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BIOSYNTHESIS OF PTEROCARPAN PHYTOALEXINS IN *TRIFOLIUM PRATENSE*

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Key Word Index—*Trifolium pratense*; Leguminosae; red clover; biosynthesis; phytoalexin; pterocarpan; demethylhomopterocarpin; maackiain; isoflavonoid; coumestan.

Abstract—Feeding experiments have demonstrated that 7,2′-dihydroxy-4′-methoxy-isoflavone-[^{14}C-Me] and -isoflavanone-[^{14}C-Me] are extremely efficient precursors of the phytoalexin demethylhomopterocarpin in Cu^{2+}-treated red clover seedlings. Neither of these compounds, nor demethylhomopterocarpin-[^{14}C-Me], was incorporated into a second pterocarpan phytoalexin, maackiain. 3-Hydroxy-9-methoxypterocarp-6a-ene-[^{14}C-Me] was a poor precursor of both pterocarpans. A biosynthetic pathway to demethylhomopterocarpin via 2′-hydroxylation of formononetin (7-hydroxy-4′-methoxyisoflavone) and subsequent reduction to the isoflavanone is proposed. The conversion of this isoflavanone into the pterocarpan may involve the corresponding isoflavanol and a carbonium ion intermediate. The branch-point to maackiain is probably at the formononetin stage. The presence of two coumestans, 9-*O*-methylcoumestrol and medicagol, previously unreported in red clover, is demonstrated. Biosynthetic implications are discussed.

INTRODUCTION

In response to fungal infection [1], or on treatment with heavy metal ions [2], seedlings of red clover (*Trifolium pratense* L.) synthesise two pterocarpan phytoalexins, (6aR,11aR)-demethylhomopterocarpin (medicarpin) (**1**) and (6aR,11aR)-maackiain (**2**). Feeding experiments utilising Cu^{2+}-treated seedlings have demonstrated [2] that labelled 2′,4′,4-trihydroxychalcone (**3**) and 7-hydroxy-4′-methoxyisoflavone (formononetin) (**6**) were readily incorporated into both pterocarpans, and that the 2,4- and 2,4,5-oxygenation patterns in the D-ring of demethylhomopterocarpin and maackiain respectively are presumably built up from the simple 4′-*O*-methyl of formononetin. However, 2′,4′-dihydroxy-4-methoxychalcone (**4**) and 7,4′-dihydroxyisoflavone (daidzein) (**5**) were inefficient biosynthetic precursors, results which may be rationalised if methylation is an integral part of the aryl migration process involved in the biosynthesis of isoflavones [3,4].

Further labelled isoflavonoids have been tested as precursors of demethylhomopterocarpin and maackiain in Cu^{2+}-treated red clover seedlings, to investigate the biosynthetic route to these pterocarpans, compounds which may well play an important role in the disease resistance of this and other plants [5]. Some of the results have been published in a preliminary communication [6].

RESULTS

Compounds tested as biosynthetic precursors were 7,2′-dihydroxy-4′-methoxyisoflavone-[^{14}C-Me] (**7**), (±)-7,2′-dihydroxy-4′-methoxyisoflavanone-[^{14}C-Me] (**8**), 3-hydroxy-9-methoxypterocarp-6a-ene-[^{14}C-Me] (**9**) and (±)-3-hydroxy-9-methoxypterocarpan-[^{14}C-Me] (demethylhomopterocarpin) (**1**).

Base condensation of 4′-benzyloxy-2′-hydroxyacetophenone with 4-benzyloxy-2-methoxybenzaldehyde yielded 4,4′-dibenzyloxy-2′-hydroxy-2-methoxychalcone which was converted via thallium nitrate oxidation and treatment with acid [7] into 7,4′-dibenzyloxy-2′-methoxyisoflavone. Debenzylation followed by selective monobenzylation produced 7-benzyloxy-4′-hydroxy-2′-methoxyisoflavone, which was methylated using methyl iodide-[^{14}C]. The product, 7-benzyloxy-2′,4′-dimethoxyisoflavone-[4′-^{14}C-Me] was debenzylated/selectively demethylated by treating with $AlCl_3$ in MeCN[8] to give 7,2′-dihydroxy-4′-methoxyisoflavone-[^{14}C-Me] (**7**). Catalytic hydrogenation of this isoflavone (via the acetate) produced the corresponding (±)-isoflavanone-[^{14}C-Me] (**8**), and the latter compound was converted into 3-hydroxy-9-methoxypterocarp-6a-ene-[^{14}C-Me] (**9**) by heating with mineral acid. $NaBH_4$ reduction of 7,2′-dihydroxy-4′-methoxyisoflavone-[^{14}C-Me] followed by treatment with acid gave (±)-demethylhomopterocarpin-[^{14}C-Me] (**1**).

Small quantities (*ca* 0.5 mg) of these labelled compounds were separately administered as their Na salts in phosphate buffer to batches of 4-day-old dark-grown red clover seedlings [2], which had been treated with aqueous $CuCl_2$ for 8 hr. After a feeding period of 16 hr in the dark, the seedlings were worked up, and the pterocarpan phytoalexins were isolated from the extract. As described previously [2], the pterocarpans were isolated together, individual concentrations were assayed by UV, then half of the fraction was diluted with inactive (±)-demethylhomopterocarpin carrier, and the other half with (±)-maackiain carrier. The individual ptero-

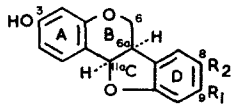
Table 1. Incorporation of [*methyl*-^{14}C]-labelled isoflavonoids into pterocarpans in *Trifolium pratense* seedings*

Compound fed	Unabsorbed precursor (%)	Demethylhomopterocarpin sp. act. (dpm/mM)	Dilution	Incorp† (%)	Maackiain sp. act. (dpm/mM)	Dilution	Incorp† (%)
For mononetin	30	3.45×10^7	34	0.62	2.00×10^7	59	0.51
7,2'- Dihydroxy-4'-methoxyisoflavone	22	4.35×10^8	2.7	12.8	1.45×10^5	7900	0.0035
(±)-7,2'-Dihydroxy-4'-methoxyisoflavanone	27	3.80×10^8	3.2	7.6	1.02×10^5	11900	0.0019
3-Hydroxy-9-methoxypterocarp-6a-ene	19	3.02×10^6	420	0.040	2.28×10^5	5600	0.0044
(±)-Demethylhomopterocarpin	20	7.92×10^8	1.5	35.6	1.86×10^5	6100	0.0035

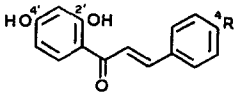
* Four-day-old, $CuCl_2$ inducer applied for 8 hr, feeding period 16 hr. † Incorporation figures are not corrected for unabsorbed precursor, or possible utilisation of only one enantiomer from racemic mixtures.

carpans were methylated and purified to constant specific activity. The results of these comparative feeding experiments are summarised in the Table 1, which also includes incorporation data for 7-hydroxy-4'-methoxyisoflavone-[^{14}C-Me] (formononetin) (**6**), administered in the same set of experiments. Since this compound is a good precursor of both pterocarpans in red clover [2], these data act as a standard.

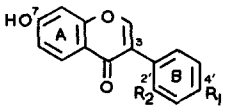
The incorporation figures indicate that isoflavone (**7**) and (±)-isoflavanone (**8**) are both excellent precursors of



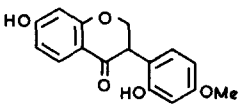
(1) R_1 = OMe, R_2 = H
(2) R_1R_2 = O-CH_2-O



(3) R = OH
(4) R = OMe



(5) R_1 = OH, R_2 = H
(6) R_1 = OMe, R_2 = H
(7) R_1 = OMe, R_2 = OH



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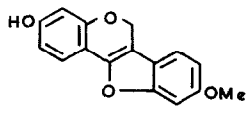

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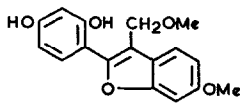

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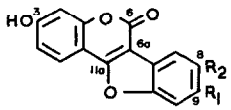
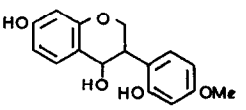


(11) R_1 = OH, R_2 = H
(12) R_1 = OMe, R_2 = H
(13) R_1R_2 = O-CH_2-O



(14)

demethylhomopterocarpin, considerably better than formononetin (**6**). Neither of these compounds was significantly incorporated into maackiain. The incorporation of pterocarp-6a-ene (**9**) into demethylhomopterocarpin was very small by comparison, and into maackiain was negligible. (±)-Demethylhomopterocarpin was not incorporated into maackiain.

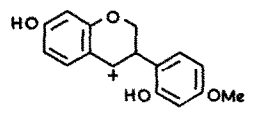
Autooxidation of pterocarp-6a-enes to the corresponding 6-oxo derivatives (coumestans) in the presence of air has frequently been reported [9–11]). To ascertain whether this process was occurring during the feeding period, thus resulting in unusually low incorporation figures for 3-hydroxy-9-methoxypterocarp-6a-ene, the stability of this pterocarp-6a-ene was investigated. An acetone solution of (**9**), after being stirred overnight in the presence of air showed little reaction, only very faint traces of the coumestan oxidation product being observed. In contrast, a methanolic solution treated similarly contained hardly any of the original pterocarp-6a-ene. Traces of coumestan were present, but the major product was found to be 2-(2,4-dihydroxyphenyl)-6-methoxy-3-methoxymethyl-benzofuran (**10**). A similar reaction has been noted [12] for anhydropisatin (3-methoxy-8,9-methylenedioxypterocarp-6a-ene), the conversion being the result of photolytic ring fission and then alcoholysis. Samples of (**9**) dissolved in the feeding mixture of NaOH/phosphate buffer also disappeared over 24 hr to give faint traces of the coumestan, plus other unidentified products. Maximum stability of (**9**) in an aqueous medium was attained by using a mixture of 2-methoxyethanol–deoxygenated water containing either NaOH or Tween-20, and keeping the solution in an N_2 atmosphere. Such solutions after a period of 24 hr in the light contained about half of the original pterocarp-6a-ene concentration, together with a product which was presumably the 2-methoxyethanol analogue of (**10**). Further feeding experiments were carried out with 3-hydroxy-9-methoxypterocarp-6a-ene-[^{14}C-Me] employing these solvent systems, and conducting the first 3.5 hr of the feeding period in an N_2 atmosphere (all feedings performed in the dark). Although such feeding conditions were abnormal for the plant, uptake of precursor and production of the pterocarpans were not noticeably different from those observed in the other experiments carried out under "normal" conditions. Incorporation data, however, were virtually identical to those shown in the Table, and demonstrate that 3-hydroxy-9-methoxypterocarp-6a-ene is not significantly utilised for pterocarpan production in red clover.

Chromatography of the seedling extracts during these experiments consistently revealed the presence of a zone exhibiting a strong bright-blue fluorescence under UV light. Such fluorescence is characteristic of coumestans,

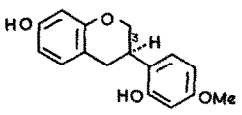
but the only reported coumestan in *T. pratense* is coumestrol (**11**) [13], and the observed zone ran at a considerably higher R_f than (**11**). UV data (λ_{max}^{EtOH} 343, 309, 302 nm) indicated the presence of two coumestans. Comparison with authentic specimens by TLC and UV spectroscopy (shifts in the presence of NaOAc, NaOEt, NaOAc–H_3BO_3) suggested these were 3-hydroxy-9-methoxycoumestan (9-*O*-methylcoumestrol) (**12**) and 3-hydroxy-8,9-methylenedioxy-coumestan (medicagol) (**13**). This was confirmed by the mass spectrum of the acetylated mixture. The acetates could be partially separated by TLC [14]. Reference material was synthesised by DDQ oxidation [11] of the corresponding (±)-pterocarpans [2].

DISCUSSION

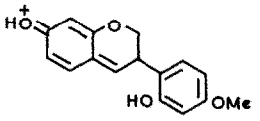
Preliminary feeding experiments using DL-phenylalanine-[1-^{14}C] [2] had suggested that the red clover phytoalexins demethylhomopterocarpin (**1**) and maackiain (**2**) were synthesised *de novo* on treatment of the plant tissue with Cu^{2+} ions, and the similar incorporation



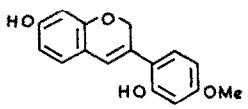
(**15**)



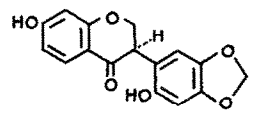
(**16**)



(**17**)



(**18**)



(**19**)

profiles observed over a period of time indicated that it was unlikely that (**1**) was a precursor of (**2**). The latter conclusion is confirmed by the negligible incorporation of labelled (**1**) into (**2**).

7,2′-Dihydroxy-4′-methoxy-isoflavone (**7**) and -isoflavanone (**8**) are extremely efficient precursors of demethylhomopterocarpin, and may well be natural intermediates on its biosynthetic pathway. Although isoflavone (**7**) has not been reported in nature, 7,2′-dihydroxy-4′-methoxyisoflavanone has recently been isolated [15] from *Onobrychis viciifolia* (Leguminosae). It appears likely therefore that formononetin (**6**) may be 2′-hydroxylated to (**7**) which is then reduced to the isoflavanone (**8**). 2′-Hydroxylation of isoflavones has been previously demonstrated [16] in *Phaseolus aureus*, and this, or the corresponding hydroxylation of isoflavanones is regarded [17] as a probable step in the biosynthetic pathway to coumestans in this and other plants. The conversion of (**8**) into (**1**) however clearly does not proceed via the pterocarp-6a-ene (**9**). Pterocarp-6a-enes, formed by dehydration of 2′-hydroxyisoflavanones, or oxidative cyclisation of 2′-deoxyisoflavanones, have been postulated [9, 16–18] as key intermediates in the biosynthesis of other isoflavonoid compounds. Thus, reduction might give pterocarpans, addition of water could produce 6a-hydroxypterocarpans, whilst allylic oxidation would serve as a route to coumestans. The incorporation of (**9**) into (**1**) (0.040%) is insignificant by comparison with other precursors, and even this small amount may be due to prior hydrolysis to (**8**), a good precursor. Incorporation into maackiain was negligible. Thus, a hypothesis for pterocarpan biosynthesis not involving pterocarp-6a-ene intermediates is clearly necessary. From these results, the most likely route from (**8**) to (**1**) would be by further reduction to the isoflavanol (**14**) which could lose water and cyclise to the pterocarpan [19]. Such a pathway would be analogous to laboratory syntheses [20] of pterocarpans by $NaBH_4$ reduction of 2′-hydroxyisoflavanones, followed by treatment with acid. This synthetic route probably involves a carbonium ion (**15**) [21], and the same entity could occupy a place in the biosynthetic pathway. Strong support comes from the results of feeding experiments [22] in lucerne (*Medicago sativa*) where demethylhomopterocarpin and the isoflavan vestitol (**16**), phytoalexins of lucerne, have been shown to be interconvertible, although they appear to be synthesised simultaneously from a common intermediate. Such an intermediate could be the carbonium ion (**15**), or its mesomeric counterpart (**17**), presumably enzyme-bound. Cyclisation and loss of a proton would lead to the pterocarpan (**1**), whilst addition of hydride ion would produce the isoflavan (**16**). Reversal of these pathways would explain the interconversion. Structure (**17**) represents the protonated form of the quinone-methide intermediate postulated [23] in the chemical conversion of (**16**) into (**1**), a reaction proposed as a chemical analogy for pterocarpan biosynthesis.

We have tested (±)-vestitol-[^{14}C-Me] as a precursor of the pterocarpans in red clover [22]. It was incorporated into demethylhomopterocarpin (0.90%, dilution 19) but not into maackiain (0.0018%, dilution 8400). Comparative figures for (±)-7,2′-dihydroxy-4′-methoxyisoflavanone-[^{14}C-Me] were: demethylhomopterocarpin (2.7%, dilution 4·5), maackiain (0.0047%, dilution 3200). These figures also suggest that vestitol is not a normal biosynthetic precursor of demethylhomopterocarpin, but may be converted into that compound by some common intermediate, e.g. carbonium ion (**15**). An alternative, uncharged intermediate, not yet excluded by feeding experiments, might be the isoflav-3-ene (**18**). However, if isoflav-3-enes are intermediates in the biosynthesis of pterocarpans and isoflavans, it is surprising that no naturally occurring example of this class of isoflavonoid has yet been reported.

Although the results strongly suggest the biosynthetic route to demethylhomopterocarpin involves 2′-hydroxyisoflavone (**7**) and 2′-hydroxyisoflavanone (**8**), isoflavanol (**14**) may be produced from formononetin via a 'metabolic grid' [24] of isoflavones, isoflavanones and isoflavanols. The further elaboration of the B ring of formononetin to produce the 2,4,5 substitution pattern in maackiain proceeds by a route, yet to be determined, which does not involve (**7**), (**8**), (**9**) or (**1**). Formononetin probably represents the branch-point in the pathways to (**1**) or (**2**).

The two pterocarpan phytoalexins produced by red

clover have large negative specific optical rotations (see Experimental) and can thus be assigned the 6aR,11aR configuration [25]. Thus, the enzymic reduction processes leading from isoflavone (**7**) to demethylhomopterocarpin via (**8**) and (**14**) are presumably stereospecific. Incorporation data for racemic substrates in the Table are, however, uncorrected for possible utilisation of only one enantiomer. In particular, if only one enantiomer of isoflavanone (**8**) is involved in the biosynthesis of demethylhomopterocarpin, and epimerisation does not occur, the percentage incorporation figures may be doubled. Relatively few naturally occurring isoflavanones are known, and of these, only two, (3S)-7,4′-dihydroxyisoflavanone [26] and (3R)-sophorol (**19**) [27] have been isolated in optically active form. (3R)-Sophorol may well occupy an important position in the biosynthetic pathway to (6aR,11aR)-maackiain.

The co-occurrence in red clover of the coumestans 9-*O*-methylcoumestrol (**12**) and medicagol (**13**), together with the corresponding pterocarpans demethylhomopterocarpin and maackiain is of biosynthetic significance, and suggests common intermediates in their biosynthetic pathways. These could well be 7,2′-dihydroxy-4′-methoxyisoflavanone (**8**) for the methoxy compounds, and sophorol (**19**) for the methylenedioxy series. Although pterocarp-6a-enes are apparently not involved in pterocarpan biosynthesis, they could still be precursors of coumestans, and would probably be derived by dehydration of 2′-hydroxyisoflavanones. The ready oxidation of pterocarp-6a-enes by such mild oxidants as atmospheric oxygen has so far precluded their use in feeding experiments as possible precursors of coumestans.

EXPERIMENTAL

General. The growing of plant material, feeding techniques, isolation and purification of metabolites, and counting of radioactive samples were all as previously described [2]. 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–EtOH, 92:8; D, toluene–Et_2O, 1:1; E, C_6H_6–EtOAc, 32:1; F, Et_2O–petrol (60–80°), 4:1; G, petrol (60–80°)–EtOAc, 7:3. Me_2CO (Analar) was used for elution of TLC zones. The synthesis of formononetin-[*methyl*-^{14}C] (sp. act. 0 535 mCi/mM) has been reported [4].

7-Benzyloxy-4′-hydroxy-2′-methoxyisoflavone. 4′-Benzyloxy-2′-hydroxyacetophenone (2g) and 4-benzyloxy-2-methoxybenzaldehyde (2g) in EtOH (100 ml) were stirred at R° overnight with KOH (20g) in H_2O (20 ml). The mixture was poured into H_2O, acidified with conc HCl, then extracted with EtOAc (2x). The EtOAc extract was evaporated, and the residue crystallised from $CHCl_3$–MeOH to give *4,4′-dibenzyloxy-2′-hydroxy-2-methoxychalcone* (1.4g), mp 143–4°. (Found: C, 77.62; H, 5.75. $C_{30}H_{26}O_5$ requires: C, 77.2; H, 5.58%). The above chalcone (1.4g) was acetylated (dry Py–Ac_2O, R° overnight), and the reaction mixture poured into H_2O and extracted with EtOAc (2x). The extract was washed with dil HCl(2x), then H_2O, and evaporated and dried. The acetate, without further purification, was dissolved in MeOH (500 ml) and stirred at R° overnight with $Tl(NO_3)_3$, $3H_2O$ (1.5g). Solid KOH (4g) was then added, and the mixture was stirred for a further 1 hr. After neutralisation with conc HCl, dil HCl (10%, 30 ml) was added, and the mixture heated under reflux for 2 hr, then filtered hot. Filtrate was concentrated under red pres, diluted with H_2O and extracted with EtOAc(2x). The EtOAc extract, on evaporation yielded *7,4′-dibenzyloxy-2′-methoxyisoflavone* which was recrystallised from MeOH. Yield 1.0g, mp 133–5°, 149–50° (dimorphic). (Found: C, 77.36; H, 5.48. $C_{30}H_{24}O_5$ requires: C, 77.6; H, 5.17%). This isoflavone (0.5g) was debenzylated by heating at 80° for 4 hr with HOAc (40 ml) and conc HCl (20 ml). The mixture was poured into H_2O, extracted with EtOAc (2x), and the extracts washed with aq. $NaHCO_3$, then H_2O, and evaporated. The product was recrystallised from MeOH to give *7,4′-dihydroxy-2′-methoxyisoflavone* (0.17g), mp 283–5°. (Found: C, 67.08; H, 4.34. $C_{16}H_{12}O_5$ requires: C, 67.6; H, 4.23%). A further 0.10g was obtained by concentration of the mother liquors. This isoflavone (0.14 g) was stirred at 80° for 1.5 hr with BzCl (48 μl), dry K_2CO_3 (3 g) and KI (0.3 g) in dry DMF (20 ml). The mixture was poured into H_2O, extracted with EtOAc (2x), and the extract washed with H_2O and evaporated. The product was dissolved in hot MeOH (150 ml), then concentrated to *ca* 40 ml, and allowed to crystallise, to yield *7-benzyloxy-4′-hydroxy-2′-methoxyisoflavone* (0.11g), mp 251–5°. (Found: C, 72.55; H, 5.06. $C_{23}H_{18}O_5$ requires: C, 73.8; H, 4.81%).

7,2′-Dihydroxy-4′-methoxyisoflavone-[^{14}*C–Me*]. 7-Benzyloxy-4′-hydroxy-2′-methoxyisoflavone (30.2 mg) was stirred and heated under reflux in dry Me_2CO (50 ml) with dry K_2CO_3 (5g), MeI (4 μl, 9.1 mg) and MeI-[^{14}C] (500 μCi, sp. act. 55 mCi/mM; 1.31 mg) overnight. Excess MeI (0.1 ml) was then added, and the reaction was continued for a further 1 hr. The reaction mixture was filtered, evaporated and the product, *7-benzyloxy-2′,4′-dimethoxyisoflavone-*[*4′-*^{14}*C–Me*] purified by TLC (solvent A), then dried under vacuum. This material was heated under reflux with $AlCl_3$ (1g) in dry MeCN (10 ml) overnight. The mixture was evaporated, treated with dil HCl (10%, 10 ml), then extracted with EtOAc (3 × 30 ml), and the evaporated extracts purified by TLC (solvent A). Inactive 7,2′-dihydroxy-4′-methoxyisoflavone (89.7 mg) prepared by a similar treatment of 7-benzyloxy-2′,4′-dimethoxyisoflavone derived from 4′-benzyloxy-2′-hydroxyacetophenone and 2,4-dimethoxybenzaldehyde, was added to the product, and the material was recrystallised from aq. MeOH to yield *7,2′-dihydroxy-4′-methoxyisoflavone-*[^{14}*C-Me*] (88.8 mg). The mother liquors yielded a further 14.0 mg of product, which was purified to constant sp. act. (0.518 mCi/mM) using TLC (solvents A, B and C). UV λ_{max}^{EtOH} nm (log ε): 240 (4.31), 249 (4.34), 259 *sh* (4 26), 292 (4.15), 305 inf (4.07).

(±)-*7,2′-Dihydroxy-4′-methoxyisoflavanone-*[^{14}*C-Me*] 7,2′-Dihydroxy-4′-methoxyisoflavone-[^{14}C-Me] (60.6 mg) was acetylated in dry Py (3 ml) with Ac_2O (0.3 ml) at R° overnight. The mixture was poured into H_2O, cooled, and *7,2′-diacetoxy-4′-methoxyisoflavone-*[^{14}*C-Me*] (68·3 mg) filtered off and dried. This isoflavone (56.0 mg) was hydrogenated overnight at R° and atmospheric pressure in EtOAc (20 ml) and HOAc (0.5 ml) over Pd–C (10%, 27 mg) catalyst. The reaction mixture was filtered, evaporated, then stirred at R° with KOH (0.5 g) in EtOH (10 ml) for 3 hr. The mixture was then concentrated to a small bulk, diluted with H_2O (25 ml) and acidified to pH4 with 10% HCl. The product, (±)-*7,2′-dihydroxy-4′-methoxyisoflavanone-*[^{14}*C-Me*] (37.8 mg) was obtained by extraction with EtOAc (3 × 25 ml), then TLC (solvent A). A portion (12.6 mg) was purified to constant sp. act. (0 546 mCi/mM) by TLC (solvents B and C). Unlabelled material (recrystd. aq. MeOH) had mp 212–4°, MS (probe) 70 eV *m/e* (rel. int.): 286 M^+ (17), 150 (100), 137 (65), 136 (27), 121 (27), 108 (47), 107 (24); M^+ 286.0832, $C_{16}H_{14}O_5$ requires 286.0841. UV λ_{max}^{EtOH} nm (log ε: 227 *sh* (4.26), 278 (4.23), 311 (3.93).

3-Hydroxy-9-methoxypterocarp-6a-ene-[^{14}*C-Me*]. A stream of N_2 was passed through refluxing MeOH (20 ml) for 0.5 hr. (±)-7,2′-Dihydroxy-4′-methoxyisoflavanone-[^{14}C-Me] (25.2 mg) and conc HCl (0.2 ml) were added, and the mixture was heated under reflux in an N_2 stream for 2 hr. The soln was evaporated and the product, *3-hydroxy-9-methoxypterocarp-6a-ene-*[^{14}*C-Me*] (9.9 mg) was isolated by TLC (solvent A) and purified to constant sp. act. (0.572 mCi/mM) by TLC (solvent D). Unlabelled material (recrystd aq. MeOH) had mp 171–3°, MS (probe) 70 eV *m/e* (rel. int.): 268 M^+ (100), 267 M^+-1 (65), 253 M^+-Me (55), 252 M^+-1-Me (13); M^+ 268.0734, $C_{16}H_{12}O_4$ requires 268.0735. UV λ_{max}^{EtOH} mm (log ε): 231 (4.21), 242 (4.19), 250 sh (4 15), 295 inf (3.92), 335 (4.49), 353 (4.44).

(±)-*3-Hydroxy-9-methoxypterocarpan-*[^{14}*C-Me*]. (*Demethyl-*

homopterocarpin-[^{14}C-Me]). 7,2'-Dihydroxy-4'-methoxyisoflavone-[^{14}C-Me] (19.8 mg) in dry THF (5 ml) was stirred at R° and treated slowly with $NaBH_4$ (50 mg) in dry EtOH (5 ml). After 2 hr, further solid $NaBH_4$ (50 mg) was added. The mixture was stirred overnight, Me_2CO (2 ml) was added, and solvents were then evaporated. The residue was treated with HCl (10%, 20 ml), and extracted with EtOAc (3 × 20 ml). TLC (solvent A) of the evapd. extracts yielded (±)-*demethylhomopterocarpin-[^{14}C-Me]* (15.3 mg) which was purified to constant sp. act. (0.514 mCi/mM) by TLC (solvent D).

Solvolysis of 3-hydroxy-9-methoxypterocarp-6a-ene. The pterocarp-6a-ene (39.2 mg) in MeOH (50 ml) was stirred vigorously in a stream of air in the light for 24 hr. The soln was evaporated and purified by TLC (solvent A). The major band was rechromatographed (solvent E), to give a product, *2-(2,4-dihydroxyphenyl)-6-methoxy-3-methoxymethylbenzofuran* (**10**) which crystallised on cooling. mp 123–4°, MS (probe) 70 eV *m/e* (rel. int.): 300 M^+ (30), 268 M^+-MeOH (100), 267 M^+-MeOH-1 (88), 253 M^+-MeOH–Me (64); M^+ 300.1001, $C_{17}H_{16}O_5$ requires 300.0998. UV λ_{max}^{EtOH} nm: 267,309. NMR (60 MHz, $CDCl_3$, TMS): δ 3.43 (3H, *s*, OMe), 3.80 (3H, *s*, OMe), 4.50 (2H, *s*, CH_2OMe), 6.07 (1H, OH), 6.40 (1H, *dd*, *J* = 9, 3 Hz, H-5), 6.45 (1H, *d*, *J* = 3 Hz. H-7), 6.83 (1H, *dd*, *J* = 9, 3 Hz, H-5'), 6.95 (1H, *d*, *J* = 3 Hz, H-3'), 7.25 (1H, *d*, *J* = 9 Hz, H-4), 7.42 (1H, *d*, *J* = 9 Hz, H-6'), 7.55 (1H, *s*, OH).

Optical rotation of pterocarpans. Demethylhomopterocarpin and maackiain were isolated [2] from $CuCl_2$-treated red clover seedlings (from 15g seeds), and purified further by TLC (solvent E). UV analysis [2] showed this fraction to contain demethylhomopterocarpin (1.08 mg) and maackiain (1.175 mg). The measured optical rotation, α_D^{20} of an EtOH (1 ml) solution was −0.041°. Lit. values of $[\alpha]_D$ for ethanolic solutions of (6aR, 11aR)-demethylhomopterocarpin and (6aR,11aR)-maackiain are −192° [28] and −214° [29] respectively. Using these values, the calculated rotation for the above pterocarpan mixture was −0·046°.

Isolation of coumestans from T pratense. Red clover seedlings (6-day-old, wet wt 650 g) were homogenised with H_2O (1l) and left for 5 hr at R° to permit autolysis of glycosides present. The slurry was worked up as previously [2]. The coumestan zone could not be obtained pure by TLC alone, and was thus acetylated (Py–Ac_2O) and the acetates purified by TLC (solvents F and G). MS (probe) 70 eV *m/e*: 338.0421, $C_{18}H_{10}O_7$ requires 338.0427; 324.0634, $C_{18}H_{12}O_6$ requires 324.0634. The coumestans were regenerated by hydrolysis (KOH/MeOH) and purified by TLC (solvent A). The coumestan content was *ca* 50 μg.

Synthesis of coumestans. The appropriate (±)-pterocarpan [2] (100 mg) was dissolved in dioxan (5 ml) and treated with DDQ (300 mg). The mixture was left at R° overnight. The pptd coumestan was filtered off and recrystallised from THF–MeOH. Yield *ca* 80 mg. mp's: *9-O-methylcoumestrol* 338–40° (lit. [30] 337–8°); *medicagol* 325–6° (lit. [31] 326–7°).

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