Biosynthesis of the A/B/C/D-Ring System of the Rotenoid Amorphigenin by *Amorpha fruticosa* Seedlings

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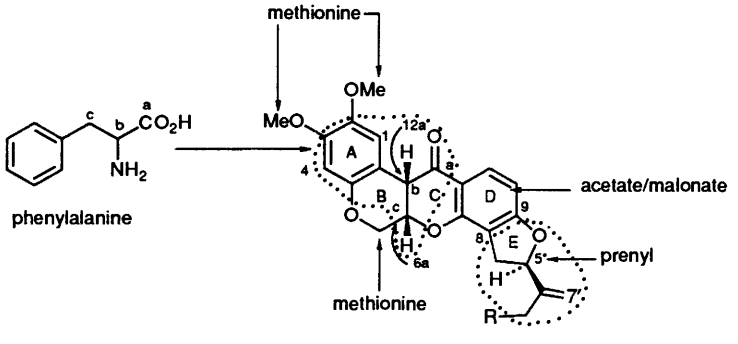
With phenylalanine as the starting point, the biosynthesis of the characteristic rotenoid A/B/C/D-ring system of amorphigenin is studied using *Amorpha fruticosa* seedlings. The course of the biosynthesis can be divided into four phases represented by the bordered and interconnecting Schemes 1, 3, 6 and 7 which summarise the Chalcone–Flavanone Phase, the Flavanone–Isoflavone Phase, the Hydroxylation/Methoxylation Phase and the Rotenoid Phase. By using an INADEQUATE NMR experiment involving the administration of [1,2-^{13}C]acetate, the type of folding forming ring-D is demonstrated by ^{13}C–^{13}C coupling and is interpreted as involving a polyketide containing a glutaconate segment which cyclises by a Claisen condensation. The resulting chalcone is cyclised, enzymically and stereospecifically, to 4′,7-dihydroxyflavanone.

The latter flavanone undergoes aryl migration, in a manner similar to that found in isoflavone biosynthesis, to give 7-hydroxy-4′methoxyisoflavone. Possible mechanisms for the flavanone–isoflavone rearrangement are discussed, including a proposal that the initiating step involves attack on ring-A and is similar to the first stage of the aromatic hydroxylation of tyrosine to dopa. Although possessing no 4′-hydroxy group in ring-A, the mechanism is also applicable to the recently discovered rotenoids of the Boerhaavia and Iris type, and it provides an explanation for the biogenesis of natural spirobenzocyclobutanes from dihydroeucominoids.

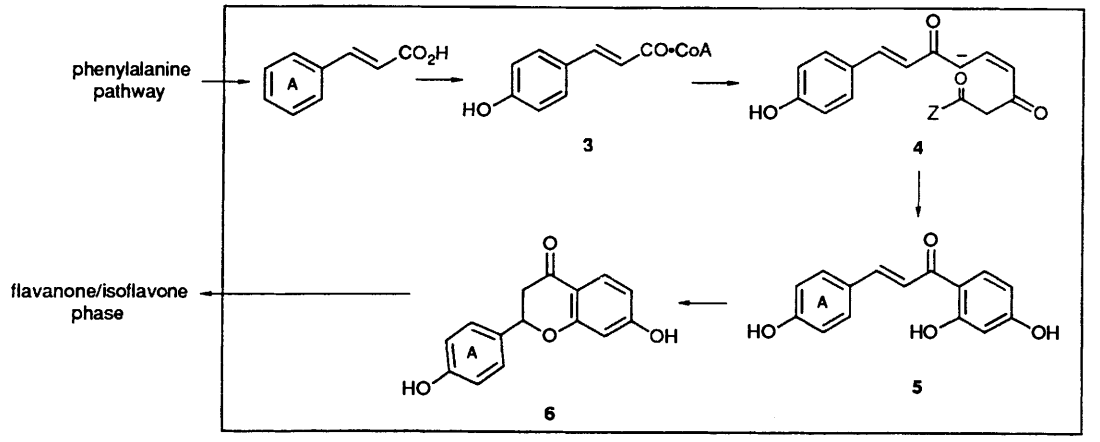
Six suitably substituted isoflavonoids labelled with ^{13}C or ^{3}H are synthesized and are used to show that the next hydroxylation (and probably methylation) involves C-3′ rather than C-2′ in 7-hydroxy-4′-methoxyisoflavone. Whilst the methylations involve *S*-adenosylmethionine, the hydroxylating enzymes are probably very similar to the flavanone–isoflavone-rearranging enzyme. The closure of ring-B to form finally the rotenoid system probably involves conjugate addition of a methoxyl radical. Prenylation and oxidative modifications are characteristically late-stage processes.

A delineation of the biosynthetic pathways to the rotenoid group of natural products has been a major objective of our laboratory.[1] The biological systems used (all members of the Leguminosae) have been rooted cuttings of *Derris elliptica* and germinated seedlings of *Amorpha fruticosa* and *Tephrosia vogellii*, together with enzymes obtained from the last two sources. Early work using ^{14}C-labelling and specific-atom extraction from the metabolites showed a basic derivation as in structure **1** for rotenone and structure **2** for amorphigenin.[2–4] In this paper we consider aspects of the chalcone–flavanone phase of the biosynthesis (Scheme 1) and the flavanone/isoflavone phase of the biosynthesis (Scheme 3). This is followed (Scheme 6) by study of ring-A hydroxylation and methylation, and finally comment is made on the ring-B closure (Scheme 7) to form 9-demethylmunduserone **69**, thereby completing the formation of rotenoid rings A–D. Methylation or prenylation and oxidative modification follow the construction of 9-demethylmunduserone, and these processes will be discussed in later papers.

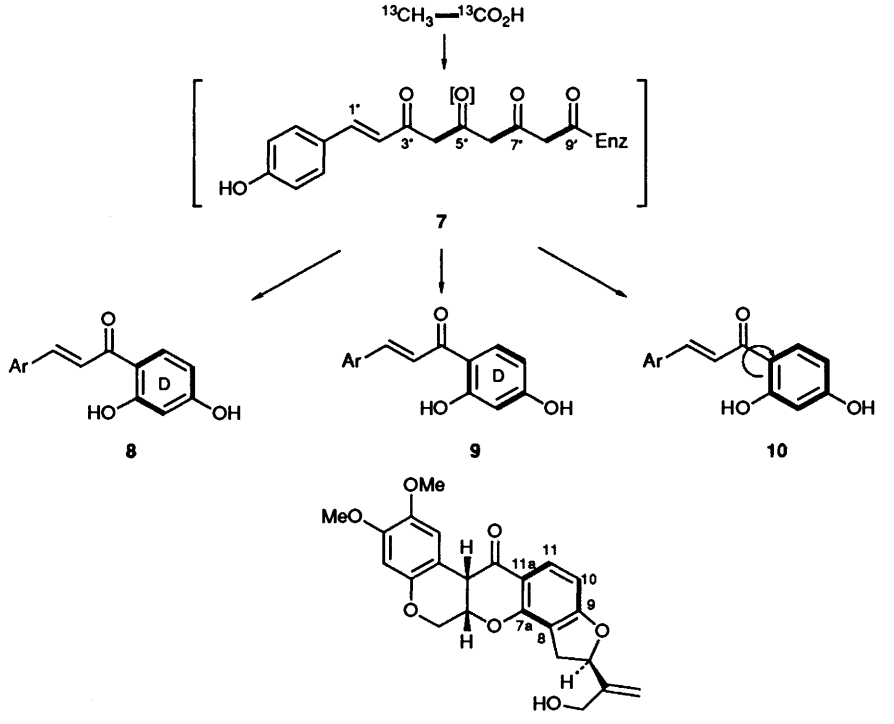
The Chalcone–Flavanone Phase (*Scheme 1*).—The entry point, and possible regulatory step, for the chalcone phase of rotenoid biosynthesis is the conversion of L-phenylalanine into cinnamic acid, catalysed by L-phenylalanine ammonia-lyase (PAL: EC 4.3.1.5).[5] This involves the enzymic elimination of ammonia in an antiperiplanar fashion to produce (*Z*)-cinnamic acid. Subsequent hydroxylation by cinnamate 4-hydroxylase (EC 1.14.13.11) leads on *via* 4-coumarate: CoA ligase (EC 6.2.1.12) to 4-coumaric acid-CoA ester **3**. Condensation of the latter with three acetate units derived from malonyl-CoA is



1 R = H, Rotenone
2 R = OH, Amorphigenin



Scheme 1 The chalcone–flavanone phase



Scheme 2 ^{13}C–^{13}C Coupling pattern for amorphigenin biosynthetically derived from ^{13}C–^{13}C-acetate

catalysed by chalcone synthase (EC 2.3.1.74), the key enzyme of flavonoid biosynthesis, forming the chalcone **5** (Scheme 1), which is ultimately built into rotenoid structures, *e.g.* amorphigenin, *via* the flavanone **6**. The formation of the latter is enzyme mediated by chalcone isomerase (EC 5.5.1.6).[5,6]

For the formal folding of the hypothetical polyketide **7** (Scheme 2), one can distinguish three situations which may be explored using 1,2-^{13}C-labelled acetate precursor as a malonate equivalent. These are substrates **8**, **9** and **10**. In compound **8** the folding is clockwise with the loss of 5′-[O], in isomer **9** it is anticlockwise with the loss of 9′-[O], and in compound **10** either 5′-[O] or 9′-[O] is lost after a freely rotating phloroglucinol unit has been formed. The use of ^{13}C–^{13}C coupling in NMR experiments involving micro-organisms is well established,[7] but in higher plants administration of an early precursor such as [1,2-^{13}C]acetate often gives very low incorporations into complex natural products, particularly when the acetate–malonate pathway provides only part of the structure. Some preliminary experiments with sodium [1-^{14}C]acetate guided our work. Table 1 shows that maximal incorporation occurs in excess of 48 h, but increasing infection causing wilting and eventual death of the seedlings occurred with prolonged incubation periods, and a compromise of 36 h was used. Table 2 indicates that a batch size of ~800 seedlings is needed to give ~30 mg amorphigenin. Administration of 200 mg of the [1-^{14}C]acetate at a specific activity of 32.5 μCi mg^{-1} gave an incorporation of 0.032% into amorphigenin at an uptake of 56% and a dilution of 510, whilst administration of 310 mg of the precursor at a specific activity of 7.9 μCi mg^{-1} gave an incorporation of only 0.009% at an uptake of 52% and a dilution of 208.

In our first experiment a batch of 800 seedlings was germinated and incubated with [1,2-^{13}C]acetate (210 mg) ('spiked' with 50 μCi of [1-^{14}C]acetate) for 36 h. Amorphigenin (31 mg) was obtained with an incorporation of 0.034% (uptake 42%) and a dilution value of 212, giving a specific enrichment over three pairs of aromatic sites of 0.13% per carbon atom. This isotopic enrichment was too low for observation of satellite peaks, and the experiment was repeated employing [1,2-^{13}C]acetate (115 mg) and 1000 *A. fruticosa* seedlings, with administration of the acetate in five 5 h pulses with a total incubation time of 36 h. An improved incorporation of 0.048% (uptake 65%), dilution 156, giving a maximal specific enrichment of 0.18% over the three pairs of aromatic sites was obtained. The two samples of [^{13}C]amorphigenin were united

Table 1 Incorporation of [1-^{14}C]acetate into amorphigenin by *Amorpha fruticosa* seedlings as a function of time

Total activity of [1-^{14}C]acetate (μCi)	Incubation period (t/h)	Uptake (%)	Incorporation (% × 10^2)
45.0	12	62	0.09
51.2	24	79	0.35
39.6	36	60	0.46
52.0	48	61	0.74
50.5	60	75	0.79
51.5	72	82	0.86

Table 2 Effect of number of seedlings on weight and radioactivity of amorphigenin isolated

Total activity of [1-^{14}C]acetate (μCi)	Number of *A. fruticosa* seedlings	Amorphigenin isolated (mg)	Incorporation (%)
50.0	100	8.1	0.081
48.1	200	10.2	0.052
51.2	600	22.5	0.071
50.2	800	33.5	0.092

Table 3 Assignments to the ^{13}C–^{13}C-coupled atoms of amorphigenin resulting from administration of [^{13}C–^{13}C]acetate

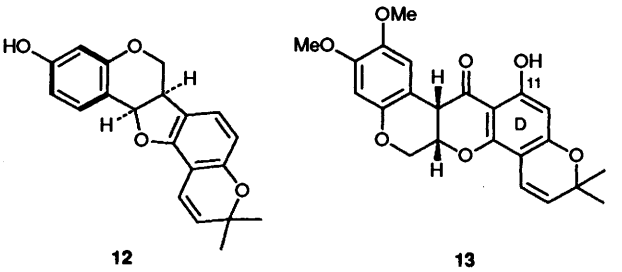
Resonance (ppm)[a]	Carbon atom in compound **11**	J_{C-C}/Hz
166.9	9	68
158.0	7a	77
130.0	11	62
113.5	11a	62
112.9	8	78
104.9	10	68

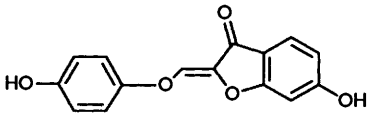
[a] These agree reasonably well with literature data if digitisation is taken into account. An apparent satellite was noted near C-6 at δ_C 146.7 (J 47 Hz).

(51 mg). Despite prolonged pulsing (63 MHz, FT-NMR), ^{13}C–^{13}C coupling could not be detected from satellite peaks in the normal broad-band decoupled spectrum and the sample was successfully examined using the INADEQUATE pulse sequence originally developed by Bax et al.[8]

The results are shown in Table 3 and demonstrate a C-7, -8, C-9, -10 and C-11, -11a coupling pattern in amorphigenin **11** and hence in substrate **8**.[9] Carbonyl reduction and dehydration clearly take place prior to cyclisation, giving the glutaconate anion **4**, which cyclises by a Claisen-type mechanism. It would seem that the attachment of the first acetate/malonate unit follows a pathway similar to fatty acid biosynthesis, with carbonyl reduction and dehydration (but not followed by reduction as in the build-up of a saturated fatty acid chain). The next two acetate/malonate condensations do not undergo this reduction/dehydration. The direction of cyclisation is similar to that deduced for the phytoalexin pisatin **12**.[10] The latter is an experimentally more favourable case for study as a phytoalexin elicitor could be employed giving an enhanced ^{13}C enrichment of 2.3% (± 0.2%) per labelled carbon: the INADEQUATE technique was not employed. Rotenoids such as α-toxicarol **13** contain a phloroglucinol ring-D and it would seem that their biosynthesis is similar except for the omission of the carbonyl reduction and dehydration step.

Isomerisation of the chalcone **5** to the more thermodynamically stable[11] liquiritigenin, the flavanone **6** (Scheme 1), is known to be enzyme catalysed in the Leguminosae and we have isolated and partially purified (activity 261-fold) the chalcone isomerase from *A. fruticosa*: it occurs as two isoenzymes and





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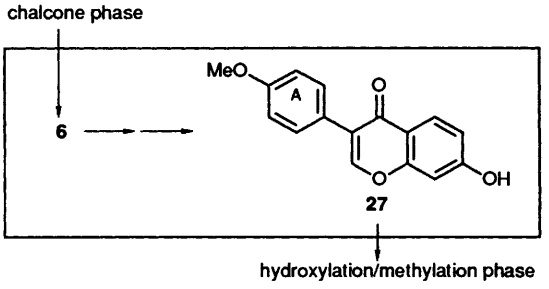
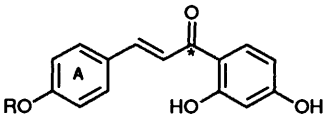
forms the (2*S*)-stereoisomer.[12] The chalcone **5** in this plant is also involved in an enzymic conversion into a novel heterocyclic enol ether, chalaurenol **14**, but we have no evidence that the latter is directly implicated in the rotenoid pathway and this interesting compound has been discussed by us elsewhere.[12]

The Flavanone/Isoflavone Phase (*Scheme 3*).—There are two major problems to be addressed in the flavanone–isoflavone phase. The first of these is the level of oxidation at which hydroxylation/methoxylation in the chalcone–flavanone–isoflavone sequence takes place during the formation of amorphigenin, and the second is the mechanism of the aryl migration, a problem that remains incompletely resolved. In earlier work[4] we addressed the first problem by making [^{14}C-*carbonyl*]-labelled specimens **15**–**19**. All of the last four chalcones showed poor incorporations into amorphigenin by *A. fruticosa* seedlings (0.006, 0.019, 0.002 and 0.003% respectively). Only compound **15** was acceptably incorporated (0.3%), and comparison with its monomethyl ether **16** indicates the requirement for a free *p*-hydroxy group on ring-A for successful aryl migration. On the other hand, whilst the [4-^{14}C]-4′-methoxylated isoflavone **21** gave a very good incorporation (0.42%) into amorphigenin, the [4-^{14}C]-4′-hydroxy analogue **20**, as well as the [4-^{14}C]-2′, 4′-dihydroxy analogue **22**, gave only poor incorporations (0.013 and 0.002% respectively). This suggested that entry to the aryl rearrangement required a free 4′-hydroxy group, but that the exit product on the rotenoid pathway was not the 4′-hydroxy- but the 4′-methoxy-isoflavone **27** (Scheme 3). From among the various mechanistic speculations for the flavanone–isoflavone change, our experimental results were most consistent with the decomposition of a spiro-intermediate **28**[13] by enzyme-mediated methylation, leading to the methyl ether **27** rather than to the phenol **20**.

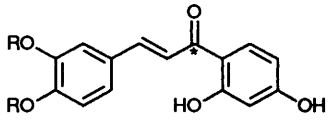


Scheme 3 The flavanone/isoflavone phase

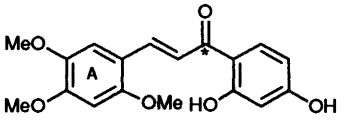
More recently a microsomal preparation from elicitor-challenged soya bean cell-culture was discovered which was capable of converting liquiritigenin **23** (≡ **6**) into daidzein **25**, or naringenin **24** into genistein **26**.[14,15] The reaction was NADPH and dioxygen dependent, and there was convincing evidence



15 R = H
16 R = Me



17 R = H
18 R = Me



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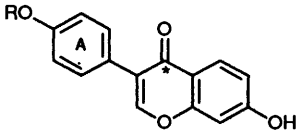
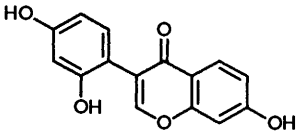
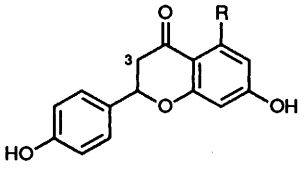


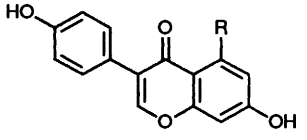
20 R = H [4-^{14}C]
21 R = *Me [4'-^{14}C]



22 Wilzbach ^{3}H-labelling

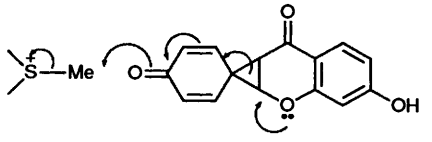


23 R = H [≡ **6**]
24 R = OH



25 R = H
26 R = OH

that the mono-oxygenase enzyme involved belonged to the cytochrome P450 group. The reaction apparently leads initially to the hydroxyisoflavanone **34**, which is then dehydrated by a second water-soluble enzyme.[15] Hagmann and Grisebach[14] suggest that expoxidation of the enolate occurs, leading to intermediate **30**, which is decomposed by protonation to the spiro-intermediate **28**. It appeared to us that acid-catalysed rearrangement of the epoxy enol **30** would be more likely to form the flavonol **31** rather than to induce an aryl shift, and so we have suggested a modification.[9,*] The enzyme system involved resembles an aryl hydroxylase of the kind that converts phenyl alanine into tyrosine (NIH shift)[16] and tyrosine into dopa (though it is uncertain if these processes are to be represented as cationic or radical).[17] *ortho*-Hydroxylation, when performed on a 3',5'-tritiated phenol, involves complete loss of one of the two tritium atoms and may be viewed as decomposition of a protonated aryl epoxide **33** as shown, or as proceeding *via* a radical mechanism (attack at C-4' by $[Fe]^{IV}O^{\cdot}$). In a flavanone, because of the structural situation, a pathway alternative to *ortho*-hydroxylation is available as in intermediates **32a**–**32b** (enolisation is known to occur in the flavone through deuterium-exchange experiments).[18] This leads to the spiro-compound **28** which can be decomposed (in ionic representation) by the equivalent of H^+ or Me^+, leading to the



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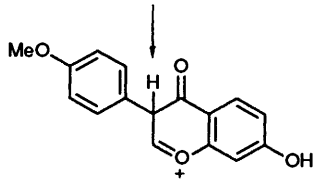

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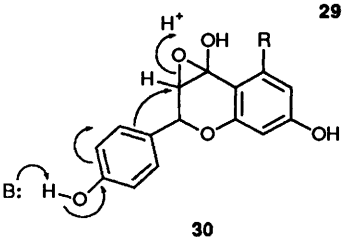

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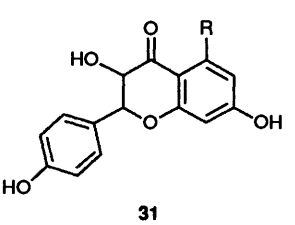

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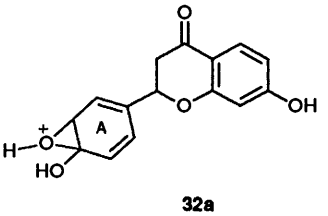
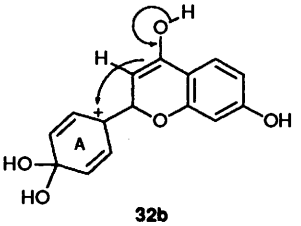
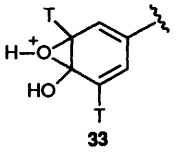


32a



32b



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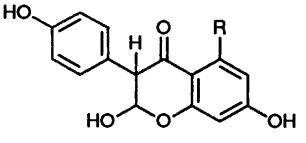

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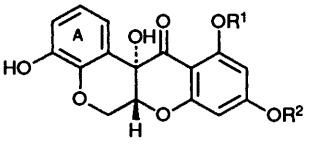

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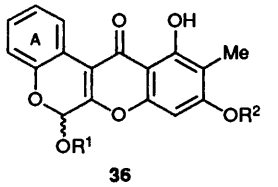

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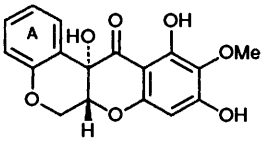

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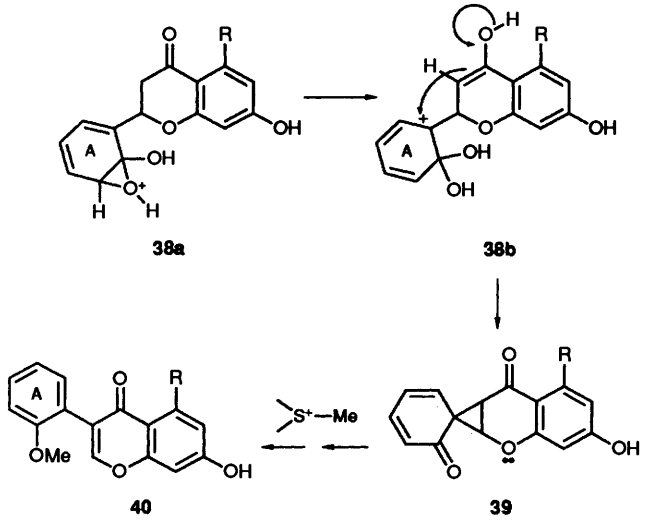
intermediate **29** in the latter case: this can either react to form the hemiacetal **34** or lose a proton to give compound **27**.

Rotenoid formation is concentrated in the Papilionatae division of the Leguminosae (Fabaceae) and all the known structural examples of this kind carry the necessary 3-oxygenation, deriving from the 4'-hydroxylation of the flavanone, which provides for formation of a spiro-ketone intermediate. However, in recent years rotenoids lacking such 3-oxygenation have been found in the Nyctaginaceae and Iridaceae, well removed in botanical classification from the Leguminosae. For example, compounds **35** (R^1 = R^2 = H and R^1 = Me, R^2 = H) occur in *Boerhaavia coccinea*[19] and the boeravinones A and B (**36**; R^1 = Me, R^2 = H and R^1 = H, R^2 = Me) in *B. diffusa*[20] (family Nyctaginaceae). Irispurinol **37** is found in *Iris spuria*[21] (Iridaceae) and another example has been reported from *B. repens*.[21] Their biosynthesis falls into a

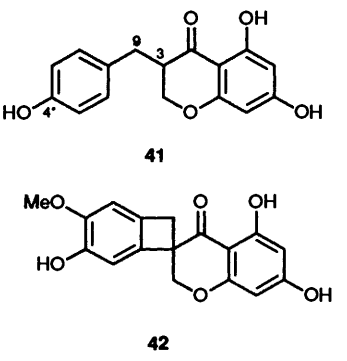
* The scheme attributed to us without a literature reference on page 406 of ref. 5 does not represent our view of the mechanism.

scheme (Scheme 4) similar to that proposed for the Leguminosae if *o*- rather than *p*-coumaric acid is involved in the early biosynthetic stages. Thus the aryl rearrangement would now involve an *ortho*-hydroxy group leading from intermediates **38a**/**38b** to spiro-compound **39** and hence the isoflavone **40**. Such a mechanism also throws light on the relationship between homoisoflavonoids biosynthetically derived from 2′-methoxychalcones, *e.g.* 4′-demethyl-3,9-dihydroeucomin **41**[22] and natural spirobenzocyclobutanes such as muscomosin **42**[22] *e.g.* the bis-spirocyclopropane **43** would undergo rearrangement to the spirocyclobutane **44** (Scheme 5).

However, recent work using an elicitor-challenged cell culture of *Pueraria lobata* indicates that in this plant system the 2-hydroxy group of compound **34** (R = OH), lost on dehydration (but none of the other oxygens), comes from an ^{18}O atmosphere rather than from water.[23] Sankawa and his colleagues[23] therefore propose removal of the 3-hydrogen in the flavanone **24** by the P450 enzyme to give a radical which undergoes a 1,2-aryl shift, the resulting radical at position 2 then being discharged by hydroxylation employing the enzyme in an $[Fe]^{IV}OH$ state. No specific function is assigned to the unmethylated *para*-hydroxy group of the aryl substituent, which appears to be essential for

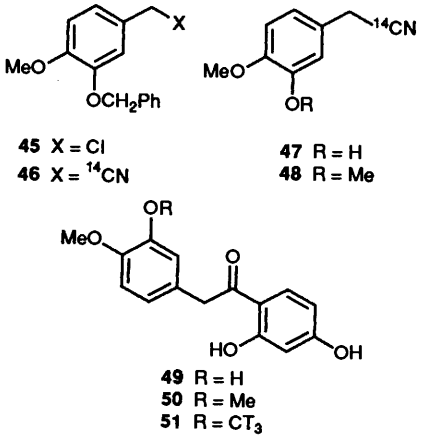


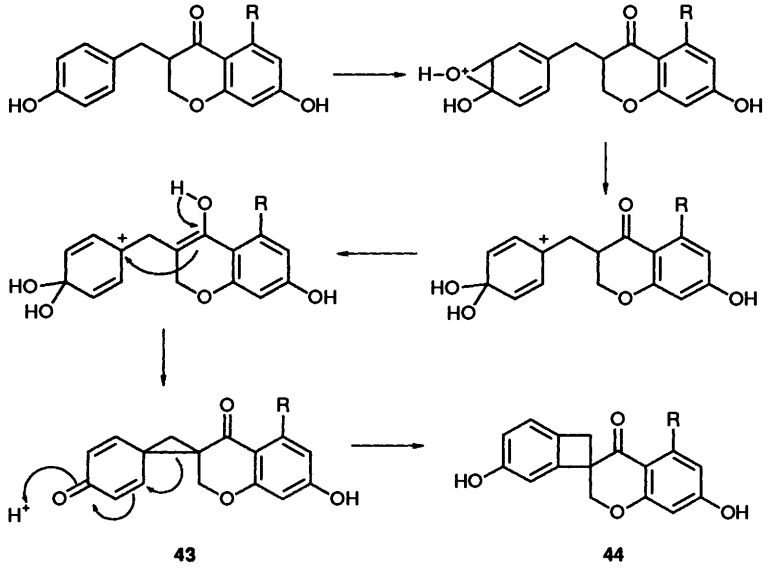
Scheme 4 Hypothesis for the flavanone–isoflavone rearrangement for monocotyledonous rotenoids lacking 3-hydroxylation



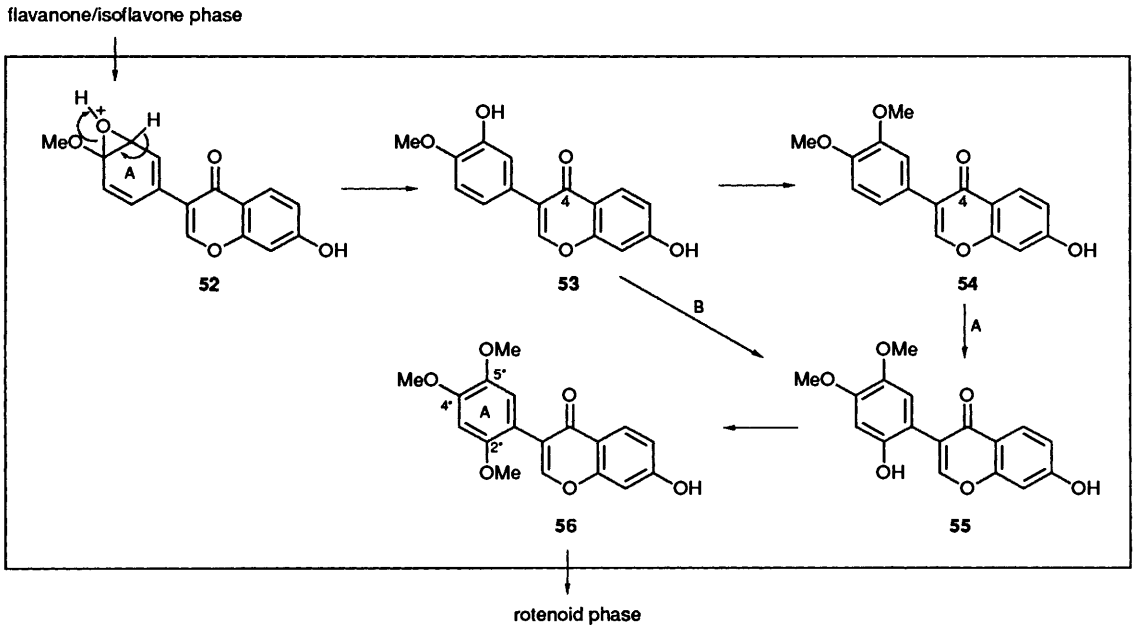
the rearrangement (it is known that 4-methoxycinnamic acid destined for isoflavone biosynthesis is first demethylated),[24] and, as judged by radical reactivities towards a sequence of substituted olefins,[25] the postulated radical formed at C-3 moves to a less stabilised position at C-2. More experimental information is desirable to establish an acceptable mechanism for the 1,2-aryl shift in the flavanone–isoflavone change.

The Hydroxylation/Methoxylation Phase (*Scheme 6*).—As mentioned above, 7-hydroxy-4′-methoxyisoflavone **27** is a good precursor (0.42%) of amorphigenin and so too is 7-hydroxy-2′,-4′,5′-trimethoxyisoflavone **56** (incorporation into amorphigenin 1.87%).[4] Between these two intermediates lies a phase in which the 2′- and 5′-methoxy groups are inserted and in this

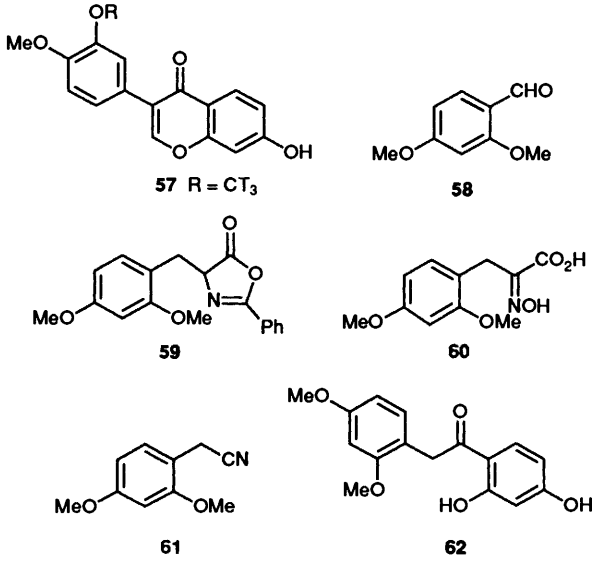




Scheme 5 Hypothesis for the formation of spiro-benzocyclobutanes from homoisoflavonides



Scheme 6 The hydroxylation/methoxylation phase



section we consider the ordering of these processes. In order to pursue this aim, a group of radiolabelled molecular tools was prepared. These were the 3′,7-dihydroxy-4′-methoxy-[4-^{14}C]-**53** and 7-hydroxy-3′,4′-dimethoxy-[4-^{14}C]-**54** isoflavones, together with the [3′-O(CH_3,CH_2T)] (abbreviated to [3′-OCT_3])-labelled substrate **57**. Further, 2′,7-dihydroxy-4′-methoxy-[2-^{14}C]isoflavone **64**, 7-hydroxy-2′,4′-dimethoxy-[2-^{14}C]isoflavone **63** and [4′-OCT_3]-7-hydroxy-4′-methoxyisoflavone **66**, were prepared and administered to *A. fruticosa*.

The [4-^{14}C]isoflavones **53** and **54** were made from the benzyloxy(methoxy) halide **45** by using displacement with sodium [^{14}C]cyanide to give nitrile **46**, which was debenzylated to give the phenol **47**, part of which was fully methylated to give **48**. Hoesch reaction with resorcinol then gave the two deoxybenzoins **49** and **50**, which were cyclised to the [4-^{14}C]-labelled isoflavones corresponding to **53** and **54** by using ethyl orthoformate and piperidine. The isoflavone **57** labelled with ^{3}H on the methyl group was made similarly by using [^{3}H]methyl iodide at the 3-hydroxy-4-methoxybenzyl cyanide methylation stage. 7-Hydroxy-2′,4′-dimethoxy-[2-^{14}C]isoflavone **63** was made from the aldehyde **58**, which was elaborated to the nitrile **61** *via* the azlactone **59** and the oxime of the derived α-keto acid (oxime **60**). Hoesch reaction gave compound **62**, which was converted into compound **63** by using ethyl [^{14}C]orthoformate and piperidine. The 2′-methoxy group was then selectively demethylated with aluminium chloride to give diol **64**. [4′-OCT_3]-7-Hydroxy-4′-methoxyisoflavone **66** was prepared *via* the Hoesch reaction of [4-OCT_3]-4-methoxybenzyl cyanide, itself made through methylation using tritiated diazomethane. Isoflavone formation was completed by using ethyl orthoformate.

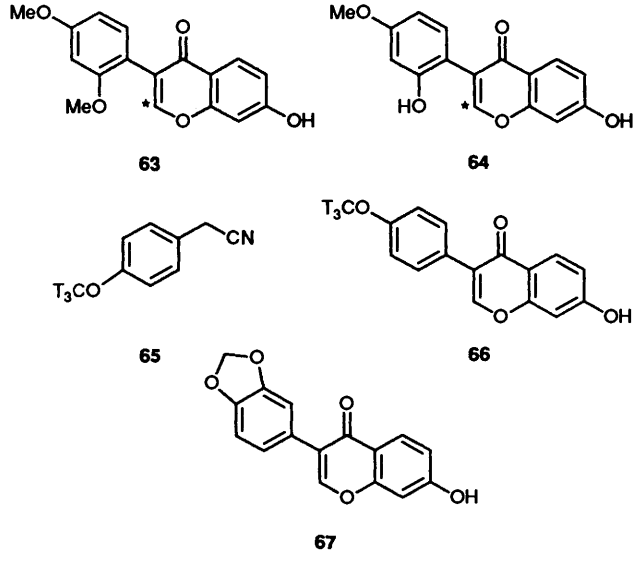
In the event 3′,7-dihydroxy-4′-methoxy-[4′-^{14}C]isoflavone **53** proved to be an excellent precursor of amorphigenin in the *A. fruticosa* germinating seedling system (0.97% incorporation) and the labelled 7-hydroxy-3′,4′-dimethoxyisoflavone **54** (≡**57**) also gave a satisfactory incorporation (0.17%) (Table 4). On the other hand, the labelled 7-hydroxy-2′,4′-dimethoxy compound **63** gave a very low incorporation (0.007%) and cannot be accepted as an intermediate. The 2′,7-dihydroxy-4′-methoxy compound **64** gave a slightly higher but still poor incorporation (0.03%). These results established that oxygenation occurs at C-3′ prior to C-2′ and were confirmed and supplemented by some competitive administration experiments which provide better comparisons than experiments carried out on different batches of seedlings at different times.

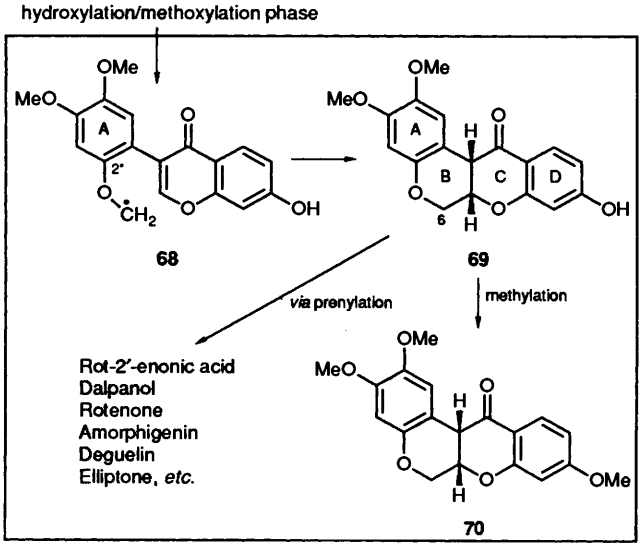
[3′-OCT_3]-3′,4′-Dimethoxy-7-hydroxyisoflavone **57** was administered along with 2′,4′-dimethoxy-7-hydroxy-[2-^{14}C]isoflavone **63** and whilst the former showed a satisfactory incorporation (0.30%), the latter was converted into amorphigenin in negligible yield. Administration of [3′OCT_3]-3′,4′-dimethoxy-7-hydroxyisoflavone **57** along with 3′,7-dihydroxy-4′-methoxy-[4-^{14}C]isoflavone **53** showed that they were somewhat similar as amorphigenin precursors (incorporations 0.086 and 0.067% respectively): although the ratio is significant, incorporations were markedly lower than usual and this is attributed to unfavourable growing conditions for the seedlings. Both substrates **66** and **53** were somewhat similarly incorporated into amorphigenin, giving very satisfactory figures (Table 4).

The major pathway in the hydroxylation/methoxylation is thus considered by us to be as in Scheme 6. Now that rearrangement to the isoflavone series has taken place (Scheme 2), *ortho*-hydroxylation **52** by the usual mechanism leads to 3′,7-dihydroxy-4′-methoxyisoflavone **53**, which is then methylated at O-3′ by *S*-adenosylmethionine in the usual way to give

Table 4 Administration of labelled candidate precursors having ring-A substitution variations at the isoflavone level: Incorporation into amorphigenin

Isoflavone	No of seedlings	Radioactivity administered (dpm)	Dose/ seedling (μg)	Uptake (%)	Amorphigenin isolated (mg)	Incorporation (%)
[4-^{14}C](**53**; 7-OH, 3′-OH, 4′-OMe)	150	9.52×10^5	6.7	71.4	8.2	0.97
[3′-OCT$_3$](**57**; 7-OH, 3′-OMe, 4′-OMe)	480	4.07×10^7	4.4	76.6	30.0	0.17
[2-^{14}C](**64**; 7-OH, 2′-OH, 4′-OMe)	600	2.26×10^5	16.7	72.4	30.8	0.03
[2-^{14}C](**63**; 7-OH, 2′-OMe, 4′OMe)	600	2.16×10^5	16.7	78.2	31.4	0.007
[3′OCT$_3$](**57**; 7-OH, 3′-OMe, 4′-OMe)	360	9.3×10^5	0.14	81.0	28.0	0.30
[2-^{14}C](**63**; 7-OH, 2′-OMe, 4′-OMe)	360	1.28×10^5	19.1	91.0	28.0	<0.001
[3′-OCT$_3$](**57**; 7-OH, 3′-OMe, 4′-OMe)	500	3.20×10^7	3.3	82.0	29.5	0.085
[4-^{14}C](**53**; 7-OH, 3′-OH, 4′-OMe)	500	4.45×10^6	2.0	90.0	29.5	0.066
[4′-OCT$_3$](**66**; 7-OH, 4′-OMe)	250	1.30×10^7		76.1	12.8	1.08
[4-^{14}C](**53**; 7-OH, 3′OH, 4′-OMe)	250	1.49×10^6		74.2	12.8	1.02





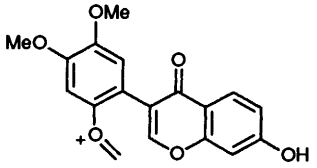
Scheme 7 The rotenoid phase

diether **54**. 6′-Hydroxylation of compound **54**, followed by another methylation, of the diphenol, **55**, then leads to triether **56** which, as mentioned above, is an excellent amorphigenin precursor. Both isoflavones **53** and **54** are good precursors, but the weight of evidence is not adequate to say if the pathway *must* proceed through intermediate **54** (route A) or whether there is also a direct pathway (route B) leading to 2′-oxygenation followed by methylation. It is possible, though unlikely, that diether **54** is 3′-demethylated to monoether **53** before passing on to diol **55**. There is room in our results for subsidiary processes operating on a grid pattern. Thus there could be a small contribution (Table 4) involving conversion of diol **64** into (labelled) isoflavones **55** or **56**, though diol **64** might be a non-natural substrate. One group of rotenoids is based on a 2,3-methylenedioxy rather than a 2,3-dimethoxy sequence and it is likely that this departure takes place on intermediate **53**, giving compound **67** *via* a methoxy radical.[26]

The Rotenoid Phase (*Scheme 7*).—Our earlier radiochemical results show clearly that the [^{14}C]-labelled 2′-methoxy carbon of the isoflavone **56** becomes the [^{14}C]-labelled 6-methylene of amorphigenin or rotenone, and that the corresponding isoflavanone is much less acceptable as a precursor.[4] The cyclisation process shows some similarity to methylenedioxy formation[26] and is represented in Scheme 7 as a radical process in which a methoxyl radical **68** adds to the carbonyl-conjugated double bond. On the other hand a mechanism involving an ionic species **71** is an alternative possibility.[4] Electrocyclisation then leads to a carbonium ion **72** which is stabilised by electron release from ring-A despite the unfavourable adjacent carbonyl. Such a reaction would be completed by hydride donation at C-12a. It may be noted, however, that an ionic mechanism would be disfavoured for non-Leguminous natural rotenoids in which electron release from ring-A is much diminished, and this leads us to prefer the radical interpretation. Natural rotenoids are known only in the thermodynamically stable 6a–12a *cis*-form and at present there is no evidence that they are formed in a labile *trans*-form which can, however, be made synthetically.[27]

9-Demethylmunduserone **69**, the outcome of this phase of amorphigenin biosynthesis, is well incorporated into amorphigenin by *A. fruticosa* {1.14% for synthetic (±)-[6-^{3}H] material}[4] and can be viewed as the prototype of a group of rotenoid cores having variation in the oxygen-substitution pattern of ring-A (methylenedioxy, 4-methoxy, or no substitution) and in ring-D (11-hydroxy, 8-methoxy). These head natural rotenoid sub-series.[1,4] In the case of 9-demethylmunduserone, the structure is found in the Leguminosae as munduserone **70** from *Mundulea sericea*, methylated at O-9.[28] In this state it may be viewed as a biosynthetic terminus since a free 9-hydroxy group is probably necessary for prenylation at C-8 or C-10 to take place. More commonly in rotenoids, prenylation at C-8 occurs giving rot-2′-enonic acid **73** (whose presence and rapid turnover in *Amorpha fruticosa* can be demonstrated by isotope dilution).[29]

In *Milletia pachycarpa* rot-2′-enonic acid is found as an



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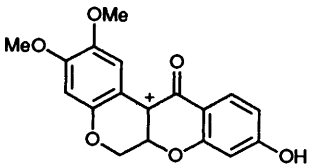

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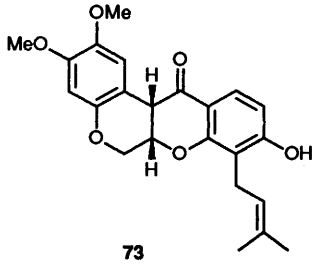
accumulation product,[30] but usually the prenyl residue undergoes further oxidative modification leading, in the case of rot-2′-enonic acid, to dalpanol, rotenone, amorphigenin, deguelin, elliptone, *etc.* Investigation of these prenyl oxidations is considered further in our following papers.

All our evidence indicates that prenylation is a late-stage process occurring after the formation of the complete A/B/C/D rotenoid ring system. Thus the labelled isoflavonoids **74** and **75**, having fully formed E-rings with the necessary appendages, are not incorporated into amorphigenin by *A. fruticosa*.[4] Closure of the B-ring must precede prenylation and further elaboration of the prenyl group. Making up the whole family of natural rotenoids are examples in which the B/C-ring system is modified by further hydroxylation at C-6 or -12a (structure **76**) or by formal dehydrogenation at C-6a, -12a (structure **77**): occasionally the 12-carbonyl is found reduced as in dihydrodalbinol. It is likely that modifications of this type occur after the construction of the rotenoid core, though there is evidence that 12aβ-hydroxyamorphigenin does not originate solely through hydroxylation of fully formed amorphigenin.[29] Whilst there is still considerable scope for further understanding of the pre-prenylation processes of rotenoid formation, their enzymology and control, the main outline presented in Schemes 1, 3, 6 and 7 now seems fairly well established experimentally as a working system.



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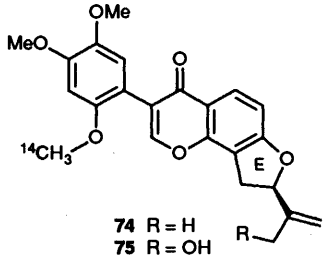
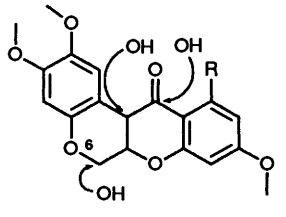


74 R = H
75 R = OH



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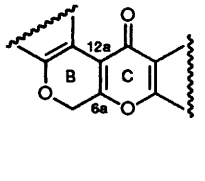

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Experimental

Radioactivity was measured using an Intertechnique SL 3000 liquid scintillation counter, samples being counted in duplicate in Nuclear Enterprises Ne 250 Fluid (10 cm^3) in plastic vials. Counting efficiency was determined using Amersham ^{14}C and ^{3}H external standards. Light petroleum refers to the fraction boiling in the range 60–80 °C, unless otherwise stated.

Preparation of A. fruticosa *Seeds for Germination.*—To ensure rapid and efficient germination it is necessary to remove the protective husk. This was done by light mechanical grinding between two layers of sandpaper, followed by sieving (0.75 mm mesh) to remove fine debris and then separation of seeds from husks by flotation on water. Seeds were sterilised by washing in ethanol (2 × 15 min) and then aq. copper(II) sulphate (8%; 10 min). Alternatively, prevention of mould growth could be attained by keeping the incubation temperature below 31 °C and by resowing sound seedlings on moist tissue paper daily. Where fungal infection caused difficulty, seedlings were washed with sterile, distilled water (Millipore 0.22 μm filter unit) containing the systemic fungicide 'Benylate' (0.1%). Germination was initiated by heating the seeds to 50 °C for ~15 min.

Germination of seeds.—The pretreated seeds were sown on moist tissue paper in plastic trays covered by foil. Trays were kept in the dark at 28–31 °C for 3–7 days depending on the rate of development, any unhealthy seedlings being discarded. Seedlings were watered daily (tap water) or with 0.1% 'Benylate' if fungal infection was apparent. At the end of the germination period, seedlings were transferred to sterile plastic Petri dishes with the desired volume of sterile buffer solution containing the radioactive precursor. Dormant seeds contain amorphin (amorphigenin vicianoside): the aglycone is formed, with turnover, on germination.[4]

Work-up Procedure for Radioactive Tracer Experiments.—Seedlings and buffer were transferred from their Petri dishes to a Buchner funnel and washed repeatedly with water. The aqueous washings were made up to a standard volume and an aliquot was added to dioxane-based scintillation fluid and counted to obtain the percentage uptake. The seedlings were ground with sand or broken glass in a mortar containing ethanol–water (1:1) (10–15 cm^3). Ethanol (100 cm^3) was added and the mixture was heated under reflux (20 min) and then filtered through a cotton-wool plug: the process was repeated 3–4 times. The combined ethanolic extracts were evaporated to dryness and the residue was taken up into water (20–50 cm^3) and washed with light petroleum (3 × 50 cm^3). The aqueous layer was extracted with diethyl ether (3 × 60 cm^3). Drying and evaporation of the ether extracts gave a green solid, which was purified by preparative layer chromatography (PLC), developed first with methanol–chloroform (1:20) and then benzene–ethyl acetate–methanol–propan-1-ol (6:4:1:3) (BEMP). The bands corresponding to amorphigenin were removed from the plates and extracted with methanol–chloroform (3:1). Filtration through cotton wool and evaporation gave a solid, which was taken up in chloroform (2 cm^3) and forced through a cotton-wool plug contained in the end of a Pasteur pipette. Evaporation gave a solid, which was crystallised, dried *in vacuo* and counted.

The above procedure was modified a little for batches of <300 seedlings. When dealing with small amounts of radioactive amorphigenin (1–2 mg) cold carrier amorphigenin was added to enable crystallisation to be carried out on a 10 mg scale. Cold amorphigenin was obtained as follows. *A fruticosa* seeds (350 g) were ground and Soxhlet extracted with light petroleum, then with methylene dichloride, then with chloroform–methanol (9:1), each for 24 h. The last extract was evaporated to dryness and dried *in vacuo* for 10 h. Trituration with hot benzene yielded a granular solid, which was filtered off through a thin Kieselghur pad and washed with benzene. Crystallisation from methanol–water (10:1) (charcoal) gave crude amorphin (17.3 g). The latter was hydrolysed under reflux (3 h) with 5% aq. sulphuric acid. Extraction with chloroform and crystallisation from methanol–chloroform gave amorphigenin (1.2 g), m.p. 194 °C (lit.,[2] m.p. 195–196 °C).

Preparation and NMR Investigation of ^{13}C*-Amorphigenin from* ^{13}C*-Acetate Administrations.*—The administration and work-up conditions were similar to those above, and other in-

formation is given in Tables 1 and 2. The ^{13}C-labelled specimen, examined by NMR spectroscopy using overnight accumulation of 41 088 pulses, recycle delay 2.5 s, τ 5.2 ms (optimised for 47 Hz couplings as a compromise value), showed the couplings of C-9, -10, -11 and -11a. When the recycle delay was increased (5 s) the couplings of C-7a and -8 became visible after 8000 pulses. The spectral width used was 25 kHz and the spectrum was acquired over 32K data points. It was Fourier transformed over 128K data points by using a simple exponential line broadening of 1 Hz. The spectrometer employed was a Bruker AM400 operating at 100.61 MHz. A picture of the spectrum is included in our prelimary communication.[9]

3-Benzyloxy-4-methoxybenzyl Chloride **45**.—3-Benzyloxy-4-methoxybenzaldehyde was prepared from the corresponding 3-hydroxy-4-methoxy compound (7.6 g, 0.05 mol) by treatment with sodium ethoxide [from sodium (1.26 g, 0.055 g-atom) and ethanol (50 cm^3)] followed by benzyl bromide (9.45 g, 0.55 mol). Reflux (2 h) gave the 3-benzyloxy aldehyde (11.9 g, 98%), which was crystallised from propan-2-ol as needles, m.p. 61–63 °C (lit.,[31] 63 °C).

Sodium borohydride (1 g) in water (20 cm^3) was added to a solution of 3-benzyloxy-4-methoxybenzaldehyde (9.68 g, 0.04 mol) in ethanol (40 cm^3) at 20–25 °C. After stirring (1 h) and work-up (pH 5) the solid 3-benzyloxy-4-methoxybenzyl alcohol product (9.7 g, almost theoretical) was filtered off and crystallised from ethyl acetate–light petroleum, m.p. 71–72 °C (lit.,[32] 69–71 °C).

A solution of the alcohol (4.88 g, 0.02 mol) in dry diethyl ether (40 cm^3) containing pyridine (0.4 cm^3) was treated dropwise with a solution of thionyl dichloride (2.97 g, 0.022 mol) in dry diethyl ether (20 cm^3) while the temperature was kept below 5 °C and the reaction mixture was stirred for 30 min. Iced water was then added and the ether layer was separated and washed. The organic layer was dried ($MgSO_4$) and evaporated, and the oil which crystallised (5.08 g, 97%) was recrystallised from ethyl acetate–light petroleum as cubes. 3-Benzyloxy-4-methoxybenzyl chloride **45** had m.p. 77–78 °C. (lit.,[32] 79 °C).

3-Benzyloxy-4-methoxybenzyl [^{14}C]Cyanide **46**.—A solution of 3-benzyloxy-4-methoxybenzyl chloride (525 mg, 2 mmol) in dry acetone (4 cm^3) was refluxed (2 h) with potassium cyanide (43 mg), potassium iodide (14.3 mg) and urea (143 mg). Potassium [^{14}C]cyanide (60 mg; 0.325 mCi $mmol^{-1}$) was then added and the mixture was heated under reflux for a further 6 h. More 'cold' potassium cyanide (40 mg) was then added and the mixture was refluxed for 4 h more. TLC examination [developer: toluene–ethanol (9:1)] showed one spot, R_f 0.47, corresponding with the unlabelled nitrile below. Water was added and the product was extracted with methylene dichloride to give the crude nitrile **46** (475 mg; specific activity 0.125 mCi $mmol^{-1}$) which was used without further purification. In a second preparation the product was purified by chromatography on silica gel, with chloroform as eluent, when it was crystallised in needles (293 mg, 58%). Using a similar method of preparation, a 'cold' specimen of the nitrile was prepared in 92% yield, crystallising from propan-2-ol, m.p. 80–81 °C (lit.,[33] 80 °C).

3-Hydroxy-4-methoxybenzyl [^{14}C]Cyanide **47**.—This was made as an oil in almost theoretical yield from the labelled benzyl ether above (457 mg, 1.82 mmol) by hydrogenolysis in dry acetone (10 cm^3) over 10% palladium on carbon (50 mg) under room conditions for 1 h. A 'cold' specimen was also prepared. The benzoyl derivative was made from 'cold' nitrile **47** (148 mg) in almost theoretical yield by stirring it in 10% aq. sodium hydroxide (1 cm^3) and benzoyl chloride (141 mg) and was crystallised from ethyl acetate–light petroleum (40–60 °C) (1:4) as needles m.p. 62–63 °C, ν_{max} 1740 cm^{-1}. It was indistinguishable (TLC) from the same derivative made from the [^{14}C]-labelled compound.

3,4-Dimethoxybenzyl [^{14}C]Cyanide **48**.—Dimethyl sulphate (139 mg, 1.1 mmol) was added to a stirred solution of 3-hydroxy-4-methoxybenzyl [^{14}C]cyanide **47** (148 mg, 0.91 mmol) in 10% aq. sodium hydroxide (1 cm^3) at 0 °C. After the mixture had been stirred for 1 h, the product was extracted with methylene dichloride and the extract was evaporated to give the [^{14}CN]-compound **48** (143 mg, 89%), which showed only one spot on TLC [toluene–ethanol (9:1)] identical with that given by authentic crystalline 'cold' material. The latter, made by a similar procedure, had m.p. 62–65 °C (lit.,[34] 68 °C).

[3-OCT_3]-3-4-Dimethoxybenzyl Cyanide (cf. **48**).—A solution of 'cold' 3-hydroxy-4-methoxybenzyl cyanide (330 mg, 2.02 mmol) in dry acetone (12 cm^3) containing anhydrous potassium carbonate was refluxed (15 min), methyl iodide (132 mg, 0.94 mmol) was added, and the mixture was refluxed for a further 15 min. A solution of tritiated methyl iodide in toluene (0.5 cm^3) was then added and the mixture was refluxed (30 min). Unlabelled methyl iodide (165 mg) was again added and the whole reaction mixture was refluxed for 2 h. The product was filtered and evaporated to dryness. The oil was chromatographed over silica gel, and eluted with chloroform, to give [3-OCT_3]-3,4-dimethoxybenzyl cyanide (290 mg, 81%), as needles from methanol, m.p. 61–63 °C, specific activity 32 mCi $mmol^{-1}$.

2,4-Dihydroxyphenyl 3'-Hydroxy-4-methoxybenzyl [^{14}C]-Ketone **49**.—3-Benzoyloxy-4-methoxybenzyl [^{14}C]cyanide (200 mg, 0.75 mmol) and resorcinol (165 mg, 1.50 mmol) were added to a stirred solution of fused zinc chloride (265 mg, 2.0 mmol) in dry diethyl ether (10 cm^3), cooled to 0 °C, and saturated with dry hydrogen chloride. The mixture was kept overnight and the ether was then decanted to leave a red oil, which was boiled with water for 1 h. The crude benzoyloxy ketone was filtered off and crystallised from propan-2-ol.

The benzoyloxy ketone was hydrolysed with soldium hydroxide in methanol–water to give 2,4-dihydroxyphenyl 3'-hydroxy-4'-methoxybenzyl [^{14}C]ketone **49** (52 mg, 31%) after crystallisation from ethyl acetate–light petroleum (40–60 °C). The yield in a second [^{14}CN]-labelled preparation was 35%. A 'cold' specimen had m.p. 166–168 °C (lit.,[35] 167 °C).

2,4-Dihydroxyphenyl 3',4'-Dimethoxybenzyl [^{14}C]Ketone **50**.—3,4-Dimethoxybenzyl [^{14}C]cyanide (133 mg, 0.75 mmol) and resorcinol (165 mg, 1.5 mmol) were added to fused zinc chloride (265 mg) in dry diethyl ether (10 cm^3). The mixture was cooled to 0–5 °C and saturated with hydrogen chloride gas. Work-up gave the title compound (82 mg, 38%) after crystallisation from ethanol. A 'cold' specimen had m.p. 183–184 °C (lit.,[36] 182–183 °C). On TLC (ethyl acetate) both specimens gave a single spot, R_f 0.70.

2,4-Dihydroxyphenyl [3-OCT_3]3',4'-Dimethoxybenzyl Ketone **51**.—Hoesch reaction as above, using [3-OCT_3]-3,4-dimethoxybenzyl cyanide (200 mg, 1.13 mmol), resorcinol (250 mg, 2.26 mmol) and fused zinc chloride (408 mg, 3 mmol) gave the title compound (170 mg, 67%) as needles from methanol, m.p. 181–183 °C (lit.,[36] 182–183 °C).

3',7-Dihydroxy-4'-methoxy-[4-^{14}C]isoflavone **53**.—A solution of 2,4-dihydroxyphenyl 3'-hydroxy-4'-methoxybenzyl [^{14}C]ketone (50 mg, 0.18 mmol) in dry dimethylformamide (DMF) (1 cm^3) containing piperidine (0.01 cm^3) and triethyl orthoformate (0.1 cm^3) was refluxed under nitrogen (1 h). Water was added and the solid which precipitated was crystallised from ethanol to constant activity, to give title compound **53**, (25 mg, 47%), m.p. 245–246 °C. A 'cold' sample made similarly (62%

yield) had m.p. 242–243 °C (lit.,[35] 245–247 °C). On TLC (ethyl acetate) both samples gave one spot, R_f 0.52. A second labelled specimen was also prepared (24 mg, 60%), crystallised to constant activity, 6.0 mCi mmol^{-1}.

7-Hydroxy-3′,4′-dimethoxy-[4-^{14}C]*isoflavone* **54**.—Following the method above, 2,4-dihydroxyphenyl 3′,4′-dimethoxybenzyl [^{14}C]ketone (75 mg, 0.26 mmol) was allowed to react with ethyl orthoformate (0.1 cm^3) in dry DMF (1 cm^3 containing piperidine) to give the isoflavone **54** (42 mg, 54%), m.p. 258–259 °C after crystallisation from methanol to constant activity. A 'cold' sample, prepared similarly, had m.p. 258–259 °C (lit.,[36] 257–258 °C). On TLC (ethyl acetate) both samples gave a single spot, R_f 0.54.

7-Hydroxy-[3′-*OCT*$_3$]*3′,4′-dimethoxyisoflavone* **57**.—2,4-Dihydroxyphenyl-[3′*OCT*$_3$]3′,4′-dimethoxybenzyl ketone (130 mg, 2.2 mmol) and triethyl orthoformate (0.2 cm^3) in DMF (1 cm^3) containing piperidine (0.02 cm^3) gave, when treated as above, the title compound **57** (65 mg, 50%), m.p. 256–258 °C, needles from methanol (lit.,[36] 257–258 °C).

2,4-Dimethoxybenzyl Cyanide **61**.—2,4-Dimethoxybenzaldehyde (4.98 g, 0.03 mol), hippuric acid (5.90 g, 0.033 mol) and potassium carbonate (2.07 g, 0.015 mol) in dry DMF (15 cm^3) were added to acetic anhydride (6.14 cm^3). The product was warmed to initiate reaction and then set aside (6 h). Water (60 cm^3) was added and after storage overnight, the azlactone **59** (8.7 g, 94%) was filtered off.

Azlactone **59** (6.2 g, 0.02 mol) was refluxed for 2 h with sodium hydroxide (4 g) in water (20 cm^3) and, after cooling, a solution of hydroxylamine hydrochloride (2.1 g, 0.03 mol) in water (5 cm^3) was added and the mixture was stirred for 2 h at 20 °C. The product was cooled to 0 °C and acidified with conc. hydrochloric acid. The precipitated mixture of the oxime of the pyruvic acid, compound **60**, and benzoic acid was collected and dried and then added to acetic anhydride (15 cm^3) at 40–50 °C, the mixture was stirred for 1 h. It was then poured into water (100 cm^3) and treated with sodium carbonate to dissolve benzoic acid and the 2,4-dimethoxybenzyl cyanide was filtered off. The latter was crystallised from propan-2-ol (0.80 g, 22%), m.p. 74–75 °C (lit.,[37] 76 °C).

2,4-Dihydroxyphenyl 2′,4′-Dimethoxybenzyl Ketone **62**.—2,4-Dimethoxybenzyl cyanide (1.5 g, 8 mmol), resorcinol (1.84 g, 16.8 mmol) and zinc chloride were subjected to the Hoesch reaction as described above. The title ketone (0.92 g, 38%) was crystallised from ethanol, as plates, m.p. 153–155 °C (lit.,[37] 154 °C).

7-Hydroxy-2′,4′-dimethoxy-[2-^{14}C]*isoflavone* **63**.—2,4-Dihydroxyphenyl 2′,4′-dimethoxybenzyl ketone (140 mg, 0.48 mmol) was dissolved in dry DMF (1.5 cm^3) containing piperidine (0.04 cm^3) and triethyl [^{14}C]orthoformate (70 mg, 0.47 mmol) (0.5 mCi, ex Amersham International) and the mixture was refluxed under nitrogen for 1 h. Since TLC monitoring showed a little unchanged starting material, 'cold' triethyl orthoformate (20 mg) was added and the mixture was refluxed 1 h. After the mixture was poured into water (4 cm^3) the isoflavone **63** was filtered off, and crystallised from DMF–water (1 : 1) to constant activity (78 mg, 55%), m.p. 263–264 °C. A 'cold' specimen, similarly prepared (65% yield), had m.p. 262–264 °C (lit.,[37] 265–267 °C).

2′,7-Dihydroxy-4′-methoxy-[2-^{14}C]*isoflavone* **64**.—7-Hydroxy-2′,4′-dimethoxy-[2-^{14}C]isoflavone **63** (50 mg, 0.17 mmol) and anhydrous aluminium chloride (33 mg, 0.25 mmol) were heated under reflux (3 h) in dry acetonitrile (1 cm^3) under nitrogen. The product was poured into water (3 cm^3) and the crude mixture was filtered off. Crystallisation to constant activity gave the title isoflavone **64** (23 mg, 49%), m.p. 211–212 °C. A 'cold' specimen prepared in 73% yield had m.p. 210–212 °C (lit.,[37] 212–215 °C).

[4-*OCT*$_3$]-4-*Methoxybenzyl Cyanide* **65**.—A solution of diazomethane in diethyl ether was prepared from *N*-methyl-*N*-nitrosourea (0.52 g) and 50% aq. potassium hydroxide (1.5 cm^3) in the usual way and dried over potassium hydroxide pellets. The dried solution was stirred with tritium oxide (0.05 cm^3; specific activity 90 mCi mmol^{-1}) and sodium hydroxide (5 mg) for 1 h at 0 °C. Meanwhile, 4-hydroxybenzyl cyanide (0.5 g, 3.8 mmol) in dry tetrahydrofuran (THF) (5 cm^3) was equilibrated with stirred tritium oxide (0.05 cm^3; specific activity 90 mCi mmol^{-1}) for 30 min at 0 °C. The latter solution was added to the diazo[^{3}H]methane solution and kept at 0 °C for 1 h. Most of the solvent was evaporated off, water (10 cm^3) was added, and the product was extracted with chloroform. Work-up gave crude [4-OCT$_3$]-4-methoxybenzyl cyanide (0.52 g; specific activity 16.2 mCi mmol^{-1}). This material was used in the Hoesch reaction without further purification. A 'cold' sample was also isolated as an oil.

7-Hydroxy-[4′-*OCT*$_3$]*-4′-methoxyisoflavone* **66**.—Using [4-OCT$_3$]-4-methoxybenzyl cyanide **65** (0.5 g, 3.4 mmol) and resorcinol (0.7 g, 6.8 mmol), Hoesch reaction as described above gave 2,4-dihydroxyphenyl-[4′-OCT$_3$]-4′-methoxybenzyl ketone (0.34 g, 39%), m.p. 160–161 °C. A 'cold' sample, made similarly (42% yield), had m.p. 159–160 °C (lit.,[38] 161–162 °C). 2,4-Dihydroxyphenyl-[4′-OCT$_3$]-4′-methoxybenzyl ketone (0.34 g, 1.32 mmol) was refluxed with triethyl orthoformate (0.35 cm^3) and piperidine (0.1 cm^3) in DMF for 1 h to give title isoflavone **66** (253 mg, 69%), crystallised from ethanol to constant activity, m.p. 258–259 °C, specific activity 16.0 mCi mmol^{-1}. A 'cold' specimen, similarly prepared, had m.p. 258–259 °C (lit,[39] 257–258 °C).

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References

1 L. Crombie, *Nat. Prod. Rep.*, 1984, **1**, 3.
2 L. Crombie and M. B. Thomas, *J. Chem. Soc. C*, 1967, 1796.
3 L. Crombie, C. L. Green and D. A. Whiting, *J. Chem. Soc. C*, 1968, 3029.
4 L. Crombie, P. M. Dewick and D. A. Whiting, *J. Chem. Soc., Perkin Trans. 1*, 1973, 1285.
5 W. Heller and G. Forkman, *The Flavonoids*, ed. J. B. Harborne, Chapman and Hall, London and New York, 1988, ch. 11.
6 J. Ebel and K. Hahlbrock, *The Flavonoids, Advances in Research*, ed. J. B. Harborne and T. J. Mabry, Chapman and Hall, London and New York, 1982, ch. 11.
7 E. Haslam, *Metabolites and Metabolism*, Clarendon Press, Oxford, 1985.
8 A. Bax, R. Freeman and S. P. Kempsall, *J. Am. Chem. Soc.*, 1980, **102**, 4849.
9 For a preliminary communication see L. Crombie, I. Holden, N. Van Bruggen and D. A. Whiting, *J. Chem. Soc., Chem. Commun.*, 1986, 1063.
10 A. Stoessl and J. B. Stothers, *Z. Naturforsch., Teil C*, 1979, **34**, 87.
11 M. J. Boland and E. Wong, *Eur. J. Biochem.*, 1975, **50**, 383.
12 M. J. Begley, L. Crombie, A. M. London, J. Savin and D. A. Whiting, *J. Chem. Soc., Perkin Trans. 1*, 1987, 2775.
13 A. Pelter, *Tetrahedron Lett.*, 1968, 897; A. Pelter, J. Bradshaw and R. E. Warren, *Phytochemistry*, 1971, **10**, 835.
14 M. Hagmann and H. Grisebach, *FEBS Lett.*, 1984, **175**, 199.
15 G. Kochs and H. Grisebach, *Eur. J. Biochem.*, 1986, **155**, 311.

16 U. Weiss and J. M. Edwards, *The Biosynthesis of Aromatic Compounds*, Wiley-Interscience, New York, 1980; R. B. Herbert, *The Biosynthesis of Secondary Metabolites*, Chapman and Hall, London, 1981; E. Haslam, *The Shikimate Pathway*, Butterworths, London, 1974.
17 P. R. Ortiz de Montellano in *Cytochrome P450*, Ed. P. R. Ortiz de Montellano, Plenum Press, New York, 1986.
18 H. Grisebach and H. Zilg, *Z. Naturforsch., Teil B*, 1968, **23**, 494.
19 I. Messana, F. Ferrari and A. E. Goulart Sant'Ana, *Phytochemistry*, 1986, **25**, 2688.
20 S. Kadota, N. Lami, Y. Tezuka and T. Kikuchi, *Chem. Pharm. Bull.*, 1988, **36**, 834.
21 A. S. Shawl, N. Mengi, L. N. Misra and Vishwapaul, *Phytochemistry*, 1988, **27**, 3331; M. Ahmed, B. K. Datta and A. S. S. Rouf, *Phytochemistry*, 1990, **29**, 1709.
22 L. Camarda, L. Merlini and G. Nasini, *Heterocycles*, 1983, **20**, 39; M. Adinolfi, G. Barone, M. Belardini. R. Lanzetta, G. Laonigro and M. Parrilli, *Phytochemistry*, 1984, **23**, 2091; M. Adinolfi, G. Barone, R. Lanzetta, G. Laonigro, L. Mangoni and M. Parrilli, *Phytochemistry*, 1985, **24**, 624.
23 T. Hakamatsuka, M. F. Hashim, Y. Ebizuka and U. Sankawa, *Tetrahedron*, 1991, **47**, 5969; M. F. Hashim, T. Hakamatsuka, Y. Ebizuka and U. Sankawa, *FEBS Lett.*, 1990, **271**, 219.
24 E. Wong, *Phytochemistry*, 1968, **7**, 1751; J. Ebel, H. Achenbach, W. Barz and H. Grisebach, *Biochim. Biophys. Acta*, 1970, **215**, 203; W. Barz and H. Grisebach, *Z. Naturforsch., Teil B*, 1967, **22**, 627.
25 T. M. Lowry and K. S. Richardson, *Mechanism and Theory in Organic Chemistry*, Harper and Row, New York, 1976.
26 D. H. R. Barton, G. W. Kirby and J. B. Taylor, *Proc. Chem. Soc.*, 1962, 340.
27 M. J. Begley, L. Crombie. A. Hamid bin A. Hadi and J. L. Josephs, *J. Chem. Soc., Perkin Trans. 1*, 1989, 204.
28 N. Finch and W. D. Ollis, *Proc. Chem. Soc.*, 1960, 176.
29 L. Crombie, I. Holden, G. W. Kilbee and D. A. Whiting, *J. Chem. Soc., Perkin Trans. 1*, 1982, 789.
30 A. K. Singhal, R. P. Sharma, J. N. Baruha, S. V. Govindan and W. Herz, *Phytochemistry*, 1982, **21**, 949.
31 A. Lovecy, R. Robinson and S. Sugasawa, *J. Chem. Soc.*, 1930, 817.
32 C. Schopf, and L. Winterhalder, *Justus Liebigs Ann. Chem.*, 1940, **544**, 62.
33 E. Wong, *Tetrahedron Lett.*, 1963, 159.
34 T. Fujii, Y. Ueno and M. Mitsukuchi, *Chem. Pharm. Bull.*, 1971, **19**, 1374.
35 L. Farkas, A. Wolfner and V. Olechnowicz-Stepien, *Acta Chim. Acad. Sci., Hung.*, 1972, **74**, 367 (*Chem. Abstr.*, 1973, **78**, 16433).
36 M. Shamma and L. D. Stiver, *Tetrahedron*, 1969, **25**, 3887.
37 W. Cocker, T. B. H. McMurry and P. A. Staniland, *J. Chem. Soc.*, 1965, 1034.
38 H. Grisebach, *Z. Naturforsch., Teil B*, 1959, **14**, 802.
39 W. Baker, J. Chadderton, J. B. Harborne and W. D. Ollis, *J. Chem. Soc.*, 1953, 1852.

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