Randomization of the Flavonoid A Ring during Biosynthesis of Kaempferol from [1,2-$^{13}C_2$]Acetate in Cell Suspension Cultures of Parsley

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Cell suspension cultures of parsley (*Petroselinum hortense*) were incubated with [1,2-$^{13}C_2$]acetate during a period of active flavonoid production. The flavonoid glycosides were extracted with ethanol, hydrolyzed in dilute acid, and the aglycones purified by chromatography. Apigenin, a flavone, and kaempferol, a flavonol, were analyzed at 67.9 MHz by ^{13}C FT NMR. The ^{13}C enrichment confirmed that acetate contributes primarily to the flavonoid A ring. The coupling patterns between adjacent ^{13}C atoms of the A ring indicate that the cyclization direction of the A ring is random in both compounds. While randomization of apigenin could have occurred chemically through opening of the pyrone ring under the acid conditions used for glycoside hydrolysis, randomization of the more stable flavonol must have occurred biosynthetically. The latter result supports the conclusion that a chalcone is an intermediate in flavonoid biosynthesis.

Introduction

Flavonoid biosynthesis involves the condensation of 4-coumaroyl-CoA, derived from phenylalanine, with three molecules of malonyl-CoA. This condensation is catalyzed by the enzyme chalcone synthase (previously called flavanone synthase) (Fig. 1) [1–3]. In crude extracts, the flavanone naringenin (**2**) is the first observable product, formed presumably by the action of chalcone isomerase [4]. One can observe the chalcone intermediate (**1**) only in highly purified preparations of the synthase [1], and so it is not absolutely certain whether appearance of the chalcone results from removal of last traces of isomerase, or from the artificial assay conditions for the purified enzyme. Other "early release" side products have been observed at various stages during enzyme purification [5, 6].

In the experiment reported here, we have used the ^{13}C-NMR technique with [1,2-$^{13}C_2$]acetate [7] to test whether the acetate incorporated into the A ring of flavonoids has been specifically or randomly oriented during biosynthesis. Random orientation would indicate that the chalcone (**1**) is an *in vivo* flavonoid biosynthetic intermediate in parsley cell cultures. The non-random orientation observed in pisatin biosynthesis by Stoessl and Stothers [8] must therefore have occured, as those authors suggested, by reduction of a polyketide precursor prior to cyclization of the A ring.

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Experimental Section

Cell suspension cultures of *Petroselinum hortense* were propagated in the dark at 27 °C on rotary shakers in 2–1 conical flasks containing 400 ml of fully synthetic medium [9] (note that 1 mmol/l $CaCl_2 \cdot 2\,H_2O$ was omitted by mistake in [9]). Inocula (40 ml) were taken from 7-day-old cultures. After growth for 6 days, 14 2-l flasks were transferred to a shaker exposed to light of about 20000 lux from fluorescent lamps (Phillips K 40 W/18). Five h after the transfer to light, 83 µmol of [1,2-$^{13}C_2$]acetate (87% enrichment, CEA, Gif-sur-Yvette, France) in 0.2 ml of 50% ethanol solution was added to each flask, and incubation was continued in the light for an additional 21 h. A small amount of [1-^{14}C]acetate (2 µCi per flask) was added along with the [^{13}C]acetate to determine the incorporation level.

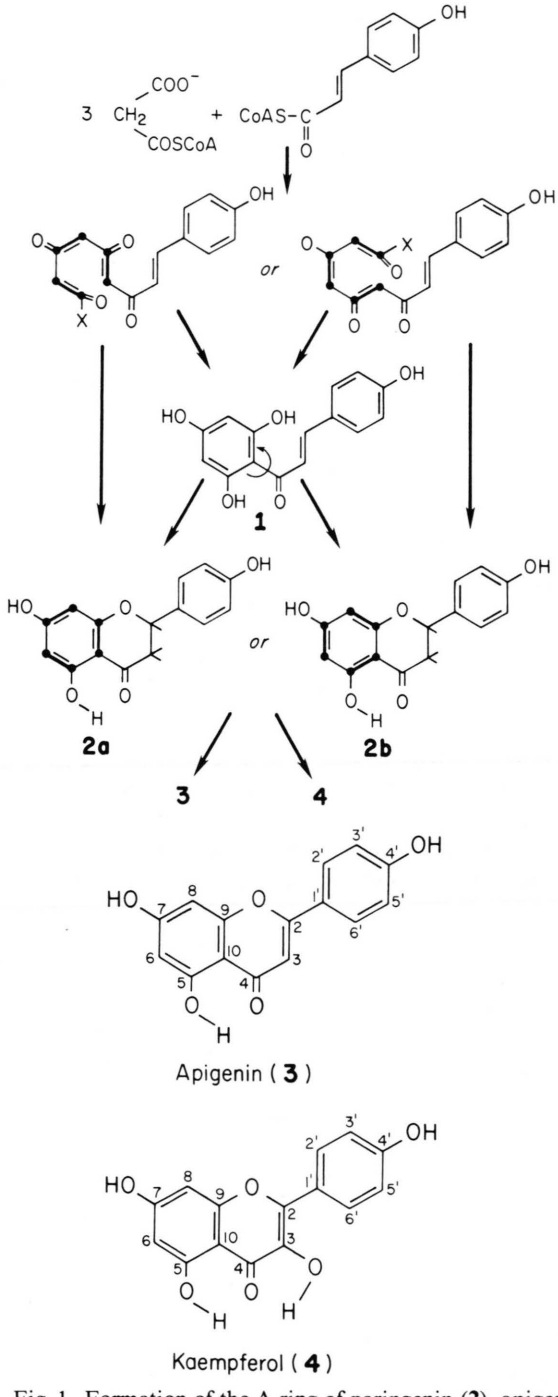

Fig. 1. Formation of the A ring of naringenin (**2**), apigenin (**3**), and kaempferol (**4**). Cyclization of the six-carbon polyketide chain could occur in either of two orientations to give **2a** or **2b**. If naringenin chalcone (**1**) is an intermediate, rotation about the indicated bond would produce a random mixture of **2a** and **2b**. A molecule of [1,2-$^{13}C_2$]-acetate incorporated into naringenin would occupy only one of the positions indicated by the heavy lines.

Cells were harvested by filtering and washed successively with 2 *l* of 0.75% sodium acetate, pH 5, followed by two 2-*l* portions of water. Cells were then extracted with two successive portions of boiling 80% ethanol (1500 ml and 1250 ml). Thin layer chromatographic analysis of the water washes and the ethanol extracts on Merck silica gel prepared plates showed that the majority of the flavonoid glycosides were in the first ethanol extract, though a small amount appeared in the second water wash and the second ethanol extract. Plates were developed in butanol/acetic acid/water (8:2:10, upper phase), and flavonoids were visualized under ultraviolet light. The second water wash was filtered over 30 g of polyamide to remove as much of the flavonoid glycosides as possible, and the polyamide was extracted with 2 *l* of 80% aqueous methanol. The ethanol extracts of the cells, and the polyamide methanol extract were each evaporated to dryness to yield a total residue of 11.0 g. The total residue was then extracted with about 500 ml of methanol, removing 9.6 g of material containing most of the flavonoid glycosides.

A major portion of this material (80%) was further enriched in glycosides by evaporating to a volume of 20 ml, cooling, and filtering, to yield 5.4 g of residue, which was then subjected to hydrolysis in one liter of 2 mol/l HCl/methanol (1:1) heated at reflux for 4 h. The solution was evaporated to 200 ml in a rotary evaporator, 400 ml of water was added, and the flavonoid aglycones were extracted with three 200-ml portions of ethyl acetate. The residue of the combined ethyl acetate extracts (0.97 g) was dissolved in 5 ml of methanol/ethyl acetate (1:1) and applied to ten silicic acid preparative thin layer plates (500 μ thickness). Plates were developed in chloroform/methanol (9:1) and the area corresponding to apigenin (**3**) and kaempferol (**4**), which have similar R_f values, was scraped from the plates and eluted with methanol. The methanol eluate (70 mg) was applied to a 75 × 2.5 cm column of Sephadex LH-20 (Pharmacia) [10]. The column was eluted with methanol at 4 °C and monitored by absorbance at 340 nm and by measuring radioactivity of aliquots. Apigenin eluted in the major peak with an elution volume of 760 ml, well separated from kaempferol eluting at 970 ml. Both peaks were skewed, probably because of chelation with contaminating metal ions, so material from each peak was combined, evaporated, shaken with dilute HCl,

and extracted with ethyl acetate. Rechromatography of the extracted material from each fraction gave a single sharp peak absorbing at 340 nm that coincided with the ^{14}C profile. The combined fractions yielded 21.3 mg of apigenin and 4.9 mg of kaempferol, each with a specific activity of 1500–1700 cpm/μmol, corresponding to a ^{13}C isotope enrichment of 1.6% in each of the acetate derived positions of the A ring.

Results and Discussion

Table I lists the chemical shifts found in the FT-^{13}C NMR spectrum of apigenin and kaempferol enriched by the incorporation of [1,2-$^{13}C_2$]acetate. Symmetrically placed satellite peaks resulting from the coupling of adjacent ^{13}C atoms of the incorporated acetate are found only in enriched positions, and these positions, as expected from early work with [^{14}C]acetate [11], are located only in the A ring.

Assignments of the signals are based on those made by Markham and Ternai [12] for apigenin, with two exceptions. Since the signal at 164.98 ppm shows the satellite peaks from ^{13}C enrichment, this signal is assigned to C-7, and the signal at 164.60 ppm must therefore belong to C-2. Distinctions between C-6 and C-8 and between C-5 and C-9 are difficult to make, though Wherli [13] assigned these positions in naringenin by looking at long range coupling to the C-5 hydroxyl proton before and after deuterium exchange. He assigned the lower field signal in each of the pairs to C-5 and C-6 of naringenin, respectively, while Ternai and Markham [14] originally had the lower field signals of each pair assigned to C-9 and C-8 of apigenin. They later reversed the assignment of the C-6, C-8 pair [12], and we are suggesting that the C-5, C-9 pair should also have been reversed on the basis of Wehrli's observations, and on the basis of more recent work by Pelter *et al.* [15], who have assigned the lower field signal to C-5 of a series of isoflavones. The ^{13}C-^{13}C coupling patterns we observe, however, would be interpreted the same way regardless of the C-5, C-9 and the C-6, C-8 assignments.

Table I. ^{13}C-NMR chemical shifts and coupling constants for apigenin (**3**) and kaempferol (**4**).

Proton noise decoupled FT ^{13}C-NMR spectra were obtained at 67.9 MHz on a Bruker HX-270 spectrometer. Samples were dissolved in 0.25 ml of $(CD_3)_2SO$ contained in a 5-mm NMR tube, and pulses were at 3-sec (apigenin) or 4-sec (kaempferol) intervals with 90° pulses (11 μsec, apigenin; 14 μsec, kaempferol). The apigenin sample was scanned 1000 times, the kaempferol sample 15000 times. A spectral width of 15000 Hz was obtained with line broadening of 1.5 Hz (apigenin) or 3 Hz (kaempferol) applied to the 32 K spectrum (16 K real points), and the spectrum recorded at 10 Hz/cm.

Carbon No.	Apigenin (**3**)		Kaempferol (**4**)	
	Shift[a] (ppm)	J_{cc} (Hz)	Shift[a] (ppm)	J_{cc} (Hz)
1′	122.09	–	121.59	–
2′,6′	129.28	–	129.38	–
4′	162.00	–	159.07	–
3′,5′	116.85	–	115.35	–
2	164.60	–	146.76	–
3	103.72	–	135.53	–
4	182.57	–	175.77	–
5	162.32	62,72	160.60	62,72
6	99.74	68,71	98.14	67,72
7	164.98	67,67	163.76	66,66
8	94.86	72,67	93.40	73,68
9	158.18	73,64	156.09	73,63
10	104.62	63,63	102.97	62,62

[a] Chemical shifts are recorded relative to tetramethylsilane.

The chemical shift assignments for kaempferol have been made by analogy with those for apigenin and those for other flavonols [14]. As expected, only C-2, C-3 and C-4 differ markedly between the two compounds, while most of the rest of the signals are found at only 1–2 ppm higher field in kaempferol as compared to apigenin.

The satellite peaks of the signals for C-5 and C-9 clearly show two sets of doublets, while the satellite peaks for C-6 and C-8 are broader (7–8 Hz at half-height) compared to the central peaks (4–5 Hz at half-height), also indicating two sets of doublets with slightly different coupling constants. These data show that each enriched position of the A ring is coupled to both of its neighbors, and the six possible coupling combinations of the A ring can be seen by comparing the coupling constants of each set of doublets given in Table I.

These data indicate that acetate is found in both the clockwise and counter-clockwise orientations of ring A as shown in Fig. 1. The random orientation is to be expected for apigenin, because the conditions used to hydrolyze the flavonoid glycosides (2 mol/l HCl/methanol, 1:1, refluxing for 4 h) are sufficient to open the pyrone ring of flavones, allowing a randomization of the A ring by the Wessely-Moser rearrangement [16, 17]. Flavonols require much stronger acidic conditions to undergo the Wessely-Moser rearrangement, not isomerizing even during

demethylation of methoxy ethers with hydroiodic acid [17]. Apparently the hydroxyl group at C-3 greatly reduces the electrophilic properties of C-2, where water attack must occur in order to open the pyrone ring and to cause isomerization.

Apigenin, therefore, serves as a control, showing what the random spectrum should look like. Since the satellite peaks of kaempferol are essentially identical to those in apigenin, acetate must also be randomly oriented in the A ring of kaempferol. Randomization of kaempferol must have occurred during biosynthesis, indicating that the chalcone is a biosynthetic intermediate *in vivo* (Fig. 1). This data supports the conclusion that the occurrence of the chalcone intermediate with the purified chalcone synthase [1] is not an artifact created during the purification of the enzyme.

These results should be compared with the recent work of Stoessl and Stothers on the biosynthesis of pisatin [8], where the same ^{13}C-coupling method established that the A ring of pisatin has a non-random, clockwise orientation. Our data support their conclusion that reduction of a polyketide precursor to pisatin must have occurred before, rather than after, cyclization of ring A, because reduction might have occurred after cyclization had the chalcone not been an intermediate. In addition, their result serves as control for our work, showing that a non-random orientation in kaempferol could have been observed had it occurred.

The specific activity of the isolated apigenin and kaempferol was about 4.8% that of the [^{14}C]acetate fed to the cells, so that each acetate position of the A ring should be enriched by about 1.6% with a [$^{13}C_2$]-acetate. This value is in good agreement with the sizes of the satellite peaks compared to the central natural abundance peak. To rule out the remote possibility that the administered acetate had not been diluted during incorporation, thereby yielding apigenin and kaempferol molecules derived from three molecules of [$^{13}C_2$]acetate, the mass spectrum of the enriched apigenin was compared to that of an unenriched sample. The molecular ion (M) is the base peak in the electron impact spectrum of apigenin [18]. Table II lists the relative intensities of the M, M + 2, M + 4 and M + 6 ions from the two apigenin samples. On the basis of the ^{14}C incorporation data, one would expect ^{13}C enrichment to increase the M + 2 peak by 4.8% if only one acetate molecule is found in each enriched apigenin molecule; to increase the M + 4 peak by 2.4% if two acetates per molecule are found, or to increase the M + 6 peak by 1.6% if three acetates per molecule are found. The data clearly show that acetate is distributed as expected, primarily as single [$^{13}C_2$]-acetate units in each enriched molecule (5% increase for the M + 2 peak).

Table II. Relative intensities of the molecular ion and higher isotope peaks of ^{13}C-enriched and unenriched apigenin.

The low resolution electron impact mass spectra of both samples were obtained on a G.E.C.-A.E.I. MS-902 mass spectrometer with a source temperature of 175 °C and an ionization voltage of 70 eV. The molecular ion (M) is at 270 *m/e*.

Ion	Relative intensity	
m/e	Unenriched [%]	^{13}C-Enriched [%]
270 (M)	100	100
272 (M + 2)	2.6	7.6
274 (M + 4)	0.04	0.72
276 (M + 6)	0.03	0.14

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