Pre-isoflavonoid Stages in the Biosynthesis of Amorphigenin: Ring-D Formation and Ring-A Migration

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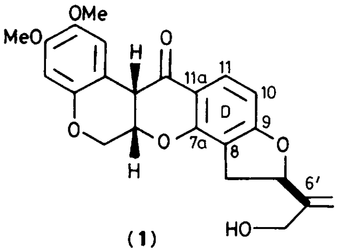
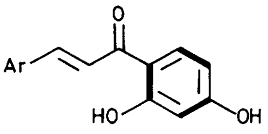
Despite low incorporations of doubly labelled [^{13}C]acetate during the biosynthesis of amorphigenin by *Amorpha fruticosa* seedlings, it has been possible to solve the ring-D folding by an INADEQUATE n.m.r. experiment; new proposals are made for the mechanism of the enzyme catalysed flavanone–isoflavone transformation step.

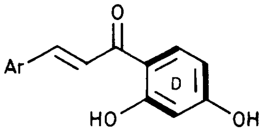
The use of ^{13}C–^{13}C couplings in n.m.r. experiments involving micro-organisms is well established, but in higher plants administration of an early precursor such as [^{13}C, ^{13}C]acetate often gives very low incorporations into complex natural products, particularly when the acetate–malonate pathway provides only part of the structure. ^{13}C–^{13}C Couplings then become extremely difficult to observe. However, using the INADEQUATE pulse sequence,[1] we have been able to observe ^{13}C–^{13}C satellite peaks indiscernible in the normal broad band decoupled off-resonance spectrum; the method basically nulls the single quantum transitions of the large ^{13}C natural abundance signal, permitting observation of the



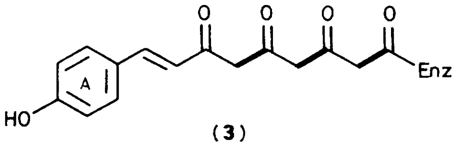
(1)



(2a)



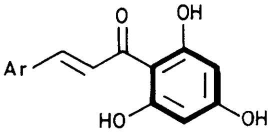
(2b)



(3)

$^{13}CH_3-^{13}CO_2H$

(4)



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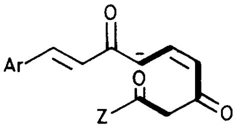
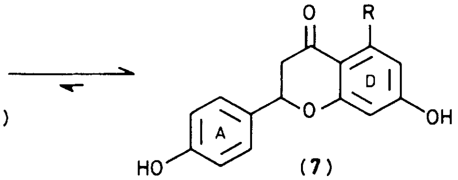


(6)

(2)

(Ar = p-HOC$_6$H$_4$)



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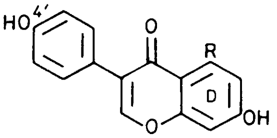
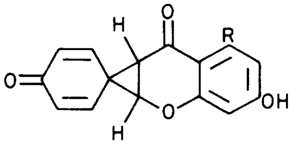
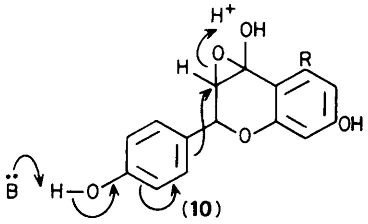


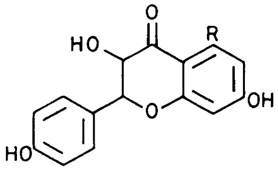
(8) a; R = H
b; R = OH



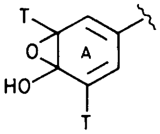
(9)



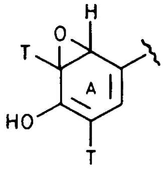
(10)



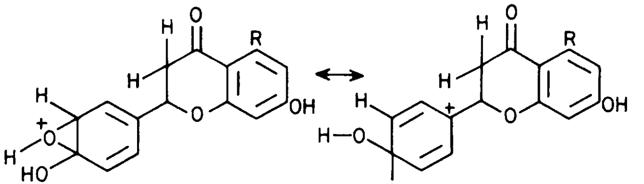
(11)



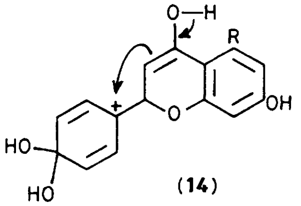
(12a)



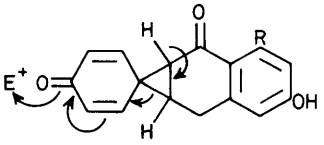
(12b)



(13a) **(13b)**



(14)



(15)

double quantum transitions of the satellites. We have employed the method to investigate the details of ring-D formation in the rotenoid amorphigenin (**1**).

Sodium [1,2-^{13}C]acetate (210 and 115 mg) was incubated with batches of 5-day old *Amorpha fruticosa* seedlings (800 and 1000) for 36 h to give, on work-up, 31 and 28 mg of pure amorphigenin respectively. Samples were spiked with approx. 50 μCi of [1-^{14}C]acetate to measure uptakes and incorporations. For the two experiments these were: uptakes, 42 and 65%; incorporations, 0.034 and 0.048%. Dilutions were 212 and 156 giving maximal specific ^{13}C enrichments of 0.14 and 0.19% respectively over the three pairs of aromatic sites. The samples of amorphigenin were united (51 mg) and the results of an INADEQUATE experiment† are shown in Figure 1, whilst the ring-D resonances and coupling constants are given in Table 1. The data demonstrate a C-7a–8, C-9–10, C-11–11a coupling pattern.

Our earlier work[2] has shown that the chalcone (**2**), originating from p-coumaric acid by three acetate–malonate extensions, is the precursor to amorphigenin and its ring-D. A hypothetical polyketide entity (**3**), from labelled acetate (**4**), might cyclise to a phloroglucinol (**5**) followed by elision of one hydroxy group. This course is clearly not taken as it would lead to no specific ^{13}C–^{13}C coupling pattern, each carbon of the aromatic ring being equivalently coupled to another, structure (**5**). Dehydration before cyclisation clearly takes place, and this occurs with the terminal carboxy group forming C-7a and not C-11 of amorphigenin [*i.e.* (**2b**) rather than (**2a**) is the precursor of the rotenoid].‡ It seems probable that (**2b**) itself is the product of cyclisation of a *cis*-glutaconate type anion (**6**).

The post-chalcone stage in rotenoid biosynthesis involves conversion of the chalcone–flavanone system (**2**, Ar = p-HOC$_6$H$_4$)–(**7**) into an isoflavone (**8a**). This process involves an aryl shift,[5,6] though its mechanism has remained obscure. A

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† Overnight accumulation of 41 088 pulses, recycle delay 2.5 s, τ 5.2 ms (optimised for a 47 Hz coupling), showed the couplings of C-9, 10, 11, and 11a. On increasing the recycle delay (5 s) the couplings of C-7a and -8 became visible after 8000 pulses. A spectral width of 25,000 Hz was used. The spectrum was acquired over 32 K data points and Fourier transformed over 128 K data points using a simple exponential line broadening of 1 Hz. A Bruker AM400 spectrometer operating at 100.61 MHz was employed.

‡ The direction of cyclisation is the same as for the pterocarpan pisatin.[4] In the latter case, because of its special nature as phytoalexin, an elicitor could be used, giving an enhanced ^{13}C enrichment level of 2.3 (±0.2)% per labelled carbon.

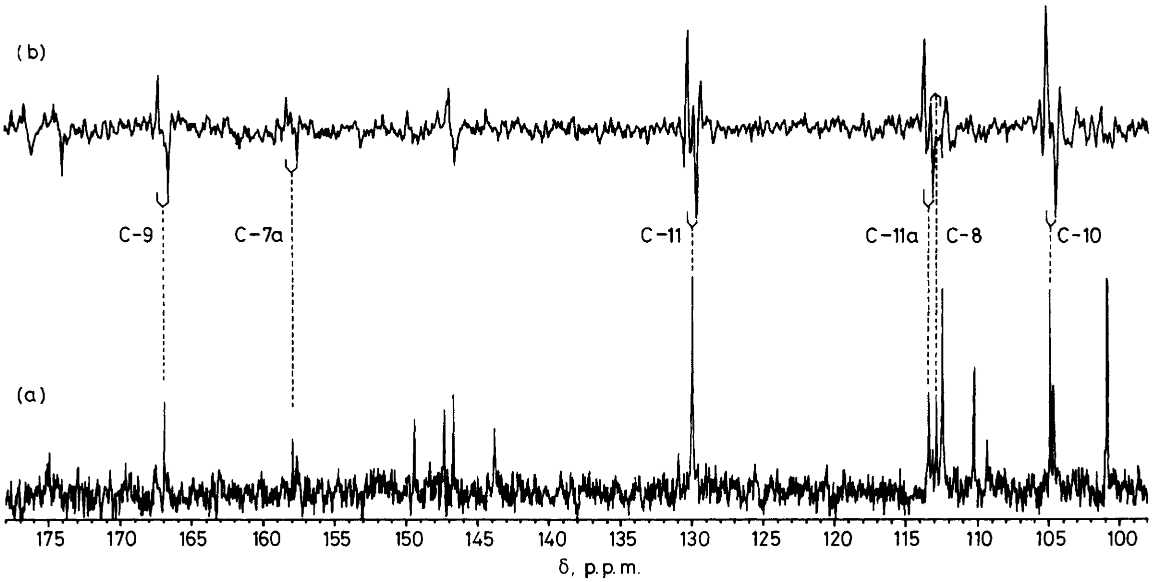

Figure 1. (a) ^{13}C N.m.r. spectrum of amorphigenin (**1**) arising from [^{13}C, ^{13}C]acetate adminstration; (b) INADEQUATE n.m.r. spectrum of the same sample

Table 1. Assignments to the ^{13}C–^{13}C coupled atoms of amorphigenin (**1**) resulting from the administration of [^{13}C, ^{13}C]acetate.

Resonance[a] δ, ppm	Carbon atom	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 with literature data[3] if digitisation error is taken into account. An apparent satellite was noted near C-6′ at δ 146.7 (J 47 Hz), but is unpartnered.

spiro-dienone intermediate (**9**)[7] is consistent with our earlier experimental data on amorphigenin biosynthesis[8] but nothing is known of the enzymes involved. Recently Hagmann and Grisebach[9,10] have isolated from an elicitor-challenged soya bean cell culture a microsomal preparation capable of converting (**7**, R = H) or (**7**, R = OH) into (**8a**) or (**8b**) respectively. This is an NADPH and dioxygen dependent reaction and there is evidence that the mono-oxygenase may belong to the cytochrome P-450 group. Hagmann and Grisebach interpret the reaction leading to (**9**) as involving epoxidation of an enolate of (**7**) to give (**10**), rearranging as shown.

On chemical grounds one might expect (**10**) not to rearrange in this way but to form the flavanol (**11**) rather than undergo aryl migration. We therefore suggest that the important experimental observations may be interpreted in a different way. The enzyme system involved bears a strong resemblance to the aryl hydroxylases which convert phenylalanine into tyrosine and then tyrosine into dopa: the first reaction involves an NIH shift with retention of most of the tritium whilst the second, *ortho*-hydroxylation, when performed on a 3′,5′-tritiated phenol, involves the complete loss of one of these tritium atoms.[11,12] This indicates that an epoxide (**12a**) rather than (**12b**) prefaces the second hydroxylation. In the isoflavanone system protonation of such an epoxide leads to (**13**), written as its resonance forms (**13a**) $\longleftrightarrow$ (**13b**). Loss of a proton from (**13b**), perhaps *via* the enolate (**14**), then gives the spiro-compound (**9**). The latter is decomposed by an electrophile (**15**) to give the isoflavone; the electrophile, if a proton, gives isoflavone (**8**), or if the equivalent of CH_3^+ (from *S*-adenosylmethionine), the 4′-methoxy derivative. There is evidence that the latter is the situation in rotenoid biosynthesis.[2,8]

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