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ISOFLAVONE BIOSYNTHESIS IN *ONOBRYCHIS VICIIFOLIA*: FORMONONETIN AND TEXASIN AS PRECURSORS OF AFRORMOSIN

HAKIM A. M. AL-ANI and PAUL M. DEWICK
Department of Pharmacy, University of Nottingham, Nottingham, NG7 2RD, U.K.

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

Abstract—4,2′,4′-Trihydroxychalcone-[carbonyl-^{14}C], formononetin-[Me-^{14}C] and texasin-[Me-^{14}C] were all good precursors of afrormosin (7-hydroxy-6,4′-dimethoxyisoflavone) in *Onobrychis viciifolia* seedlings, and a biosynthetic pathway involving these intermediates is proposed. 2′,4′-Dihydroxy-4-methoxychalcone-[carbonyl-^{14}C] and daidzein-[carbonyl-^{14}C] were poor precursors. Incorporations into formononetin were also recorded.

INTRODUCTION

Earlier feeding experiments in seedlings of sainfoin (*Onobrychis viciifolia*) [1] have shown that phenylalanine, 4,2′,4′-trihydroxychalcone (**5**) and formononetin (**3**) are good precursors of 7-hydroxy-6,4′-dimethoxyisoflavone (afrormosin) (**1**). The incorporation of formononetin demonstrated that introduction of the 6-hydroxy/methoxy grouping may be a late stage in the biosynthesis of afrormosin, and 6,7-dihydroxy-4′-methoxyisoflavone (texasin) (**2**) was suggested as a likely intermediate. In the present studies, labelled texasin has been synthesized and tested as a biosynthetic precursor of afrormosin.

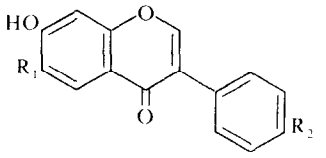
RESULTS AND DISCUSSION

Texasin-[Me-^{14}C] was synthesized by $Tl(NO_3)_3$ oxidation of 2′,4′,5′-tribenzyloxy-4-methoxychalcone-[Me-^{14}C], obtained by methylation of the corresponding 4-hydroxychalcone with $^{14}CH_3I$. Since this chalcone could not be obtained by normal base-catalysed condensation of 2′,4′,5′-tribenzyloxyacetophenone and 4-hydroxybenzaldehyde, it was prepared via 2′,4′,5′-tribenzyloxy-4-methoxymethoxychalcone which was readily synthesized from the acetophenone and 4-methoxymethoxybenzaldehyde. This isoflavone (*ca* 0.5 mg) was fed through the roots to batches of 30 6-day-old *Onobrychis viciifolia* seedlings for 48 hr. The plant material was worked up and afrormosin was isolated, quantified by UV spectroscopy, then diluted with synthetic carrier. After methylation, the material was purified by TLC, recrystallized to constant activity and counted. Formononetin, another isoflavone produced by *O. viciifolia* seedlings [2], was also isolated and treated similarly. Comparative feeding experiments were carried out simultaneously in which 4,2′,4′-trihydroxychalcone-[carbonyl-^{14}C] (**5**), 2′,4′-dihydroxy-4-methoxychalcone-[carbonyl-^{14}C] (**6**), daidzein-[carbonyl-^{14}C] (**4**) and formononetin-[Me-^{14}C] (**3**) were tested as precursors of the two isoflavones. The results are summarized in Table 1.

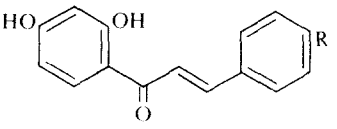
The results show that, as in the earlier experiments [1], the trihydroxychalcone and formononetin were good precursors of afrormosin, and that texasin was also well incorporated. In contrast, the dihydroxymethoxychalcone and daidzein were poor precursors. 4,2′,4′-Trihydroxychalcone was also an excellent precursor of formononetin, whereas the methylated chalcone was poorly incorporated. The incorporation of daidzein into formononetin was significant, but much less than might be expected if it was an

Table 1. Incorporation of labelled compounds into afrormosin and formononetin in *Onobrychis viciifolia* seedlings

Compound fed	Afrormosin			Formononetin		
	Sp. act. (dpm/mmol)	Dilution	Incorp. (%)	Sp. act. (dpm/mmol)	Dilution	Incorp. (%)
4,2′,4′-Trihydroxychalcone-[carbonyl-^{14}C]	3.05×10^6	162	0.25	2.28×10^7	22	1.8
2′,4′-Dihydroxy-4-methoxychalcone-[carbonyl-^{14}C]	3.39×10^4	13 300	0.0035	1.83×10^5	2460	0.0082
Daidzein-[carbonyl-^{14}C]	5.09×10^3	14 700	0.0015	2.59×10^5	289	0.098
Formononetin-[Me-^{14}C]	5.07×10^6	295	0.17	6.72×10^8	2.2	54
Texasin-[Me-^{14}C]	9.65×10^7	34	1.14	3.14×10^5	10 400	0.0025



1 $R_1 = R_2 = OMe$
2 $R_1 = OH$, $R_2 = OMe$
3 $R_1 = H$, $R_2 = OMe$
4 $R_1 = H$, $R_2 = OH$



5 R = OH
6 R = OMe

obligatory intermediate in the biosynthetic pathway to this isoflavone. These observations parallel results in other plants [3–7], and are again consistent with a hypothesis that 4′-*O*-methylation in isoflavonoids is associated with the aryl migration step [4]. Thus, the biosynthetic pathway to afrormosin in *O. viciifolia* is probably via 4,2′,4′-trihydroxychalcone and formononetin, involving methylation during the aryl migration, followed by 6-hydroxylation to texasin and then 6-methylation to yield afrormosin.

The possible involvement of 2′,4′,5′-trioxygenated chalcones as precursors of afrormosin, though not completely ruled out, is made much less likely by the excellent incorporations of formononetin and texasin. Despite a number of attempts, we were unable to synthesize 4,2′,4′,5′-tetrahydroxychalcone for feeding experiments. However, from results now available [1, 4, 7–9], it would appear that further hydroxylation in either aromatic ring takes place at the isoflavone rather than at chalcone level during the biosynthesis of isoflavonoids.

EXPERIMENTAL

General. TLC was carried out using 0.5 mm layers of Si gel (Merck TLC-Kieselgel 60 GF_{254}, mean particle size 15 μm) in the solvent systems: A, hexane–EtOAc (1:1); B, $CHCl_3$–MeOH (9:1); C, toluene–HCO_2Et–HCO_2H (5:4:1). Radioactive samples were counted as previously [3].

Plant material, feeding techniques and isolation of isoflavones. Methods were as previously described [1] with minor modifications as follows. Seeds of *O. viciifolia* were germinated in moist vermiculite and the feeding period was 48 hr. Solvent A was used in TLC of the Et_2O extract from the plant, except in the daidzein feeding experiment, where afrormosin was further separated from unmetabolized daidzein by TLC using solvent B. 6,7,4′-Trimethoxyisoflavone was purified by TLC (solvent A) prior to recrystallization. Treatment of formononetin also isolated was as in other studies [3], but using solvent A for the TLC of the Me ether.

Radiochemicals. The syntheses of 4,2′,4′-trihydroxychalcone-[carbonyl-^{14}C] (0.223 mCi/mmol) [4], 2′,4′-dihydroxy-4-methoxychalcone-[carbonyl-^{14}C] (0.203 mCi/mmol) [4], daidzein-[carbonyl-^{14}C] (0.034 mCi/mmol) [7] and formononetin-[Me-^{14}C] (0.676 mCi/mmol) [7] have been described. $^{14}CH_3I$ (59.1 mCi/mmol) was purchased (Amersham).

Texasin-[Me-^{14}C]. 4-Hydroxybenzaldehyde (1.1 g) was dissolved in aq. KOH (5%, 10 ml), the soln evapd to dryness, and the residue dried under vacuum. Dry MeCN (25 ml) and 18-Crown-6 (0.26 g) were added and the mixture stirred at room temp. for 30 min. Chloromethylmethyl ether (1 g) was added, and the mixture stirred a further 1.5 hr, then filtered. The filtrate was evapd, taken up in Et_2O (100 ml) and the Et_2O soln washed with H_2O (100 ml). Unreacted phenol was removed from the Et_2O soln by washing with dil aq. NaOH, then H_2O, and the extract was then evapd to give 4-methoxymethoxybenzaldehyde (0.86 g) as an oil. ^{1}H NMR (60 MHz, $CDCl_3$, TMS): δ 3.45 (3 H, *s*, OMe), 5.22 (2 H, *s*, OCH_2O), 7.07 (2 H, *d*, J = 9 Hz, H-3,5), 7.76 (2 H, *d*, J = 9 Hz, H-2,6), 9.83 (1 H, *s*, CHO).

Without further purification, this aldehyde (0.25 g) and 2′,4′,5′-tribenzyloxyacetophenone [10] (0.44 g) were dissolved in warm EtOH (60 ml), then treated with aq. KOH (1:1 w/w, 10 g) and stirred at room temp. for 18 hr. 2′,4′,5′-Tribenzyloxy-4-methoxymethoxychalcone (0.47 g) was obtained by filtration, washed with H_2O, then recrystallized from $CHCl_3$–MeOH. Mp 143–146°. ^{1}H NMR (100 MHz, $CDCl_3$, TMS): δ 3.48 (3 H, *s*, OMe), 5.0, 5.15, 5.19 and 5.21 (4 × 2 H, *s*'s, $OC\underline{H}_2Ph$ and OCH_2O), 6.63 (1 H, *s*, H-3′), 6.91 (2 H, *d*, J = 9 Hz, H-3,5), 7.25 (2 H, *d*, J = 9 Hz, H-2,6), *ca* 7.3 (15 H, *m*, Ph), 7.58 (2 H, *s*, H-α,β), 7.59 (1 H, *s*, H-6′).

This chalcone (0.30 g) in MeOH (150 ml) was heated under reflux with dil HCl (7%, 5 ml) for 3 hr. The mixture was concd, poured into H_2O, extracted with EtOAc (2 ×), and the extracts washed with H_2O and evapd. 2′,4′,5′-Tribenzyloxy-4-hydroxychalcone (0.22 g) was recrystallized from MeOH. Mp 152–154°. ^{1}H NMR (100 MHz, $CDCl_3$, TMS): δ 5.0, 5.14, 5.21 (3 × 2 H, *s*'s, $OC\underline{H}_2Ph$), 6.63 (1 H, *s*, H-3′), 6.72 (2 H, *d*, J = 9 Hz, H-3,5), 7.18 (2 H, *d*, J = 9 Hz, H-2,6), *ca* 7.3 (15 H, *m*, Ph), 7.57 (2 H, *s*, H-α,β), 7.58 (1 H, *s*, H-6′).

2′,4′,5′-Tribenzyloxy-4-hydroxychalcone (101 mg) in dry Me_2CO (20 ml) was stirred under reflux over dry K_2CO_3 (2 g) with MeI (10 μl, 22.8 mg) and $^{14}CH_3I$ (100 μCi, 0.244 mg) for 2.5 hr. Excess MeI (50 μl) was added and the reaction continued for a further 2 hr. The mixture was filtered, the filtrate evapd, and the residue recrystallized from $CHCl_3$–MeOH to give 2′,4′,5′-tribenzyloxy-4-methoxychalcone-[Me-^{14}C] (99 mg). Inactive material had mp 130–132°. ^{1}H NMR (100 MHz, $CDCl_3$, TMS): δ 3.82 (3 H, *s*, OMe), 5.0, 5.15, 5.21 (3 × 2 H, *s*'s, $OC\underline{H}_2Ph$), 6.63 (1 H, *s*, H-3′), 6.78 (2 H, *d*, J = 9 Hz, H-3,5), 7.25 (2 H, *d*, J = 9 Hz, H-2,6), *ca* 7.3 (15 H, *m*, Ph), 7.57 (2 H, *s*, H-α,β), 7.59 (1 H, *s*, H-6′). An identical product was obtained by the base-catalysed condensation of 2′,4′,5′-tribenzyloxycetophenone and anisaldehyde.

The labelled chalcone (99 mg) was dissolved in MeOH–$CHCl_3$ (2:1, 20 ml) and stirred at room temp. for 3 hr with Tl $(NO_3)_3 \cdot 3H_2O$ (95 mg). The soln was evapd, treated with H_2O (25 ml), and extracted with $CHCl_3$ (3 × 20 ml). The extracts were evapd, then heated at 80° with HOAc (10 ml) and conc HCl (5 ml) for 16 hr. The mixture was poured into H_2O, extracted with EtOAc (3 × 25 ml), the extracts washed free of acid with aq. $NaHCO_3$, washed with H_2O and evapd. Texasin-[Me-^{14}C] was isolated and purified by TLC (solvent C). Yield 8.5 mg, sp. act. 1.48 mCi/mmol. Inactive material had mp 275° dec., lit. [11] 275–278°, [12] 285–287°.

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