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PTEROCARPAN BIOSYNTHESIS: CHALCONE AND ISOFLAVONE PRECURSORS OF DEMETHYLHOMOPTEROCARPIN AND MAACKIAIN IN *TRIFOLIUM PRATENSE*

PAUL M. DEWICK

Department of Pharmacy, University of Nottingham, Nottingham NG7 2RD, England

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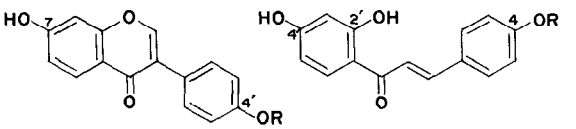
Key Word Index—*Trifolium pratense*; Leguminosae; red clover; pterocarpan; demethylhomopterocarpin; maackiain; phytoalexin; biosynthesis; formononetin; isoflavone; chalcone.

Abstract—Feeding experiments with DL-phenylalanine-[1-^{14}C] have demonstrated the *de novo* synthesis of the pterocarpan phytoalexins demethylhomopterocarpin and maackiain in $CuCl_2$-treated *Trifolium pratense* L. seedlings. 2′,4′,4-Trihydroxychalcone-[*carbonyl*-^{14}C] and 7-hydroxy-4′-methoxyisoflavone-[*methyl*-^{14}C] (formononetin) were readily incorporated into demethylhomopterocarpin and maackiain, but 2′,4′-dihydroxy-4-methoxychalcone-[*carbonyl*-^{14}C] and 7,4′-dihydroxyisoflavone-[T] (daidzein) proved inefficient precursors. The trihydroxychalcone was also an excellent precursor of formononetin in *T. pratense*, but the dihydroxymethoxychalcone and daidzein were poorly incorporated. These observations offer further evidence that methylation may be an associated part of the mechanism for aryl migration in the biosynthesis of formononetin.

INTRODUCTION

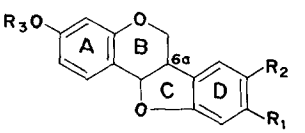
Pterocarpans have been recognized as natural products for many years, mainly as heartwood constituents of various leguminous plants [1]. Recent observations that certain pterocarpans function as phytoalexins, antifungal compounds produced in growing tissues in response to stress such as viral or fungal infection [2], have opened the way to biosynthetic studies. Simple precursors such as phenylalanine and cinnamic acid were readily incorporated into the 6a-hydroxypterocarpan pisatin in *Pisum sativum* [3] and the isoflavonoid nature of pterocarpans was demonstrated by the incorporation of 7,4′-dihydroxyisoflavone (daidzein) (**1**) into phaseollin [4], a phytoalexin produced by *Phaseolus vulgaris*. More recently, 2′,4′,4-trihydroxychalcone (isoliquiritigenin) (**3**) was established as a precursor of 6a-hydroxyphaseollin in *Glycine max* [5].

This communication reports the results of experiments conducted to study the biosynthesis of the two phytoalexin pterocarpans produced by red clover [6] (*Trifolium pratense* L.), demethylhomopterocarpin (**5**) and maackiain (**6**).



(**1**) R = H
(**2**) R = Me

(**3**) R = H
(**4**) R = Me



(**5**) R_1 = OMe, R_2 = R_3 = H
(**6**) R_1R_2 = O–CH_2–O, R_3 = H
(**7**) R_1 = OMe, R_2 = H, R_3 = Me
(**8**) R_1R_2 = O–CH_2–O, R_3 = Me

RESULTS

Demethylhomopterocarpin and maackiain have been identified [6] as the phytoalexins produced in red clover when leaves were inoculated with *Helminthosporium turcicum*. Our initial studies indicated that production of these two pterocarpans could also be stimulated by treating the roots of red clover seedlings with a dilute solution of

$CuCl_2$. No trace of the pterocarpans could be detected in untreated tissues. The chemical stimulation of phytoalexin production has been utilized in earlier biosynthetic studies [3, 4]. Pterocarpan production was monitored by UV absorption of the appropriate fraction of the phenolic extract of the seedlings. The two pterocarpans can be separated only with difficulty, by a procedure [6] inappropriate in biosynthetic studies, although separation of the methyl ethers homopterocarpin (**7**) and pterocarpin (**8**) is easily accomplished [7] by fractional crystallization. However, the amount of each pterocarpan in a mixture of the two can readily be assessed from the UV spectrum, which exhibits a band at 310 nm due exclusively to maackiain, and two peaks at 287 and 282 nm due to the combined absorptions of demethylhomopterocarpin and maackiain. The contribution from each can be assessed with a knowledge of the extinction coefficients for the individual compounds. The relative proportions of maackiain to demethylhomopterocarpin varied between 0·85 and 1·53 in the feeding experiment (Table 1), increasing steadily as the period between induction and sampling lengthened, the maximum total pterocarpan content occurring some 24 hr after treatment of the seedlings. After this time, the content decreased fairly quickly (Table 1).

To investigate the biosynthesis of demethylhomopterocarpin and maackiain, batches of 4-day-old seedlings (from 1 g dry seeds) were treated with $CuCl_2$ for 12 hr, after which time the inducer solution was replaced by a solution of DL-phenylalanine-[1-^{14}C]. The seedlings were grown on and worked up after various feeding times. The pterocarpan concentrations of the seedlings were assayed by UV, then half of this fraction was diluted with inactive (±)-demethylhomopterocarpin carrier, and the other half with (±)-maackiain carrier. The individual pterocarpans were methylated, then purified to constant activity by TLC and recrystallization. Table 1 contains details of the pterocarpan content and incorporation data for the various feedings. Also included are data for formononetin (**2**), the main phenolic component of *T. pratense*, which might be expected to play a role in the metabolic processes leading to the pterocarpans. Formononetin was isolated, assayed, methylated and purified by similar methods to those employed for the pterocarpans. The incorporation of considerable activity into both pterocarpans and into formononetin indicated that *de novo* synthesis of these compounds was occurring.

Similar experiments were carried out using other possible pterocarpan precursors. The labelled chalcones 2′,4′,4-trihydroxychalcone-[*carbonyl*-^{14}C] (**3**) and 2′,4′-dihydroxy-4-methoxychalcone-[*carbonyl*-^{14}C] (**4**), and the isoflavones daidzein-[T] (**1**) and formononetin-[*methyl*-^{14}C] were administered as their Na salts to $CuCl_2$-treated seedlings. After 12 hr, the seedlings were worked up and the incorporations of activity into demethylhomopterocarpin, maackiain and formononetin were established. The results of these experiments are shown in Table 2 and clearly indicate that only 2′,4′,4-trihydroxychalcone and formononetin are good precursors of the pterocarpans. This chalcone is also an excellent precursor of formononetin.

DISCUSSION

The feeding of DL-phenylalanine-[1-^{14}C] to *T. pratense* was conducted mainly to establish that *de novo* synthesis of demethylhomopterocarpin and maackiain was occurring, and also to find suitable feeding conditions for subsequent experiments. Both of these objectives were realized, and in addition, certain other deductions concerning pterocarpan biosynthesis can be made. At the start of the experiment, *T. pratense* contains a fairly

Table 1. Feeding of DL-phenylalanine-[1-^{14}C] to *Trifolium pratense* seedlings*

Time (hr)	Demethylhomopterocarpin			Maackiain			Formononetin		
	μM	sp. act. (dpm/mM)	Incorporation (%)	μM	sp. act. (dpm/mM)	Incorporation (%)	μM	sp. act. (dpm/mM)	Incorporation (%)
$t = -12$†	0			0			1·10		
$t = 0$‡	0·452			0·383			0·96		
$t = 6$	0·532	$3·97 \times 10^7$	0·076	0·556	$4·31 \times 10^7$	0·086	1·03	$6·02 \times 10^6$	0·022
$t = 12$	0·596	$3·82 \times 10^7$	0·082	0·675	$4·35 \times 10^7$	0·105	1·07	$5·06 \times 10^6$	0·020
$t = 24$	0·320	$2·12 \times 10^7$	0·024	0·488	$2·34 \times 10^7$	0·041	1·12	$4·92 \times 10^6$	0·020

* Four-day-old. † $CuCl_2$ inducer added. ‡ Inducer removed. DL-phenylalanine-[1-^{14}C] added.

Table 2. Feeding of labelled chalcones and isoflavones to *Trifolium pratense* seedlings*

Precursor	Unabsorbed precursor (%)	Demethylhomopterocarpin sp. act. (dpm/mM)	Dilution	Incorp.† (%)	Maackiain sp. act. (dpm/mM)	Dilution	Incorp.† (%)	Formononetin sp. act. (dpm/mM)	Dilution	Incorp.† (%)
2',4',4-Trihydroxychalcone‡	29	$1{\cdot}26 \times 10^7$	39	0·43	$1{\cdot}05 \times 10^7$	47	0·40	$6{\cdot}25 \times 10^6$	79	0·44
2',4'-Dihydroxy-4-methoxychalcone‡	27	$1{\cdot}01 \times 10^5$	4470	0·006	$1{\cdot}24 \times 10^5$	3640	0·008	$2{\cdot}42 \times 10^5$	1860	0·019
Daidzein§	50	$4{\cdot}76 \times 10^5$	1280	0·018	$1{\cdot}06 \times 10^6$	575	0·048	$1{\cdot}59 \times 10^6$	384	0·091
Formononetin‖	28	$3{\cdot}71 \times 10^7$	32	0·91	$2{\cdot}79 \times 10^7$	42	0·77	$3{\cdot}83 \times 10^8$	3·1	24·9

* Four-day-old, $CuCl_2$ inducer applied for 12 hr, feeding period 12 hr. † Incorporation figures are not corrected for unabsorbed precursor. ‡ [*carbonyl*-^{14}C]. § [T]. ‖ [*methyl*-^{14}C].

large pool of formononetin (Table 1, $t = -12$) which could perhaps be used for phytoalexin synthesis. Since the specific activities of demethylhomopterocarpin and maackiain always exceed the specific activity of formononetin, which remains fairly constant during the experiment, it may be concluded that this pool of formononetin cannot be used as a whole for pterocarpan synthesis. If the formononetin pool is metabolically subdivided, the picture becomes more complex; the fall in total formononetin content during the initial synthesis of pterocarpans might offer evidence for this possibility. However, it is clear that the bulk of pterocarpan synthesis is *de novo*.

The specific activities of the two pterocarpans decrease markedly between $t = 12$ and $t = 24$, indicating that both of these compounds are subject to turnover. During this period, the degradation rate must exceed the rate of synthesis since the total pterocarpan content is decreasing rapidly. Such observations are in keeping with their role as phytoalexins. By contrast, turnover in the formononetin pool is relatively slow. The similar changes occurring in the specific activities of demethylhomopterocarpin and maackiain during the course of the experiment suggest that it is unlikely that demethylhomopterocarpin is a biosynthetic precursor of maackiain.

The experiments summarized in Table 2 clearly indicate that formononetin is an excellent precursor of both demethylhomopterocarpin and maackiain in *T. pratense*. Total incorporation into the pterocarpans was 1·68% in 12 hr, a figure which is uncorrected for the unabsorbed precursor (28%), or the large amount of unchanged formononetin (25%) present in the tissue at the end of the experiment. Although degradations were not performed, the *O*-methyl group of formononetin presumably supplies the *O*-methyl of demethylhomopterocarpin and the methylenedioxy grouping of maackiain. The unmethylated isoflavone, daidzein is a much less acceptable precursor for the pterocarpans, even after allowances have been made for unabsorbed precursor (50%) and the possibility of losing some of the tritium label (especially that at position 2'). Conversely, the unmethylated chalcone, 2',4',4-trihydroxychalcone is also an excellent precursor, whereas the methylated compound, 2',4'-dihydroxy-4-methoxychalcone is poorly incorporated. Similar results are noted for the formononetin isolated, daidzein and 2',4'-dihydroxy-4-methoxychalcone being poor precursors, 2',4',4-trihydroxychalcone being a good precursor. These observations parallel those reported earlier in other areas of isoflavonoid biosynthesis. 2',4',4-Trihydroxychalcone, as the glucoside, was readily incorporated into formononetin [8], but 4-methylated compounds were incorporated *via* prior demethylation [9, 10], and daidzein was a poor precursor [11]. 2',4',4-Trihydroxychalcone and formononetin were excellent precursors of the rotenoid amorphigenin [12] whereas 2',4'-dihydroxy-4-methoxychalcone and daidzein were hardly incorporated. All of these results suggest that when the aryl migration step characteristic of isoflavonoid biosynthesis takes place in the production of formononetin, it must be associated with 4'-methylation. Any acceptable hypothesis [12] for the aryl migration step should account for these observations and assign a role [13] to the 4-hydroxy group of the migrating aryl. However, the alternative route to formononetin, by methylation of daidzein, as exemplified by the recent isolation [14] from *Cicer arietinum* of an enzyme catalysing the 4'-methylation of daidzein and genistein, is probably also of some importance. Incorporation of daidzein into formononetin and the pterocarpans could be lessened by poor transport and/or

rapid turnover. The more complex 2,4- and 2,4,5-oxygenation patterns in the D ring of demethylhomopterocarpin and maackiain respectively would appear to be built up subsequently from the simple 4'-*O*-methyl group of formononetin, by analogy with biosynthetic observations in the formation of the related isoflavonoids the coumestans [15–17] and rotenoids [12, 17].

EXPERIMENTAL

General. Seeds of *Trifolium pratense* L. were purchased. UV spectra were measured in EtOH solution. TLC was carried out using 0·5 mm layers of Si gel (Merck Kiesel gel GF_{254}) in: A, C_6H_6–EtOAc–MeOH–petrol (60–80°), 6:4:1:8; B, C_6H_6–EtOAc–MeOH–petrol (60–80°), 6:4:1:6; C, C_6H_6. Radioactive samples were counted with a Tracerlab Corumatic liquid scintillation spectrometer in a dioxan-based scintillator. Efficiencies were measured by means of toluene-[^{14}C] or −[T] internal standards.

Radiochemicals. DL-Phenylalanine-[1-^{14}C] (sp. act. 60 mCi/mM) was purchased. The syntheses of 2',4',4-trihydroxychalcone-[*carbonyl*-^{14}C] (0·223 mCi/mM), 2',4'-dihydroxy-4-methoxychalcone-[*carbonyl*-^{14}C] (0·203 mCi/mM) and formononetin-[*methyl*-^{14}C] (0·535 mCi/mM) have been described in [12]. Daidzein-[T] (0·275 mCi/mM) was generously donated by Professor W. Barz, University of Münster, Westf., Germany, whom we thank.

Pterocarpans. (±)-Demethylhomopterocarpin [18] and (±)-maackiain [19] were prepared by $NaBH_4$ reduction and subsequent acid catalysed cyclization [20] of the appropriate 2'-hydroxyisoflavones which were synthesized by $Tl(NO_3)_3$ oxidation of chalcones according to the general method of Farkas *et al.* [21] The pterocarpans were purified by TLC (solvent A) and crystallization from aq. Me_2CO mp (+)-demethylhomopterocarpin 192–5° (lit. [18] 194 195°); (±)-maackiain 195 198° (lit. [19] 194–195°).

Plant material, feeding techniques and isolation of metabolites. Seeds of *T. pratense* (1 g) were placed on layers of moist filter paper (Whatman seed test, 9 cm dia) in a Petri dish and germinated in the dark at 25° for 4 days. Seedlings were removed from paper and placed in a smaller Petri dish (i.d. 6·5 cm) containing 10 ml of aq. $CuCl_2$ (3×10^{-3} M), and grown in the dark for a further 12 hr. The inducer soln was removed from the roots, the seedlings washed with H_2O and the labelled precursor solution added. Phenylalanine was fed in H_2O (3 ml) and the labelled phenols (*ca* 0·5 mg) were administered as their Na salts in phosphate buffer (0·1 M, pH 7·0, 3 ml). Seedlings were transferred to the light for the feeding period, additional H_2O being added as required. After the appropriate time, unassimilated precursor was removed from the roots by washing (using first H_2O then EtOH in the case of phenolic compounds), and the seedlings homogenized by grinding in a mortar with ground glass and H_2O (*ca* 20 ml). The slurry was then poured into boiling EtOH (100 ml). After filtering, the tissue was re-extracted with hot EtOH (2×100 ml) and the combined extracts were evaporated, taken up in H_2O (50 ml) and extracted with Et_2O (100 ml and then 4×50 ml). The Et_2O extracts were evaporated to dryness and separated by TLC (solvent B). Bands corresponding to demethylhomopterocarpin + maackiain, and formononetin were eluted with Me_2CO (Analar), and their isoflavonoid content was assayed by UV absorption of EtOH solutions: demethylhomopterocarpin, ν_{max} 44·29 × 10^3 sh (ε 14380), 35·21 × 10^3 sh (7760), 34·75 × 10^3 cm^{-1} (8690)*; maackiain, ν_{max} *ca* 44·0 × 10^3 (ε 10200), 35·34 × 10^3 sh (3760), 34·71 × 10^3 (4470)*, 31·99 × 10^3 cm^{-1} (7370)*; formononetin, ν_{max} 40·24 × 10^3 cm^{-1} (27600) [22]*. (* Bands used for quantitative assay.)

The pterocarpan extract was divided into two parts, inactive (±)-demethylhomopterocarpin (25 mg) being added to one, and inactive (±)-maackiain (20 mg) to the other. The individual samples were then methylated in dry Me_2CO (10 ml) over K_2CO_3 (1 g) with MeI (0·5 ml) for 1 hr. The products were isolated in the usual manner, and purified by TLC (solvent C) and recrystallization to constant activity from MeOH. The products, (±)-homopterocarpin and (±)-pterocarpin had mps 128–129° (lit. [23] 123–125°) and 187–189° (lit. [24] 185–186°) respectively.

The formononetin extract was diluted with carrier formononetin (20 mg) and methylated as above. 7,4'-Dimethoxyisoflavone was purified by TLC (solvent A) and recrystallization to constant activity from aq. MeOH, mp 162–163° (lit. [25] 162–164°).

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