Biosynthesis of Rotenoids. Chalcone, Isoflavone, and Rotenoid Stages in the Formation of Amorphigenin by *Amorpha fruticosa* Seedlings

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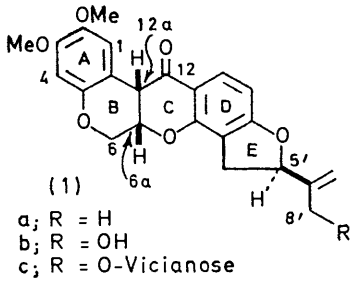
[2'-^{14}C]-7-Hydroxy-2',4',5'-trimethoxyisoflavone (2b) is shown to be excellently converted into [6-^{14}C]-amorphigenin (1b) by *Amorpha fruticosa* seedlings, this being the first direct demonstration of the biosynthetic link between isoflavones and rotenoids. Isoflavones rather than isoflavanones are implicated and prenylation is evidently a post-rotenoid phase. In agreement, (±)-[6-^{3}H]-9-demethylmunduserone (8a) is a good precursor for amorphigenin. This places 9-demethylmunduserone in a key biosynthetic position as parent of a sub-family of rotenoids, similar unprenylated rotenoids heading other sub-families. (±)-[6-^{3}H]-8-Dimethylallyl-9-demethylmunduserone and [6-^{3}H]rotenone (as 6a*S*,12a*S*,5'*R*- and 6a*R*,12a*R*,5'*R*-diastereoisomers) are each satisfactorily incorporated into amorphigenin. Post-rotenoid stages are thus likely to be dimethylallylation, epoxidation, cyclisation to dalpanol (17), dehydration to rotenone (1a), and 8'-oxidation to amorphigenin. Administration of chalcones shows that the 2',4,4'-trihydroxy-member is the most acceptable precursor and a mechanism for 2,3-aryl migration in biosynthesis takes account of the free 4-hydroxy-group requirement. The product from the rearrangement appears to be 7-hydroxy-4'-methoxyisoflavone rather than the 4',7-dihydroxy-compound. Methylation accompanying rearrangement of a spirodienone intermediate (14) can accommodate this. Acetate is incorporated into rings D and E, but the radioactivity from serine and glycine is found in risic acid (16) obtained by degrading amorphigenin. This is discussed.

CIRCUMSTANCES (structural relationships, occurrence, and distribution in nature) [1,2] connect the biosynthesis of rotenoids with that of isoflavonoids. In previous work [3,4] we have shown that the biosyntheses of rotenone (1a) and amorphigenin (1b) follow the characteristics of isoflavonoid formation and involve aryl migration from C-6a to C-12a of the final rotenoid. The 'extra' methylene (C-6), as well as the methoxy-methyls, can be supplied by methionine.[4,5] Speculation on the possibilities of methylenation by *S*-adenosylmethionine has led to a new synthesis of rotenoids [6] using dimethylsulphoxonium methylide and the method is readily adapted to afford labelled rotenoids which are used in the current work. We now present evidence establishing a direct link between rotenoids and 2'-methoxyisoflavones. Information on both the pre-rotenoid and post-isoflavonoid stages of biosynthesis is then developed by further radiochemical experiments. Preliminary communication [7] of some of the results has been made.

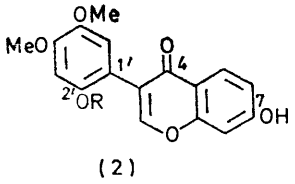
Earlier work employed two plant systems for precursor administration experiments: whole *Derris elliptica* plants (producing rotenone), and germinating *Amorpha fruticosa* seeds.[3,4] Dormant seeds of the latter contain predominantly the vicianoside amorphin [8] (1c); the aglycone amorphigenin is formed on germination.[3] To improve understanding of the situation in *A. fruticosa* seedlings, a uniform batch (after 7 days germination) was grown for 24 h on aqueous (±)-[1-^{14}C]phenylalanine. Unabsorbed precursor (18%) was removed and batches of seedlings were assayed for weight and activity of free and bound amorphigenin (the latter probably as amorphin) after various time intervals. The results are shown in Table 1. It can be seen that the free amorphigenin content is at a fairly constant level during the experimental period, building up a little. Bound amorphigenin also remains fairly constant, but is always present in much lower quantity (1/10 — 1/40). The specific and total activity of amorphigenin reaches a maximum after a 24 h grow-on period (total time from beginning of feeding 48 h) and then declines, showing a definite turn-over of rotenoid in the seedlings. The fate of amorphigenin in further metabolism is as yet undetermined, but the rotenoid is not an end product. Similar

[1] L. Crombie, *Fortschr. Chem. org. Naturstoffe*, 1963, **21**, 275.
[2] S. H. Harper, *J. Chem. Soc.*, 1940, 1178; S. H. Harper and W. G. E. Underwood, *ibid.*, 1965, 4203; H. Grisebach and W. D. Ollis, *Experientia*, 1961, **17**, 4.
[3] L. Crombie and M. B. Thomas, *J. Chem. Soc.* (*C*), 1967, 1796.
[4] L. Crombie, C. L. Green, and D. A. Whiting, *J. Chem. Soc.* (*C*), 1968, 3029.
[5] M. Hamada and M. Chubachi, *Agric. and Biol. Chem.* (*Japan*), 1969, **33**, 793.
[6] L. Crombie, P. W. Freeman, and D. A. Whiting, preceding paper; *Chem. Comm.*, 1970, 563.
[7] L. Crombie, P. M. Dewick, and D. A. Whiting, *Chem. Comm.*, 1970, 1469; 1971, 1182, 1183.
[8] J. Claisse, L. Crombie, and R. Peace, *J. Chem. Soc.*, 1964, 6023.



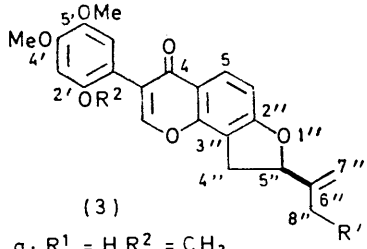
(1)

a; R = H
b; R = OH
c; R = O-Vicianose



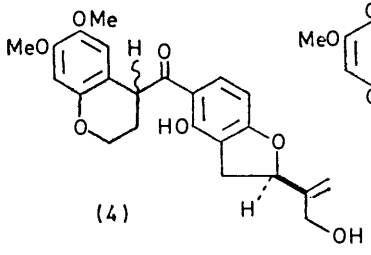
(2)

a; R = CH_3
b; R = $^{14}CH_3$
c; R = CT_3
d; R = H

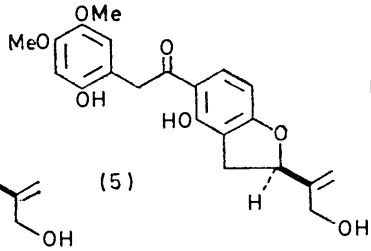


(3)

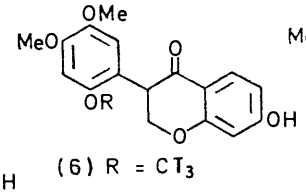
a; R^1 = H, R^2 = CH_3
b; R^1 = OH, R^2 = CH_3
c; R^1 = R^2 = H
d; R^1 = OH, R^2 = H
e; R^1 = H, R^2 = $^{14}CH_3$
f; R^1 = OH, R^2 = $^{14}CH_3$



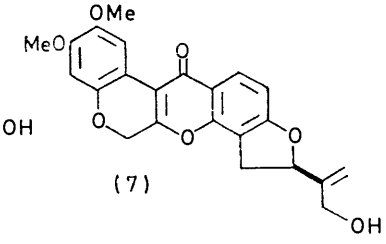
(4)



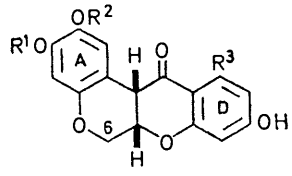
(5)



(6) R = CT_3

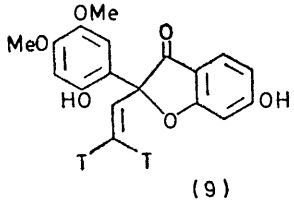


(7)

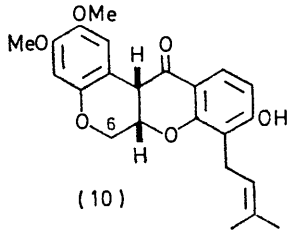


(8)

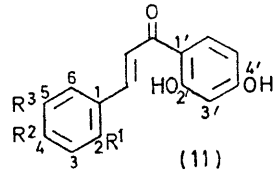
a; R^1 = R^2 = CH_3, R^3 = H
b; R^1 = R^2 = CH_3, R^3 = OH
c; R^1, R^2 = CH_2, R^3 = OH
d; R^1, R^2 = CH_2, R^3 = H



(9)

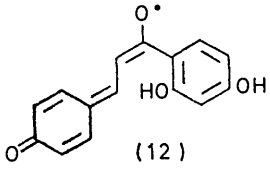


(10)



(11)

a; R^1 = R^3 = H, R^2 = OH
b; R^1 = H, R^2 = R^3 = OH
c; R^1 = H, R^2 = R^3 = OMe
d; R^1 = R^2 = R^3 = OMe
e; R^1 = R^3 = H, R^2 = OMe



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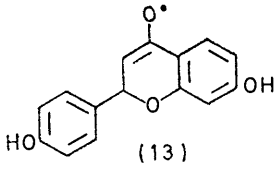
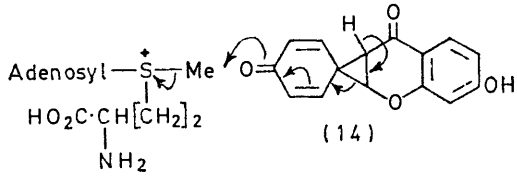
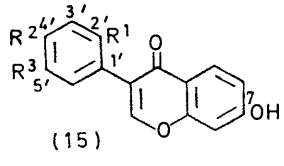


(13)

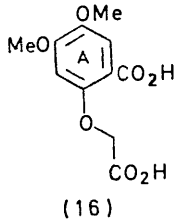


(14)



(15)

a; R^1 = R^3 = H, R^2 = OH
b; R^1 = R^3 = H, R^2 = OMe
c; R^3 = H, R^1 = R^2 = OH



(16)

observations have been reported with isoflavones and coumestans.[9-11] Dilutions for bound amorphigenin were always greater than for free amorphigenin, indicating that the former is synthesised (relatively slowly) from amorphigenin, and not *vice versa*. A large pool of metabolically inactive amorphin could also explain the

[9] W. Barz, *Z. Naturforsch.*, 1969, **24b**, 234.
[10] W. Barz and B. Roth-Lauterbach, *Z. Naturforsch.*, 1969, **24b**, 638.
[11] W. Barz, Ch. Adamek, and J. Berlin, *Phytochemistry*, 1970, **9**, 1735.

large dilutions but this would have been apparent on analysis. The *A. fruticosa* seedling system was used in all *in vivo* experiments described in this paper.

Current views of methylation and methylenation [12] have led us to attempt to convert suitable 2'-methoxyisoflavones into rotenoids. Three likely candidates were prepared with specific labelling. 7-Hydroxy-2',4',5'-[2'-*O*-^{14}C]trimethoxyisoflavone (2b) was obtained by methylation with $^{14}CH_3I$ of 7-benzyloxy-2'-hydroxy-4',5'-dimethoxyisoflavone followed by debenzylation in phigenin, *via* treatment with zinc metal in alkali, gave the rotenol and derritol analogues (4) and (5), respectively. The former retained 94% of the activity, with only 5% in the deoxybenzoin (5). Thus virtually all the radioactivity is located at C-6 or C-6a. As the origin of C-6a from C-3 of phenylalanine has been previously demonstrated,[4] the 2'-isoflavonoid methoxy-methyl group is likely to be specifically incorporated into the rotenoid as C-6. The process bears some resemblance to the biological conversion of an *o*-methoxyphenol into

TABLE 1

Administration of (±)-[1-^{14}C]phenylalanine to *Amorpha fruticosa* [a]

Metabolite	Grow-on time (days)	Weight (mg) of amorphigenin	Specific activity (d.p.m. mM^{-1})	Dilution	Incorporation (%)
Free amorphigenin	0	1·548	$7·43 \times 10^6$	14,950	0·14
	1	1·245	$1·47 \times 10^7$	7550	0·22
	2	1·870	$1·14 \times 10^7$	9740	0·26
	4	2·015	$9·19 \times 10^6$	12,090	0·23
	7	2·005	$7·66 \times 10^6$	14,500	0·19
Bound amorphigenin	0	0·135	$1·59 \times 10^4$	69,800	0·0026
	1	0·082	$4·20 \times 10^6$	26,450	0·0042
	2	0·049	$2·67 \times 10^6$	41,700	0·0016
	4	0·108	$2·29 \times 10^6$	48,500	0·0030
	7	0·068	$1·48 \times 10^6$	75,000	0·0012

[a] Fed in aqueous solution for 24 h; 40 seedlings per batch, 7 days old.

TABLE 2

Administration of [2'-*O*-^{14}C]isoflavones and mevalonic acid to *A. fruticosa* [a]

Precursor	Weight (mg) of amorphigenin	Specific activity (d.p.m. mM^{-1})	Uptake (%)	Dilution	Incorporation (%)
Derritol isoflavone methyl ether (3e) [b]	1·460	$<2 \times 10^2$	13	$>2 \times 10^6$	$<1 \times 10^{-4}$
8''-Hydroxyderritol isoflavone methyl ether (3f) [b]	1·920	$3·97 \times 10^3$	22	381,000	0·001
7-Hydroxy-2',4',5'-trimethoxyisoflavone (2b) [b]	1·245	$7·22 \times 10^5$	38	725	0·25
7-Hydroxy-2',4',5'-trimethoxyisoflavone (2b) [c]	0·781	$6·13 \times 10^5$	58	854	0·13
7-Hydroxy-2',4',5'-trimethoxyisoflavone (2b) [c]	11·60 [d]	$1·32 \times 10^6$	54·5	397	0·75
[2-^{14}C]Mevalonic acid [c]	1·92	$6·24 \times 10^4$	50	208,000	0·0003

[a] Administration period 48 h; 80 seedlings per batch, 3 days old. [b] Applied in ethylene glycol monomethyl ether–Tween 20–water. [c] Administered as sodium salt in 0·05M-phosphate buffer, pH 7. [d] 200 Seedlings.

acid. Synthesis of the isoflavone followed established procedures.[13] Derritol [2'-*O*-^{14}C]isoflavone methyl ether (3e) and 8''-hydroxyderritol [2'-*O*-^{14}C]isoflavone methyl ether (3f) were prepared by methylation (with $^{14}CH_3I$) of derritolisoflavone [13f] (3c) and 8''-hydroxyderritol isoflavone [13f] (3d). On administration of labelled isoflavones (3e) and (3f), no acceptable conversion into amorphigenin was observed (Table 2). However, very satisfactory incorporation of the unprenylated isoflavone (2b) was obtained. In the best experiment an incorporation of 0·75% (dilution 397) was achieved, the isoflavone being administered as sodium salt in phosphate buffer (48 h; uptake 54·5%). Degradation of the [6-^{14}C]amor-

[12] *Cf.* E. Lederer, *Quart. Rev.*, 1969, **23**, 453.

[13] (*a*) A. Robertson and G. H. Rusby, *J. Chem. Soc.*, 1935, 1371; (*b*) K. Fukui, M. Nakayama, M. Hatanaka, T. Okamoto, and Y. Kawase, *Bull. Chem. Soc. Japan*, 1963, **36**, 397; (*c*) K. Fukui, M. Nakayama, and T. Harano, *ibid.*, 1969, **42**, 233; (*d*) J. Harley-Mason and A. H. Jackson, *J. Chem. Soc.*, 1954, 1165; (*e*) O. H. Emerson and E. M. Bickoff, *J. Amer. Chem. Soc.*, 1958, **80**, 4381; (*f*) M. B. Thomas, Ph.D. Thesis, University of London, 1965.

a methylenedioxybenzene.[14] This experiment provides the first direct demonstration of a biosynthetic link between isoflavones and rotenoids.

The presence of isoflavones in *Amorpha fruticosa* seedlings has been investigated by isotope dilution experiments, in which the unlabelled isoflavones (2a), (3a), and (3b) were added to an extract of *A. fruticosa* seedlings which had metabolised (±)-[1-^{14}C]phenylalanine over 48 h. The isoflavones were reisolated and purified. Results are shown in Table 3. Clearly, isoflavone (2a) occurs in the seedlings as a natural product. However, neither isoflavone (3a) nor isoflavone (3b) could be detected at an acceptable level.

These results strongly indicate that prenylation and subsequent modification to form ring E of amorphigenin occur not at the isoflavonoid stage, but after closure of rotenoid ring B. Since isoflavone (2a) can, as shown above, penetrate to the site of rotenoid biosynthesis, it

[14] D. H. R. Barton, G. W. Kirby, and J. B. Taylor, *Proc. Chem. Soc.*, 1962, 340.

is somewhat surprising that mevalonic acid, a potential precursor of ring E, is only poorly incorporated. A typical experiment is included in Table 2 (*cf.* ref. 5).

TABLE 3

Administration of (±)-[1-^{14}C]phenylalanine to *A. fruticosa*; dilution analyses with isoflavones [a]

Diluent	Total activity (d.p.m.)	Incorporation (%)
Amorphigenin (1b)	$8{\cdot}67 \times 10^5$ [c]	0·39
Derritol isoflavone methyl ether (3a)	20 [b]	$<1 \times 10^{-5}$
8″-Hydroxyderritol isoflavone methyl ether (3b)	337 [b]	$<1{\cdot}5 \times 10^{-4}$
7-Hydroxy-2′,4′,5′-trimethoxy-isoflavone (2a)	$1{\cdot}38 \times 10^4$ [c]	0·006

[a] Feeding period 48 h, 285 seedlings; 3 days old. [b] Activity still decreasing, after 7 recrystallisations. [c] Purified to constant activity.

Similar low incorporations of mevalonic acid into hemiterpenoid units of other phenolic compounds have been noted,[15] and often explained in terms of compartmentation.

are appropriately corrected. To test the specificity of labelling, the [6-^{3}H]amorphigenin was degraded to 8′-hydroxyderritol (5), which proved to be inactive (Table 5). Tritium is thus located at C-6, -6a, or -12a. Dehydrogenation using iodine–ethanolic sodium acetate [8] gave 6a,12a-didehydroamorphigenin (7), retaining only 73% of the activity; however loss of label is almost certainly occasioned by leakage from C-6, rather than by scrambling of tritium among C-6, -6a, and -12a. In support, further similar treatment of 6a,12a-didehydro-[6-^{3}H]amorphigenin with ethanolic sodium acetate continued the exchange and reduced the activity to 51% (compared with starting amorphigenin, Table 5).

The important role now allotted to isoflavone (2a) in amorphigenin biosynthesis places the rotenoid 9-demethylmunduserone (8a) in a key position as parent to the sub-family of rotenoids with 2,3-dimethoxylation in ring A, and resorcinol oxygenation in ring D (*e.g.* munduserone,[19] rotenone, amorphigenin, deguelin, elliptone).[1] By analogy other rotenoid sub-families would be headed by 9-demethylsermundone (8b) [20] (2,3-dimethoxylation

TABLE 4

Administration of 7-hydroxy-2′,4′,5′-[2′-*OC*^{3}H$_3$]trimethoxyisoflavone and (±)-7-hydroxy2′,4′,5-[2′-*OC*^{3}H$_3$]trimethoxyisoflavanone to *A. fruticosa* [a]

Precursor	Time (h)	Uptake (%)	Weight (mg) of amorphigenin	Specific activity (d.p.m. mM^{-1})	Dilution [b]	Incorporation [b] (%)
Isoflavone(2c)	6	56	*c*			0·049
	12	63	0·352	$7{\cdot}17 \times 10^6$	2300	0·057
	24	57	0·564	$1{\cdot}45 \times 10^7$	1130	0·19
	48	66	1·060	$3{\cdot}51 \times 10^7$	470	0·84
Isoflavanone(6)	6	40	*c*			0·016 [d]
	12	54	0·318	$2{\cdot}84 \times 10^6$	6100	0·020 [d]
	24	50	0·583	$1{\cdot}95 \times 10^6$	8900	0·024 [d]
	48	59	0·908	$2{\cdot}38 \times 10^6$	7280	0·045 [d]

[a] Administered as sodium salt in 0·05M-phosphate buffer (pH 7·0), 40 seedlings per batch, 3 days old. [b] Corrected for loss (1/3) of tritium on ring closure. [c] Not measured. [d] Corrected for utilisation of one enantiomorph.

The possibility arises that isoflavone (2a) may be transformed into rotenoid *via* the corresponding isoflavanone (6). There is evidence that isoflavanones and isoflavones are readily interconverted in plants [16] and the former are implicated in coumestrol biosynthesis.[17,18] To test this possibility, 7-hydroxy-2′,4′,5′-[2′-*OC*^{3}H$_3$]-trimethoxyisoflavone was prepared (using the above method but substituting C^{3}H$_3$I), and a sample was reduced to the corresponding (±)-isoflavanone (6). The incorporations of these two were followed in *A. fruticosa* seedlings, over various feeding times, in parallel experiments to cancel any tritium isotope effect on rotenoid ring B closure. The results (Table 4) show that the isoflavone (2c) is used considerably more efficiently than the isoflavanone (6). Utilisation of only one enantiomorph of the latter might be expected, and the results in Table 4

in ring A, phloroglucinol oxygenation in ring D; *e.g.* toxicarol, sumatrol),[1] and by the 2,3-methylenedioxy-

TABLE 5

Degradation of amorphigenin from feeding of 7-hydroxy-2′,4′,5′-[2′-*OC*^{3}H$_3$]trimethoxyisoflavone

Compound	Specific activity (d.p.m. mM^{-1})	Activity (%)
Amorphigenin (1b)	$5{\cdot}50 \times 10^4$	100
8′-Hydroxyderritol (5)	0	0
6a,12a-Didehydroamorphigenin (7)	$4{\cdot}03 \times 10^4$	73
6a,12a-Didehydroamorphigenin [a]	$2{\cdot}79 \times 10^4$	51

[a] After exchange with sodium acetate–refluxing ethanol, 3 h.

analogues of 9-demethylsermundone [*i.e.* (8c)] (2,3-methylenedioxy-ring A, phloroglucinol ring D) and of 9-demethylmunduserone [*i.e.* (8d)] (2,3-methylenedioxy

[15] *Inter alia* S. A. Brown, *Phytochemistry*, 1970, **9**, 2471; H. G. Floss and U. Mothes, *ibid.*, 1966, **5**, 161.
[16] H. Grisebach and H. Zilg, *Z. Naturforsch.*, 1968, **23b**, 494.
[17] P. M. Dewick, W. Barz, and H. Grisebach, *Phytochemistry*, 1970, **9**, 775.
[18] J. Berlin, P. M. Dewick, W. Barz, and H. Grisebach, *Phytochemistry*, 1972, **11**, 1689.
[19] N. Finch and W. D. Ollis, *Proc. Chem. Soc.*, 1960, 176.
[20] W. D. Ollis in ' Proceedings of the Symposium on Phytochemistry,' Hong Kong, Sept. 1961, ed. H. R. Arthur, Hong Kong Univ. Press.

ring A, and resorcinol ring D, *e.g.* dolineone, milletone).[21,22]

To confirm the position of 9-demethylmunduserone * (previously unknown), the (±)-form has now been synthesised, tritiated at C-6, using the ylide rotenoid synthesis.[6] Tritiated dimethylsulphoxonium methylide was used to prepare the vinylcoumaranone (9) from the 2'-hydroxyisoflavone (2d), which rearranged in pyridine to [6-^{3}H]-9-demethylmunduserone (8a). Administration of the last product (Table 6) to germinating *A. fruticosa* seeds resulted in a very satisfactory conversion (1·14%) into amorphigenin.

The same method was employed to produce [6-^{3}H]-rotenone from derritol isoflavone (3c). The product is a mixture (' mutarotenone ')[23] of (6a*S*,12a*S*,5'*R*)-(natural) and (6a*R*,12a*R*,5'*R*)-diastereomers. 1,4-Hydrogenolysis in ring E then yielded (±)-[6-^{3}H]rotenonic acid [6] (10), the expected product of 8-prenylation of 9-demethylmunduserone. Administration to the seedling system (Table 6) showed good conversion (0·76%) into amorphigenin; [6-^{3}H]rotenone (as mutarotenone) was also converted (1·00%).

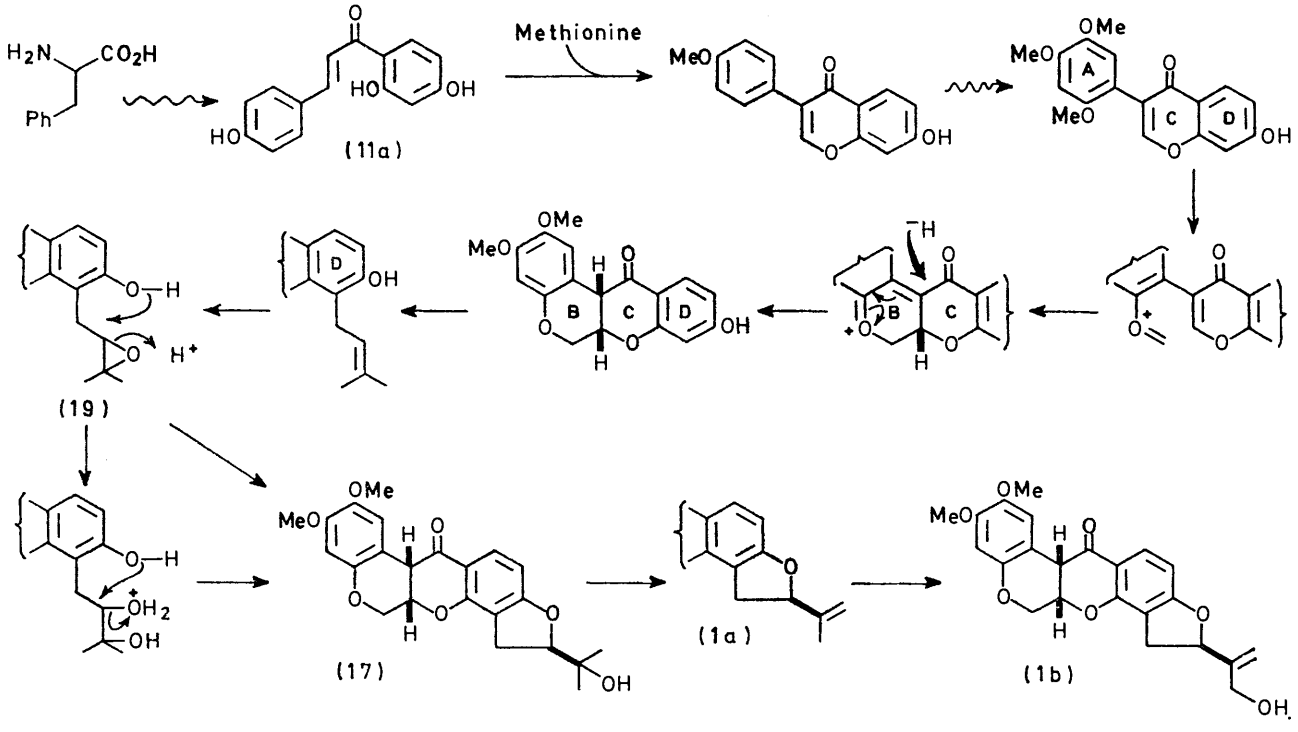
These observations suggest that the post-isoflavonoid stages in amorphigenin biosynthesis are likely to be 8-dimethylallylation, epoxidation, and cyclisation, either *via* the epoxide or its derived diol, to dalpanol (17), recently found in nature.[24] Dehydration then gives rotenone (1a), and 8'-hydroxylation leads to amorphigenin. These transformations are illustrated in Scheme 1.

TABLE 6

Incorporation of [^{3}H]rotenoids by *A. fruticosa* [a]

Precursor	Weight (mg) of amorphigenin	Specific activity (d.p.m. mM^{-1})	Uptake (%)	Dilution	Incorporation (%)
(±)-9-[6-^{3}H]Demethylmunduserone (8a) [b,d]	2·200	3·96 × 10^6	70	244	1·14
(±)-[6-^{3}H]Rotenonic acid (10) [b,d]	1·815	1·44 × 10^6	67	293	0·76
[6-^{3}H]Rotenone (1a) [c]	2·000	1·24 × 10^6	57	320	1·00
Isoflavone (2b) [b]	1·815	3·32 × 10^6	70	158	1·81

[a] Administration period 48 h, 40 seedlings per batch, 5 days old. [b] Feeding as sodium salts in 0·05M-phosphate buffer (pH 7·0). [c] Applied as ' mutarotenone,' in ethylene glycol monomethyl ether–Tween 20–0·05M-phosphate buffer; results corrected arbitrarily for utilisation of one diastereomer in a 1 : 1 mixture. [d] Corrected for utilisation of one enantiomer.



SCHEME 1 Biosynthesis of amorphigenin in *Amorpha fruticosa* seedlings

* For full description of the syntheses of (±)-9-demethylmunduserone, rotenone, and (±)-rotenonic acid, and their characterisation, see ref. 6.

[21] L. Crombie and D. A. Whiting, *J. Chem. Soc.*, 1963, 1569.

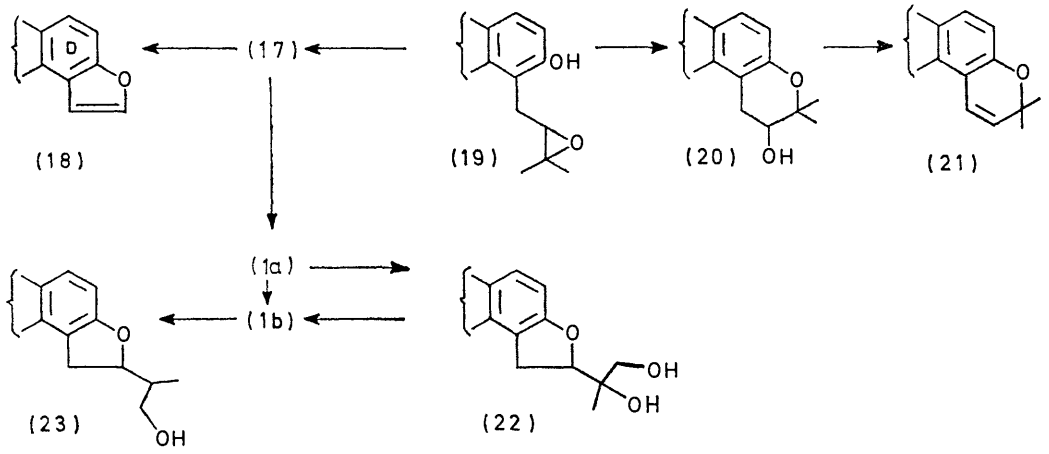
[22] W. D. Ollis, C. A. Rhodes, and I. O. Sutherland, *Tetrahedron*, 1967, **23**, 4741.

[23] L. Crombie, P. J. Godin, D. A. Whiting, and K. S. Siddalingaiah, *J. Chem. Soc.*, 1961, 2876.

[24] D. Adinarayana, M. Radhakrishniah, J. Rajasekhara Rao, R. V. M. Campbell, and L. Crombie, *J. Chem. Soc.* (*C*), 1971, 29.

Speculating beyond the experimental evidence at present available, it seems reasonable (Scheme 2) that elliptone (18), might be derived from dalpanol (17) and that the alternative cyclisation of the epoxide (19) should lead *via* (20) to deguelin (21).[1] Under acid conditions the conversion (19) ⟶ (20) has been realised *in vitro*.[25] Similar pathways for the modification of hemiterpenoid dihydrofuran attachments to coumarins [26,27] and quinoline alkaloids [28] have been postulated. More than one route to amorphigenol (22) [29] can be envisaged, among them direct 6,7-hydroxylation of rotenone, but we have no direct evidence on this, or the possible derivation of dihydroamorphigenin (23) [30] from amorphigenin. It is relevant, however, that both 8'-hydroxylation and 6',7'-dihydroxylation of rotenone have been demonstrated in its detoxification by mammalian and insect tissue preparations.[31] Interconnections other than those shown are possible, and further work is required to unravel the post-dalpanol stages with certainty.

From these late stages of amorphigenin biosynthesis attention is now turned back to an earlier phase leading up to isoflavone (2a) (the pre-rotenoid stages). Isoflavones are known to share with flavonoids a biosynthetic origin involving the elaboration of aromatic amino-acids, through substituted cinnamic acids, to a chalcone.[32] The hydroxylation pattern of the acetate–malonate-derived ring is frequently determined before or during chalcone formation, but oxygenation and/or methylation, *etc.*, of the phenylalanine-derived ring may occur before, at, or after the chalcone stage. For isoflavone (2a), there are some 27 2',4'-dihydroxychalcones possible as precursors. Five likely representatives were synthesised: 2',4,4'-trihydroxychalcone (11a), 2',3,4,4'-tetrahydroxychalcone (11b), 2',4'-dihydroxy-3,4-dimethoxychalcone (11c), 2',4'-dihydroxy-2,4,5-trimethoxychalcone (11d), and 2',4'-dihydroxy-4-methoxychalcone (11e). Each



SCHEME 2 Late stages in rotenoid biosynthesis

TABLE 7

Incorporation of chalcones and isoflavones by *A. fruticosa* [a]

Precursor	Weight (mg) of amorphigenin	Specific activity (d.p.m. mM^{-1})	Uptake (%)	Dilution	Incorporation (%)
Chalcone(11a) [b,e]	3·075	4·61 × 10^5	87	1080	0·30
Chalcone(11e) [b,e]	2·015	1·03 × 10^4	86	44,000	0·006
Chalcone(11b) [b,e]	1·973	3·20 × 10^4	81	13,900	0·019
Chalcone(11c) [b,g]	0·583	2·33 × 10^4	43	19,500	0·002
Chalcone(11d) [b,e]	1·840	6·42 × 10^3	78	70,200	0·003
Isoflavone(15b) [c,e]	1·908	2·29 × 10^6	83	515	0·42
Isoflavone(2b) [e]	1·815	3·32 × 10^6	70	158	1·87
Chalcone(11a) [b,f]	8·08	6·78 × 10^5	89	732	1·22
Isoflavone(15a) [b,f]	9·04	2·04 × 10^4	80	94,600	0·013
Isoflavone(15c) [e,f]	5·56	5·60 × 10^4	89	743,000	0·002
Isoflavone(2b) [f]	8·42	3·48 × 10^6	78	150	8·70

[a] Compounds administered as sodium salts in 0·05M-phosphate buffer (pH 7), 48 h. [b] [*carbonyl*-^{14}C]. [c] [4'-*O*-^{14}C]. [d] Tritiated by Wilzbach method, followed by removal of alkaline exchangeable tritium; <3% of ^{3}H at C-2.[18] [e] 40 Seedlings, 5 days old. [f] 100 Seedlings, 4 days old. [g] 70 Seedlings, 5 days old.

[25] L. Crombie, P. W. Freeman, and D. A. Whiting, unpublished observations.

[26] S. A. Brown, M. El-Dakhakhny, and W. Steck, *Canad. J. Biochem.*, 1970, **48**, 863.

[27] W. Steck and S. A. Brown, *Canad. J. Biochem.*, 1970, **48**, 872; P. G. Harrison, B. K. Bailey, and W. Steck, *ibid.*, 1971, **49**, 964.

[28] R. M. Bowman, J. F. Collins, and M. F. Grundon, *Chem. Comm.*, 1967, 1131.

[29] A. U. Kasymov, E. S. Kondratenko, Ya. V. Rashkes, and N. K. Abubakirov, *Khim. prirod. Soedinenii*, 1970, 197.

[30] A. U. Kasymov, E. S. Kondratenko, and N. K. Abuvakirov, *Khim. prirod. Soedinenii*, 1972, 115.

[31] J. Fukami, I. Yakamoto, and J. E. Casida, *Science*, 1967, **155**, 713.

[32] H. Grisebach and W. Barz, *Naturwiss*, 1969, **56**, 538 and references therein.

was synthesised from the appropriate aldehyde by base catalysed condensation with [*carbonyl*-^{14}C]resacetophenone, conveniently prepared [33] from sodium [1-^{14}C] acetate, and administered (as sodium salts) to *Amorpha fruticosa*. Results (Table 7) show that much the best incorporation was found for 2',4,4'-trihydroxychalcone (11a). Chalcones with hydroxylation–methoxylation patterns approaching that of the shikimate-derived ring of isoflavone (2a) were rather poor precursors of amorphigenin. This indicates that the substitution pattern of (2a) is built up after the aryl migration involved in the chalcone ⟶ isoflavone transformation and that chalcone (11a) is the final pre-migration chalcone. Chalcone (11a) is also a better precursor than 2,2',4,4'-tetrahydroxychalcone in coumestrol biosynthesis.[17] The present results are consistent with the observation that a loss. It is consistent that chalcone (11e), with the same substitution pattern as formononetin was inappreciably converted (Table 7) into amorphigenin.

These pre-flavonoid stages are included in Scheme 1. The first recognisable intermediate is the simple chalcone (11a). Aryl migration is associated with 4'-methylation, placing formononetin next in sequence. Oxygenation and methylation, in an undetermined order, but involving *S*-adenosylmethionine, and not apparently proceeding through 2',4',7-trihydroxyisoflavone (15c) (Table 7), are presumed to lead to 7-hydroxy-2',4',5-trimethoxyisoflavone, at which stage conversion to the rotenoid occurs involving the 2'-methoxy-group. It is attractive to invoke oxidation of the 2'-methoxy-group to the cation (shown), followed by electrocyclic ring closure and return of hydrogen anion from coenzyme to yield the rotenoid,

TABLE 8

Administration of [*u*-^{14}C]amino-acids and sodium [2-^{14}C]acetate to *Amorpha fruticosa* [a]

Precursor	Weight (mg) of amorphigenin	Specific activity (d.p.m. mM^{-1})	Uptake (%)	Dilution	Incorporation (%)
Sodium [2-^{14}C]acetate	6·15	$1·75 \times 10^{7}$	93	6980	0·22
L-[*u*-^{14}C]Threonine	4·92	$7·65 \times 10^{5}$	91	290,000	0·038
L-[*u*-^{14}C]Leucine	5·69	$3·35 \times 10^{5}$	91	994,000	0·019
L-[*u*-^{14}C]Isoleucine	5·79	$1·34 \times 10^{5}$	90	2,490,000	0·0079
L-[*u*-^{14}C]Serine	6·13	$9·56 \times 10^{6}$	93	17,500	0·59
[*u*-^{14}C]Glycine	4·74	$3·23 \times 10^{6}$	89	34,400	0·16

[a] Administered in water over 48 h to batches of 60 seedlings, 4 days old.

TABLE 9

Degradation of amorphigenin from feeding of sodium [2-^{14}C]acetate, L-[*u*-^{14}C]serine, and [*u*-^{14}C]glycine

Precursor	Specific activity amorphigenin (1b) [d.p.m. mM^{-1} (%)]	Specific activity didehydroamorphigenin (7) [d.p.m. mM^{-1} (%)]	Specific activity risic acid (16) [d.p.m. mM^{-1} (%)]
Sodium [2-^{14}C]acetate	$1·13 \times 10^{5}$ (100)	$1·08 \times 10^{5}$ (96)	$2·48 \times 10^{3}$ (2·2)
L-[*u*-^{14}C]Serine	$4·31 \times 10^{4}$ (100)	$4·15 \times 10^{4}$ (96)	$4·14 \times 10^{4}$ (96)
[*u*-^{14}C]Glycine	$1·24 \times 10^{4}$ (100)	$1·16 \times 10^{4}$ (94)	$1·26 \times 10^{4}$ (102)

4-methoxylated cinnamic acid destined for isoflavonoid biosynthesis becomes demethylated.[32,34,35] Preliminary demethylation may be implicated in the limited incorporation found for some of the chalcones of Table 7.

Many hypotheses have been suggested for the mechanism of aryl migration in chalcones, and various *in vitro* analogies have been attempted.[2,34,36] The suggestion of Pelter [37] is chemically acceptable, and assigns a role to the 4-hydroxy-group, which the above evidence shows must not be blocked by methylation. One electron transfer from chalcone (11a, anion) leads *via* (12) to (13), and hence to spirodienone (14) by another one-electron oxidation. However, the product of proton catalysed decomposition of spirodienone (14), 4',7-dihydroxyisoflavone [(15a), daidzein] is unimpressively accepted by *A. fruticosa* seedlings for conversion into amorphigenin (Table 7). On the other hand, 7-hydroxy-4'-methoxyisoflavone [(15b), formononetin] [11,17] is very satisfactorily incorporated. This situation is explained if spirodienone (14) is decomposed by methylating agent (*e.g. S*-adenosylmethionine) with accompanying proton without overall change in oxidation level. A radical version of this may be written.

Earlier in this paper, we pointed to the poor incorporations of mevalonic acid into amorphigenin, in *A. fruticosa* and into rotenone in *Derris elliptica* plants.[5,13f] In search of better precursors to ring E other compounds connected with mevalonate metabolism were fed to the *A. fruticosa* seedling system, including L-[*u*-^{14}C]leucine, sodium [2-^{14}C]acetate, and, in view of the reported [3,5] low incorporations of acetate into rotenoids, two *in vivo* sources [38] of acetyl-CoA, [*u*-^{14}C]glycine and L-[*u*-^{14}C]-serine. In addition L-[*u*-^{14}C]isoleucine and L-[*u*-^{14}C]-threonine were tested as precursors. Results are shown in Table 8. Good incorporations were noted for acetate, glycine, and serine. Amorphigenin isolated from

[33] E. Wong, *Phytochemistry*, 1968, **7**, 1751.

[34] J. Ebel, H. Achenbach, W. Barz, and H. Grisebach, *Biochim. Biophys. Acta*, 1970, **215**, 203.

[35] W. Barz and H. Grisebach, *Z. Naturforsch.*, 1967, **22b**, 627.

[36] *Inter alia* K. Freudenberg, G. Carrara, and E. Cohn, *Annalen*, 1926, **446**, 87; G. W. K. Cavill, F. M. Dean, A. McGookin, B. M. Marshall, and A. Robertson, *J. Chem. Soc.*, 1954, 4573; S. C. Bhrara, A. C. Jain, and T. R. Seshadri, *Tetrahedron*, 1965, **21**, 963; H. Grisebach and W. Barz, *Chem. Ber.*, 1964, **97**, 1688; W. D. Ollis, K. L. Ormand, and I. O. Sutherland, *J. Chem. Soc.* (*C*), 1970, 119; W. D. Ollis, K. L. Ormand, B. T. Redman, R. J. Roberts, and I. O. Sutherland, *ibid.*, p. 125.

[37] A. Pelter, *Tetrahedron Letters*, 1968, 897; A. Pelter, J. Bradshaw, and R. E. Warren, *Phytochemistry*, 1971, **10**, 835.

[38] S. P. J. Shah and L. J. Rogers, *Biochem. J.*, 1969, **114**, 395 and references therein.

these experiments was degraded *via* 6,12a-didehydroamorphigenin (7) to risic acid (16) which contains ring A and C-6, -6a, and -12a of amorphigenin. It is apparent that acetate is incorporated into ring D and/or E, in accord with expectation. However, the activity from both [*u*-^{14}C]-serine and -glycine was entirely localised in the risic acid part of the molecule (Table 9). Metabolism of serine and glycine to acetyl-CoA proceeds through pyruvate;[38] this may be incorporated into amorphigenin ring A by way of shikimic acid, or into C-6a, -12, and -12a by involvement in the shikimic acid ⟶ phenylalanine sequence. Since degradation shows C-12 to be inactive, the second route is feasible only if carboxy-exchange with inactive carbon dioxide occurs (*via* the ' dicarboxylic shuttle ').[39]

EXPERIMENTAL

General Procedures.—*Amorpha fruticosa* seeds were dehusked, soaked in ethanol (2 × 5 min), washed with sterile water, soaked in sodium hypochlorite solution (12% free chlorine; 2 × 10 min), and washed again with sterile water. They were allowed to germinate at 20° in the dark on moist filter paper in a closed tank, under sterile conditions. After 7 days, seeds at a similar stage of development were transferred to Petri dishes containing the appropriate precursor, and allowed to grow in the light for the appropriate administration period. The germination rate was improved by heating the seeds in sterile water at 60° for 10 min before germination; in this case, seeds were germinated for 3—5 days. Air was passed through a sterile pad over the seeds, and humidity was maintained by adding sterile water as necessary. After the administration period, the seeds were washed with sterile water; unassimilated precursor in the washings was counted. The seedlings were harvested by grinding with broken glass under aqueous ethanol (1 : 1), and pouring the homogenate into boiling ethanol. The mixture was refluxed for 10 min and filtered. The residue was re-extracted twice with hot ethanol. The combined extracts were evaporated to dryness. Water was added to the residue and the mixture was extracted with ether. To isolate amorphigenin the ether extracts were evaporated and separated by t.l.c. using chloroform–isopropanol (10 : 1). Amorphigenin bands were eluted with acetone (AnalaR) and repurified by t.l.c., using benzene–ethyl acetate–methanol–light petroleum (b.p. 60—80°) (B.E.M.P.) (6 : 4 : 1 : 3). Amorphigenin content was assessed by u.v. absorption of an ethanol solution [$\nu_{max.}$ 34·12 × 10^3 cm^{-1} (ε 16,680)]. Carrier (20 mg) was added and the diluted amorphigenin crystallised to constant activity from methanol (crystallising as the monomethanol solvate).[8] T.l.c. was carried out with Kieselgel G (0·5 mm layers). Radioactive samples were counted in a dioxan-based scintillator (NE 250) using Nuclear Enterprises NE8310 liquid scintillation counter. Efficiencies were measured using [^{14}C]-toluene internal standards. Phenolic precursors were administered as sodium salts in 0·05M-phosphate buffer (pH 7·0). Neutral compounds (*ca.* 0·5 mg) were dissolved in methyl cellosolve (ethylene glycol monomethyl ether), (0·1 cm^3) and Tween 20 (1 drop, *ca.* 14 mg) and diluted with sterile water (5 ml). (±)-[1-^{14}C]Phenylalanine (50 mCi mM^{-1}), [2-^{14}C]mevalonic acid (5·85), sodium [2-^{14}C]acetate (55), L-[*u*-^{14}C]threonine (100), L-[*u*-^{14}C]isoleucine (150), L-[*u*-^{14}C]leucine (150), L-[*u*-^{14}C]serine (75), and [*u*-^{14}C]-glycine (40) were obtained from the Radiochemical Centre, Amersham.

Formation and Turnover of Free and Bound Amorphigenin (Table 1).—(±)-[1-^{14}C]Phenylalanine (0·05 mCi) was administered to 240 seedlings for 24 h. The washed seedlings were allowed to grow-on, and batches of 40 seedlings were removed after 0, 1, 2, 4, and 7 days. Free amorphigenin was measured as described above (total ethanol used in extraction, 300 cm^3). The aqueous layers after removal of free amorphigenin were diluted with water to 50 cm^3, and heated under reflux with ethanol (50 cm^3) and hydrochloric acid (10 cm^3) for 1 h. After removal of ethanol by evaporation, the amorphigenin was assayed as before.

Administration of [2′-*O*-^{14}C]*Isoflavones and Mevalonic Acid* (Table 2).—(a) *2′-Hydroxyisoflavones.* Authentic samples of derritol isoflavone [13f] and 8″-hydroxyderritol isoflavone [13f] were available. 7-Benzyloxy-2′-hydroxy-4′,5′-dimethoxyisoflavone was prepared from 2,4,5-trimethoxybenzaldehyde (25 g), rhodanine (17 g), anhydrous sodium acetate (63·5 g), and acetic acid (85 ml) heated at 120—130° for 30 min. The red precipitate (2,4,5-trimethoxybenzalrhodanine) was collected, washed, dried, and heated for 90 min under nitrogen with sodium hydroxide (64 g) and sodium sulphide (16 g) in water (450 cm^3). The cooled mixture was acidified and the precipitated 2,4,5-trimethoxyphenylthiopyruvic acid (32 g) was extracted into ethyl acetate. The recovered acid was converted into the corresponding oxime using hydroxylamine liberated from its hydrochloride (25 g). Treatment of the oxime with acetic anhydride (25 cm^3) gave 2,4,5-trimethoxybenzyl cyanide (5·1 g) after dilution with water. Hydrolysis of the nitrile was effected under reflux with potassium hydroxide (3 g) in water (30 cm^3) for 3 h. On acidification 2,4,5-trimethoxyphenylacetic acid (4 g) was obtained, m.p. 78—80° (from water) (lit.,[13a] 81°). The acid was condensed with resorcinol (6 g) in polyphosphoric acid (40 g) at 100° for 15 min. Dilution with water and extraction with ether gave, after isolation from solvent, 2,4-dihydroxyphenyl 2,4,5-trimethoxybenzyl ketone (1·83 g), m.p. 200—203° (from methanol) (lit.,[13b] m.p. 201—202°). The deoxybenzoin (1·83 g) was refluxed overnight in dry pyridine (20 cm^3) with triethyl orthoformate (4 cm^3) and piperidine (1 cm^3). The cooled mixture was poured onto ice. 7-Hydroxy-2′,4′,5′-trimethoxyisoflavone was collected by filtration, and purified by p.l.c. [chloroform–isopropanol (10 : 1)] (0·85 g), m.p. 244—245° (lit.,[13b] 244—245°). 2′-Demethylation of this isoflavone (150 mg) was accomplished by heating overnight in acetonitrile (5 cm^3) with aluminium chloride (400 mg). The solvent was evaporated and the residue decomposed with dilute hydrochloric acid. Filtration yielded 2′,7-dihydroxy-4′,5′-dimethoxyisoflavone (120 mg), m.p. 235—236° (lit.,[13c] 236—237°). Finally, selective benzylation of the isoflavone (110 mg) was achieved by stirring overnight in acetone (15 cm^3) with anhydrous potassium carbonate (1 g), potassium iodide (10 mg), and benzyl chloride (44 mg, 1 mol. equiv.). The filtered mixture was evaporated and the residue crystallised from methanol to give 7-benzyloxy-2′-hydroxy-4′,5′-dimethoxyisoflavone (30 mg), m.p. 160° (lit.,[13c] 172—173°). A trace of unbenzylated isoflavone was detected by t.l.c.

(b) *2′-Methoxy*[^{14}C]*isoflavones.* [^{14}C]Methyl iodide (0·5 mCi, 51·5 mCi mM^{-1}, 0·6 mm^3) was dissolved in dry acetone

[39] M. F. Utter and K. Kurahashi, *J. Biol. Chem.*, 1954, **207**, 821.

(3 cm^3) containing inactive methyl iodide (5 mm^3). The 2′-hydroxyisoflavone (15—30 mg) was stirred under reflux in dry acetone (7 cm^3) with dry potassium carbonate (1 g) and the solution of $^{14}CH_3I$ (1 cm^3) for 6 h. Excess of methyl iodide (0·5 cm^3) was added and the reaction was continued overnight. After filtration, the acetone solution was evaporated to dryness and the product purified by t.l.c. Derritol isoflavone [$\nu_{max.}$ 45·11 × 10^3 (ε 29,220), 40·75 × 10^3 (28,780), 39·67 × 10^3 (29,850), and 33·47 × 10^3 cm^{-1} (16,520)] and 8″-hydroxyderritol isoflavone [$\nu_{max.}$ 45·14 × 10^3 (ε 29,560), 40·72 × 10^3 (29,070), 39·63 × 10^3 (30,260), and 33·46 × 10^3 cm^{-1} (16,810)] methyl ethers were purified to constant activity (0·245 and 0·676 mCi mM^{-1}, respectively) by repeated t.l.c. using B.E.M.P., chloroform–isopropanol (10 : 1), and benzene–ethanol (92 : 8) solvent systems in turn. 7-Benzyloxy-2′,4′,5′-[2′-*O*-^{14}C]trimethoxyisoflavone was debenzylated with acetic acid (2 cm^3) and concentrated hydrochloric acid (2 cm^3) at 100° for 30 min. 7-Hydroxy-2′,4′,5′-trimethoxyisoflavone was isolated *via* ethyl acetate extraction and purified to constant activity (0·236 mCi mM^{-1}) by t.l.c., using three solvent systems as above.

Detection of Natural 7-Hydroxy-2′,4′,5′-trimethoxyisoflavone in A. fruticosa *Seedlings, by Dilution Analysis* (Table 3). —(±)-[1-^{14}C]Phenylalanine (0·1 mCi, 48 mCi mM^{-1}) was applied to *A. fruticosa* seedlings for 4 h. The seedlings were macerated and extracted as above. The ether extract was chromatographed (t.l.c.). Derritol isoflavone methyl ether (22·5 mg) (3a), 7-hydroxy-2′,4′,5′-trimethoxyisoflavone (15·5 mg) (2a), and 8″-hydroxyderritol isoflavone methyl ether (22 mg) (3b) were added separately to eluates of appropriate zones of the thin layer chromatogram. The isoflavones were purified using t.l.c. (B.E.M.P.), and then recrystallised from methanol or aqueous methanol to constant m.p.

Degradation of [6-^{14}C]*Amorphigenin.*—Zinc dust (330 mg) and hot 20% aqueous potassium hydroxide (1·5 cm^3) were added to a refluxing solution of amorphigenin (165 mg) in ethanol (4·5 cm^3). The mixture was heated under reflux with stirring for 2·5 h, cooled, and filtered. The filtrate was acidified and extracted with ether (3 × 20 cm^3). The concentrated extract was separated by t.l.c., 8′-hydroxyderritol and 8′-hydroxyrotenol were extracted from the appropriate bands (comparison with authentic specimens), rechromatographed (B.E.M.P.), and crystallised to constant activity from aqueous methanol.

The Role of Isoflavanone (6) *in Amorphigenin Biosynthesis* (Table 4).—(a) *7-Hydroxy-2′,4′,5′-[2′-OC*^{3}*H*$_3$*]trimethoxyisoflavone.* 7-Benzyloxy-2′-hydroxy-4′,5′-dimethoxyisoflavone (28·6 mg) was methylated using [$^3H_3$]methyl iodide (25 mCi, 100 mCi mM^{-1}), in dry acetone (10 cm^3) over anhydrous potassium carbonate (1 g), as described for the ^{14}C analogue. The product was diluted with 7-benzyloxy-2′,4′,5′-trimethoxyisoflavone (28·9 mg) and debenzylated with hydrochloric and acetic acids (as above). The product was diluted with inactive 7-hydroxy-2′,4′,5′-trimethoxyisoflavone (29·8 mg), and crystallised to constant activity (2·47 × 10^{10} d.p.m. mM^{-1}, 11·1 mCi mM^{-1}).

(b) *(±)-7-Hydroxy-2′,4′,5′-[2′-OC*^{3}*H*$_3$*]trimethoxyisoflavanone.* 7-Hydroxy-2′,4′,5′-trimethoxyisoflavone (36·1 mg) in acetic acid (10 cm^3) was hydrogenated at atmospheric temperature and pressure over platinum catalyst [from platinum oxide (536 mg) in acetic acid (10 cm^3)]. Uptake of 1 mol. equiv. of hydrogen was complete in 75 min. The mixture was filtered and evaporated to dryness. The residue was purified by repeated t.l.c. [B.E.M.P., chloroform–isopropanol (10 : 1), and benzene–ethanol (92 : 8)] to yield the required *isoflavanone* (6) (23 mg), m.p. 205—206° (Found: C, 65·55; H, 5·7. $C_{18}H_{18}O_6$ requires C, 65·45; H, 5·5%), $\nu_{max.}$ (KBr) 3279, 1673, 1600, and 1518 cm^{-1}, $\nu_{max.}$ (EtOH) 49·24 × 10^3, (ε 46,500), 46·26 × 10^3 (27,300), 43·06 × 10^3 (19,100), 35·86 × 10^3 (16,400), and 32·21 × 10^3 cm^{-1} (9270), specific activity 2·60 × 10^{10} d.p.m. mM^{-1} (11·7 mCi mM^{-1}).

Degradation of [6-^{3}H]*Amorphigenin.*—(a) 6a,12a-*Didehydroamorphigenin.* Amorphigenin (85 mg) and freshly fused sodium acetate (250 mg) were heated in ethanol (3 cm^3). Iodine (80 mg) in ethanol (1 cm^3) was added during 1 h, and the mixture was refluxed for 1 h more. The product was concentrated and cooled. 6a,12a-Didehydroamorphigenin crystallised and was collected by filtration, purified by t.l.c. [chloroform–isopropanol (10 : 1)] and recrystallised from chloroform–methanol (35 mg).

(b) 8′-*Hydroxyderritol.* This was obtained as described above.

Synthesis and Administration of [6-^{3}H]*Rotenoids* (Table 6). —Administration techniques were as described above.

(a) [$^3H_9$]*Trimethylsulphoxonium iodide.* Trimethylsulphoxonium iodide (156·5 mg) and potassium carbonate (9·1 mg) were heated at 100° for 3 h in a sealed vessel with tritiated water (2 Ci; 0·4 cm^3). The solvent was transferred (vacuum line) to a second batch of the salt (104·7 mg) and potassium carbonate (9·1 mg) and the exchange was repeated. The combined residues after evaporation of tritiated water were dried overnight in vacuum.

(b) (±)-9-[6-$^3H_2$]*Demethylmunduserone.* Sodium hydride (50% in oil; 71·7 mg, 1·5 mM) was washed with dry ether and dried under a stream of nitrogen. [$^3H_9$]Trimethylsulphoxonium iodide (261·2 mg, 1·19 mM) in dry dimethyl sulphoxide (5 cm^3) was added and the mixture stirred under nitrogen for 30 min, when a clear solution was obtained. 2′,7-Dihydroxy-4′,5′-dimethoxyisoflavone (369·3 mg, 1·18 mM) was added. Stirring under nitrogen at ambient temperature was continued for 3·5 h. The mixture was diluted with water, acidified, and extracted with ethyl acetate (3 × 20 cm^3). The extracts were washed with water, dried, and evaporated. The residue was heated in dry pyridine (2 cm^3) in a sealed vessel at 100° for 2 h. The product, precipitated on dilution with water and acidification, was collected in ethyl acetate (2 × 20 cm^3). The concentrated extracts were separated by t.l.c. (B.E.M.P.). A band at R_F 0·5 (purple colouration on spraying with dilute HI) was removed and eluted with acetone. After evaporation the residue was purified by t.l.c. to constant activity (0·87 mCi mM^{-1}), using B.E.M.P., chloroform–isopropanol (10 : 1), and benzene–ethanol (92 : 8), to yield (±)-9-[6-$^3H_2$]*demethylmunduserone* (12 mg), m.p. 148—150° (Found: M^+, 328·095. $C_{18}H_{16}O_6$ requires M^+, 328·095), $\nu_{max.}$ (EtOH) 42·98 × 10^3 (ε 13,610), 35·55 × 10^3 (13,920), and 31·67 × 10^3sh cm^{-1} (7700). This product was spectroscopically and chromatographically identical with an inactive specimen prepared by a similar method. See ref. 6 for a full discussion of the spectra, and the course of the reaction.

(c) [6-$^2H_2$]*Mutarotenone.*—Derritol isoflavone (3c) (259·5 mg) was converted into [6-$^3H_2$]mutarotenone (79 mg) using the above method, with trimethylsulphoxonium iodide (149·1 mg). The product was purified by t.l.c., using benzene–ethyl acetate–methanol–light petroleum (b.p. 60—80°) (6 : 4 : 1 : 7) and benzene–ethanol (92 : 8), to constant activity (7·93 × 10^8 d.p.m. mM^{-1}, 0·357 mCi mM^{-1}).

Mutarotenone [23] from this experiment, $\nu_{max.}$ (EtOH) $45{\cdot}72 \times 10^3$sh (ε 27,030), $42{\cdot}16 \times 10^3$ (15,600), $41{\cdot}11 \times 10^3$sh (13,830), and $34{\cdot}07 \times 10^3$ cm^{-1} (16,520), was treated as a 1 : 1 mixture of diastereoisomers.

(d) (±)-[6-$^3H_2$]*Rotenonic acid*. [6-$^3H_2$]Mutarotenone (50 mg) was hydrogenated in pyridine (5 cm^3) over palladium–barium sulphate (5%, 30 mg), at ambient temperature and pressure, until 1·1 mol. equiv. of hydrogen was absorbed. The solvent was evaporated and the residue was dissolved in methanol, filtered, and concentrated. After dilution with water the product was extracted with ethyl acetate (2 × 20 cm^3). The concentrated extract was purified by t.l.c. using chloroform–isopropanol (10 : 1). (±)-Rotenonic acid (17 mg) was obtained after extraction of the major band with acetone, $\nu_{max.}$ (EtOH) $45{\cdot}52 \times 10^3$sh (ε 25,980), $42{\cdot}53 \times 10^3$ (16,180), and $34{\cdot}18 \times 10^3$ cm^{-1} (17,500), and was purified by t.l.c. [B.E.M.P., benzene–ethanol (9 : 1)] to constant activity ($8{\cdot}45 \times 10^8$ d.p.m. mM^{-1}, 0·380 mCi mM^{-1}). Authentic (±)-rotenonic acid was available.[6]

Synthesis of Labelled Chalcones and Isoflavones (Table 7).—(a) *Preparation of* [*carbonyl*-^{14}C]*chalcones*. [*carbonyl*-^{14}C]-Resacetophenone, prepared * by the method of Wong,[33] (50 mg) was heated with the appropriate aldehyde (50 mg), ethanol (0·1 cm^3), and aqueous potassium hydroxide (100% w/v; 1 cm^3) in a sealed flask for 30 min at 100°, and then set aside overnight. The mixture was acidified and extracted with ether. The required chalcone was separated from the ether extracts by t.l.c. using B.E.M.P., and purified to constant activity using t.l.c. with B.E.M.P., benzene–ethanol (92 : 8), benzene–ethyl acetate–methanol (6 : 4 : 1), and chloroform–isopropanol (10 : 1). In this way the following chalcones were prepared (10—20 mg): 2′,4,4′-trihydroxy[*carbonyl*-^{14}C]chalcone, $\nu_{max.}$ (EtOH) $44{\cdot}94 \times 10^3$ (ε 9800), $43{\cdot}35 \times 10^3$ (9800), $41{\cdot}77 \times 10^3$ 9980), $33{\cdot}39 \times 10^3$infl. (8230), $32{\cdot}21 \times 10^3$ (9940), and $26{\cdot}72 \times 10^3$ cm^{-1} (32,100) ($4{\cdot}96 \times 10^8$ d.p.m. mM^{-1}, 0·223 mCi mM^{-1}); 2′,3,4,4′-tetrahydroxy[*carbonyl*-^{14}C]-chalcone, $\nu_{max.}$ (EtOH) $49{\cdot}16 \times 10^3$ (ε 35,290), $37{\cdot}99 \times 10^3$ (10,650), $30{\cdot}75 \times 10^3$ (11,400), and $25{\cdot}97 \times 10^3$ cm^{-1} (31,790) ($4{\cdot}45 \times 10^8$ d.p.m. mM^{-1}, 0·200 mCi mM^{-1}); 2′,4′-dihydroxy-3,4-dimethoxy[*carbonyl*-^{14}C]chalcone, $\nu_{max.}$ (EtOH) $49{\cdot}01 \times 10^3$ (ε 36,400), $40{\cdot}64 \times 10^3$ (9600), $38{\cdot}57 \times 10^3$ (10,600), $31{\cdot}57 \times 10^3$ (11,600), and $26{\cdot}53 \times 10^3$ cm^{-1} (32,000) ($4{\cdot}55 \times 10^8$ d.p.m. mM^{-1}, 0·205 mCi mM^{-1}); 2′,4′-dihydroxy-2,4,5-trimethoxy[*carbonyl*-^{14}C]chalcone, $\nu_{max.}$ $49{\cdot}13 \times 10^3$ (ε 32,710), $40{\cdot}23 \times 10^3$ (9200), $37{\cdot}78 \times 10^3$ (9520), $31{\cdot}89 \times 10^3$ (11,100), and $25{\cdot}15 \times 10^3$ cm^{-1} (26,330) ($4{\cdot}53 \times 10^8$ d.p.m. mM^{-1}, 0·204 mCi mM^{-1}); 2′,4′-dihydroxy-4-methoxy[*carbonyl*-^{14}C]chalcone, $\nu_{max.}$ $43{\cdot}81 \times 10^3$ (ε 10,790), $42{\cdot}21 \times 10^3$sh (10,340), $32{\cdot}51 \times 10^3$infl. (10,900), and $27{\cdot}12 \times 10^3$ cm^{-1} (27,790) ($4{\cdot}51 \times 10^8$ d.p.m. mM^{-1}, 0·203 mCi mM^{-1}).

* We thank Dr. P. A. Firth for this preparation.

(b) *Isoflavones* (15a—c). 2′,4′,7-Trihydroxy[^{3}H]isoflavone ($1{\cdot}93 \times 10^9$ d.p.m. mM^{-1}, 0·87 mCi mM^{-1}) was prepared [18] by tritiation using the Wilzbach method, followed by removal of alkaline-labile tritium. Less than 3% of activity is located at C-2.[18] [4-^{14}C]Daidzein ($4{\cdot}16 \times 10^{10}$ d.p.m. mM^{-1}, 18·65 mCi mM^{-1}) was generously provided by Dr. W. Barz, Freiburg i.Br., whom we thank.

[*O*-^{14}C]*Formononetin*. Daidzein diacetate (180 mg), dry acetone (15 cm^3), anhydrous potassium carbonate (1 g), dry potassium iodide (100 mg), and benzyl chloride (0·2 cm^3) were heated together under reflux with stirring for 24 h. The mixture was filtered and the filtrate was evaporated. The residue was deacetylated by heating in acetone (30 cm^3) with hydrochloric acid (0·5 cm^3) for 1 h. After concentration, the product was precipitated with water, purified by t.l.c. [chloroform–isopropanol (10 : 1)], and crystallised from methanol to yield 7-*O*-benzyldaidzein (120 mg), m.p. 222—224° (lit.,[11] 169—170°). Despite the discrepancy in m.p., spectral details were in complete agreement with those published.[11] This isoflavone (45·5 mg, 0·132 mM) in dry acetone (10 cm^3) over anhydrous potassium carbonate (1 g) was treated with [^{14}C]methyl iodide (0·1 mCi, 55 mCi mM^{-1}). Inactive methyl iodide (6 mm^3, 0·0965 mM) was added and the mixture stirred and heated under reflux for 3·5 h. Excess of methyl iodide (50 mm^3) was added and the reaction continued for 1·5 h. The mixture was filtered and the filtrate evaporated to dryness. The residue was heated under reflux with acetic acid (5 cm^3) and hydrochloric acid (5 cm^3) for 1 h. After evaporation of the solvents, [*O*-^{14}C]-formononetin, identical in all respects with an authentic specimen, was separated by t.l.c. (B.E.M.P.) (33 mg) and purified by further t.l.c. [chloroform–isopropanol (10 : 1) and benzene–ethanol (92 : 8)] to constant activity ($1{\cdot}18 \times 10^9$ d.p.m. mM^{-1}, 0·534 mCi mM^{-1}).

Degradation of Amorphigenin to Risic Acid.—Amorphigenin (450 mg) was dehydrogenated with iodine to 6a,12a-didehydroamorphigenin as described above. The didehydrorotenoid (160 mg) was added to a stirred solution of potassium hydroxide (0·8 g) in water (3·5 cm^3) and ethanol (15 cm^3) at 40°. 30% Hydrogen peroxide (2 cm^3) was added over 15 min and stirring was continued for 20 min. More peroxide (4 × 1 cm^3) was added at 10 min intervals and the solution was stirred for 30 min more. After heating briefly at 70°, the solution was cooled, acidified, and extracted with ethyl acetate (3 × 25 cm^3). The extracts were evaporated. The residue crystallised from methanol to yield risic acid (22 mg) which was recrystallised from aqueous methanol and sublimed at 225° and 1 mmHg. It was spectrally (i.r.) identical with an authentic sample.

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