4'-trihydroxychalcone-[carbonyl-^{14}C], 2',4'-dihydroxy-4-methoxychalcone-[carbonyl-^{14}C] and formononetin-[Me-^{14}C] have been described previously [18]. Daidzein-[4-^{14}C] has been synthesized for earlier feeding experiments [23, 25] by demethylation of formononetin-[4-^{14}C], derived by reaction of triethylorthoformate with 2,4-dihydroxyphenyl-4-methoxybenzylketone-[carbonyl-^{14}C]. This ketone was the product of a Hoesch reaction between resorcinol and 4-methoxyphenylacetonitrile-[1-^{14}C], conveniently prepared using labelled KCN. Limitations of this method as a general synthetic route arise from difficulties associated with the Hoesch condensation on a very small scale, and the synthesis of labelled phenylacetonitriles from K^{14}CN.



13

For the synthesis of a variety of isoflavones bearing different substituents in the B-ring, the $Tl(NO_3)_3$ oxidation of appropriate chalcones [26] derived from 4'-benzyloxy-2'-hydroxyacetophenone-[carbonyl-^{14}C] has been applied [27]. Similarly, $Tl(NO_3)_3$ oxidation of 4'-benzyloxy-2'-hydroxy-4-methoxychalcone-[carbonyl-^{14}C] (**19**) prepared by base condensation of the above acetophenone with anisaldehyde yielded 7-*O*-benzylformononetin-[4-^{14}C] (**8**). Daidzein-[4-^{14}C] was obtained by dealkylation of this isoflavone with HI. A number of other approaches to daidzein-[4-^{14}C] from resacetophenone-[carbonyl-^{14}C] were explored. $Tl(NO_3)_3$ oxidation sequences involving 4,4'-dibenzyloxy-2'-hydroxychalcone, 2',4,4'-tribenzyloxychalcone and 2',4,4'-triacetoxychalcone were all found to be inferior to the route described above. The latter sequence seemed particularly attractive, since 2,4,4'-trihydroxychalcone may be synthe-

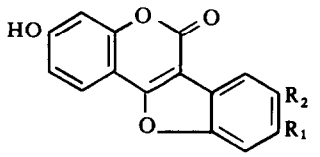
isoflavanones into isoflavonoids in *Medicago sativa* seedlings*

	Sativan			9-*O*-Methylcoumestrol		
Incorp.† (%)	Sp. act. (dpm/mM)	Dilution	Incorp.† (%)	Sp. act. (dpm/mM)	Dilution	Incorp.† (%)
0.59	3.37×10^7	45	0.71	1.08×10^8	14	0.45
0.21	8.19×10^6	165	0.21	2.96×10^7	46	0.10
1.12	2.37×10^7	49	0.89	9.48×10^7	12	0.68
0.67	2.04×10^7	59	0.49	1.11×10^8	11	0.68

sized in excellent yields from resacetiphenone, and good yields (up to 50%) of daidzein could be obtained in the $Tl(NO_3)_3$ oxidation using only acetylation for protection of the hydroxyls. Difficulties were experienced in purification of the daidzein however, and the method was not adopted for the radiochemical synthesis. For the preparation of (±)-7-hydroxy-4′-methoxyisoflavanone-[Me-^{14}C], although isoflavanones are usually synthesized by catalytic hydrogenation of isoflavones (often as their acetates) [28], this method when applied to formononetin consistently yielded the fully-reduced isoflavan rather than the required isoflavanone (**10**). Recent reports [29] suggest the precise pre-treatment of the catalyst is an important criterion for successful hydrogenation of isoflavones. Thus, the isoflavanone (**10**) was synthesized by $NaBH_4$ reduction of formononetin-[Me-^{14}C], a process which produced satisfactory yields of the required compound; formononetin-[Me-^{14}C] recovered from the reaction mixture was re-treated to improve the overall yield. Surprisingly, the product obtained in the reaction which involved a large excess of $NaBH_4$ was in fact the isoflavanone and not the isoflavanol (**12**). Attempts to produce the isoflavanol for incorporation studies were totally unsuccessful; isoflavanol (**11**) is so readily cyclized to the pterocarpan that it too could not be tested in feeding experiments.

DISCUSSION

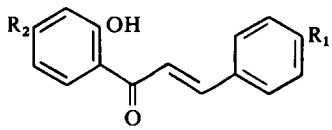
The incorporation data show the ready incorporation of 2′,4,4′-trihydroxychalcone and formononetin into the three phytoalexins, demethylhomopterocarpin, vestitol and sativan, and also into 9-*O*-methylcoumestrol. In contrast, 2′,4′-dihydroxy-4-methoxychalcone and daidzein are relatively inefficient precursors of these compounds. Synthesis of these four compounds is greatly stimulated by the abiotic treatment, and the incorporation of precursors is quite substantial. Coumestrol synthesis is however not noticeably affected by the treatment, and consequently incorporation of precursors into this compound is not nearly as great. However, 2′,4,4′-trihydroxychalcone and daidzein were incorporated into coumestrol in agreement with earlier studies in mature specimens of *M. sativa* [21, 22], and in *Phaseolus aureus* seedlings [23, 24]. There thus appears to be a stimulation of biosynthesis of 4′-methoxy rather than 4′-hydroxy derivatives (isoflavone numbering) by this induction process, and the inability to detect medicagol (or maackiain) suggests that synthesis of methylenedioxy derivatives is similarly not stimulated. The incorporation results parallel those reported earlier in other areas of isoflavonoid biosynthesis. 2′,4,4′-Trihydroxychalcone is readily incorporated into formononetin [16, 21, 30], demethylhomopterocarpin [16], maackiain [16] and the rotenoid amorphigenin [18], whereas daidzein and 2′,4′-dihydroxy-4-methoxychalcone are poor precursors [16, 18, 22, 31]. Methylated compounds destined for formononetin synthesis were in fact demethylated prior to incorporation [31, 32]. All of these results support the hypothesis that the aryl migration step characteristic of isoflavonoid biosynthesis is dependent on the participation of a 4-hydroxy group on the migrating aryl, and that this group may be methylated as an integral part of the process [17, 18]. In such cases, 4′-hydroxyisoflavones (proton catalysed decomposition products of the postulated [17] spirodienone intermediate) would not be obligatory intermediates in the biosynthesis of 4′-methoxyisoflavones, which could arise by *S*-adenosylmethionine mediated decomposition of the spirodienone [18]. Although the isolation of an enzyme catalysing the 4′-methylation of daidzein and genistein [33] demonstrates the existence of an alternative pathway, and probably accounts for the observed small incorporations of daidzein into more complex 4′-methoxy isoflavonoids, the concensus of results indicates this process may only be a minor biosynthetic route to formononetin. Moreover, the present results where daidzein is incorporated



14 R_1 = OH; R_2 = H
15 R_1 = OMe; R_2 = H
16 R_1R_2 = OCH_2O

into coumestrol, via a synthetic pathway which appears to be non-stimulated, yet is not incorporated into 9-*O*-methylcoumestrol whose synthesis is greatly stimulated by the abiotic treatment, suggest the non-incorporation is unlikely to be caused by poor transport and/or rapid turnover. Such comments must, of course, be limited by the relatively low rate of coumestrol synthesis and the consequent poor incorporation figures, but similar conclusions can be drawn from the earlier biosynthetic experiments in *M. sativa*, where daidzein proved a poor precursor of formononetin, whilst at the same time bing well-incorporated into coumestrol [22].

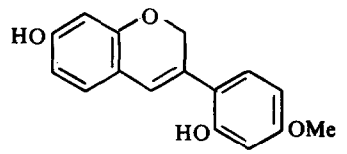
The good incorporations of formononetin, 2′,7-dihydroxy-4′-methoxyisoflavone, (±)-7-hydroxy-4′-methoxyisoflavanone and (±)-2′,7-dihydroxy-4′-methoxyisoflavanone into demethylhomopterocarpin, vesitol,



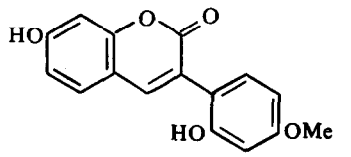
17 R_1 = R_2 = OH
18 R_1 = OMe; R_2 = OH
19 R_1 = OMe; R_2 = OCH_2Ph

sativan and 9-*O*-methylcoumestrol offer substantial evidence that the transformation of formononetin to 2′,7-dihydroxy-4′-methoxyisoflavanol (**11**) may in fact be accomplished via a metabolic grid of isoflavones, isoflavanones and isoflavanols. In the absence of incorporation data for 7-hydroxy-4′-methoxyisoflavanol (**12**) though, the incorporation of isoflavanone (**10**) via formononetin cannot be excluded completely, since isoflavanones may be oxidized to isoflavones *in vivo* [34]. The incorporation data for the isoflavanones are not corrected for the possible utilization of only one enantiomer from the racemate fed, and may be doubled if epimerisation does not occur. The reduction sequence from 2′,7-dihydroxy-4′-methoxyisoflavone to demethylhomopterocarpin has been shown to be stereospecific [7], and a number of optically active isoflavanones have now been isolated from nature [35–39]. Thus suitable

correction may well be justified. Even after correction though, incorporation data for 7-hydroxy-4'-methoxyisoflavanone are still the lowest of the four compounds tested, and if a metabolic grid is operative, this could mean the major route is probably via 2',7-dihydroxy-4'-methoxyisoflavone. This contrasts with the proposed major pathway to 2',4',7-trihydroxyisoflavanone from daidzein in the biosynthesis of coumestrol [24], where a sequence via 4',7-dihydroxyisoflavanone seemed more important. The proposed biosynthetic pathway to coumestrol must now be modified in the light of further studies [40] on 9-*O*-methylcoumestrol biosynthesis in *M. sativa*, but 2',4',7-trihydroxyisoflavanone still figures as a key intermediate. The excellent incorporation of 2'-hydroxyisoflav-3-ene and 2'-hydroxyisoflav-3-ene-2-one precursors into 9-*O*-methylcoumestrol rules out the role previously ascribed to pterocarp-6a-enes in coumestan biosynthesis. A pathway to 9-*O*-methylcoumestrol involving dehydration of isoflavanol (**11**) to isoflav-3-ene (**20**), allylic oxidation to **21** followed by oxidative ring cyclization has now been proposed [40]. Isoflav-3-ene (**20**) could also be derived by loss of a proton from carbonium ion (**13**). Thus, it seems likely that demethylhomopterocarpin, vestitol, sativan and 9-*O*-methylcoumestrol share a common biosynthetic pathway to isoflavanol (**11**) or carbonium ion (**13**), and stimulation of this pathway accounts for the increased synthesis of **15** as well as the phytoalexins. Which enzymes are actually involved in the regulation are at present unknown. A similar pathway involving hydroxy derivatives presumably operates for the biosynthesis of coumestrol, but the enzymes on this pathway are seemingly not affected by the abiotic treatment.



20



21

EXPERIMENTAL

General. 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, 32:1; B, C_6H_6–EtOAc–MeOH–petrol (60–80°), 6:4:1:3; C, $CHCl_3$–*iso*-PrOH, 10:1; D, C_6H_6–EtOH, 9:1; E, C_6H_6–EtOAc–MeOH–petrol (60–80°), 6:4:1:6; or 0.5 mm layers of cellulose (Whatman CC41) in solvent system F, HOAc–H_2O, 1:1. Me_2CO was used for elution of TLC zones. Radioactive samples were counted as previously [16]. The syntheses of carrier (±)-demethylhomopterocarpin [16], (±)-vestitol [6], (±)-sativan [6] and 9-*O*-methylcoumestrol [5] have been described. Coumestrol was purchased (Eastman).

Radiochemicals. The syntheses of 2',4,4'-trihydroxychalcone-[carbonyl-^{14}C] (0.223 mCi/mM) [18], 2',4'-dihydroxy-4-methoxychalcone-[carbonyl-^{14}C] (0.203 mCi/mM) [18], 2',7-dihydroxy-4'-methoxyisoflavone-[Me-^{14}C] (0.518 mCi/mM) [5] and (±)-2',7-dihydroxy-4'-methoxyisoflavanone-[Me-^{14}C] (0.546 mCi/mM) [5] have been described. Formononetin-[Me-^{14}C] (0.676 mCi/mM) was synthesized according to published procedures [18, 25].

Daidzein-[4-^{14}C]. 4'-Benzyloxy-2'-hydroxyacetophenone-[carbonyl-^{14}C] [27] (0.316 mCi/mM, 80 mg) and anisaldehyde (140 mg) in EtOH (3 ml) were stirred at room temp. overnight with KOH (0.8 g) in H_2O (0.75 ml). The mixture was poured into H_2O, acidified with conc HCl, then extracted with EtOAc (3 ×). The EtOAc extract was evapd, and the residue separated by TLC (solvent A) to give 4'-benzyloxy-2'-hydroxy-4-methoxychalcone-[carbonyl-^{14}C] (100 mg). Inactive material, recrystallized from MeOH had mp 128–132°, lit. [41] 132–3°. This chalcone (100 mg) was acetylated (dry Py–Ac_2O, room temp. overnight), and the reaction mixture poured into H_2O and extracted with EtOAc (3 ×). The extract was washed with dil. HCl (2 ×), then H_2O, and evapd and dried. The acetate, without further purification, was dissolved in MeOH (30 ml) and stirred at room temp. overnight with $Tl(NO_3)_3.3H_2O$ (200 mg). Solid KOH (0.3 g) was then added, and the mixture was stirred for a further 1 hr. After neutralization with conc HCl, dil. HCl (10%, 2 ml) was added, and the mixture was heated under reflux for 2 hr, then filtered hot. The filtrate was concd under red. press., diluted with H_2O, and extracted with EtOAc (3 ×). The EtOAc extract, on evapn yielded 7-benzyloxy-4'-methoxyisoflavone-[carbonyl-^{14}C] which was purified by TLC (solvent A). Inactive material, recrystallized from MeOH had mp 181–2°, lit. [42] 180–2°. This isoflavone was dealkylated by heating under reflux with HI (5 ml) overnight. The mixture was poured into H_2O, decolourized by the addition of solid $Na_2S_2O_5$, and extracted with EtOAc (3 ×). The evapd extracts were separated by TLC (solvent B) to yield daidzein-[4-^{14}C] (30 mg), which was purified to constant sp. act. (0.322 mCi/mM) by TLC (solvents C and D), and was chromatographically and spectrally identical to an authentic sample.

(±)-7-Hydroxy-4'-methoxyisoflavanone-[Me-^{14}C]. A mixture of formononetin-[Me-^{14}C] (0.676 mCi/mM, 8.5 mg), EtOH (5 ml) and $NaBH_4$ (100 mg) was stirred at 60° overnight. The soln was concd, diluted with H_2O and extracted with EtOAc (3 ×). The evapd EtOAc extracts were separated by TLC (solvent B) to give (±)-7-hydroxy-4'-methoxyisoflavanone-[Me-^{14}C] (2.3 mg) and unreacted formononetin-[Me-^{14}C], which was re-treated as above to give further isoflavanone (0.6 mg). The combined product was purified to constant sp. act. (0.608 mCi/mM) by TLC (solvents C and D). Inactive material, recrystallized MeOH, had mp 194–7°, lit. [43] 197°.

Plant material, feeding techniques and isolation of metabolites. Seeds of *M. sativa* (4g batches) were germinated as described previously [6]. Phytoalexin synthesis was stimulated by treating the 4-day-old seedlings with aq. $CuCl_2$ [6] over 8 hr and exposing them to UV light (254 nm, Mineralight UVSL-58, Ultra-Violet Products Inc., 5 cm from seedlings) for 0.5 hr at the beginning of this period. Labelled compounds were fed as before [6], and extractions were also carried out in a similar manner. The Et_2O extracts obtained were separated by TLC (solvent B). Bands corresponding to demethylhomopterocarpin plus sativan, vestitol plus 9-*O*-methylcoumestrol, and coumestrol were eluted. The demethylhomopterocarpin/sativan band was treated as previously described [6]. The vestitol/9-*O*-methylcoumestrol band was applied to TLC plates which were developed twice for a distance of *ca* 2 cm (solvent B), thus concentrating the compounds into a narrow band. The plates were then developed using solvent C. Bands corresponding to vestitol and 9-*O*-methylcoumestrol were eluted. The coumestrol band was

purified further by cellulose TLC (solvent F). Isoflavonoid content was assayed by UV absorption of EtOH solns: demethylhomopterocarpin, λ_{max} 288 nm, log ε 3.94 [16], sativan, λ_{max} 284 nm, log ε 3.80 [6], vestitol, λ_{max} 281 nm, log ε 3.86 [6], 9-*O*-methylcoumestrol, λ_{max} 341 nm, log ε 4.40, coumestrol, λ_{max} 343 nm, log ε 4.45 [23].

Demethylhomopterocarpin, vestitol and sativan were diluted with inactive carrier and treated as previously. 9-*O*-Methylcoumestrol and coumestrol were separately diluted with carrier (25 mg), then methylated by heating and stirring under reflux overnight with MeI (1 ml), dry Me_2CO (30 ml) and dry K_2CO_3 (1 g). The reaction mixture was filtered, the inorganics washed thoroughly with dry Me_2CO, the combined filtrates evapd and purified by TLC (solvent E). Di-*O*-methylcoumestrol was eluted, recrystallized from MeOH and sublimed at 180°/0.05 mm, procedures which were repeated until the material was of constant sp. act. Inactive material had mp 198–200°, lit. [44] 200–1°.

Acknowledgement—We thank the Agricultural Research Council for financial support.

REFERENCES

1. Smith, D. G., McInnes, A. G., Higgins, V. J. and Millar, R. L. (1971) *Physiol. Plant Pathol.* **1**, 41.
2. Ingham, J. L. and Millar, R. L. (1973) *Nature* **242**, 125; Ingham, J. L., personal communication.
3. Van Etten, 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.
4. Gross, D. (1977) *Fortschr. Chem. Org. Naturst.* **34**, 187.
5. Dewick, P. M. (1977) *Phytochemistry* **16**, 93.
6. Dewick, P. M. and Martin, M. (1979) *Phytochemistry* **18**, 591.
7. Dewick, P. M. and Ward, D. (1977) *J. Chem. Soc. Chem. Commun.* 338.
8. Bu'Lock, J. D. (1965) *The Biosynthesis of Natural Products*, p. 82. McGraw-Hill, London.
9. Bickoff, E. M., Loper, G. M., Hanson, C. H., Graham, J. H., Witt, S. C. and Spencer, R. R. (1967) *Crop. Sci.* 7, 259.
10. Olah, A. F. and Sherwood, R. T. (1971) *Phytopathology* **61**, 65.
11. Wong, E. and Latch, G. C. M. (1971) *Phytochemistry* **10**, 466.
12. Keen, N. T., Zaki, A. I. and Sims, J. J. (1972) *Phytochemistry* **11**, 1031.
13. Hijwegen, T. (1973) *Phytochemistry* **12**, 375.
14. Rich, J. R., Keen, N. T. and Thomason, I. J. (1977) *Physiol. Plant Pathol.* **10**, 105.
15. Gnanamanickam, S. S. and Patil, S. S. (1977) *Physiol. Plant Pathol.* **10**, 159.
16. Dewick, P. M. (1975) *Phytochemistry* **14**, 979.
17. Pelter, A., Bradshaw, J. and Warren, R. E. (1971) *Phytochemistry* **10**, 835.
18. Crombie, L., Dewick, P. M. and Whiting, D. A. (1973) *J. Chem. Soc. Perkin Trans.* 1 1285.
19. Wong, E. (1975) in *The Flavonoids* (Harborne, J. B., Mabry, T. J. and Mabry, H., eds.) p. 780 and refs. cited therein. Chapman & Hall, London.
20. Livingston, A. L., Witt, S. C., Lundin, R. E. and Bickoff, E. M. (1965) *J. Org. Chem.* **30**, 2353.
21. Grisebach, H. and Barz, W. (1964) *Z. Naturforsch. Teil. B* **19**, 569.
22. Barz, W. and Grisebach, H. (1966) *Z. Naturforsch. Teil B* **21**, 1113.
23. Dewick, P. M., Barz, W. and Grisebach, H. (1970) *Phytochemistry* **9**, 775.
24. Berlin, J., Dewick, P. M., Barz, W. and Grisebach, H. (1972) *Phytochemistry* **11**, 1689.
25. Barz, W., Adamek, C. and Berlin, J. (1970) *Phytochemistry* **9**, 1735.
26. Farkas, L., Gottsegen, A., Nogradi, M. and Antus, S. (1974) *J. Chem. Soc. Perkin Trans.* 1 305.
27. Dewick, P. M. and Ward, D. (1978) *Phytochemistry* **17**, 1751.
28. Farkas, L. (1975) in *The Flavonoids* (Harborne, J. B., Mabry, T. J. and Mabry, H., eds.) p. 192 and refs cited therein. Chapman & Hall, London.
29. Lamberton, J. A., Suares, H. and Watson, K. G. (1978) *Aust. J. Chem.* **31**, 455.
30. Grisebach, H. and Patschke, L. (1960) *Chem. Ber.* **93**, 2326.
31. Barz, W. and Grisebach, H. (1967) *Z. Naturforsch. Teil B* **22**, 627.
32. Ebel, J., Achenbach, H., Barz, W. and Grisebach, H. (1970). *Biochim. Biophys. Acta* **215**, 203.
33. Wengenmayer, H., Ebel, J. and Grisebach, H. (1974) *Eur. J. Biochem.* **50**, 135.
34. Grisebach, H. and Zilg, H. (1968) *Z. Naturforsch. Teil B* **23**, 494.
35. Suginome, H. (1959) *J. Org. Chem.* **24**, 1655.
36. Suginome, H. (1966) *Bull. Chem. Soc. Jpn* **39**, 1544.
37. Fitzgerald, M. A., Gunning, P. J. M. and Donnelly, D. M. X. (1976) *J. Chem. Soc. Perkins Trans.* 1 186.
38. Delle Monache, G., Delle Monache, F., Marini-Bettolo, G. B., Machado Fernandez de Albuquerque, M., De Mello, J. F. and Goncalves de Lima, O. (1977) *Gazz. Chim. Ital.* **107**, 189.
39. Kalra, A. J., Krishnamurti, M., and Nath. M. (1977) *Ind. J. Chem.* **15B**, 1084.
40. Martin, M. and Dewick, P. M. (1978) *Tetrahedron Letters* 2341.
41. Mahol, H. S., Rai, H. S. and Venkataraman, K. (1935) *J. Chem. Soc.* 866.
42. Bhrara, S. C., Jain, A. C. and Seshadri, T. R. (1964) *Tetrahedron* **20**, 1141.
43. Inoue, N. (1961) *Sci. Rep. Tohoku Univ. Ser.* 1 **45**, 63; *Chem. Abstr.* (1963) **58**, 5619.
44. Bickoff, E. M. and Booth, A. M. (1962) Am. Patent; *Chem. Abstr.* **56**, 5936.