Phytochemistry, 1978, Vol. 17, pp 1751–1754. © Pergamon Press Ltd Printed in England. 0031-9422/78/1001-1751 $02.00/0

ISOFLAVONE PRECURSORS OF THE PTEROCARPAN PHYTOALEXIN MAACKIAIN IN *TRIFOLIUM PRATENSE*

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(*Received* 1 *March* 1978)

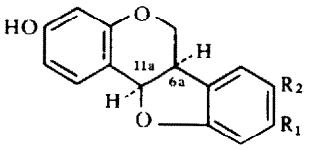
Key Word Index—*Trifolium pratense*; Leguminosae; red clover; biosynthesis; phytoalexin; pterocarpan; isoflavone; maackiain.

Abstract—Feeding experiments in Cu^{2+}-treated red clover seedlings have demonstrated that ^{14}C-labelled isoflavones formononetin, 7,3′-dihydroxy-4′-methoxyisoflavone, 7-hydroxy-3′,4′-methylenedioxyisoflavone and 7,2′-dihydroxy-4′,5′-methylenedioxyisoflavone are all good precursors of the pterocarpan phytoalexin maackiain (incorporations 1.8–7.6%). These compounds represent a logical biosynthetic sequence for elaboration of the substitution pattern of maackiain.

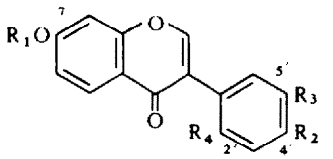
INTRODUCTION

Demethylhomopterocarpin (medicarpin) (**1**) is perhaps the most common of the isoflavonoid phytoalexins produced by leguminous plants when their tissues are challenged by a variety of biotic or abiotic agents [1, 2]. Frequently, isoflavans having the same oxygenation pattern as demethylhomopterocarpin are also produced. Less frequently, a second pterocarpan, maackiain (**2**) is synthesized simultaneously with (**1**) on such treatment. The ability of plants to synthesize this chemically more 'advanced' pterocarpan has been used in the taxonomic classification of various *Trigonella* species [3].

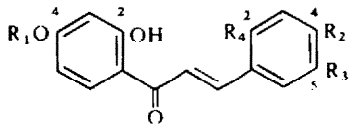
The biosynthesis of (6aR, 11aR)-demethylhomopterocarpin has been investigated by feeding experiments in chemically-treated seedlings of red clover (*Trifolium pratense*) [4, 5], lucerne (*Medicago sativa*) [6] and fenugreek (*Trigonella foenum-graecum*) [7]. In all of these plants, a biosynthetic pathway from 2′,4′,4-trihydroxychalcone (**3**) via formononetin (**7**) and 7,2′-dihydroxy-4′-methoxyisoflavone (**8**) appears to be operative. Isoflavone (**8**) is then probably reduced, firstly to 7,2′-dihydroxy-4′-methoxyisoflavanone (**16**) and then to the corresponding isoflavanol. This reductive sequence is stereospecific [7]. The isoflavanol then cyclizes to yield the pterocarpan, and an intermediate carbonium ion (**17**), or its mesomeric counterpart, has been postulated [6]. Support for the intermediacy of this carbonium ion has come from feeding experiments in *M. sativa* [6], where demethylhomopterocarpin and the isoflavan vesti-



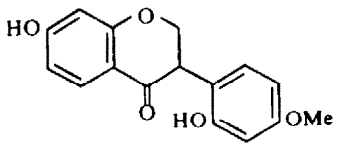
(**1**) R_1 = OMe, R_2 = H
(**2**) R_1R_2 = O–CH_2–O



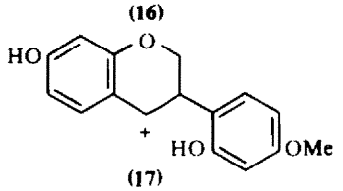
	R_1	R_2	R_3	R_4
(**7**)	H	OMe	H	H
(**8**)	H	OMe	H	OH
(**9**)	H	OCH_2O		OH
(**10**)	H	OMe	OH	H
(**11**)	H	OCH_2O		H
(**12**)	H	OMe	OH	OH
(**13**)	CH_2Ph	OMe	OCH_2Ph	H
(**14**)	CH_2Ph	OCH_2O		H
(**15**)	CH_2Ph	OCH_2O		OCH_2Ph



	R_1	R_2	R_3	R_4
(**3**)	H	OH	H	H
(**4**)	CH_2Ph	OMe	OCH_2Ph	H
(**5**)	CH_2Ph	OCH_2O		H
(**6**)	CH_2Ph	OCH_2O		OCH_2Ph



(**16**)



(**17**)

tol are interconvertible, although they appear to be synthesized simultaneously from such a common intermediate. The pathway to (6aR, 11aR)-maackiain (**2**) in red clover, however, branches from that to demethylhomopterocarpin, probably at formononetin. This isoflavone is well-incorporated, as is 2',4',4-trihydroxychalcone (**3**) [4]. Other compounds tested, viz. (**8**), (**16**) and (**1**), were not incorporated to any significant extent [5].

To investigate further the biosynthetic pathway to maackiain, further labelled isoflavones have been synthesized and tested as precursors of this pterocarpan phytoalexin in Cu^{2+}-treated red clover seedlings.

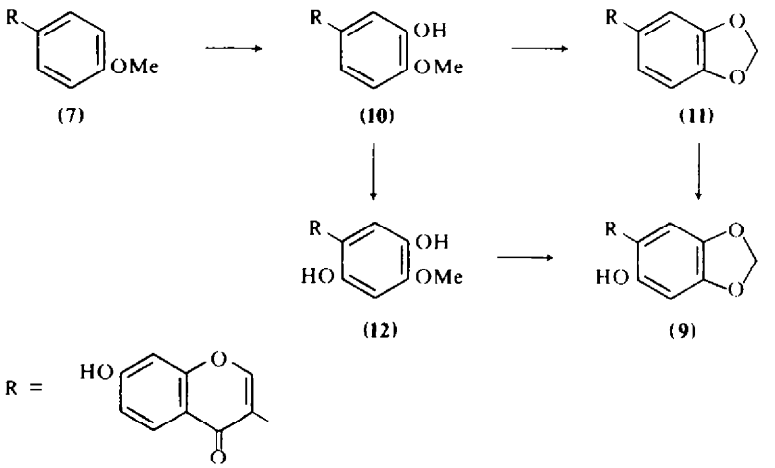
RESULTS

From the results obtained in the study of demethylhomopterocarpin biosynthesis, it is reasonable to suggest that maackiain may be derived by a reductive sequence from 7,2'-dihydroxy-4',5'-methylenedioxyisoflavone (**9**). The substitution pattern of this isoflavone must be built up from the 7-hydroxy-4'-methoxy of formononetin, which is readily incorporated into maackiain. Since 7,2'-dihydroxy-4'-methoxyisoflavone is not incorporated, 7,3'-dihydroxy-4'-methoxyisoflavone (3'-hydroxyformononetin or calycosin) (**10**) is a likely intermediate in the pathway immediately after the branch-point. From this isoflavone, two routes might exist for elaborating the substitution pattern of (**9**): cyclisation of the 3'-hydroxy-4'-methoxy of (**10**) to a methylenedioxy group, yielding 7-hydroxy-3',4'-methylenedioxyisoflavone (ψ-baptigenin) (**11**), followed by 2'-hydroxylation, or alternatively, 2'-hydroxylation of (**10**) followed by formation of the methylenedioxy group, thus involving 7,2',5'-trihydroxy-4'-methoxyisoflavone (**12**) as an intermediate (Scheme 1).

Compounds tested as biosynthetic precursors of maackiain were 7,3'-dihydroxy-4'-methoxyisoflavone-[*carbonyl*-^{14}C] (**10**), 7-hydroxy-3',4'-methylenedioxyisoflavone-[*carbonyl*-^{14}C] (**11**) and 7,2'-dihydroxy-4',5'-methylenedioxyisoflavone-[*carbonyl*-^{14}C] (**9**). Formononetin-[*methyl*-^{14}C] was fed in a parallel experiment as a standard. The facile oxidation of 7,2',5'-trihydroxy-4'-methoxyisoflavone (**12**) to the corresponding *p*-benzoquinone precluded its use in feeding experiments.

These compounds were synthesized from resacetophenone-[*carbonyl*-^{14}C] prepared by Hoesch reaction of resorcinol with acetonitrile-[1-^{14}C]. Although an excess of labelled acetonitrile was necessary for good yields, this reaction proved to be a more reliable route to labelled resacetophenone than Friedel–Crafts syntheses using acetic anhydride or acetyl chloride previously employed [8, 9], reactions which can sometimes fail for no readily apparent reason. Monobenzylation of resacetophenone-[*carbonyl*-^{14}C] yielded 4'-benzyloxy-2'-hydroxyacetophenone-[*carbonyl*-^{14}C], which was condensed in basic solution with 3-benzyloxy-4-methoxybenzaldehyde, piperonal, or 2-benzyloxy-4,5-methylenedioxybenzaldehyde to give a series of [*carbonyl*-^{14}C]-labelled chalcones, 3,4'-dibenzyloxy-2'-hydroxy-4-methoxychalcone (**4**), 4'-benzyloxy-2'-hydroxy-3,4-methylenedioxychalcone (**5**), and 2,4'-dibenzyloxy-2'-hydroxy-4,5-methylenedioxychalcone (**6**), respectively. These chalcones, as their acetates, were oxidized with thallium nitrate [10] to give the corresponding isoflavones, 7,3'-dibenzyloxy-4'-methoxyisoflavone (**13**), 7-benzyloxy-3',4'-methylenedioxyisoflavone (**14**) and 7,2'-dibenzyloxy-4',5'-methylenedioxyisoflavone (**15**). The [*carbonyl*-^{14}C]-labelled isoflavones (**10**), (**11**) and (**9**) were obtained by debenzylation of these compounds using HOAc–HCl.

The labelled isoflavones (*ca* 0.5 mg) were separately administered as their Na salts in phosphate buffer to batches of 4-day-old dark-grown red clover seedlings which had been treated with aqueous $CuCl_2$ for 8 hr, as described previously [4]. After a feeding period of 16 hr in the dark, the seedlings were worked up, and the pterocarpan phytoalexins demethylhomopterocarpin and maackiain were isolated together from the extract. The maackiain content was established by UV [4], then the fraction was diluted with synthetic (±)-maackiain carrier. The samples were counted after methylation and purification to constant specific activity. The demethylhomopterocarpin content of the extract was not studied further in these experiments. The results of these comparative feeding experiments are summarized in Table 1. The incorporation figures indicate that all four isoflavones were very good precursors of maackiain.



Scheme 1. Possible routes for elaboration of the 2'-hydroxy 4',5'-methylenedioxy substitution pattern in isoflavones.

DISCUSSION

The four isoflavones tested represent a logical biosynthetic sequence for elaborating the 2'-hydroxy-4',5'-methylenedioxy grouping from a 4'-methoxy substituent. All compounds were very good precursors of maackiain, and it thus seems likely that all could be involved in the biosynthesis of this pterocarpan. Ideally, one would expect to see a steady increase in incorporation/reduction in dilution value as the compound tested became biosynthetically nearer to the final product. Under the conditions used, the incorporation of 7,2'-dihydroxy-4',5'-methylenedioxyisoflavone, although extremely good for a whole-plant biosynthetic experiment, is somewhat lower than might be expected if it is an intermediate between 7-hydroxy-3',4'-methylenedioxyisoflavone and maackiain. An incorporation some three to four times larger might have been predicted. Simple biosynthetic feeding experiments are far from ideal, however, and the incorporation of any particular compound can be drastically reduced by poor transport and/or rapid turnover. Such may be the case in the present experiments. An alternative possibility is that a 'metabolic grid' [11] of isoflavones, and their reduction products isoflavanones and isoflavanols is operative. If this is so, there may be no unique route from formononetin to maackiain, although some pathway may be 'preferred'. A 'metabolic grid' in isoflavonoid biosynthesis has been implicated in the production of the coumestan coumestrol in *Phaseolus aureus* [12], and our studies [13] in *Medicago sativa* have also demonstrated that such a situation exists for the biosynthesis of pterocarpan, isoflavan and coumestan metabolites. Further studies are required to establish the function of a 'metabolic grid' in *T. pratense*. Although 7,2',5'-trihydroxy-4'-methoxyisoflavone could not be tested as a precursor of maackiain in these experiments, its role as an intermediate seems unlikely since such an excellent incorporation of 7-hydroxy-3',4'-methylenedioxyisoflavone (ψ-baptigenin) was observed. Nevertheless, it cannot be excluded completely since it could perhaps be involved in a more complex form of 'metabolic grid'.

By analogy with these results, logical suggestions can be made about the biosynthetic pathway involved in rotenoid formation. Feeding experiments [8] have established that formononetin and 7-hydroxy-2',4',5'-trimethoxyisoflavone are good precursors of amorphigenin (8'-hydroxyrotenone) in *Amorpha fruticosa*, rotenone itself being an intermediate. The elaboration of the 2',4',5'-trimethoxy system could arise by a number of pathways, but the most likely would now seem to be the route: formononetin → 7,3',dihydroxy-4'-methoxyisoflavone → 7-hydroxy-3',4'-dimethoxyisoflavone → 7,2'-dihydroxy-4',5'-dimethoxyisoflavone → 7-hydroxy-2',4'-5'-trimethoxyisoflavone. These suggestions require experimental verification.

The production of maackiain *as a phytoalexin* in leguminous plants is almost always accompanied by synthesis of demethylhomopterocarpin [2, 3, 14]. One reported exception is in *Pisum sativum* where maackiain is produced in small quantities along with the structurally similar pisatin [15]. This seemingly obligatory co-occurrence of the methylenedioxy-pterocarpan with its methoxy analogue is unusual since the branch-point to these two compounds lies so far back on the biosynthetic pathway, at formononetin, which is most probably the first-formed isoflavonoid intermediate [4, 8]. Also unusual is the lack of reports of, for example, isoflavan phytoalexins related to maackiain, since demethylhomopterocarpin frequently co-occurs with isoflavans having the same substitution pattern. This may, however, reflect the ready metabolism of maackiain and its derivatives, particularly in fungus-infected plants [16].

A very recent paper [17] reports the presence of calycosin and ψ-baptigenin in *Trifolium pratense*. This lends considerable support to the proposed biosynthetic pathway.

Table 1. Incorporation of labelled isoflavones into maackiain in *Trifolium pratense* seedlings*

Compound fed	Sp. act. maackiain (dpm/mM)	Dilution	Incorp. (%)
Formononetin†	2.76×10^7	43	1.8
7,3'-Dihydroxy-4'-methoxyisoflavone‡	1.63×10^6	37	2.2
7-Hydroxy-3',4'-methylenedioxyisoflavone‡	6.80×10^6	9.0	7.6
7,2'-Dihydroxy-4',5'-methylenedioxyisoflavone‡	1.47×10^6	40	2.8

* Four-day old, $CuCl_2$ inducer applied for 8 hr, feeding period 16 hr. †[*methyl*-^{14}C]; ‡[*carbonyl*-^{14}C].

EXPERIMENTAL

General. The growing of plant material, feeding techniques, isolation and purification of metabolites, and counting of radioactive samples were as previously described [4], except that 2 g batches of seeds were germinated for each feeding. 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; B, C_6H_6–EtOAc, 20:1; C, $CHCl_3$–*iso*-PrOH, 10:1; D, C_6H_6–EtOAc–MeOH, 6:4:1; E, C_6H_6–EtOAc–MeOH–petrol (60–80°), 6:4:1:3; F, C_6H_6–EtOH, 92:8. Me_2CO (Analar) was used for elution of TLC zones. The synthesis of formononetin-[*methyl*-^{14}C] has been reported [8].

Resacetophenone-[carbonyl-^{14}C]. Acetonitrile-[1-^{14}C] (1 mCi, sp. act. 9.7 mCi/mM) and freshly-distilled (from P_2O_5) acetonitrile (0.9 ml) was added to a 2-necked flask provided with a magnetic stirrer and $CaCl_2$ guard-tube, containing freshly-sublimed resorcinol (1 g), Na-dried Et_2O (4.5 ml) and dry $ZnCl_2$ (2 g). Dry HCl gas was passed through the stirred mixture at 0° for 2.5 hr, and the resulting viscous pale orange soln was left in the stoppered flask at 0° for 2 days. Et_2O was decanted off from the white solid ketimine hydrochloride produced, which was washed with dry Et_2O (3 × 2 ml). H_2O (20 ml) was added to the solid, and the mixture was heated at 95° with stirring for 2 hr, then cooled at 0° for 16 hr. The precipitate of resacetophenone-[*carbonyl*-^{14}C] was filtered off and dried. Yield 872 mg.

4'-Benzyloxy-2'-hydroxyacetophenone-[carbonyl-^{14}C]. Resacetophenone-[*carbonyl*-^{14}C] (800 mg), BzCl (665 mg), dry K_2CO_3 (2 g) and dry KI (0.1 g) in dry DMF (25 ml) were stirred at 80° for 2 hr. The mixture was poured into H_2O, and the precipitate filtered off, washed and recrystallized from MeOH. Yield 880 mg, mp 103–105° (lit [18] 111°), sp. act. 6.09×10^7 dpm/mM.

3,4'-Dibenzyloxy-2'-hydroxy-4-methoxychalcone-[carbonyl-^{14}C]. 4'-Benzyloxy-2'-hydroxyacetophenone-[*carbonyl*-^{14}C] (100 mg) and 3-benzyloxy-4-methoxybenzaldehyde (150 mg) were dissolved in warm EtOH (5 ml), and KOH (2 g) in H_2O (2 ml) was added. The soln was stirred for 16 hr at room temp.,

then neutralized with dil. HCl. The suspension was extracted with CH_2Cl_2 (2 × 50 ml), the combined extracts washed with H_2O and evapd. The residue was purified by TLC (solvent A) to give 3,4'-dibenzyloxy-2'-hydroxy-4-methoxychalcone-[*carbonyl*-^{14}C] (100 mg). Inactive material, crystd. $CHCl_3$–MeOH had mp 165–167°, lit [10] 166–168°.

7,3'-Dibenzyloxy-4'-methoxyisoflavone-[carbonyl-^{14}C]. The above chalcone was acetylated (dry Py–Ac_2O, room temp. 16 hr) and the reaction mixture poured into H_2O, neutralized with dil. HCl, and extracted with CH_2Cl_2 (2 × 50 ml). The extracts were washed with dil. HCl (2 × 50 ml), then H_2O (2 × 50 ml), evapd and dried. The acetate, without further purification, was dissolved in MeOH (50 ml) and stirred at room temp. for 16 hr with $Tl(NO_3)_3.3H_2O$ (0.24 g). Solid KOH (0.4 g) was then added, and the mixture stirred for a further 1 hr. After neutralization with conc HCl, dil. HCl (10%, 3 ml) was added, and the mixture heated under reflux for 2 hr, then filtered hot. The filtrate was concd under red. pres., diluted with H_2O and extracted with CH_2Cl_2 (2 ×). The CH_2Cl_2 extract was washed with H_2O, evapd, then purified by TLC (solvent B) to give 7,3'-dibenzyloxy-4'-methoxyisoflavone-[*carbonyl*-^{14}C] (36 mg). Inactive material, crystd MeOH had mp 129–130°, lit [10] 132–134°.

7,3'-Dihydroxy-4'-methoxyisoflavone-[carbonyl-^{14}C]. The above isoflavone was heated at 80° for 3 hr with HOAc (80 ml) and conc HCl (20 ml). The mixture was poured into H_2O, extracted with EtOAc (2 × 100 ml), and the extracts washed with aq. $NaHCO_3$, then H_2O, and evapd. The product, 7,3'-dihydroxy-4'-methoxyisoflavone-[*carbonyl*-^{14}C], was isolated by TLC (solvent C) and purified to constant sp. act. (6.01×10^7 dpm/mM) by TLC (solvents D, E and F). Yield 14 mg. Inactive material (crystd aq. MeOH) had mp 249–251°, lit [19] 245–247°.

7-Hydroxy-3',4'-methylenedioxyisoflavone-[carbonyl-^{14}C] and *7,2'-dihydroxy-4',5'-methylenedioxyisoflavone*-[carbonyl-^{14}C]. These were prepared by routes analogous to that described above. 4'-Benzyloxy-2'-hydroxyacetophenone-[*carbonyl*-^{14}C] (100 mg) was condensed with piperonal (170 mg) or 2-benzyloxy-4,5-methylenedioxybenzaldehyde (190 mg) to give respectively 4'-benzyloxy-2'-hydroxy-3,4-methylenedioxychalcone-[*carbonyl*-^{14}C] (103 mg), mp 172–174° (ex CH_2Cl_2–MeOH), lit [20] 174–175°, and 2,4'-dibenzyloxy-2'-hydroxy-4,5-methylenedioxychalcone-[*carbonyl*-^{14}C] (80 mg), mp 196–198° (ex HOAc), lit [21] 201–203°. These chalcones, as their acetates were treated with $Tl(NO_3)_3.3H_2O$ (240 and 130 mg, respectively) and yielded 7-benzyloxy-3',4'-methylenedioxyisoflavone-[*carbonyl*-^{14}C] (25 mg), mp 166–167° (ex MeOH), and 7,2'-dibenzyloxy-4',5'-methylenedioxyisoflavone-[*carbonyl*-^{14}C] (37 mg), mp 146–147° (ex MeOH), lit [10] 152–154°. Debenzylation produced 7-hydroxy-3',4'-methylenedioxyisoflavone-[*carbonyl*-^{14}C] (12 mg), mp 289–290° (ex MeOH), lit [19] 291–292°, and 7,2'-dihydroxy-4',5'-methylenedioxyisoflavone-[*carbonyl*-^{14}C] (15 mg), mp 247–250° (ex aq. MeOH), lit [10] 246–248°.

Acknowledgement We thank the Science Research Council for financial support.

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