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BIOSYNTHESIS OF THE 2-ARYLBENZOFURAN PHYTOALEXIN VIGNAFURAN IN *VIGNA UNGUICULATA*

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Key Word Index—*Vigna unguiculata*; Leguminosae; cowpea; biosynthesis; phytoalexin; 2-arylbenzofuran; vignafuran; isoflavonoid.

Abstract—Feeding experiments using L-phenylalanine-[U-^{14}C], DL-phenylalanine-[1-^{14}C] and -[2-^{14}C] together with degradative studies have been used to investigate the biosynthesis of the 2-arylbenzofuran phytoalexin vignafuran in UV-treated seedlings of cowpea (*Vigna unguiculata*). During the biosynthetic process, C-3 of phenylalanine appears to be lost, and the resulting labelling pattern is consistent with vignafuran being derived from an isoflavonoid precursor, but the phenylalanine-derived aromatic ring becomes the 2-aryl substituent and not part of the benzofuran system. A previously proposed pathway to 2-arylbenzofurans by loss of C-6 from a coumestan is thus excluded. Alternative routes are suggested.

INTRODUCTION

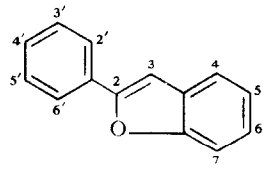
The number of known naturally-occurring 2-arylbenzofurans is not large, but the variety of structures encountered suggests that they arise not by a single biosynthetic pathway, but by a number of different routes. Thus, egonol (**1**) and homoegonol (**2**) isolated from *Styrax* species [1] are envisaged as being of bisarylpropanoid (lignan [2] or neolignan [3]) origin, derived by a process involving loss of one carbon atom. The close structural similarity with lignans/neolignans which retain this carbon atom, e.g. eupomatene (**3**) from *Eupomatia laurina* [2], enhances this view. Other 2-arylbenzofurans bear striking resemblances to stilbenes isolated from the same or related sources, and may well be derived by oxidative cyclization of hydroxystilbenes in a process analogous to laboratory conversions [4]. In this group may be placed 2-(3,5-dihydroxyphenyl)-6-hydroxybenzofuran (**4**) which co-occurs with oxyresveratrol (**34**) in *Morus laevigata* [5], and the recently reported phytoalexins moracin A (**5**) and moracin B (**6**) which are produced on infection of *Morus alba* with *Fusarium solani* f. sp. *mori* [6]. The stilbene oxyresveratrol has also been isolated from *M. alba* [5]. A third group of 2-arylbenzofurans, observed in the Leguminosae, appears to be related to isoflavonoids, since isoflavonoids having similar substitution patterns co-occur in these plants. These include pterofuran (**7**) from *Pterocarpus indicus* [7], neoraufurane (**8**) from *Neorautanenia edulis* [8], 2-(2,4-dihydroxyphenyl)-5,6-dimethoxybenzofuran (**9**) from *Myroxylon balsamum* [9], and 2-(2,4-dihydroxyphenyl)-5,6-methylenedioxybenzofuran (**10**) and its methyl ether (**11**) from *Sophora tomentosa* [10]. To this group must be added the phytoalexins vignafuran (**12**) from leaves of cowpea, *Vigna unguiculata* infected with *Colletotrichum lindemuthianum* [11] and *Lablab niger* infected with *Helminthosporium carbonum* [12], and 6-demethylvignafuran (**13**) from *H. carbonum*-infected leaves of *Tetragonolobus maritimus*, *Anthyllis vulneraria* and *Coronilla emerus* [13]. A biosynthetic sequence involving loss of one carbon atom (C-6) from a coumestan, by analogy with alkaline degradation of coumestans [14], is generally postulated [9, 15], and the co-occurrence of **9** with 3-hydroxy-8,9-dimethoxycoumestan (**35**) [9] and **10** and **11** with medicagol (**36**) [10] lend support for this view. However, plausible sequences involving loss of a carbon atom from isoflavone, pterocarpan or pterocarpene precursors may also readily be devised. Neoraufurane (**8**) though, co-occurs with similarly substituted pterocarpan, isoflavene and isoflavan derivatives [8, 16], e.g. neorauflavane (**17**) and edudiol (**23**), and this type of biosynthetic origin is thus probably excluded. In this case, the benzofuran moiety is presumably related to the acetate-derived ring of the isoflavonoid, not the shikimate-derived ring as above. The biosynthetic route to 2-(6-hydroxy-2-methoxy-3,4-methylenedioxyphenyl)-benzofuran (**14**) isolated from bakers' yeast [17] is not readily apparent. It is structurally similar to betavulgarin (**28**) a phytoalexin of sugar beet, *Beta vulgaris* (Chenopodiaceae) infected with *Cercospora beticola* [18], but an isoflavonoid origin in bakers' yeast seems unlikely. Biosynthetic suggestions also include acetate chain extension of a phenylacetyl starter group [19].

To extend our investigations into the biosynthesis of isoflavonoid phytoalexins and related compounds in leguminous plants [20–26], and to establish if there exists any relationship between certain 2-arylbenzofurans and the isoflavonoids, we report here preliminary results concerning the biosynthesis of vignafuran (**12**) in *Vigna unguiculata* seedlings.

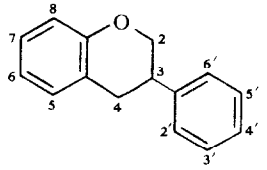
RESULTS AND DISCUSSION

Vignafuran production in cowpea

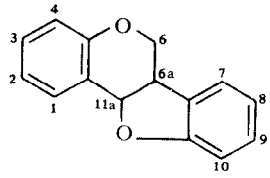
A number of phytoalexins have been isolated from



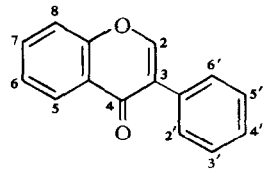
Trivial name	Substituent at position 3	4	5	6	7	2′	3′	4′	5′	6′
1 Egonol	—	—	$(CH_2)_3OH$	—	OMe	—	OCH_2O		—	—
2 Homoegonol	—	—	$(CH_2)_3OH$	—	OMe	—	OMe	OMe	—	—
3 Eupomatene	Me	—	$CH{=}CHCH_3$	—	OMe	—	OCH_2O		—	—
4	—	—	—	OH	—	—	OH	—	OH	—
5 Moracin A	—	—	OH	OMe	—	—	OH	—	OMe	—
6 Moracin B	—	OMe	—	OMe	—	—	OH	—	OH	—
7 Pterofuran	—	—	—	OH	—	OMe	OH	OMe	—	—
8 Neoraufurane	—	OMe	$CH{=}CHC(Me)_2O$		—	OH	—	OH	—	—
9	—	—	OMe	OMe	—	OH	—	OH	—	—
10	—	—	OCH_2O		—	OH	—	OH	—	—
11	—	—	OCH_2O		—	OH	—	OMe	—	—
12 Vignafuran	—	—	—	OMe	—	OMe	—	OH	—	—
13 6-Demethylvignafuran	—	—	—	OH	—	OMe	—	OH	—	—
14	—	—	—	—	—	OMe	OCH_2O		—	OH
15 Methylvignafuran	—	—	—	OMe	—	OMe	—	OMe	—	—
16	—	OMe	$CH_2CH{=}C(Me)_2$	OMe	—	OMe	—	OMe	—	—



Trivial name	Substituent at position 5	6	7	2′	3′	4′
17 Neorauflavane	OMe	$CH{=}CHC(Me)_2O$	OH	—		OH
18 2′-*O*-Methylphaseollinisoflavan	—	—	OH	OMe	$CH_2CH{=}CMe_2$	OH
19 Vestitol	—	—	OH	OH	—	OMe
20 Sativan	—	—	OH	OMe	—	OMe
21 Isovestitol	—	—	OH	OMe	—	OH
22 Demethylvestitol	—	—	OH	OH	—	OH



Trivial name	Substituent at position 1	2	3	9	10
23 Edudiol	OMe	$CH_2CH{=}CMe_2$	OH	OH	—
24 Phaseollin	—	—	OH	$OC(Me)_2CH{=}CH$	
25 Phaseollidin	—	—	OH	OH	$CH_2CH{=}CMe_2$
26 Demethylhomopterocarpin	—	—	OH	OMe	—
27 Edulane	OMe	$CH_2CH_2C(Me)_2O$		OMe	—



Trivial name	Substituent at position 5	6	7	2′	4′
28 Betavulgarin	OMe		OCH_2O	OH	—
29	—	—	OCH_2Ph	OCH_2Ph	OMe
30	—	—	OH	OH	OMe
31 Daidzein	—	—	OH	—	OH
32 Formononetin	—	—	OH	—	OMe
33	—	—	OMe	OMe	OH

cowpea (*Vigna unguiculata*) after fungal or viral infection. Kievitone (**38**) [27], phaseollin (**24**) [27], phaseollidin (**25**) [27], 2′-*O*-methylphaseollidinisoflavan (**18**) [28] and demethylhomopterocarpin (**26**) [29] are all isoflavonoids; vignafuran (**12**) [11] represents the first reported 2-arylbenzofuran phytoalexin. This was isolated from leaves of light- and dark-grown seedlings infected with *Colletotrichum lindemuthianum*, only trace amounts being isolated from the stems. The studies utilized cowpea seed, accession line TVu 57 obtained from the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria. Because the phytoalexin pattern produced by infected cowpea seedlings can vary markedly in different accession lines [28], our preliminary studies on vignafuran biosynthesis involved four accession lines originating from IITA. These were TVu 57 (brown seeds), TVu 57 (grey seeds), TVu 76, TVu 1035 and K 2809. 7-Day-old seedlings from these accession lines, grown in a greenhouse in a peat-based medium, all contained traces of vignafuran (presumably induced by natural fungal contamination), but largest quantities were observed in line K 2809 and further experiments were directed using plant material from this line. Reproducible abiotic induction of vignafuran synthesis in *V. unguiculata* proved surprisingly difficult to attain. Abiotic induction ensures that biosynthetic studies on phytoalexins reflect the plant's metabolic activity and not fungally-mediated transformations. Although a variety of abiotic agents may induce phytoalexin synthesis [30], the most convenient materials are probably heavy metal salts and UV radiation, and the use of cupric chloride, sometimes together with UV light, has proved an extremely reliable inducer for biosynthetic studies in *Trifolium pratense* [20, 21, 26], *Medicago sativa* [22, 23, 25] and *Trigonella foenum-graecum* [24]. With 7-day-old light-grown *V. unguiculata* seedlings, $CuCl_2$ applied to the roots proved ineffective, as did root-applied $CuCl_2$ + UV irradiation of the leaves. However, UV irradiation (254 nm, 30 min) of light-grown seedlings was found to induce vignafuran production, little or none of this phytoalexin being observed in untreated seedlings. The age of the seedlings was critical for successful induction. Seeds were germinated in the dark for 3 days, then the seedlings were transferred to small flasks containing distilled water and grown on in the light. Batches of seedlings of different ages were exposed to UV light (254 nm, 30 min), grown in the dark then extracted 16 hr later. Table 1 shows maximum phytoalexin synthesis occurred in 7/8-day-old seedlings, the quantities induced falling markedly in younger and older seedlings. The amount of vignafuran accumulated in seedlings irradiated at 7-days old did not vary significantly with longer induction periods after irradiation, similar quantities being isolated after 24, 48 and 72 hr periods. Eventually, for feeding experiments, batches of 25 7-day-old seedlings grown in the light in moist vermiculite, and a feeding period of 24 hr in the dark, were utilized. Labelled precursors were administered in aqueous solution via the roots.

Vignafuran, for use as reference and carrier material, was synthesized by a route analogous to that employed in the synthesis of 6-demethylvignafuran (**13**) [13]. This route exploited the similarity of 2-arylbenzofurans to the isoflavonoids, and involved as a key step the formation of deoxybenzoins via base hydrolysis of 2′-benzyloxyisoflavones. Debenzylation and acid-catalysed ring-closure produced 2-arylbenzofurans in reasonable yields. Base-hydrolysis of 2′,7-dibenzyloxy-4′-methoxyisoflavone (**29**) yielded the deoxybenzoin (**39**), which was methylated to **40**. Treatment of **40** with HOAc–HCl afforded vignafuran in a yield of *ca* 20% from the isoflavone, as a gummy material, which for convenience was converted into the crystalline acetate. Two other synthetic approaches to vignafuran have been reported [11, 31].

Feeding experiments

The first feeding experiment involved the administration of 2′,7-dihydroxy-4′-methoxyisoflavone-[*Me*-^{14}C]

Table 1. Vignafuran content of UV-induced *Vigna unguiculata* seedlings*

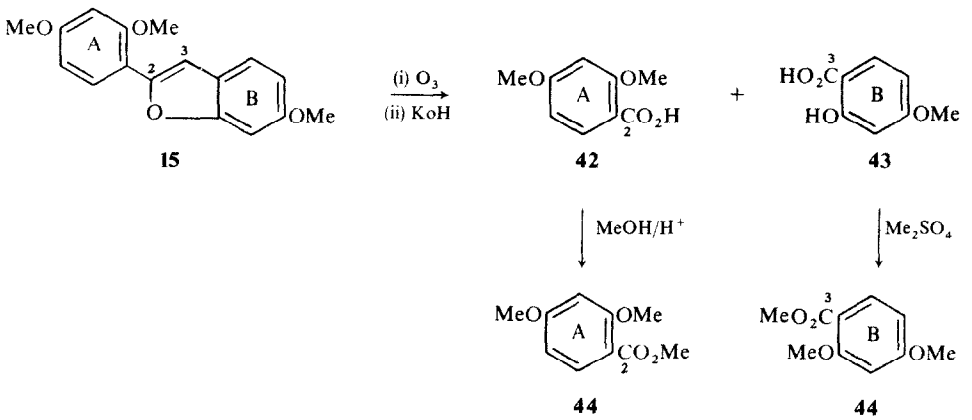
Age of seedlings (days)	µg vignafuran isolated Expt. (i)	Expt. (ii)
5	4	
6	12	29
7	34	37
8	39	26
9	24	18
10	9	

*6 seedlings, exposed to UV for 30 min, extracted 16 hr later.

Table 2. Feeding of ^{14}C-labelled phenylalanines to UV-induced *Vigna unguiculata* seedlings* and degradation of vignafuran

Phenylalanine fed	Incorp. %	Dilution	Methylvignafuran Sp. act. dpm/mM	%	Fragment ring A + C-2 Sp. act. dpm/mM	%	Fragment ring B + C-3 Sp. act. dpm/mM	%
L-[U-^{14}C]	0.022	190	1.33×10^5	100	1.19×10^5	89.5	1.88×10^4	14.1
DL-[1-^{14}C]	0.019	1060	1.66×10^5	100	0	0	1.66×10^5	100
DL-[2-^{14}C]	0.009	2570	6.12×10^4	100	6.23×10^4	102	1.20×10^3	2

* 25 seedlings, exposed to UV for 30 min, feeding period 24 hr.



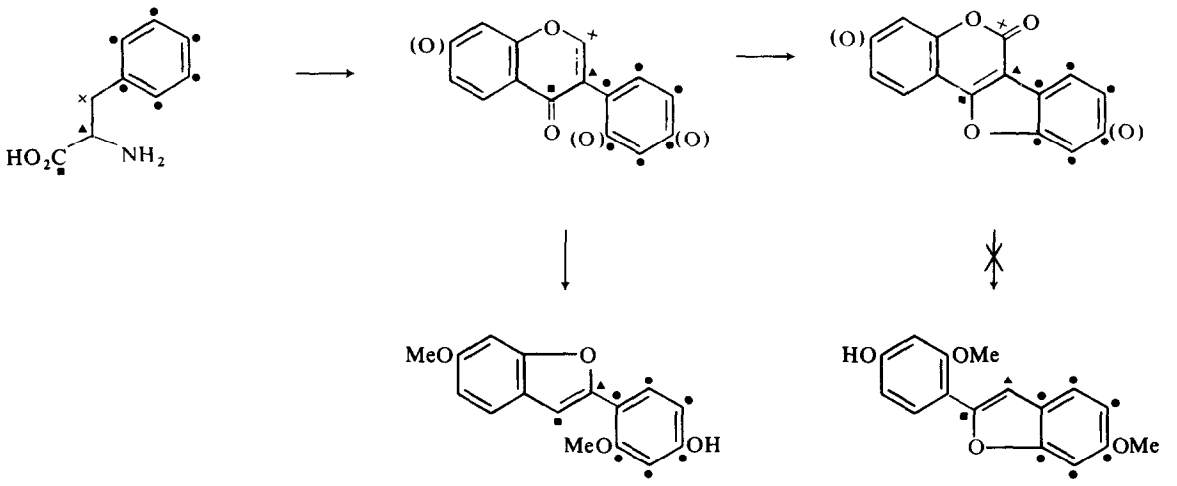
Scheme 1. Degradation of methylvignafuran.

(**30**) as its Na salt in phosphate buffer to the roots of UV-treated seedlings. This isoflavone had proved to be an excellent precursor of demethylhomopterocarpin (**26**), sativan (**20**), vestitol (**19**) and 9-*O*-methylcoumestrol (**37**) in Cu^{2+}-treated lucerne (*Medicago sativa*) seedlings [22, 23, 25] and of demethylhomopterocarpin in similarly-induced red clover (*Trifolium pratense*) [21] and fenugreek (*Trigonella foenum-graecum*) [24] seedlings. These compounds bear an obvious structural relationship to vignafuran, and incorporation of this isoflavone would have indicated a biosynthetic relationship similar to that predicted [9, 15]. Vignafuran was isolated from the seedlings after a metabolism period of 24 hr, quantified by UV spectroscopy, diluted with inactive carrier (regenerated from the acetate prior to use), then methylated to yield the methyl ether (**15**) which was purified to constant specific activity and counted. No incorporation of this isoflavone was observed. Similar experiments using 2',4,4'-trihydroxychalcone-[*carbonyl*-^{14}C] (**41**), daidzein-[4-^{14}C] (**31**) and formononetin-[*Me*-^{14}C] (**32**) were performed. Again, no significant incorporation of activity could be detected.

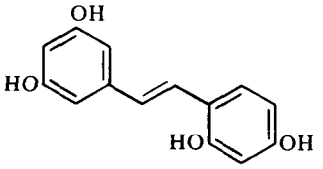
Degradation experiments

Feedings of L-phenylalanine-[U-^{14}C], DL-phenylalanine-[1-^{14}C] and DL-phenylalanine-[2-^{14}C] did result in the isolation of radioactive vignafuran, but incorporations were disappointingly low (Table 2). However, degradations of the labelled materials were conducted to establish the labelling patterns produced. Degradations of methylvignafuran (**15**) have been reported as part of a degradative sequence on coumestrol [32, 33]. A modification of the ozonolysis reaction reported previously [32] was employed in the present studies. Ozonolysis of methylvignafuran at 0°, followed by base hydrolysis yielded 2,4-dimethoxybenzoic acid (**42**) and 2-hydroxy-4-methoxybenzoic acid (**43**) (Scheme 1). No additional treatment was necessary to decompose the initial ozonide, and acid (**43**) resulted rather than the anticipated aldehyde. The two acids were separately methylated to give methyl 2,4-dimethoxybenzoate (**44**), and the two samples were purified and counted. The results of the three phenylalanine feeding experiments and degradative data are shown in Table 2 and Scheme 2.

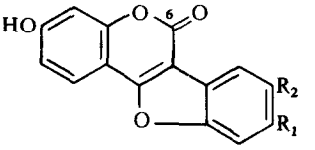
The feeding of phenylalanine-[U-^{14}C] and degradation of the methylvignafuran produced show that 14% (i.e. *ca* 1/8 (12.5%)) of the activity was located in the fragment derived from ring B + C-3, and 90% (*ca* 7/8 (87.5%)) was located in ring A + C-2. This result eliminates the proposed pathway involving loss of C-6 from a coumestan or pterocarpan, which route would locate 1/8 of the activity at C-2 and 7/8 in ring B + C-3. Vignafuran is unlikely to be a simple stilbene derivative, since demonstrated biosynthetic pathways [34] would incorporate all carbon atoms of phenylalanine and produce the two degradation fragments having activities in the ratio 2:7 or 7:2 depending on which of the aromatic rings was shikimate-derived. Also, vignafuran would appear to be unrelated to lignans, since such an origin would presumably produce similar quantities of labelling in each degradation fragment. This conclusion is borne out by the results of the phenylalanine-[1-^{14}C] feeding, which show that essentially all of the incorporated activity resides in the ring B + C-3 fragment,



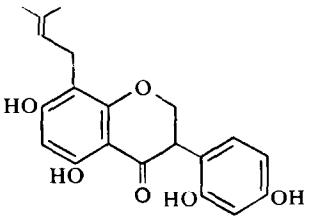
Scheme 2. Incorporation of phenylalanine into vignafuran.



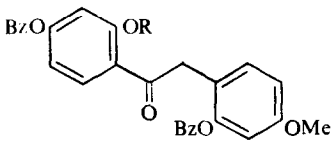
34 Oxyresveratrol



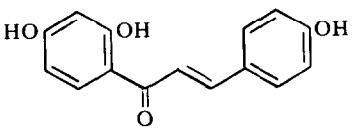
35 $R_1 = R_2 = OMe$
36 $R_1R_2 = OCH_2O$
37 $R_1 = OMe$; $R_2 = H$



38 Kievitone



39 R = H
40 R = Me



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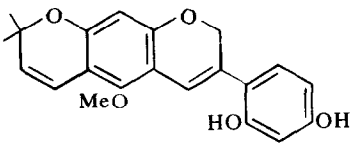
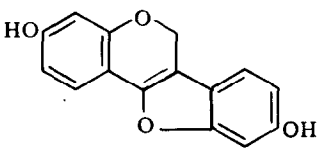


45 Neorauflavene



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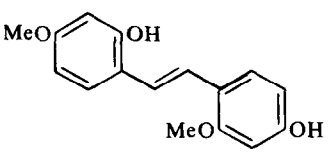

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presumably located at C-3, although further degradation to confirm this was inappropriate at this level of incorporation. Label from phenylalanine-[1-^{14}C] would be lost completely on transformation of a lignan-like compound to a simple 2-arylbenzofuran.

Possible biosynthetic pathways

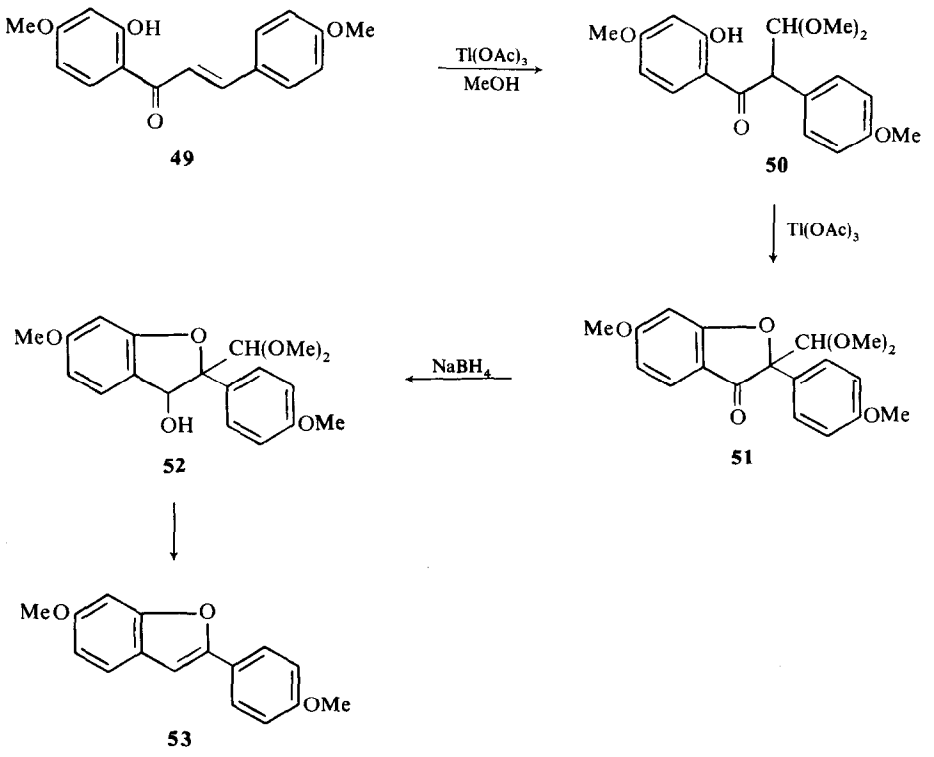
These results are however consistent with two possible biosynthetic pathways. The compound may be isoflavonoid-derived involving loss of C-2 (isoflavone numbering), but by a process in which the phenylalanine-derived aromatic ring becomes the 2-aryl moiety, not part of the benzofuran system. Alternatively, by analogy with synthetic procedures to 3-carboxy-2-arylbenzofurans by reaction of H_2O_2 on flavylium salts (part of a synthetic route to coumestans [35]) or on 2'-hydroxychalcones (by-products of the Algar–Flynn–Oyamada reaction [36]), vignafuran may be derived by decarboxylation of such an intermediate. However, if a chalcone/flavonoid precursor is involved in this manner, then the carbon atom lost in the decarboxylation would be C-3 of the flavonoid (C-α of the chalcone) [37, 38], which is biosynthetically derived from C-2 of phenylalanine. Such routes are then excluded by the incorporation of label from phenylalanine-[2-^{14}C], label which essentially all resides in the ring A + C-2 fragment (and is presumably located at C-2). This result is however consistent with the isoflavonoid-type origin outlined above.

In the absence of suitable feeding data, it can only be an assumption that ring B of vignafuran is derived from acetate–malonate. Nevertheless, vignafuran appears to be derived from phenylalanine by a pathway which involves a 1,2-aryl migration, and may therefore be produced from an isoflavonoid precursor involving loss of C-2 of the isoflavonoid, but the phenylalanine-derived aromatic ring becomes the 2-aryl substituent and not part of the benzofuran system. The observed co-occurrence of neoraufurane (**8**) with the isoflavonoids neorauflavane (**17**), neorauflavene (**45**), edulane (**27**) and edudiol (**23**) [8, 16] is thus consistent with the present labelling studies and strengthens the suggestion that neoraufurane and these isoflavonoids may have a common isoflavonoid precursor. It is also significant that in *Tetragonolobus maritimus*, 6-demethylvignafuran (**13**) co-occurs with isovestitol (**21**), together with demethylvestitol (**22**) and 3,9-dihydroxypterocarp-6a-ene (**46**) [13, 39]. Similarly, vignafuran in *Lablab niger* co-occurs with isovestitol, demethylvestitol and other 4'-hydroxyisoflavonoids, but no traces of vestitol (**19**) or demethylhomoterocarpin (**26**) were detected [12]. These observations are also in accord with the biosynthetic results concerning the origin of the benzofuran skeleton. In contrast, demethylhomopterocarpin has been reported as a phytoalexin of *Vigna unguiculata* [29], but this represents the only 4'-methoxy (isoflavonoid numbering) phytoalexin isolated from the plant. In the present studies, demethylhomopterocarpin could not be detected. The co-occurrence of the 2-arylbenzofurans **9**, **10** and **11** with the corresponding coumestans **35** and **36** [9, 10], however, appears to support the coumestan–2-arylbenzofuran relationship and may indicate an alternative biosynthetic route to these compounds, unless the observed co-occurrence is coincidental.

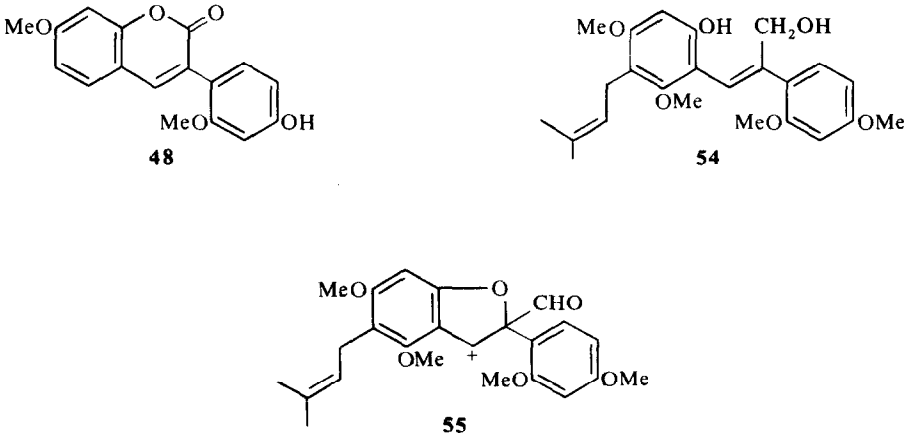
Because vignafuran is not produced by a route involving loss of C-6 from a coumestan or pterocarpan, the non-incorporation of 2',7-dihydroxy-4'-methoxyisoflavone (**30**) in the initial feeding experiment now becomes self-explanatory. If the biosynthetic pathway to vignafuran does proceed via an isoflavone precursor having a similar substitution pattern, then this intermediate is more likely to be 2',7-dimethoxy-4'-hydroxyisoflavone (**33**). Similarly, formononetin (**32**) would not now be expected to be a precursor, but the insignificant incorporations of 2',4,4'-trihydroxychalcone (**41**) and daidzein (**31**) are not readily apparent; these compounds are still likely precursors. Since vignafuran is synthesized in the leaves, and feeding experiments were conducted via the roots of the seedlings, poor transport of precursors could be to blame. Incorporations of phenylalanine were extremely poor by comparison with results from other phytoalexin studies [20, 22], so experiments to improve incorporations were carried out. Feeding experiments using L-phenylalanine-[U-^{14}C] were conducted using 7-day-old UV-induced seedlings. Comparisons were made between whole seedlings fed via the roots as before, seedlings deprived of their roots and fed via the stems, and leaf discs floated on the precursor solution. After 24 hr, the plant material was worked up. No vignafuran was isolated from the latter experiment, an incorporation of 0.010% (dilution 3200) was observed in the whole plant experiment, and an incorporation of 0.024% (dilution 1200) was recorded in the stem-fed cuttings. Thus transport of precursor had been improved, but not by a particularly large margin.

In another experiment, it was established that a major factor responsible for the low levels of incorporation of label from phenylalanine into vignafuran was because of the concomitant synthesis of another phytoalexin. During the feeding of DL-phenylalanine-[2-^{14}C] via the roots (Table 2; incorporation into vignafuran 0.009%, dilution 2570), TLC zones corresponding to markers of demethylhomopterocarpin (**26**), kievitone (**38**), phaseollin (**24**) and phaseollidin (**25**) were eluted, purified further and analysed by UV spectroscopy and counting. Demethylhomopterocarpin, phaseollin and phaseollidin were absent (UV) and no significant activity was associated with these chromatographic zones. However, kievitone was present and counting showed an incorporation of 0.23% (dilution 31). Thus, there appears to be a mechanism in the induced plant whereby suitable precursors are preferentially channelled into synthesis of kievitone rather than vignafuran. However, such a mechanism unfortunately still does not explain the negligible incorporation of the trihydroxychalcone and daidzein, since these compounds are unlikely precursors of the 5,7-dihydroxyisoflavanone kievitone. Poor transport and the use of relatively low specific activity materials may account for the insignificant incorporations, but it is also possible that these compounds are not biosynthetic precursors of vignafuran.

The present studies have broadly established the labelling pattern produced in vignafuran from phenylalanine precursors, and have demonstrated the biosynthetic similarity of the molecule with isoflavonoids. They also indicate though that established hypotheses [9, 15] involving loss of a carbon atom from a coumestan are untenable in this case. The precise pathway leading to vignafuran, however, is still obscure. One possibility involves the formation of a 2-arylbenzofuran by oxidative cyclization [4] of a 2-hydroxystilbene, e.g. **47**, which could be produced by ring opening and decarboxylation



Scheme 3. Synthesis of a 2-arylbenzofuran via $Tl(OAc)_3$ oxidation of a chalcone [42].



of a 3-arylcoumarin, e.g. **48**, rather than by the normal route to stilbenes [34]. Literature reports of natural 3-arylcoumarins are rare [40, 41] and cover only five examples, but such compounds appear to be biosynthetic intermediates in the production of coumestans [25]. An observation by Ollis and coworkers [42], however, provides an interesting chemical analogy for the present results (Scheme 3). During thallium acetate oxidation of a 2'-hydroxychalcone (**49**) to prepare an isoflavone, they isolated two products, neither of which was the required isoflavone. One proved to be a flavone and the other was a coumaranone (**51**), which was envisaged as being formed by further oxidation of the intermediate acetal (**50**). Reduction of the coumaranone with $NaBH_4$ gave **52**, which on acid treatment, eliminated formic acid to yield the 2-arylbenzofuran (**53**). This sequence of reactions shows a chemical relationship between chalcones, isoflavones (via the acetal) and 2-arylbenzofurans in a manner which is analogous to the biosynthetic findings. Kinoshita and coworkers have more recently reported the conversion of a 3-arylcoumarin into a 2-arylbenzofuran [41]. DDQ oxidation of **54**, derived by AlH_3

reduction of the corresponding 3-arylcoumarin, produced the 2-arylbenzofuran (**16**). These authors postulate a mechanism for the transformation via loss of formaldehyde, but alternatively, if **54** undergoes oxidation at the alcohol function prior to ring formation, an intermediate ion (**55**) may be involved. This ion may be regarded as analogous to **52** in the Ollis scheme.

EXPERIMENTAL

General. 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–EtOAc–MeOH–petrol (60–80°), 6:4:1:6; B, C_6H_6–EtOAc, 32:1; C, toluene–HCO_2Et–HCO_2H, 5:4:1; D, $CHCl_3$–MeOH, 50:1; E, $CHCl_3$–*iso*-PrOH, 10:1. Me_2CO (Analar) was used for elution of TLC zones. Radioactive samples were counted as previously [20].

Radiochemicals. L-Phenylalanine-[U-^{14}C] (sp. act. 10 mCi/mM), DL-phenylalanine-[1-^{14}C] (sp. act. 60 mCi/mM) and DL-phenylalanine-[2-^{14}C] (sp. act. 25 mCi/mM) were purchased (Amersham). The syntheses of 2′,7-dihydroxy-4′-methoxyisoflavone-[*Me*-^{14}C] (0.518 mCi/mM) [21], formononetin-[*Me*-^{14}C] (0.676 mCi/mM) [23], daidzein-[4-^{14}C] (0.322 mCi/mM) [23] and 2′,4,4′-trihydroxychalcone-[*carbonyl*-^{14}C] (0.223 mCi/mM) [43] have been described.

Feeding techniques and isolation of metabolites. Seeds of *Vigna unguiculata* (IITA accession line K 2809) were washed with EtOH and surface-sterilized in NaOCl soln (1% w/v Cl) for 15 min, then washed with distilled H_2O and germinated at 21° in moist vermiculite in a greenhouse for 7 days. Seedlings (25) were uprooted, transferred to a small beaker containing distilled H_2O and irradiated with UV light (254 nm, Mineralight UVSL-58) at a distance of *ca* 5 cm for 30 min before feeding in the dark. Phenylalanine precursors (100–150 μCi) were administered in H_2O (4 ml), phenolic compounds (0.5–1.0 mg) as their Na salts in Pi buffer (0.1 M, pH 7.0, 4 ml), to the roots of the plants. Distilled H_2O was added as required during the feeding period. After 24 hr, the seedlings were homogenized by grinding in a mortar with ground glass and H_2O (*ca* 20 ml). The slurry was poured into boiling EtOH (200 ml) and filtered. The tissue was re-extracted with hot EtOH (2 × 100 ml) and the combined extracts evapd to dryness. After the addition of H_2O (50 ml), the soln was extracted with Et_2O (100 ml, then 4 × 50 ml) and the extracts combined and evapd. The extract was separated by TLC (solvent A), and vignafuran located by reference to marker material. The isolated material was purified further by TLC (solvent B) and quantified by UV absorption of EtOH soln (λ_{max}, 320 nm, log ε 4.59 [11, 31]). The vignafuran was diluted with carrier material (*ca* 25 mg), dried *in vacuo*, then methylated by stirring under reflux under anhydrous conditions with MeI (0.5 ml), dry K_2CO_3 (1 g) in dry Me_2CO (10 ml) for 1 hr. The methyl ether was isolated after filtration and evapn of the filtrate, by TLC (solvent B), then recrystallized to constant sp. act. from aq. MeOH. The methyl ether was diluted further with carrier (*ca* 40 mg), prior to degradative studies.

Degradation of methylvignafuran. A gentle stream of ozone in O_2 (6–7%) was passed through a soln of the labelled methylvignafuran (*ca* 50 mg) in CH_2Cl_2 (25 ml), cooled in an ice-bath. After 1 hr, the reaction mixture was allowed to warm to R°, and was then evapd to dryness. EtOH (20 ml) and KOH (0.5 g) were added, and the mixture was heated under reflux for 1.5 hr. The solvent was evapd and the mixture acidified with dil. HCl, extracted with EtOAc (3 × 25 ml) and the extracts washed with H_2O. The evapd extract was separated by TLC (solvent C) and bands corresponding to markers of 2,4-dimethoxybenzoic acid and 2-hydroxy-4-methoxybenzoic acid were eluted. The dimethoxybenzoic acid fraction was heated under reflux in MeOH (10 ml) with conc H_2SO_4 (1 ml) for 1.5 hr, when the mixture was poured into H_2O, extracted with Et_2O (3 × 20 ml), the extracts washed with H_2O and evapd. Methyl 2,4-dimethoxybenzoate was isolated by TLC (solvent A). The hydroxymethoxybenzoic acid fraction was heated under reflux with dry K_2CO_3 (1 g) and Me_2SO_4 (0.2 ml) in dry Me_2CO (10 ml) for 1.5 hr. After filtration and evapn, methyl 2,4-dimethoxybenzoate was isolated by TLC (solvent A). Both ester samples were purified further by TLC (solvents B and D), then quantified by UV absorption: λ_{max}^{EtOH} 290 nm, log ε 3.745. Yields *ca* 5 mg from each fraction. After evapn of the EtOH, the residues were washed into counting vials with dioxan-based scintillator, and counted.

Feeding of L-*phenylalanine*-[U-^{14}C] *to parts of seedlings.* Batches (3 × 25) of cowpea seedlings were grown and irradiated with UV light as before. One batch was fed in the usual way by administration of an aq. soln (5 ml) of the precursor (16.7 μCi) to the roots. Seedlings from the second batch had their roots removed under H_2O by cutting with a scalpel. The cut stems were then placed in the feeding soln (5 ml, 16.7 μCi). Leaves from the third batch were removed, and leaf discs, 1 cm dia, were cut with a cork-borer. The discs were floated on the precursor soln (10 ml, 16.7 μCi) to which 1 drop Tween 20 had been added. The three batches were left for 24 hr in the dark and then each was extracted as before. Vignafuran was absent from the leaf discs. Vignafuran from the other two batches was worked up and counted as previously.

Vignafuran. 2-Benzyloxy-4-methoxybenzyl-4-benzyloxy-2-hydroxyphenylketone [13] (200 mg) was stirred at 60° with dry K_2CO_3 (2 g) and Me_2SO_4 (50 μl, 67 mg) in dry DMF (20 ml) for 1 hr. The mixture was poured into H_2O, extracted with EtOAc (2 × 25 ml) and the extracts washed with H_2O. The extracts were evapd, and the product, 2-benzyloxy-4-methoxybenzyl-4-benzyloxy-2-methoxyphenylketone isolated by TLC (solvent B) to yield a gum. NMR (60 MHz, $CDCl_3$, TMS): δ 3.66 (6H, *s*, OMe), 4.18 (2H, *s*, CO-C<u>H</u>$_2$-Ar), 4.88 (2H, *s*, O-C<u>H</u>$_2$-Ph), 4.96 (2H, *s*, O-CH$_2$-Ph), 6.42 (4H, *m*, H-3,3′,5,5′), 7.13 (1H, *d*, *J* = 9 Hz, H-6′), 7.23 (5H, *s*, O-CH$_2$-<u>Ph</u>), 7.36 (5H, *s*, O-CH$_2$-<u>Ph</u>), 7.76 (1H, *d*, *J* = 9 Hz, H-6). The product was stirred at 80° with HOAc (15 ml) and conc HCl (7.5 ml) for 1.5 hr then poured into H_2O, and extracted with EtOAc (3 × 25 ml). The extracts were washed with aq. $NaHCO_3$ and then H_2O. The product was isolated by TLC (solvent B) to give vignafuran as a gum, UV and NMR in agreement with those reported [11, 13]. Acetylation of the product (Py–Ac_2O, R° overnight) gave vignafuran acetate (44 mg), mp 92–4° (from MeOH), lit. [11] 94–94.5°.

Vignafuran for dilution of radioactive material was obtained by stirring vignafuran acetate (30 mg) in EtOH (15 ml) with KOH (0.1 g) at R° for 0.75 hr. The mixture was concd, acidified with dil. HCl, extracted with EtOAc (3 ×), and vignafuran purified by TLC (solvent A) and assayed by UV absorption. Methylation of vignafuran (MeI, K_2CO_3, Me_2CO) yielded methylvignafuran, mp 88–9° (from aq. MeOH). NMR (60 MHz, $CDCl_3$, TMS): δ 3.83 (6H, *s*, OMe), 3.91 (3H, *s*, OMe), 6.53 (2H, *m*, H-3′,5′), 6.78 (1H, *dd*, *J* = 9, 2 Hz, H-5), 7.08 (2H, *m*, H-3,7), 7.35 (1H, *d*, *J* = 8 Hz, H-4), 7.83 (1H, *d*, *J* = 9 Hz, H-6′).

Other phytoalexins in V. unguiculata. Kievitone was isolated from the UV-induced cowpea extract by TLC (solvent A) by reference to marker material, and purified further by TLC (solvent E). The material was eluted, quantified by UV spectroscopy (λ_{max}^{EtOH} 293 nm, log ε 4.22 [44]) and then counted. Chromatographic zones corresponding to demethylhomopterocarpin, phaseollin and phaseollidin were also eluted, then purified by TLC (solvent B). None of these compounds could be detected by UV spectroscopy.

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