Biosynthesis of Isoflavonoid Phytoalexins: Incorporation of Sodium [1,2-$^{13}C_2$]Acetate into Phaseollin and Kievitone

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^{13}C-NMR analysis of the isoflavonoid phytoalexins phaseollin and kievitone produced by feeding sodium [1,2-$^{13}C_2$]acetate to wounded bean (*Phaseolus vulgaris*) cotyledons has demonstrated the incorporation of intact acetate units into the aromatic A rings. Phaseollin shows a specific folding of the polyketide chain, whereas kievitone exhibits a randomisation of label in accordance with the intermediacy of a 2′,4′,6′-trihydroxylated chalcone during its formation. In neither case was sufficient label incorporated into the isoprenoid substituents to allow further analysis.

Introduction

The A-ring of flavonoids and isoflavonoids is derived by condensation of acetate-malonate units, and recent studies [1, 2] using sodium [1,2-$^{13}C_2$]acetate have elegantly displayed significant differences in the pathways to the 5-hydroxy and 5-deoxy series of compounds. Thus, Stoessl and Stothers [1] observed the intact incorporation of double-labelled acetate units into carbons 1a–1, 2–3 and 4–4a of the 6a-hydroxypterocarpan pisatin (**1**) (an isoflavonoid) in *Pisum sativum* demonstrating a specific folding of the polyketide chain and reduction of the "missing" oxygen function prior to ring closure (Fig. 1). In contrast, Light and Hahlbrock [2], investigating the biosynthesis of the flavonol kaempferol (**2**) in *Petroselinum hortense* cell suspension cultures, noted that A-ring signals in the ^{13}C-NMR spectrum of the enriched material were flanked not by one pair of satellites (as with pisatin), but by two pairs of satellites where sufficiently different coupling constants enabled the signals to be resolved. This indicated a mixture of the two possible products (Fig. 1) and the randomisation of label was ascribed to free rotation of a chalcone intermediate. Similar results with the flavone apigenin were less certain, since chemical randomisation could have occurred during the work up [2].

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We have carried out a similar experiment using wounded bean (*Phaseolus vulgaris*) cotyledons which accumulate the isoflavonoid phytoalexins phaseollin (**3**) and kievitone (**4**), together with smaller amounts of other related isoflavonoids [3]. Phaseollin and kievitone are representative of the 5-deoxy and 5-hydroxy series of isoflavonoids in the same plant, and in addition contain isoprenoid substituents, which may also be expected to be derived in nature from acetate precursors.

The enzymic formation of 6′-hydroxychalcones (5-hydroxyflavonoid/isoflavonoid series) is now well understood [4, 5]. A comparison of the ^{13}C A-ring labelling patterns of phaseollin and kievitone will provide an insight into the nature of the as yet uncharacterised enzyme system catalysing the formation of 6′-deoxychalcones from 4-coumaroyl coenzyme A and acetate (malonyl CoA) units.

Experimental

Seeds of dwarf French bean (*Phaseolus vulgaris* cultivar The Prince) (0.5 kg) were germinated in the dark in moist vermiculite for seven days at 25 °C. Cotyledons were excised, wounded by removing the top 2 mm of cells from the inner surface with a razor blade, and arranged in petri dishes on moist filter paper. Distilled water (50 µl) was applied to the cut surface of each cotyledon, and the petri dishes were placed in a dark, humid chamber at

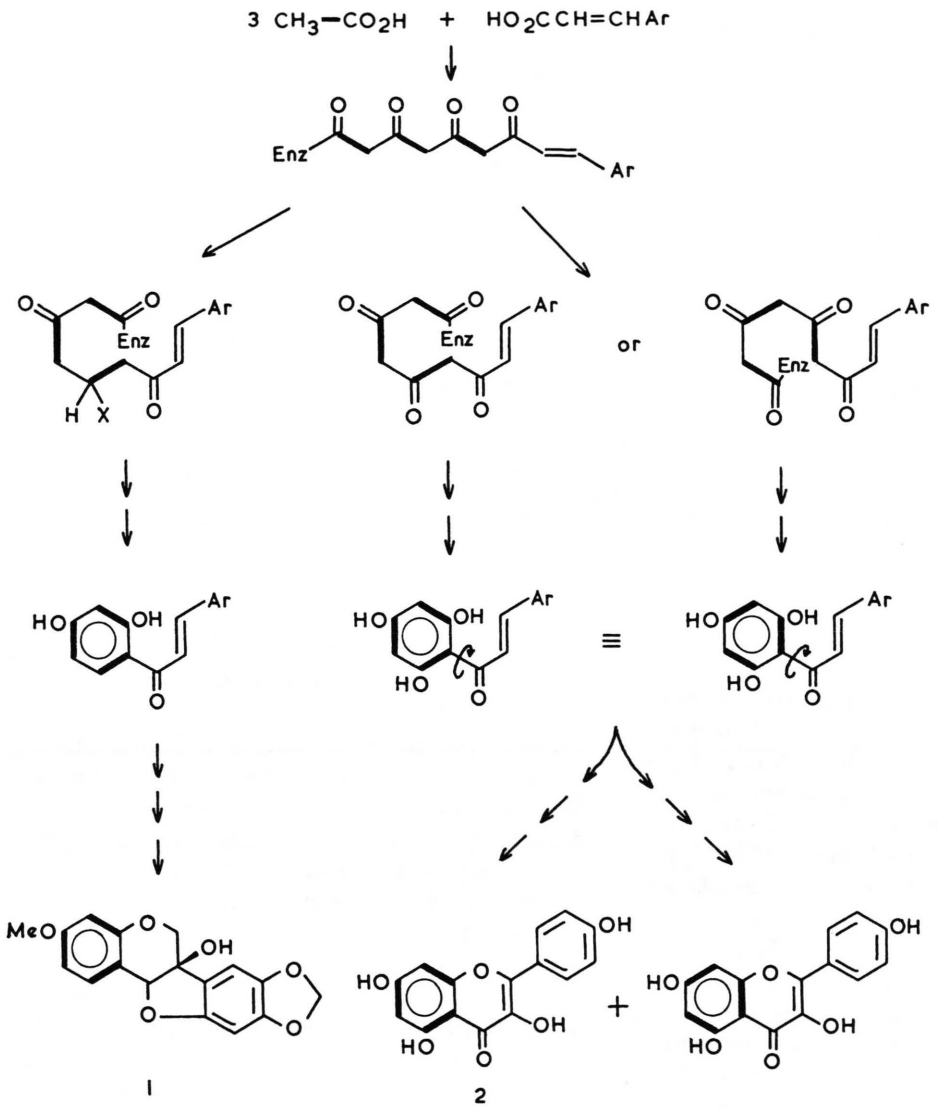
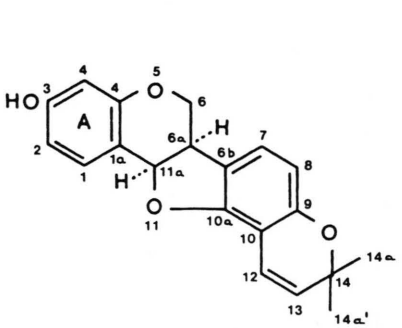


Fig. 1. Biosynthesis of pisatin (**1**) and kaempferol (**2**) from sodium [1,2-$^{13}C_2$]acetate [1, 2].



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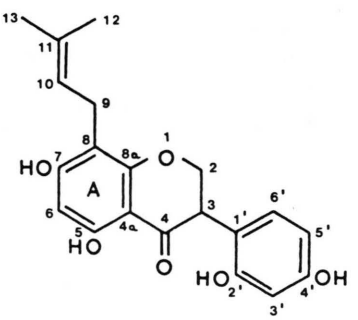

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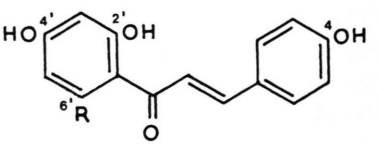


5 R = H

6 R = OH

25 °C. After 48 h, 50 µl of an aqueous solution of [1,2-$^{13}C_2$]NaOAc (5 mg ml^{-1}; 90% labelled at each carbon atom; pH 7.6) was applied to the wounded surface of each cotyledon (total volume 100 ml). The cotyledons were incubated as above with the label for a further 48 h, and were then harvested by transfer to redistilled ethanol (500 ml). The precise timing and extent of the above labelling period were selected on the basis of previous experiments on the time courses of accumulation of phaseollin and kievitone in the cotyledons [3], and on a preliminary labelling experiment using [U-^{14}C]NaOAc (5 mg ml^{-1}, 5 µCi mg^{-1}, 50 µl per cotyledon). The ^{14}C-labelling experiment, under the conditions outlined above, resulted in the isolation of ^{14}C-labelled phaseollin which, after purification to constant specific activity, showed an incorporation of label of 5.7%, with an isotope dilution of 17.7.

The EtOH extracts were worked up, and the phytoalexins isolated and purified by TLC as previously described [11]. Yields of pure phytoalexins were: phaseollin, 49 mg; kievitone, 89 mg.

Results and Discussion

Signals in the ^{13}C-NMR spectra of phaseollin and kievitone were assigned on the basis of characteristic chemical shifts, off-resonance decoupling multiplicities, comparison with other isoflavonoid derivatives [6–10], and, where necessary, analysis of carbon-carbon couplings in spectra of the enriched samples. The assignments are listed in Table I.

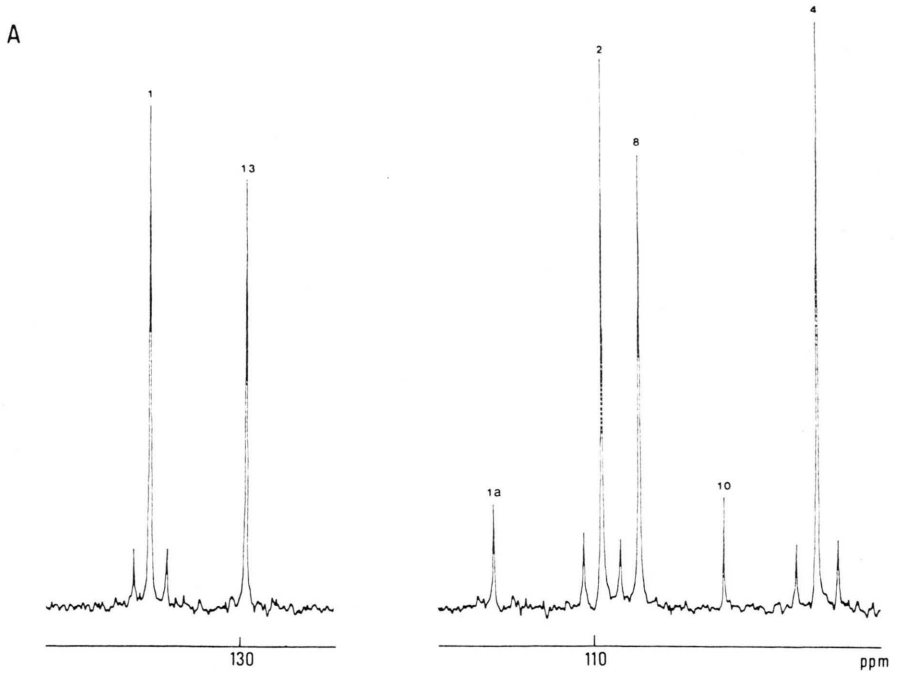
The 62.9 MHz spectrum of phaseollin derived from [$^{13}C_2$]acetate showed four signals to be flanked by satellites, and two more sets were observed with the increased sensitivity of a 100.6 MHz spectrometer (Fig. 2). These six signals correspond to the A ring carbons, and the ^{13}C-^{13}C coupling constants observed (Table I) indicate that intact acetate units are incorporated into the ring in precisely the same manner as Stoessl and Stothers observed with pisatin [1], *i.e.* into carbons 1a–1, 2–3 and 4–4a. From the relative intensities of the satellite peaks to the central signal, the enrichment level was estimated to be 0.30% at each acetate position.

In the spectrum of enriched kievitone, six signals were flanked by satellites. Two of these sets were plainly resolved as pairs of satellite signals, whereas the other four at both 62.9 MHz and 100.6 MHz were unresolved, broad peaks (Fig. 2). Clearly though, the pattern observed is analogous to that reported by Light and Hahlbrock for kaempferol [2], and is a result of two types of labelled kievitone

Table I. ^{13}C-NMR chemical shifts[a] and coupling constants for phaseollin (**3**) and kievitone (**4**).

Phaseollin (**3**)				Kievitone (**4**)			
Carbon	δ_c [ppm]	J_{cc}	[Hz]	Carbon	δ_c [ppm]	J_{cc}	[Hz]
1a	112.7	J_{1a-1}	61.0	4a	103.7	J_{4a-5}	62
1	132.3			5	163.4	J_{5-6}	72
2	109.7	J_{2-3}	64.7	6	96.5	J_{6-7}	ca. 68
3	157.1			7	164.7	J_{7-8}	ca. 68
4	103.7	J_{4-4a}	72.1	8	108.1	J_{8-8a}	73
4a	156.8			8a	161.3	J_{8a-4a}	63
6	66.6			2	71.2		
6a	39.7			3	47.2		
6b	119.1			1′	114.0		
7	123.8			6′	131.6		
8	108.6			5′	107.9		
9	155.4[b]			4′	157.0		
10	106.3			3′	103.8		
10a	153.8[b]			2′	158.8		
11a	78.7			4	198.8		
12	116.5			9	22.1		
13	129.6			10	123.9		
14	76.1			11	131.2		
14a	27.8			12	17.8		
14a′	27.8			13	25.8		

[a] ^{13}C-NMR spectra were obtained on a Bruker WM250 spectrometer operating at 62.90 MHz, or a Bruker WH400 at 100.62 MHz. Phaseollin was dissolved in $CDCl_3$, and kievitone in $(CD_3)_2CO$, and chemical shifts were recorded relative to TMS.
[b] Tentative assignments.



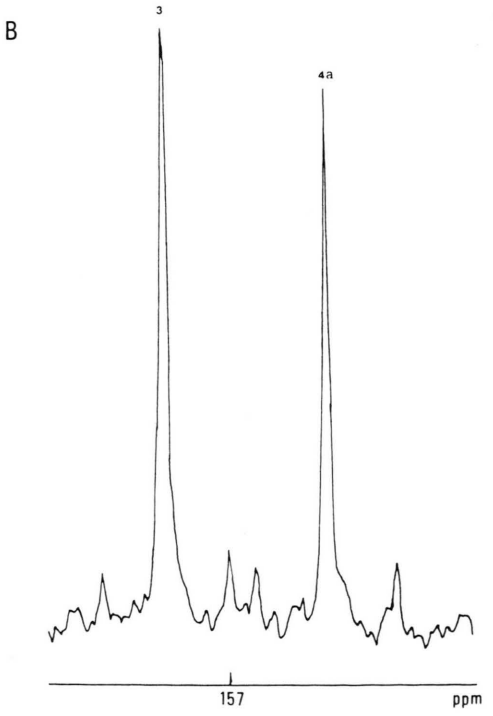


Fig. 2

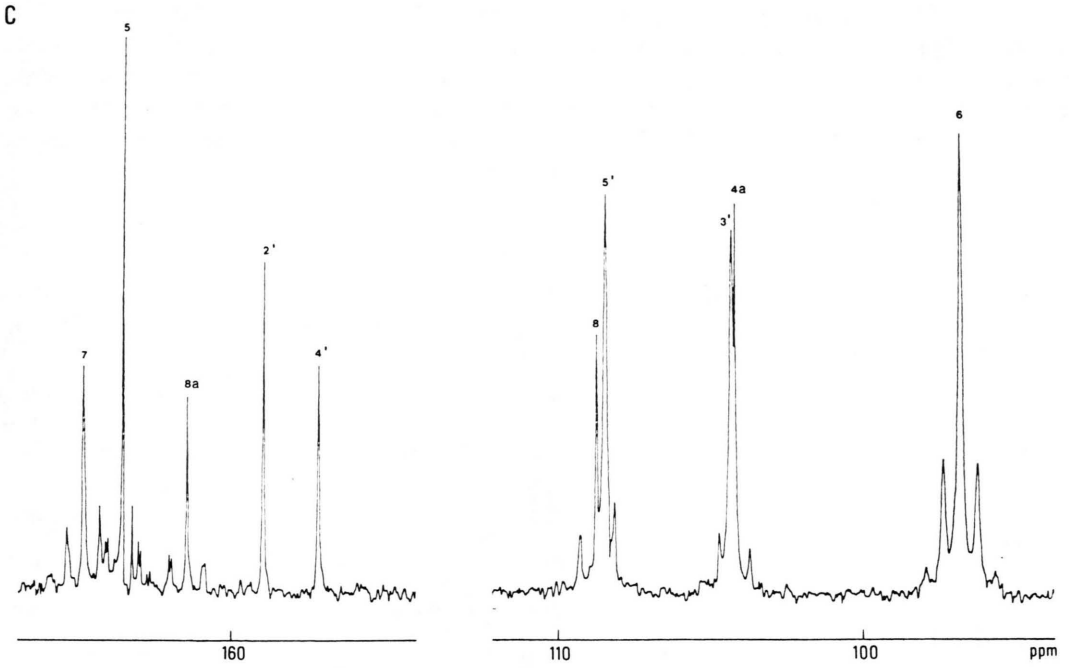


Fig. 2. ^{13}C-NMR spectra of enriched isoflavonoids: A, phaseollin, 62.9 MHz; B, phaseollin, 100.6 MHz; C, kievitone, 62.9 MHz.

being produced. From the relative intensities of the satellite peaks, the enrichment level was about 0.7% at each acetate position, more than double the figure for phaseollin.

The biosynthetic pathway to phaseollin thus involves specific folding of the polyketide chain, and the "missing" oxygen function must therefore be reduced before formation of the aromatic ring. This is in keeping with the role of 2′,4′,4-trihydroxychalcone (**5**) as an intermediate in phaseollin biosynthesis [11]. In contrast, for kievitone, the aromatic ring is formed without removal of oxygen, and if specific folding of the polyketide chain is again required, at some stage in the pathway this ring is free to rotate. A chalcone intermediate, most probably 2′,4′,6′,4-tetrahydroxychalcone (**6**) is thus implicated [12]. The biosynthetic routes to these 5-hydroxy- and 5-deoxy-isoflavonoids thus diverge prior to chalcone formation. A similar situation has also been demonstrated earlier with formononetin and biochanin A biosynthesis in *Cicer arietinum* using ^{14}C-labelled precursors [13]. The present results lend support to the involvement, in the biosynthesis of phaseollin, of a 6′-deoxychalcone synthase which catalyses the formation, reduction, dehydration and possibly cyclisation of a polyketide intermediate made from one molecule of *p*-coumaroyl coenzyme A and three molecules of malonyl CoA. The existence of such an enzyme, and the analogy of its reaction mechanism to that of 6-methylsalicylic acid synthase from *Penicillium patulum* [14], was pointed out earlier [4]. Kievitone is presumably synthesised via the normal "6′-hydroxy" chalcone synthase whose presence has been shown in French bean [15] and several other plant tissues [4, 16].

Although good incorporation of [$^{13}C_2$]acetate into the aromatic ring A was observed with both metabolites, neither spectrum gave evidence for any significant incorporation of acetate into the isoprenoid substituents, though these are undoubtedly acetate-mevalonate derived. Radiolabelling experiments have demonstrated that isoprenylation is a late stage in the biosynthesis of phaseollin [11] and the rotenoid amorphigenin [17], occurring after the basic pterocarpan or rotenoid skeletons have been built up. The same is probably true for kievitone. It is possible then that during the present experiment, the supply of precursor had been exhausted before isoprenylation occurred. However, it is more likely that there is

probably a significant difference in the efficiency of transport of acetate precursor into the acetate-malonate and acetate-mevalonate derived portions of the molecule. Mevalonate itself is poorly incorporated into phaseollin [18], and similarly into rotenoids [17] and other hemiterpenoid derivatives [19]. The incorporation of acetate into wholly terpenoid phytoalexins [20] is not affected in the same way. An enzyme catalysing the dimethylallylation of 3,6a,9-trihydroxypterocarpan has been isolated from soya bean cotyledons [21], and is possibly located in the plastid fraction. Apart from microsomal hydroxylation systems, the other known enzymes involved in the biosynthesis of isoflavonoids appear to be soluble cytoplasmic enzymes. Such subcellular compartmentation might explain the disappointing incorporation into the terpenoid fragment of the isoflavonoids in the present work.

Acknowledgements

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