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BIOSYNTHESIS OF THE 6-OXYGENATED ISOFLAVONE AFRORMOSIN IN *ONOBRYCHIS VICIIFOLIA*

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Key Word Index—*Onobrychis viciifolia*; Leguminosae; sainfoin; biosynthesis; afrormosin; isoflavone.

Abstract—DL-Phenylalanine-[2-^{14}C], 2',4',4-trihydroxychalcone-[*carbonyl*-^{14}C] and formononetin-[*Me*-^{14}C] were all good precursors of afrormosin (7-hydroxy-6,4'-dimethoxyisoflavone) in *Onobrychis viciifolia* seedlings. This demonstrates that 6-oxygenation may be a late process in the biosynthesis of isoflavones.

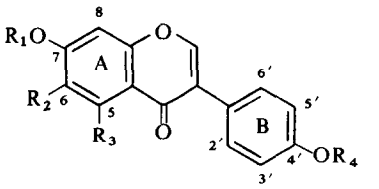
INTRODUCTION

The structural variety of isoflavonoids in nature arises both from the oxidation level in the heterocyclic ring, and also from the substituents on the aromatic rings [1]. Biosynthetic investigations have so far been primarily directed towards a correlation of the various classes of isoflavonoids arising from the different oxidation levels. Our knowledge of how complex substitution patterns are produced is less definite. Feeding experiments in the coumestan [2, 3], pterocarpan [4, 5] and rotenoid [6] fields have indicated that 2',4'- and 2',4',5'-oxygenation patterns in the B ring of isoflavones are built up sequentially from a single 4'-hydroxy or -methoxy substituent. The precise sequence for the tri-substituted compounds has not been established completely. The results are consistent with a hypothesis that the characteristic aryl migration step occurring during the biosynthesis of isoflavones generally involves only chalcones containing a single 4-hydroxy substituent [6]. Of the several hundred known naturally-occurring isoflavonoids, very few indeed lack the corresponding 4'-oxygen substituent [1].

Ring A of isoflavonoids is acetate–malonate derived, and consequently 5,7-dioxygenated compounds are fairly common. Frequently though, the 5-oxygen substituent is reduced before chalcone formation, and thus, 2',4',6'-trihydroxy- and 2',4'-dihydroxychalcones can both be accepted for aryl migration [7–9]. There is no labelling evidence to explain the production of any other oxygenation patterns in ring A of isoflavonoids. Consequently, this paper reports the results of initial radiochemical feeding experiments to investigate the origin of the 5-deoxy-6-methoxy substitution pattern in the A ring of the isoflavone afrormosin (7-hydroxy-6,4'-dimethoxyisoflavone) (**1**) using seedlings of sainfoin, *Onobrychis viciifolia* (Leguminosae).

RESULTS AND DISCUSSION

Seedlings of *Onobrychis viciifolia* accumulate significant amounts of afrormosin within a few days of germination. DL-Phenylalanine-[2-^{14}C], 2',4',4-trihydroxychalcone-[*carbonyl*-^{14}C] (**7**) and formononetin-[*Me*-^{14}C] (**2**) were tested as precursors using batches of 30 six-day-old seedlings, administering the labelled

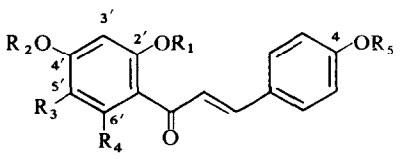


(**1**) $R_1 = R_3 = H$, $R_2 = OMe$, $R_4 = Me$
(**2**) $R_1 = R_2 = R_3 = H$, $R_4 = Me$
(**3**) $R_1 = R_3 = H$, $R_2 = OH$, $R_4 = Me$
(**4**) $R_1 = R_2 = R_3 = R_4 = H$
(**5**) $R_1 = R_2 = R_4 = H$, $R_3 = OH$
(**6**) $R_1 = R_2 = H$, $R_3 = OH$, $R_4 = Me$

compounds via the roots over 24 hr. After work-up of the plant material, afrormosin was isolated, quantitated then diluted with synthetic carrier. After methylation, the isoflavone was purified by TLC, then recrystallised to constant activity and counted. The results are shown in Table 1.

Afrormosin carrier was synthesised by thallium nitrate oxidation [10, 11] of 2',4'-dibenzyloxy-5',4-dimethoxychalcone (**8**) to the acetal, followed by treatment with HOAc–HCl. The chalcone was prepared by base condensation of the appropriate acetophenone with anisaldehyde.

The results clearly show that all three labelled compounds served as good precursors of afrormosin. The incorporation of formononetin moreover demonstrates that introduction of the 6-hydroxy/methoxy group may be a late stage in the biosynthesis, and suggests that 6,7-dihydroxy-4'-methoxyisoflavone (texasin) (**3**) is an intermediate in the process. 2',4',4-Trihydroxychalcone is an



(**7**) $R_1 = R_2 = R_3 = R_4 = R_5 = H$
(**8**) $R_1 = R_2 = CH_2Ph$, $R_3 = OMe$, $R_4 = H$, $R_5 = Me$
(**9**) $R_1 = R_2 = R_3 = R_5 = H$, $R_4 = OH$

Table 1. Incorporation of labelled precursors into afrormosin in *Onobrychis vicifolia* seedlings.

Compound fed	Unabsorbed precursor (%)	sp. act. (dpm/mM)	Dilution	Incorp.* (%)
DL-Phenylalanine-[2-^{14}C]	3	6.30×10^8	88	0.25
2′,4′,4-Trihydroxychalcone-[*carbonyl*-^{14}C]	35	8.85×10^6	56	0.10
Formononetin-[*Me*-^{14}C]	43	1.62×10^7	73	0.13

*Incorporation figures are not corrected for unabsorbed precursor.

established precursor of formononetin [4, 7, 8], and its incorporation into afrormosin is presumably via this isoflavone, rather than by further substitution at the chalcone level. Thus, further substitution in both aromatic rings can take place at the isoflavone level during biosynthesis. The aryl migration step in isoflavone biosynthesis might in general be restricted to the two chalcone precursors, 2′,4′,4-trihydroxychalcone (**7**) and 2′,4′,6′,4-tetrahydroxychalcone (**9**), giving daidzein (**4**) and genistein (**5**) respectively, or formononetin (**2**) and biochanin A (**6**) if methylation is associated with the aryl migration [6]. These four isoflavones could then, by further substitution, act as precursors of virtually all other natural isoflavonoids. In contrast, however, Inoue and Fujita [12] have shown by feeding experiments in *Pueraria thunbergiana* that although daidzein is readily 7-*O*-glucosylated to daidzin, it is poorly utilised as a precursor of the 8-*C*-glucoside puerarin, and they suggest that *C*-glucosylation occurs at the chalcone level.

EXPERIMENTAL

Seeds of *Onobrychis viciifolia* (Giant, milled) were purchased (Miln Marsters Ltd., Chester). TLC was carried out using 0.5 mm layers of Si gel (Merck Kiesel gel GF_{254}) in C_6H_6–EtOAc–MeOH–petrol (60–80°) (6:4:1·3) Radioactive samples were counted as previously [4].

Radiochemicals. DL-Phenylalanine-[2-^{14}C] (sp. act. 25 mCi/mM) was purchased (Amersham). The syntheses of 2′,4′,4-trihydroxychalcone-[*carbonyl*-^{14}C] (0.233 mCi/mM) and formonetin-[*Me*-^{14}C] (0.535 mCi/mM) have been described [6]

Plant material, feeding techniques and isolation of afrormosin Seeds of *O viciifolia* were surface sterilised (EtOH–H_2O–NaOCl, 15:3 2: 15 min) then germinated in peat-based compost (Fisons Levington) in a greenhouse for 6 days. Seedlings were carefully uprooted, thoroughly washed free of compost, then the roots of batches of 30 seedlings were dipped into the precursor solns (3 ml) in a small flask. Phenylalanine was fed in H_2O and the labelled phenols (*ca* 0 5 mg) were administered as their Na salts in phosphate buffer (0.1 M, pH 7). Seedlings were grown on for 24 hr, adding extra H_2O as required, then unabsorbed precursor was removed by washing (H_2O, then EtOH). The seedlings were homogenised by grinding in a mortar with ground glass and H_2O (*ca* 20 ml), and the slurry was poured into hot EtOH (100 ml) After filtering, the tissue was reextracted with hot EtOH (2 × 100 ml), and the combined extracts were evapd, taken up in H_2O (50 ml) and extracted with Et_2O (100 ml, and then 4 × 50 ml). The Et_2O extracts were evapd to dryness and separated by TLC. Afrormosin was eluted with Me_2CO–MeOH, 1:1, and quantitated by UV absorption of EtOH soln at 258 nm (log ε 4.49 [13]). The afrormosin extract was diluted with carrier afrormosin (*ca* 20 mg) and methylated in dry Me_2CO (20 ml) over K_2CO_3 (2 g) with Me_2SO_4 (0.05 ml) for 1 hr. The product was isolated in the usual manner, then purified by TLC and recrystallised to constant activity from MeOH. The product, 6,7,4′-trimethoxyisoflavone had mp 178–180° (lit. [13] 178–9°).

Synthesis of afrormosin. 4-Benzyloxy-2-hydroxy-5-methoxyacetophenone [14] (1.6 g) was stirred at 80° for 2 hr with benzyl chloride (0.7 g), K_2CO_3 (10 g), KI (1 g) in DMF (30 ml). The mixture was poured into H_2O, extracted with EtOAc (2 ×), the extracts washed with aq. NaOH (5%), H_2O, then evapd to give 2,4-dibenzyloxy-5-methoxyacetophenone as a dark oil. Without further purification, this was stirred at room temp for 18 hr with anisaldehyde (2 g) in EtOH (100 ml) with KOH (10 g) in H_2O (10 ml). The ppt was filtered, washed with H_2O and recrystallised from $CHCl_3$–MeOH to give 2′,4′-dibenzyloxy-5′,4-dimethoxychalcone (1.6 g) mp 128–9° (lit. [15] 125–6°). This chalcone (0.6 g) in MeOH–$CHCl_3$ (2 1, 75 ml) was stirred at room temp for 4 hr with $Tl(NO_3)_3.3H_2O$ (0.65 g), neutralised with NaOMe and evapd. The residue was taken up in H_2O (50 ml) and extracted with $CHCl_3$ (2 × 50 ml). The $CHCl_3$ extracts were evapd to dryness, then treated with HOAc (20 ml) and conc HCl (10 ml) at 80° for 1 hr, then left at room temp. for 18 hr. The mixture was poured into H_2O, filtered and the solids recrystallised from MeOH to yield afrormosin (0 20 g), mp 226–9° (lit. [13] 236–7°)

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