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BIOSYNTHESIS OF COUMESTROL IN *PHASEOLUS AUREUS*

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Abstract—Feeding experiments with 4′,7-dihydroxyisoflavone-[4-^{14}C] (daidzein), 2′,4′,7-trihydroxyisoflavone-[T] and (±)-4′,7-dihydroxyisoflavanone-[T] (dihydrodaidzein) in suspension cultures of mung bean (*Phaseolus aureus* Roxb.) roots have shown that daidzein is a better precursor of the coumestan coumestrol than is the trihydroxyisoflavone and that dihydrodaidzein can also be converted very efficiently. The results provide further evidence for the intermediacy of a pterocarp-6a-en in coumestrol biosynthesis, and also indicate the possible existence of a 'metabolic grid' of isoflavones and isoflavanones in *P. aureus*.

INTRODUCTION

IN A recent communication,[1] we reported the results of comparative feeding experiments designed to elucidate some of the later stages in the biosynthesis of coumestrol (I) in mung bean (*Phaseolus aureus* Roxb.). In particular, the information obtained from dilution analyses with 2′,4′,7-trihydroxyisoflavone (II) suggested that this isoflavone, or the corresponding isoflavanone, occupied a prominent position in the biosynthetic pathway.

We have now continued our investigations and here report on the role of the isoflavones daidzein (4′,7-dihydroxyisoflavone) (III) and 2′,4′,7-trihydroxyisoflavone and the isoflavanone dihydrodaidzein (4′,7-dihydroxyisoflavanone) (IV) in coumestrol biosynthesis. In contrast to our earlier studies[1] which were carried out in aseptic intact plants, these experiments utilised cell suspension cultures of mung bean roots, cultures which have recently been shown to synthesise coumestrol.[2] Use of these cultures allowed shorter incubation periods, and avoided the danger of microbial contaminations.[3]

RESULTS

In a first set of experiments we have used as substrate the isoflavones daidzein-[4-^{14}C] and 2′,4′,7-trihydroxyisoflavone-[T] both separately, and together in a competitive feeding experiment. The preparation of daidzein-[4-^{14}C] has been reported in an earlier communication.[3] Tritiated 2′,4′,7-trihydroxyisoflavone was prepared by a modified Wilzbach procedure,[4] and tritium at positions 3′ and 5′ (at which hydrogen may be labile at physiolocical pHs)[5] was removed by base-catalysed exchange. The proportion of label introduced at position 2, which must be lost on incorporation into coumestrol was established by alkaline degradation of the methylated isoflavone to the corresponding deoxybenzoin; only 2·5% of the total activity was present at this position.

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TABLE 1. INCORPORATION OF LABELLED ISOFLAVONES AND ISOFLAVANONES INTO COUMESTROL IN *Phaseolus aureus* ROOT CELL CULTURES*

Compound fed	Daidzein-[4 ^{14}C]	2′,4′,7-Trihydroxy-isoflavone-[T]	Daidzein-[4 ^{14}C] + 2′,4′,7-trihydroxy-isoflavone-[T]†		Dihydro-daidzein-[T]
Specific activity (dpm/mM $\times 10^{-9}$)	1·93	41·6	^{14}C:	1·93	3·30
			T:	41·6	
Activity fed (dpm $\times 10^{-6}$)	6·67	48·9	^{14}C:	6·67	38·0
			T:	48·9	
Coumestrol isolated (μg)	91	67		40	92
Specific activity of isolated coumestrol (dpm/mM $\times 10^{-6}$)	7·75	18·2	^{14}C:	2·86	5·95
			T:	3·70	
Incorporation (%)	0·035	0·0085§	^{14}C:	0·0058	0·005‡
			T:	0·001§	
Dilution of specific activity	249	2230§	^{14}C:	675	555‡
			T:	11000§	

* Uptake of the various substrates by mung bean culture was in the range of 90%.

† A 3:1 molar ratio of daidzein to 2′,4′,7-trihydroxy-isoflavone was used.

‡ These values are uncorrected for obligatory loss of T (see Results) and thus represent minimum incorporation.

§ Corrected for loss of 2·5% T activity from C-2.

Cell suspension cultures of mung bean roots were used on the 12th day after inoculation into fresh medium, during a phase of linear coumestrol accumulation.[2] After an incubation period of 24 hr, the cell material was worked up, and coumestrol was isolated, diluted with carrier and purified to constant activity as described previously.[1] The percentage incorporations and specific activity dilution values are summarised in Table 1.

The data clearly indicate that daidzein is a better precursor of coumestrol than is 2′,4′,7-trihydroxyisoflavone. In particular, the competitive feeding experiment would exclude the trihydroxyisoflavone from being an intermediate of major importance in the pathway for coumestrol biosynthesis.

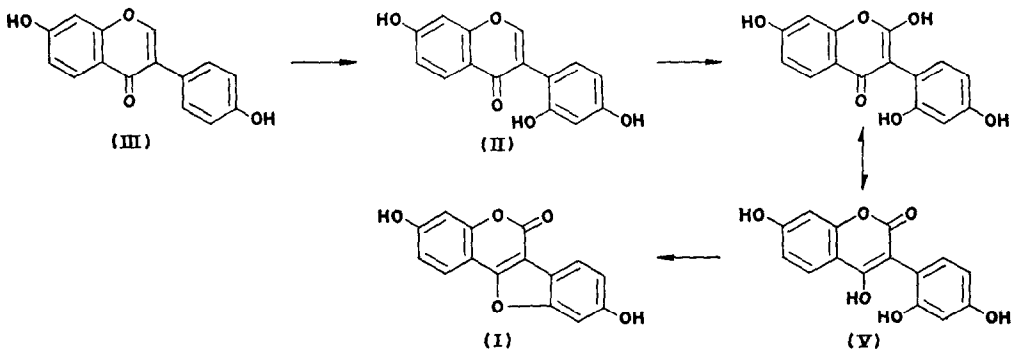
It has earlier been noted[6] that 4′,7-dihydroxyisoflavanone (dihydrodaidzein) may be converted into coumestrol in *P. aureus* seedlings; at that time, no conclusion was drawn regarding its role as a possible precursor of coumestrol. We have therefore repeated the feeding of dihydrodaidzein, but instead of using intact seedlings, have utilized the root cell-suspension cultures of *P. aureus* described above. Tritiated (±)-dihydrodaidzein was prepared by the Wilzbach procedure[4] and the labelled isoflavanone was rigorously purified by paper chromatography as described in the Experimental. The (±)-isoflavanone was fed to the plant material in exactly the same manner as the isoflavones above. The incorporation data for (±)-dihydrodaidzein-[T] are also shown in Table 1. The experiment was conducted on a qualitative basis only, since an unknown fraction of the activity must be lost on incorporation into coumestrol, namely the activity resident at C2, C3 and C2′, together with that at C3′ and C5′ if the biosynthetic route should pass through a 2′,4′-dihydroxylated isoflav(an)one. In addition, although epimerization at the C3 position of isoflavanones may be expected to occur fairly readily *in vivo*, it is still possible that only the natural epimer is

[6] H. ZILG and H. GRISEBACH, *Phytochem.* 7, 1965 (1968).

incorporated. However, the incorporation of dihydrodaidzein into coumestrol (Table) does indicate that dihydrodaidzein is a precursor of similar efficiency as daidzein.*

DISCUSSION

Examination of the percentage incorporation and specific activity dilution data in the Table clearly demonstrates that the route daidzein → 2′,4′,7-trihydroxyisoflavone →→ coumestrol cannot constitute the major biosynthetic pathway for coumestrol in *P. aureus*. From our earlier results,[1] we postulated that two routes might be utilised in the biosynthesis. One route (Scheme 1) involving 2′-hydroxylation of daidzein (III) to 2′,4′,7-trihydroxy-



SCHEME 1. POSTULATED[1] BIOSYNTHETIC PATHWAY TO COUMESTROL (I) VIA 2-HYDROXYLATION OF 2′,4′,7-TRIHYDROXYISOFLAVONE (II).

isoflavone (II), followed by 2-hydroxylation[7] and tautomerism[8] to 3-(2,4-dihydroxyphenyl)-4,7-dihydroxycoumarin (V) which on dehydration could yield coumestrol, can now be excluded on the above evidence that daidzein is a better precursor than 2′,4′,7-trihydroxyisoflavone. The alternative route (Scheme 2), suggested by a chemical analogy,[9] was dehydration/cyclisation of 2′,4′,7-trihydroxyisoflavanone (VI) to 3,9-dihydroxypterocarp-6a-en (VII) followed by allylic oxidation to coumestrol. Recent studies[10] have confirmed our suggestion[9] that pterocarp-6a-ens are extremely easily oxidized to coumestans. The present results offer further evidence for this biosynthetic route. In addition, however, they would indicate that as the major route, 2′-hydroxylation must occur, not at the isoflavone oxidation level, but at the isoflavanone level. The significant incorporation, which, as mentioned in the Results, can only be a minimum value, of the isoflavanone dihydrodaidzein supports this.*

Since conversion of daidzein into 2′,4′,7-trihydroxyisoflavone,[1] and of 2′,4′,7-trihydroxyisoflavone and dihydrodaidzein into coumestrol do occur, it appears that the situation existing in *P. aureus* may best be described by a 'metabolic grid'[11] (Scheme 2) of isoflavones

* Dihydrodaidzein is about five times more rapidly catabolized than daidzein[2] in mung bean cultures (Berlin, unpublished). The amount of dihydrodaidzein available as precursor for coumestrol will therefore decline more rapidly than that of daidzein.

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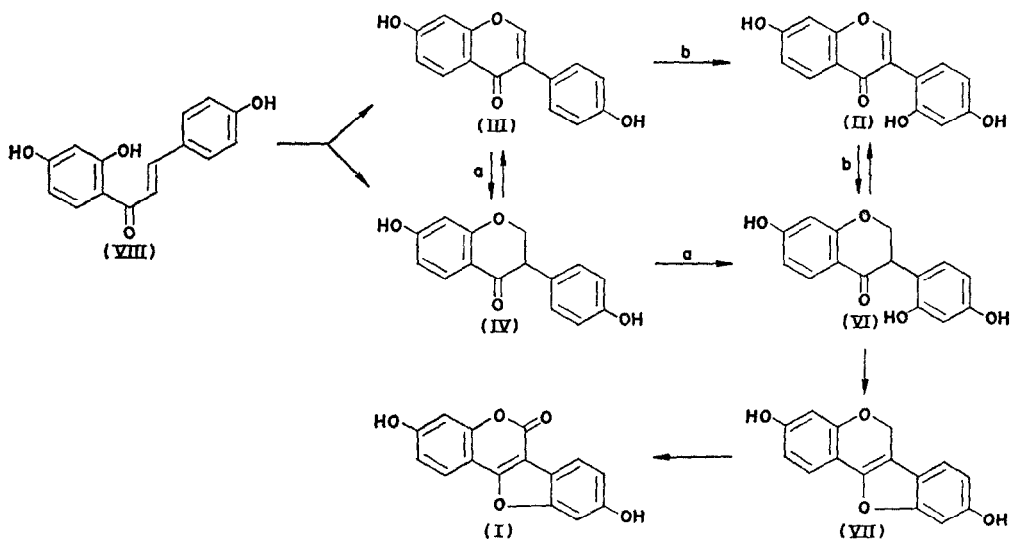

SCHEME 2. PROBABLE BIOSYNTHETIC ROUTE TO COUMESTROL (I) IN *Phaseolus aureus*, DEPICTING 'METABOLIC GRID' OF ISOFLAVONES AND ISOFLAVANONES.

and isoflavanones derived from 2',4,4'-trihydroxychalcone (VIII)[1] in which the major route daidzein to 2',4',7-trihydroxyisoflavanone is indicated 'a', and the minor route 'b'. The situation is perhaps more complex since there is evidence for the reversible interconversion of isoflavones and isoflavanones in *P. aureus*.[12] Although there is no proof at present, it is attractive to view this metabolic grid as involving enzymes of only relative specificity and that mainly kinetic factors are responsible for route 'a' acting as the major pathway. On steric grounds alone, it is apparent that 2'-hydroxylation of isoflavanones should proceed faster than a similar hydroxylation of isoflavones where the planarity of the molecule will hinder the approach of reacting entities. Studies[13] on the biosynthesis of 2'-hydroxylated flavones have similarly indicated that this additional hydroxyl group is introduced at the flavanone or dihydroflavonol level of oxidation rather than at the planar flavone level. Obviously, the real answer will only be obtained when the relevant enzymes are available for further studies.

Another possibility would be formation of the pterocarpen VII from the isoflavanone IV by direct oxidative cyclization.[19] There is, as yet, little direct evidence on whether isoflavones or isoflavanones are the earliest isolable isoflavonoids produced from chalcones and/or flavanones.[14] However, recent results[15] on the utilisation of chalcone, isoflavone and isoflavanone precursors during rotenoid biosynthesis would argue strongly for isoflavones being the first-formed.

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EXPERIMENTAL

Cell cultures and feeding techniques. The cultivation of the cell suspension cultures of *Phaseolus aureus* Roxb. and the feeding techniques were as described previously.[2]

Isolation and purification of coumestrol. Coumestrol was isolated from the cell suspensions and purified to constant specific radioactivity according to our routine methods.[1,2]

Radioactive assay. Tritium and ^{14}C were determined according to published methods.[1]

Labelled substrates. 2',4',7-Trihydroxyisoflavone-[T]. 2',4',7-Trihydroxyisoflavone[16] (30 mg) was absorbed on finely-powdered quartz (5 g), and labelled with tritium by a modified Wilzbach procedure,[4] using tritium gas (5 Ci) for a period of 6 weeks. Labile tritium was removed by repeatedly dissolving the compound in methanol and evaporating to dryness under reduced pressure. Easily-exchangeable tritium (positions 3' and 5') was removed by dissolving the isoflavone in N_2-flushed 0·1 N aq. NaOH (10 ml), and allowing to stand at R° for 2 hr. The isoflavone was obtained from the solution by acidification (dil. HCl) and EtOAc extraction (5 × 20 ml). The exchange was repeated. The isoflavone was finally purified to constant activity by repeated TLC (Kiesel-gel G, 0·4 mm layers) using $CHCl_3$–*iso*-PrOH (7:1), benzene–EtOAc–MeOH (6:4:1) and benzene–EtOH (5:1). Yield 14 mg, specific activity $4{\cdot}15 \times 10^{10}$ dpm/mM (18·7 mCi/mM). A small sample was diluted with carrier (20 mg), permethylated[1] and purified to constant activity ($4{\cdot}87 \times 10^5$ dpm/mM) via TLC[1] and repeated crystallization from MeOH. A portion (10 mg) was heated under reflux (75 min) with KOH (0·4 g) in 50% v/v aq. EtOH (8 ml). After removal of the EtOH, the mixture was acidified and extracted with EtOAc (2 × 15 ml), the product being purified by TLC (benzene–EtOAc–MeOH–petrol (6:4:1:8)). 2,4-Dimethoxybenzyl-2-hydroxy-4-methoxyphenylketone[17] was crystallized to constant activity ($4{\cdot}75 \times 10^5$ dpm/mM) from aq. MeOH.

4',7-Dihydroxyisoflavanone-[T]. 4',7-Dihydroxyisoflavanone[12] (50 mg) was tritiated as described above on quartz powder using tritium gas (5 Ci) for 4 weeks. Purification to constant specific activity was carried out by paper chromatography (prewashed[18] Whatman 3MM) using 15% aq. HOAc, 40% aq. EtOH and benzene–HOAc–H_2O (125:72:3). Yield 33 mg, specific activity $3{\cdot}30 \times 10^9$ dpm/mM (1·49 mCi/mM).

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Key Word Index—Phaseolus aureus; Leguminosae; biosynthesis; coumestrol; daidzein; dihydrodaidzein.