Studies on Plant Tissue Cultures. Part 36. Biosynthesis of a Retrochalcone, Echinatin, and Other Flavonoids in the Cultured Cells of *Glycyrrhiza echinata*. A New Route to a Chalcone with Transposed A- and B-Rings [1,2]

By **Shin-ichi Ayabe** and **Tsutomu Furuya,*** School of Pharmaceutical Sciences, Kitasato University, Minato-ku, Tokyo 108, Japan

Compounds labelled with radioisotopes were fed to the cultured cells of *Glycyrrhiza echinata* and the echinatin (1) obtained was degraded with alkali to *p*-hydroxyacetophenone (12) and 4-hydroxy-2-methoxybenzaldehyde (13) to determine the position of the incorporated radioactivity. The label from [3-^{14}C]cinnamic acid and [3,5-$^{3}H_2$]-isoliquiritigenin was found only in compound (12) while the label from [1-^{14}C]cinnamic acid and sodium [1-^{14}C]-acetate was found only in compound (13). These results establish the ' retrochalcone ' nature of compound (1), in which, contrary to normal flavonoids, the A-ring and C_3-unit are of cinnamate origin and the B-ring is of acetate-malonate origin. ^{14}C-Labelled precursors were synthesized and feeding experiments using a mutant strain of *G. echinata* cell culture revealed that licodione (9), a dibenzoylmethane, is an intermediate in the transposition of the α,β-unsaturated carbonyl group in the chalcone molecule during echinatin biosynthesis. The isotopic labelling of licodione (9) was also incorporated into 7,4'-dihydroxyflavone (6), although [carbonyl-^{14}C]isoliquiritigenin labelled the flavone (6) more efficiently, while incorporation of compound (9) was very low compared with that of isoliquiritigenin (11) into the isoflavone formononetin (5). A feeding experiment with[1, 2-$^{13}C_2$]iacetate further confirmed the polyketide moiety of the B-ring of chalcone (1) as well as the A-ring of isoflavone (5), both of which were labelled non-randomly with intact acetate units.

FLAVONOIDS are representative of higher plant phenolics, and numerous investigations of their biosynthesis have established that their C_6-C_3-C_6 skeleton comprises two parts that are different in their biosynthetic origins; *i.e.* the phenylpropanoid part (C_6-C_3), derived from the aromatic amino-acid phenylalanine, and the polyketide moiety (C_6) of acetate–malonate origin. The first C_{15} intermediate has long been discussed and recent enzymic studies conclusively demonstrated the crucial importance of chalcone; the product of the successive condensation of three molecules of malonyl CoA and *p*-coumaroyl CoA, catalyzed by a highly purified enzyme from parsley cell culture, is naringenin chalcone, and not the flavanone naringenin.[3]

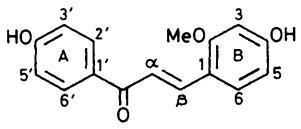
Echinatin (1),[4,5] a chalcone isolated from the callus culture of *Glycyrrhiza echinata* has, however, a strange substitution pattern of oxygen functionalities, which implicates an unusual biosynthetic process that does not comply with a flavonoid biosynthesis operating in the cultured cells. Contrary to normal flavonoids, this chalcone lacks hydroxy-groups at C-2' and C-6' in the A-ring and possesses the resorcinol type B-ring. Saitoh and Shibata isolated more unusually *O*-substituted chalcones,[6] the licochalcones A (2) and B(3) together with echinatin, from a commercial sample of the drug Sinkiang licorice, and also an isoflavone with the phloroglucinol type B-ring, namely licoricone (4),[7] from another *Glycyrrhiza* spp. Thus, a new biosynthetic scheme has been postulated in *Glycyrrhiza* that gives flavonoids in which the A-rings and C_3 units are derived from cinnamate derivatives and the B-rings are derived from the polyketide moiety. These unusual flavonoids have been designated as retroflavonoids, and, in particular, the chalcones as retrochalcones.[6]

G. echinata callus, as well as Sinkiang licorice, contains, in addition to retrochalcones, several normal flavonoids, *e.g.* the isoflavone formononetin (5), 7,4'-dihydroxyflavone (6), and its *C*-prenyl derivatives (7) and (8).[5] This implies that retrochalcone may have a close relationship to normal flavonoids; *i.e.* retrochalcones may be derived from the common C_{15} intermediate of general flavonoid biosynthesis. Further studies of minor components of the *G. echinata* callus have resulted in the isolation of a new dibenzoylmethane, licodione (9),[5,8] which is very likely to be a key compound that links normal flavonoid and retrochalcones.

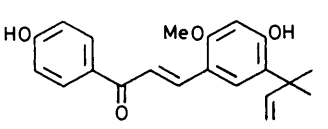
We here report incorporation studies with isotopically labelled compounds that establish a novel biosynthetic pathway to form a retrochalcone, echinatin, and we also describe some aspects of flavone and isoflavone biosynthesis in *G. echinata* cultured cells.

RESULTS AND DISCUSSION

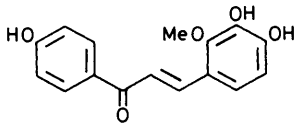
Cell Culture.—*G. echinata* callus was derived from seedlings and cultured in the dark on White's agar medium [9] which contained yeast extract (1%) and 2,4-dichlorophenoxyacetic acid (2,4-D) (0.1 p.p.m.). The cells were yellow due to echinatin, and the earlier feeding studies with radiolabelled cinnamate and isoliquiritigenin were carried out using this parent strain. However, if the culturing was continued for several years, subculturing at intervals of 6 weeks, the cells gradually became colourless and the content of echinatin decreased. The cells were then treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), which is well known as a potent mutagen and commonly used in microbial systems and, occasionally, in plant tissue cultures [10] to produce mutant strains.[11] Several cell types of *G. echinata* with different amounts and composition of constituents have been derived. Among them, the strain M-1-3, cultured under the same conditions as that of the parent strain, is considerably echinatin rich and its



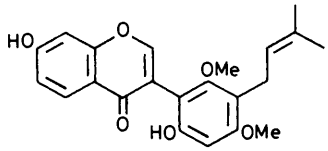
(1)



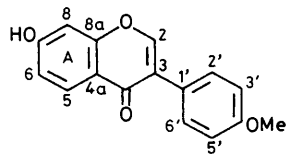
(2)



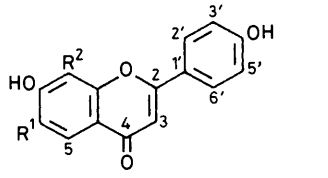
(3)



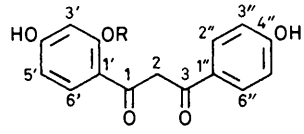
(4)



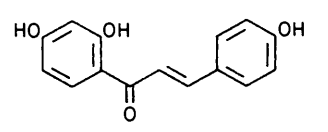
(5)



(6) $R^1 = R^2 = H$
(7) $R^1 = CH_2CH{=}CMe_2, R^2 = H$
(8) $R^1 = H, R^2 = CH_2CH{=}CMe_2$



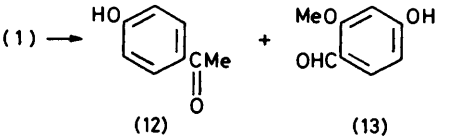
(9) R = H
(10) R = Me



(11)

growth is fast, and this was utilized in incorporation experiments with acetate and advanced precursors labelled with ^{14}C. The strain M-2-9, maintained on Murashige and Skoog's medium [12] with indole-3-acetic acid (IAA) and kinetin added, produces high amounts of formononetin (5), which provides a good material for investigation of the isoflavonoid biosynthesis.

Incorporation Experiments with Primary Precursors and Isoliquiritigenin.—The initial feeding study was carried out to test whether the echinatin molecule contains a phenylpropanoid moiety that originates from the cinnamate derivatives, and to obtain evidence for the inversion of the α,β-unsaturated carbonyl group of cinnamate, if it was incorporated, taking place in the C_3 unit of the resultant echinatin. *G. echinata* parent callus (6 weeks old) was incubated for 5 days with [1-^{14}C]- and [3-^{14}C]-cinnamic acid, respectively. Echinatin was isolated and recrystallized together with radioinactive carrier to constant specific activity. The remainder of the chalcone (1), after the radioactivity had been measured, was degraded by refluxing with aqueous KOH to yield *p*-hydroxyacetophenone (12) and 4-hydroxy-2-methoxybenzaldehyde (13) (Scheme 1). As shown in Table 1



(12) (13)

SCHEME 1 Degradation of echinatin

(*cf.* Expts. 1 and 2), the major radioactivity that exists in the acetophenone (12) originates from [3-^{14}C]cinnamate and, in contrast, the activity in the aldehyde (13) originates from [1-^{14}C]cinnamate. This result indicates that the carboxyl carbon of cinnamate specifically converted into the β-position of echinatin, while C-3 of cinnamate converted into the carbonyl of echinatin. The radioactivity of echinatin labelled with sodium [1-^{14}C]-acetate is distributed almost exclusively in the aldehyde (13) derived from the degradation reaction (*cf.* Table 1, Expt. 3), which suggests that the B-ring of echinatin is the polyketide moiety.

Now, it is apparent that echinatin (1) is biosynthesized from the common precursors of conventional flavonoids, and that the origins of A- and B-rings of the chalcone (1) are reversed with respect to normal origins. The result of a feeding study with [3,5-$^3H_2$]isoliquiritigenin is also listed in Table 1 (Expt. 4) * and reveals that isoliquiritigenin (11) is an efficient precursor of echinatin in which the A-ring is derived from the B-ring of normal chalcone (11) by the inversion of carbonyl.

Incorporation Experiments with Advanced Precursors.—Elucidation of the mechanism of the transposition of the α,β-unsaturated carbonyl unit of cinnamate origin in the chalcone (1) formed the next stage of our investigation. An oxidation–reduction process was presumed and licodione (9), a dibenzoylmethane, was regarded as the most likely intermediate for this process. This view was

TABLE 1

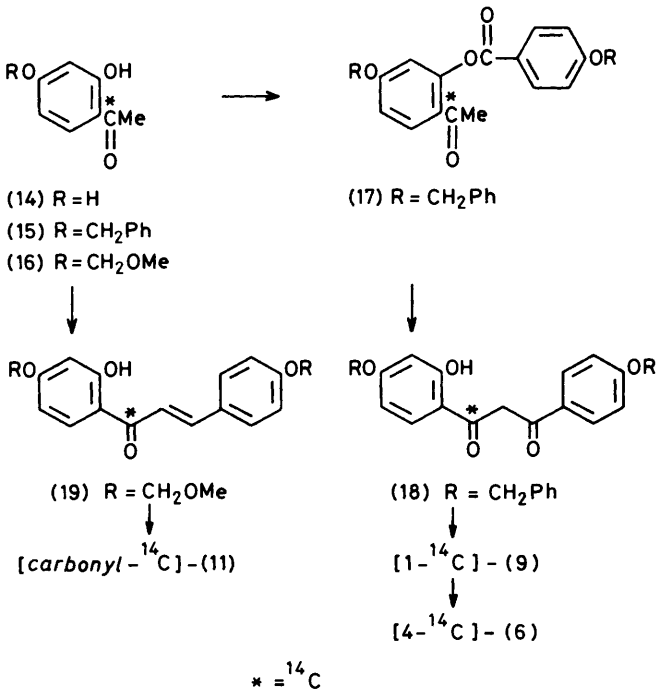
Specific incorporation of ^{14}C-labelled primary precursors and ^3H-labelled isoliquiritigenin into echinatin (1) and the distribution of radioactivity in *p*-hydroxyacetophenone (12) and 4-hydroxy-2-methoxybenzaldehyde (13), both derived from compound (1)

Expt.	Compound administered	mCi mmol^{-1}	Echinatin (1) (μCi mmol^{-1}) [%]	(12) [a] (%)	(13) [a] (%)
1	[3-^{14}C]Cinnamic acid (50 μCi)	50	0.643 [0.0013]	94	7
2	[1-^{14}C]Cinnamic acid (50 μCi)	4.19	2.33 [0.056]	0	104
3	Sodium [1-^{14}C]acetate (100 μCi)	2.50	26.7 [1.07]	0.1	112
4	[3,5-$^3H_2$]Isoliquiritigenin (8.56 mCi)	8.48	117 [1.38]	85.5	0

[a] Percentage of specific activity of echinatin.

* This was carried out by T. Saitoh, S. Shibata, and U. Sankawa and has been described in ref. 2*a*.

strongly supported by parallel studies on enzymic *O*-methylation of licodione in the same cultured cells.[13] On the other hand, both the formation and the further possible biosynthetic roles of licodione in the cultured cells were also of interest. Licodione might be generated by direct oxygenation of isoliquiritigenin (11) *via* epoxide or peroxide,[14] but the absence (t.l.c. detection) of the chalcone (11), in contrast to the presence of 7,4'-dihydroxyflavone (6), in the callus implied another possible process of licodione formation; *i.e.* hydration of the 2,3-double bond of flavone to give the 2-hydroxyflavanone structure, which is one of the tautomeric forms of dibenzoylmethane.* Such an enzymic hydration toward the 3-hydroxyflavone has been known in the reactions catalyzed by peroxidases from some plant materials,[15] including the *Mentha* cell culture.[16] The reverse reaction in the cells has also been reasonably conceived for flavone biosynthesis,[17] in analogy with the facile chemical dehydration of dibenzoylmethanes to flavones by acid treatment. Furthermore, the existence of formononetin (5) in the callus led us to suspect that licodione might be an intermediate in the conversion of the chalcone into isoflavone, in which the biosynthesis would require oxidation and phenyl migration within the chalcone molecule. Thus, incorporation of ^{14}C-labelled isoliquiritigenin (11), licodione (9), and 7,4'-dihydroxyflavone (6) into echinatin (1), and also of the chalcone (11) and licodione (9) into the flavone (6) were examined using M-1-3 strains cultured at same time and under equal conditions. Incorporations of the radioactive chalcone (11) and licodione (9) into formononetin (5) in the M-2-9 strain were lso examined.



SCHEME 2 Synthesis of advanced precursors

[4-^{14}C]-7,4'-dihydroxyflavone (34% yield). Condensation of [*carbonyl*-^{14}C]-4-*O*-methoxymethylresacetophenone and *p*-methoxymethoxybenzaldehyde followed by HCl treatment to remove the protecting group gave [*carbonyl*-^{14}C]isoliquiritigenin (68% yield from [*carbonyl*-^{14}C]resacetophenone).

TABLE 2

Specific incorporation of ^{14}C-labelled advance precursors into echinatin (1) and 7,4'-dihydroxyflavone (6) and the distribution of radioactivity in the degradation products of compound (1)

Expt.	Compound administered	mCi mmol^{-1}	Compound isolated (μCi mmol^{-1}) [%]	(12) [a] (%)	(13) [a] (%)
5	[1-^{14}C]Licodione (30 μCi)	0.710	(1) 3.65 [0.51] (6) 0.692 [0.097]	3	106
6	[*carbonyl*-^{14}C]Isoliquiritigenin (15 μCi)	0.643	(1) 0.304 [0.047] (6) 6.12 [0.95]	0.4	92
7	[4-^{14}C]-7,4'-Dihydroxyflavone (13 μCi)	0.768	(1) 0.098 [0.013]	*b*	*b*

[a] Percentage of specific activity of echinatin. [b] Not determined.

^{14}C-Labelled compounds were synthesized as shown in Scheme 2 from [*carbonyl*-^{14}C]resacetophenone (14), which was prepared from resorcinol and [1-^{14}C]acetic acid in the presence of $ZnCl_2$. Acylation of [*carbonyl*-^{14}C]-4-*O*-benzylresacetophenone (15) by *p*-benzyloxybenzoyl chloride in pyridine yielded the ^{14}C-ester (17); this was followed by a rearrangement, mediated by alkali, and hydrogenolytic deprotection to give[1-^{14}C]licodione (15% yield from [*carbonyl*-^{14}C]resacetophenone). Treatment of [1-^{14}C]licodione with HCl in MeOH afforded

The results of feeding ^{14}C-precursors into 3-week old M-1-3 strains for two days are summarized in Table 2. Licodione (9) was effectively incorporated into echinatin (1) while isoliquiritigenin (11) and 7,4'-dihydroxyflavone (6) were also incorporated, but to a lesser extent. The presence of more radioactivity in 4-hydroxy-2-methoxybenzaldehyde (13), obtained by alkaline degradation of echinatin from both cells fed with ^{14}C-labelled licodione (9) and isoliquiritigenin (11), suggests that the incorporation was again specific. It has now been confirmed that licodione serves as an advanced precursor of echinatin. A relatively low incorporation of the flavone (6) into echinatin, compared with that of isoliquiritigenin (11), implies that the chalcone is the preferred precursor of licodione, and the assumed hydration of the flavone (6) is, if present, a minor pathway to licodione.

* Until a recent correction of the false identification of the product of the ' chalcone ' synthase reaction (to be a flavanone),[3b] the possible biosynthetic scheme in *Glycyrrhiza* cells that did not involve a chalcone intermediate had been considered the more likely; *i.e.* liquiritigenin (flavanone) dehydrogenates to yield the flavone (6), which subsequently forms the dibenzoylmethane (9) and chalcone (1).

The flavone (6), isolated from the cells fed with [^{14}C]-licodione, is considerably radioactive (*cf.* Expt. 5), which suggests that a dehydrative cyclization of licodione to form the flavone is taking place in the cells. This is the first demonstration of *in vivo* preparation of the flavone from dibenzoylmethane. However, incorporation of [^{14}C]isoliquiritigenin was much higher than that of [^{14}C]licodione into flavone (*cf.* Expt. 6). This may reflect the difficulty of exogenous licodione to reach the site of biosynthesis or the existence of another pathway that does not involve enzyme-free dibenzoylmethane *e.g.* dehydration of the oxygenated flavanone (2- or 3-hydroxyliquiritigenin).*

TABLE 3

Specific incorporation of ^{14}C-labelled isoliquiritigenin (11) and licodione (9) into formononetin (5)

Expt.		Compound administered mCi mmol^{-1}	Formononetin (μCi mmol^{-1}) [%]
8	[*carbonyl*-^{14}C]Isoliquiritigenin (15 μCi)	0.643	3.64 [0.57]
9	[1-^{14}C]Licodione (15 μCi)	0.710	0.023 [0.0033]

Comparison of incorporation ratios of ^{14}C-labelled licodione and isoliquiritigenin into formononetin in the M-2-9 strain clearly indicates that licodione cannot be an intermediate in isoflavone biosynthesis (Table 3). Intermediate steps of isoflavonoid biosynthesis, after the chalcone stage, therefore still require further investigation.

Incorporation Experiments with ^{13}C-Doubly Labelled Acetate.—Further labelling experiments with [1,2-$^{13}C_2$]-acetate confirmed the polyketide moiety in the B-ring of echinatin and provided some information about the mechanism of aromatic-ring formation from three acetate units during the early stage of echinatin biosynthesis. Table 4 shows the assignments of the ^{13}C n.m.r. spectra of echinatin together with ^{13}C–^{13}C coupling constants of the biosynthetic sample obtained from [1,2-$^{13}C_2$]acetate feeding. Assignments of carbonyl, methyl, C-2′ and -6′, and C-3′ and -5′ were obvious from their characteristic chemical shifts or strong peak intensities. The α- and β-carbons were identified from their chemical shifts and splitting patterns (broad double-doublets) in the off-resonance spectra.[20] C-3, -5, and -6 were assigned according to the calculated values of a model compound, 4-hydroxy-2-methoxycinnamic acid,[21] and those of C-1′ and -1 were clearly distinguished as the latter resonates at *ca.* 15 p.p.m. higher field than the former, because of the *ortho*-methoxy-group.

A long term incubation (18 days) of a 4-week culture of the M-1-3 strain with 90% enriched sodium [1,2-$^{13}C_2$]-acetate resulted in a good incorporation of ^{13}C atoms into echinatin. Figure 1 shows the 25 MHz and 50 MHz ^{13}C n.m.r. spectra of biosynthetic echinatin. Six peaks have satellites due to ^{13}C–^{13}C spin coupling which arises from the incorporation of the intact $^{13}C_2$-acetate unit, and among them, clear coupling between C-1 and C-6 (coupling constant 59.8 Hz) is observed. Other peaks that bear satellite bands are those of C-3, -5, and additional low field peaks occur which are attributable to C-2 and C-4 which bear oxygen-containing groups. The precise value of the coupling constants of the lower field signals were measured on 50 MHz recordings. Since all the enriched carbon atoms have only one one-bond ^{13}C–^{13}C coupling and that no coupling between C-5 and C-6 was observed, the assignments of the resonances of C-2 and C-4 are as in Table 5 and the carbon pairs derived from the same acetate molecule are deduced to be [C-2, -3], [C-4, -5], and [C-6, -1].

TABLE 4

^{13}C N.m.r. chemical shifts of echinatin (1) and ^{13}C–^{13}C coupling constants in compound (1) labelled by [1,2-$^{13}C_2$]acetate [a]

Carbon No.	δ (p.p.m.) [b]	$^1J_{CC}$ (Hz)
1	114.8 (s)	59.8
2	160.3 (s)	69.6 [c]
3	99.3 (d)	68.4
4	161.8 (s)	63.5 [c]
5	108.4 (d)	63.5
6	130.3 (s)	59.8
1′	129.9 (s)	
2′,6′	131.0 (d)	
3′,5′	115.5 (d)	
4′	162.0 (s)	
CO	187.6 (s)	
α	118.4 (dd)	
β	138.2 (dd)	
Me	55.6 (q)	

[a] 25 MHz, [$^2H_6$]DMSO, TMS as internal standard. [b] Multiplicity in off-resonance spectrum is indicated in parenthesis. [c] Determined from a spectrum recorded at 50 MHz.

This result indicates that the B-ring of echinatin is of acetate–malonate origin and that the assembly of three acetate units is non-random; *i.e.* echinatin biosynthesis does not involve an enzyme-free intermediate that possesses the phloroglucinol-type oxygenated ring (*e.g.* naringenin chalcone), which would result in a random assembly of acetate units due to free-rotation about the C_2-axis.[22] Therefore, the process of acetate-derived ring formation would include either deoxygenation from the phloroglucinol-type ring of an intermediate bound to an enzyme surface before free-rotation, or, more likely, reduction of a carbonyl group at some stage during successive condensation of three malonyl CoA's to the primer, presumably *p*-coumaroyl CoA. Judging from the orientation of the acetate incorporation, the reduction must take place on the first, not the third, condensed malonyl

* Following completion of this work, characterizations of enzyme systems that convert flavanones into flavones from the flower extracts of *Antirrhinum majus* (snapdragon) [18] and *Petroselinum hortense* (parsley) cell culture [19] have recently been reported. It is suggested that both systems contain oxygenases and involve 2-hydroxyflavanone-type intermediates (although not yet identified). It appears, therefore, that licodione (9), present as the tautomeric cyclic hemiacetal structure, which is not found in n.m.r. solvents and perhaps exists only when bound to the enzyme, is likely to be an intermediate in the biosynthesis of 7,4′-dihydroxyflavone (6).

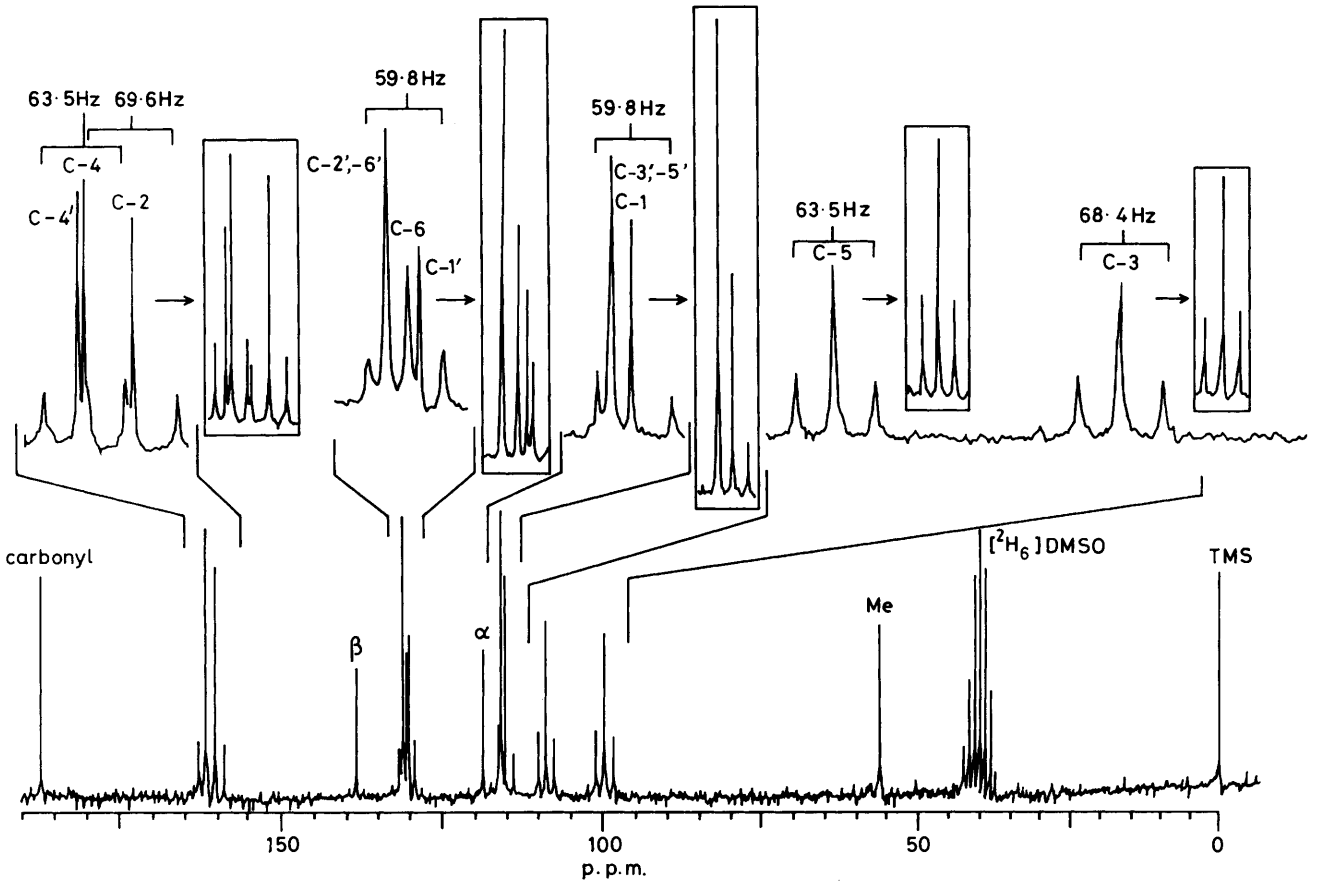


FIGURE 1 ^{13}C N.m.r. spectrum of echinatin (1) enriched with [1,2-$^{13}C_2$]acetate {25 MHz and 50 MHz (in frames), [$^2H_6$]DMSO}

CoA. The known mechanism of formation of the hydrogen-substituted C-4 atom of 6-methylsalicylic acid during its biosynthesis in *Penicillium patulum* (reduction of carbonyl to an alcohol and successive dehydration to afford the *cis*-olefin [23]) may resemble the process of resorcinol-type ring formation in *Glycyrrhiza* and other higher plants.

TABLE 5

^{13}C N.m.r. chemical shifts of formononetin (5) and ^{13}C-^{13}C coupling constants in compound (5) labelled by [1,2-$^{13}C_2$]acetate [a]

Carbon No.	δ (p.p.m.) [b]	$^1J_{CC}$ (Hz)
2	153.2 (d) [b]	
3	124.5 (s)	
4	174.9 (s)	
4a	116.9 (s)	*ca.* 62 [c]
5	127.5 (d)	58.6
6	115.3 (d)	61.0
7	162.9 (s)	61.0
8	102.3 (d)	72.0
8a	157.7 (s)	*ca.* 73 [c]
1'	123.4 (s)	
2',6'	130.3 (d)	
3',5'	113.8 (d)	
4'	159.2 (s)	
Me	55.2 (q)	

[a] 25 MHz, [$^2H_6$]DMSO, TMS as internal standard. [b] Multiplicity in off-resonance spectrum is indicated in parenthesis. [c] An approximate value because of signal overlapping (see Figure 2).

Formononetin was also enriched with ^{13}C in the same experiment. ^{13}C N.m.r. assignments (Table 5) were achieved by chemical shift rules and off-resonance measurements, using the shift values in the literature.[24] The carbon pairs that originated from the intact acetate units are shown to be [C-4a, -5], [C-6, -7], and [C-8, -8a] (*cf.* Figure 2). A similar result of non-random incorporation of acetates into the A-ring of pisatin, an isoflavonoid phytoalexin, in $CuCl_2$-treated *Pisum sativum* has been reported.[25]

The same arrangement of acetate units in the A-ring of formononetin as in the B-ring of echinatin is obvious. This fact supports a rationale that the common precursor of retrochalcone and isoflavone, and perhaps also of flavones and glycosides of all these classes of compounds found in the cultured cells, is synthesized in a single enzyme system.

Biosynthesis of Echinatin and Other Flavonoids in G. echinata *Cultured Cells.*—Scheme 3 indicates the biosynthetic routes consistent with the foregoing results. The last steps of echinatin biosynthesis would be as follows; 2'-*O*-methylation of licodione (9), subsequent reduction of the 1-keto-group of 2'-*O*-methyl-licodione

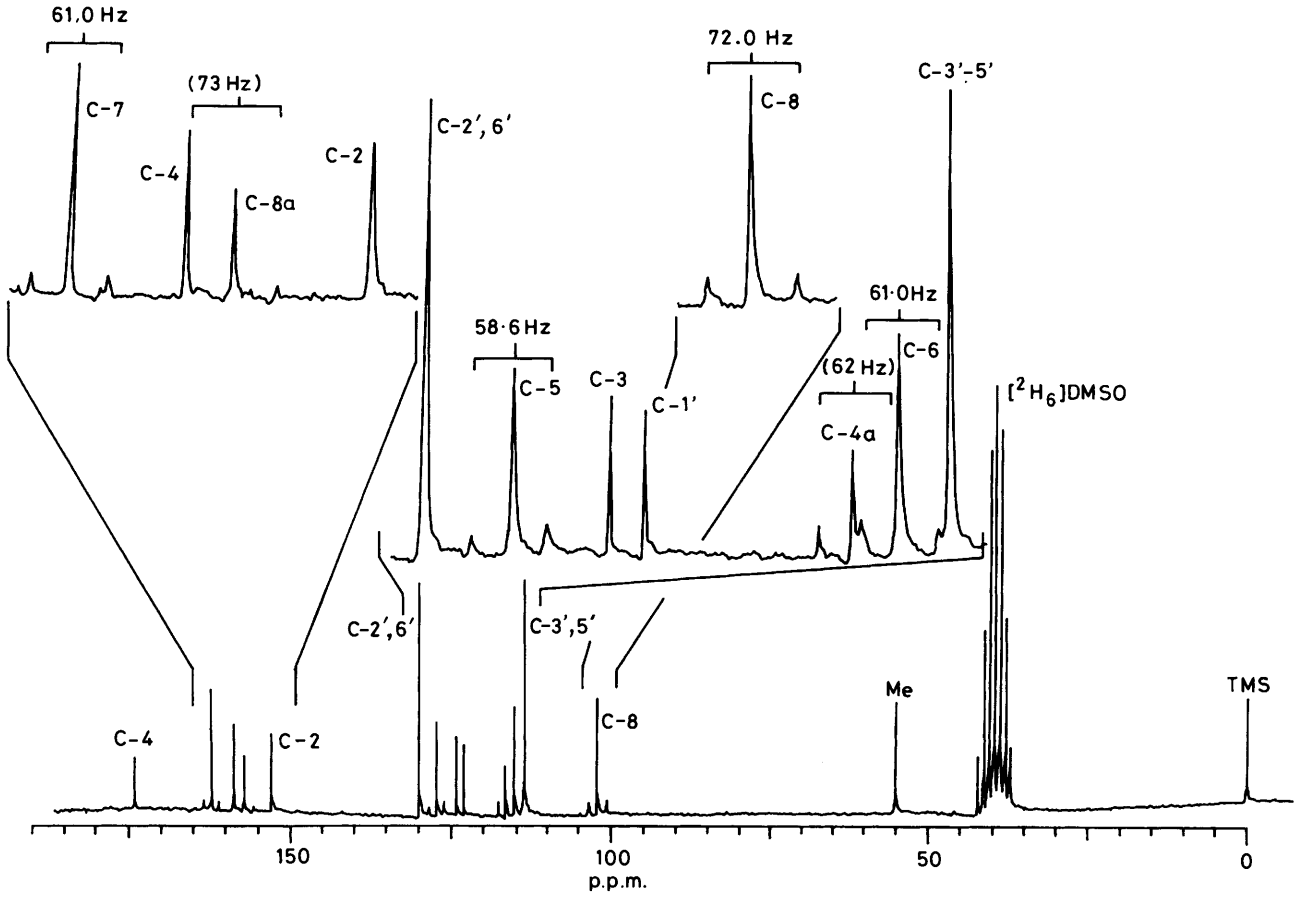
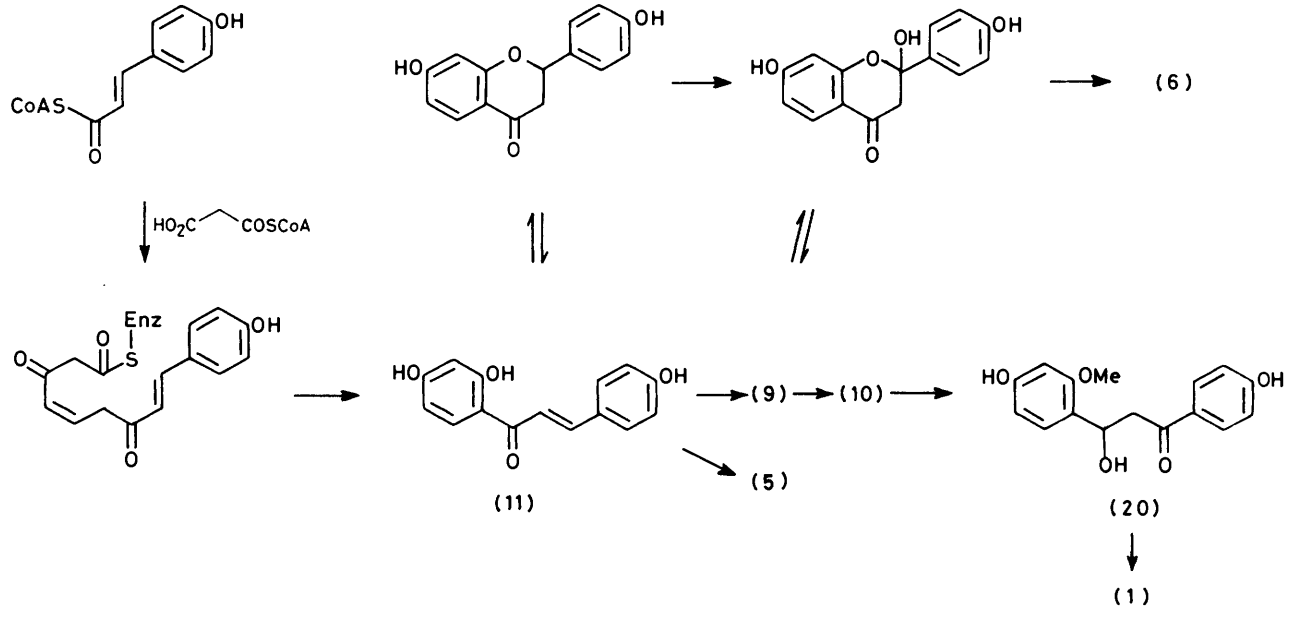


FIGURE 2 ^{13}C N.m.r. spectrum of formononetin (5) enriched with [1,2-$^{13}C_2$]acetate (25 MHz, [$^2H_6$]DMSO)



SCHEME 3 Biosynthesis of flavonoids in *Glycyrrhiza echinata* cell culture

(10) to a hypothetical benzyl alcohol (20), and final dehydration. Among these steps, formation of compound (10) from licodione has been clarified at the enzymic level; an enzyme, *S*-adenosyl-L-methionine : licodione 2′-*O*-methyltransferase, has been partially purified from the cultured cells.[13] A subsequent reduction process may be supported by the existence of β-hydroxydihydrochalcones in nature.[26]

EXPERIMENTAL

Melting points were taken on a Mitamura hot-stage micromelting point apparatus and are uncorrected. U.v. spectra were recorded on a Shimadzu double-beam spectrophotometer, UV-200. ^{1}H N.m.r. and i.r. spectra were run on a Varian EM-390 instrument and a JASCO IRA-200 spectrometer, respectively. ^{13}C N.m.r. spectra were recorded on JEOL PS-100/EC-100 and JEOL FX-200 Fourier transform spectrometers. Silica gel used in column chromatography was Wakogel C-200 (Wako Pure Chemical Ltd., Tokyo). Analytical and preparative t.l.c. were carried out using precoated (Merck, Kieselgel $60F_{254}$) or hand-made (Merck, Kieselgel GF_{254}) plates. Transfer of cultured cells and feeding of labelled precursors into cells were carried out in an aseptic manner either in an aseptic transfer room or by the use of a clean bench.

Callus Culture.—The parent strain of *Glycyrrhiza echinata* L. callus was derived from seedlings in 1965, and cultured on White's basal medium [9] to which was added 2,4-dichlorophenoxyacetic acid (2,4-D, 0.1 p.p.m.), yeast extract (Difco, 0.1%), sucrose (2%), and agar (0.8%) in the dark at 26 °C with subculture intervals of 5—6 weeks.

Derivation and culture of M-1 and M-2 strains were as follows. The parent strain was precultured in White's liquid medium, containing 2,4-D (0.1 p.p.m.) and yeast extract (0.1%), for 10 days. After removal of the medium by filtration, the cells were suspended in sterilized water and shaken for 24 h, then a solution of *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG, 100 μg/ml water) was added and incubated for a further 24 h. Precipitated cells from centrifugation at 500 × *g* for 3 min were thoroughly washed with sterilized water, dispersed on agar medium, and left in the dark at 26 °C for 4 weeks. From the resultant colonies, deep yellow cells (M-1 strain) and pale yellow cells (M-2 strain), which do not grow on White's medium, were collected, transferred onto media (40 ml) and cultured as usual (26 °C, dark). Nine M-1 strains (M-1-1—M-1-9) and four M-2 strains (M-2-1—M-2-4) were established. The medium for M-1 strains was the same as that for the parent strain, and for M-2 strains, Murashige and Skoog's [12] media were used with 3-indoleacetic acid (IAA, 1 p.p.m.), kinetin (0.1 p.p.m.), sucrose (2%), and agar (0.8%) added. Subculture intervals were 4 weeks (M-1) and 3 weeks (M-2).

Composition of liquid media used for suspension cultures in incorporation experiments was the same as that for stationary culture, but without agar.

Radiochemical Methods.—Radioisotopically labelled compounds were purchased from Schwarzmann, Orangeburg ([3-^{14}C]cinnamic acid), Mallinckrodt Chemicals Works, St. Louis ([1-^{14}C]cinnamic acid), and New England Nuclear, Boston ([1-^{14}C]acetic acid and sodium [1-^{14}C]acetate). Radioactive materials chemically synthesized or biosynthesized were purified chromatographically and/or by recrystallization, identified with authentic radioinactive samples on t.l.c. and by thin-layer radiochromatograms (giving one radioactive spot). Physicochemical data for radioinactive samples of echinatin (1), 7,4′-dihydroxyflavone (6), licodione (9), and 4′,4″-di-*O*-benzyllicodione (18) have been previously described.[5] Thin-layer radiochromatography was performed on an Aloka thin-layer chromatogram scanner, JTC-202B. Radioactivities were estimated in Aloka Liquid Scintillation Spectrometers LSC-651 and LSC-653. Composition of the scintillant was as follows: 500 ml toluene, 250 ml Triton X-100, 2 g DPO (2,5-diphenyloxazole), and 0.25 g POPOP (1,4-bis-[2-(5-phenyloxazolyl)]-benzene). Every radioactive sample was added with either 10 or 15 ml of scintillant, and counting efficiencies were measured by calibrated external standard. The term 'constant specific radioactivity' used in the following sections means that the specific activities of 2—4 samples obtained from successive recrystallization are within ±5%.

Synthesis of Radioisotopically Labelled Compounds.—[carbonyl-^{14}C]*Resacetophenone* (14). To the mixture of resorcinol (210.2 mg) and dry $ZnCl_2$ (183.8 mg), [1-^{14}C]acetic acid (1 mCi, *ca.* 30 μl) diluted with radioinactive acetic acid (*ca.* 100 μl) was added and the mixture was stirred at 150—170 °C for 30 min. When at ambient temperature, 6M hydrochloric acid (*ca.* 5 ml) was added and the resultant precipitates were collected by filtration. The red-brown solid (72.9 mg) was washed with water and subjected to column chromatography on silica gel (14 g; $CHCl_3$). The fractions that gave one spot on t.l.c. were combined, concentrated, and recrystallized from water to give ^{14}C-resacetophenone (14) (57.2 mg) as pale brown plates. The filtrate was extracted with ethyl acetate, the organic layer was evaporated off, and the residue (deep brown solid, *ca.* 180 mg) was chromatographed on silica gel (50 g; $CHCl_3$–MeOH, 97 : 3). The fractions that contained compound (14) and the mother liquor from the recrystallization above were combined and purified by preparative t.l.c. (hexane–acetone, 2 : 1) to afford an additional 64.7 mg of radioactive resacetophenone (14) (total weight 121.9 mg; 0.59 mCi).

[1-^{14}C]*Licodione*{[1-^{14}C]-(9)}. The mixture of [*carbonyl*-^{14}C]resacetophenone (14) (64.5 mg), benzyl chloride (53.9 mg), K_2CO_3 (29.3 mg), and KI (7.2 mg) in DMF (*ca.* 0.15 ml) was stirred at 50—55 °C for 1 h. Water was added to the reaction mixture, which was then extracted with ethyl acetate, and the organic layer was washed, dried, and evaporated to give a powder (103.7 mg). Preparative t.l.c. (hexane–acetone, 2 : 1) afforded radioactive 4-*O*-benzylresacetophenone (15) as a white powder (79.6 mg, 63%) identical with a radioinactive sample, m.p. 105.5—106.5 °C (from methanol) (lit.,[27] m.p. 101—102 °C), and unchanged compound (14) (14.1 mg).

p-Benzyloxybenzoyl chloride (127.1 mg) and [*carbonyl*-^{14}C]-4-*O*-benzylresacetophenone (15) (79.6 mg) in dry pyridine (1 ml) were stirred, at room temperature, for 5 h. The reaction mixture was distributed between ethyl acetate and water, and the organic layer was washed with 1M hydrochloric acid, water, aqueous Na_2CO_3, and brine, then dried and evaporated. The residue was recrystallized from hexane–acetone to give plates of [*carbonyl*(*acetyl*)-^{14}C]-4-*O*-benzyl-2-*O*-(*p*-benzyloxybenzoyl)resacetophenone (17) (69.4 mg), identical with a radioinactive sample, m.p. 142—142.5 °C; $\nu_{max.}$ (KBr) 1 725, 1 675, and 1 595 cm^{-1}; δ([^{2}H$_6$]acetone) 2.43 (3 H, s, Me), 5.23 and 5.27 (each 2 H, s, OC*H*$_2$Ph), 6.98br (1 H, s, 3-H), 7.03 (1 H, dd, *J* 2 and 9 Hz, 5-H), 7.19 (2 H, d, *J* 9 Hz, 3′, 5′-H), 7.3—7.6 (10 H, m, 2 × OCH$_2$*Ph*), 7.93br (1 H, d, *J* 8 Hz, 6-H), and 8.14 (2 H, d, *J* 9 Hz, 2′-, 6′-H); *m/e* 452 (M^+, $C_{29}H_{24}O_5$, 3%), 242(3), 211(58), and 91(100).

The mother liquor gave additional pure radioactive ester (17) by preparative t.l.c. (total weight 106.7 mg, 72%).

^{14}C-Labelled ester (17) (106.7 mg) and KOH (*ca.* 1 g) in dry pyridine (1 ml) were stirred at room temperature under nitrogen for 40 h, and the reaction mixture, after acidification with 1M hydrochloric acid, was extracted with ethyl acetate. The organic layer was washed with 5% Na_2CO_3, to remove by-products resulting from hydrolysis, and then washed with H_2O, dried, and evaporated to give radioactive 4',4''-di-*O*-benzyllicodione (18) (84.5 mg) as a yellow solid. The product (18) (82.7 mg) in EtOH–ethylene glycol monomethyl ether (1 : 1, 5 ml) was stirred at room temperture under H_2 over 10% Pd–C (17.6 mg) for 26 h. Pd–C was removed by filtration and the filtrate was concentrated and purified by preparative t.l.c. [benzene–ethyl acetate–methanol–light petroleum (b.p. 40—50 °C), 6 : 4 : 1 : 3 (BEMP)]. Recrystallization from ethanol–water gave [1-^{14}C]licodione{[1-^{14}C]-(9)} as fine yellow needles (22 mg, 34%; 0.057 mCi), identical with a radioinactive sample.

[4-^{14}C]-7,4'-*Dihydroxyflavone*{[4-^{14}C]-(6)}. To [1-^{14}C]-Licodione{[1-^{14}C]-(9)} (15.59 mg, crude material) in ethanol (2 ml) was added concentrated hydrochloric acid (4 drops) and the mixture was stirred at room temperature for 2 h. Addition of water (10 ml) caused a yellow precipitate which was collected and recrystallized from methanol to give radioactive (6) (2.45 mg) as fine, pale yellow needles. The filtrate was extracted with ethyl acetate and work-up afforded further crude ^{14}C-(6) (6.20 mg). This material, together with mother liquor obtained from the recrystallization, was purified by preparative t.l.c. (BEMP) to give compound [4-^{14}C]-(6) (2.45 mg) (total weight 4.90 mg, 34%; 0.015 mCi).

[carbonyl-^{14}C]*Isoliquiritigenin*{[carbonyl-^{14}C]-(11)]}. A mixture of ^{14}C-resacetophenone (14) (13.20 mg) and K_2CO_3 (107.7 mg) in dry acetone (0.5 ml) was stirred at room temperature for 15 min. The mixture was cooled to 0 °C, then 3 drops of chloromethyl methyl ether were added and the mixture was stirred for a further 8 min at 0 °C. The reaction mixture was filtered, the filtrate evaporated off, and the residue taken up in ethyl acetate. The organic layer was washed with water, dried, and evaporated to give [*carbonyl*-^{14}C]-4-*O*-methoxymethylresacetophenone (16) (*ca.* 30 mg) *p*-Methoxymethoxybenzaldehyde (*ca.* 30 mg) was prepared in the same manner as described above from *p*-hydroxybenzaldehyde (18.5 mg).

Potassium hydroxide (2 g) was dissolved in ethanol (8 ml). This solution (1 ml) was slowly poured into the mixture of compound (19) and *p*-methoxymethoxybenzaldehyde prepared above at 0 °C, and the resultant red solution was stirred at room temperature overnight. Addition of ice, neutralization with 1M hydrochloric acid, and subsequent ethyl acetate extraction afforded radioactive 4,4'-di-*O*-methoxymethyl-(11), (19), as an orange oil (49.90 mg). This was dissolved in methanol (2 ml), concentrated hydrochloric acid (0.2 ml) was added, and the mixture was stirred at room temperature for 3.5 h. Ice–water was then added and the resultant precipitate was collected by filtration to yield a yellow powder (13.75 mg). The filtrate was extracted with ethyl acetate, the organic layer was dried, evaporated, and subjected to preparative t.l.c. (BEMP) to afford a further 8.80 mg of crude ^{14}C-(11). Combined yellow powders were recrystallized from ethanol–water to give [*carbonyl*-^{14}C]-isoliquiritigenin as yellow needles (15.05 mg, 68%; 0.038 mCi), identical with a radioinactive sample, m.p. 198—200 °C (lit.,[28] m.p. 202—204 °C).

Administration of [1-^{14}C]- *and* [3-^{14}C]-*Cinnamic Acids to the* G. echinata *Parent Strain.*—Each ^{14}C-labelled cinnamic acid (0.05 mCi) was dissolved in a small quantity of EtOH and divided into ten equal portions. The solvent was removed by passing a nitrogen stream, and then liquid medium (400 ml) was poured into each flask, and the medium sterilized in an autoclave (120 °C, 20 min). *G. echinata* callus (parent strain) cultured on agar media (40 ml) in Erlenmeyer flasks for 6 weeks was inoculated into liquid medium that contained radioactive cinnamates. The callus from 6—7 flasks were combined in one. Incubation was carried out for 5 days by reciprocal shaking (*ca.* 85 r.p.m.) in the dark at 28 °C.

Administration of ^{14}C-*Labelled Advanced Precursors.*—*G. echinata* callus M-1-3 strain was cultured on agar media (40 ml) in Erlenmeyer flasks for 3 weeks. The callus from 10 flasks was suspended in each freshly prepared liquid medium (250 ml). Twelve flasks that contained the medium and the callus were prepared in this manner. [1-^{14}C]Licodione (30 μCi) and [*carbonyl*-^{14}C]isoliquiritigenin (15 μCi) were dissolved in Tween-80–ethanol (2 ml) (1 : 1) and [4-^{14}C]-7,4'-dihydroxyflavone (13 μCi) in 2.5 ml of Tween-80–ethanol–DMSO (3 : 3 : 1). These solutions were equally distributed into each of the 4 flasks prepared above, then the flasks were shaken (*ca.* 85 r.p.m.) at 27 °C in the dark for 2 days.

G. echinata callus M-2-9 strain, cultured for 2 weeks, was transferred into liquid medium, ^{14}C-labelled compounds were

TABLE 6

Data for incorporation experiments with radioisotope-labelled compounds and yields of the degradation products of radioactive echinatin (1)

	Incorporation experiment			Degradation		
	Callus			Echinatin [a]		
Expt.	Strain (g)	EtOAc (g)	Compound isolated (mg)	(mg)	(12) (mg)	(13) (mg)
1	Parent strain (643.5)	0.80	Echinatin (16.8)	13.65	5.30	5.50
2	Parent strain (685.3)	0.80	Echinatin (29.7)	13.10	5.05	5.35
3	M-1-3 (97.8)	0.34	Echinatin (1.14) [b]	29.05	9.35	9.20
5	M-1-3 (235.1)	0.31	Echinatin (5.50) 7,4'-Dihydroxyflavone (1.82) [b]	16.0	4.70	4.60
6	M-1-3 (237.6)	0.40	Echinatin (14.35) 7,4'-Dihydroxyflavone (0.65) [b]	13.80	4.05	4.80
7	M-1-3 (223.6)	0.17	Echinatin (2.10)			
8	M-2-9 (215.0)	0.52	Formononetin (67.75)			
9	M-2-9 (211.8)	0.51	Formonanetin (74.60)			

[a] Diluted with carrier. [b] Determined by u.v. absorption.

added and the mixture was shaken as described above. Three samples, each containing callus collected from 6—7 flasks (total: 20 flasks), were used for the feeding of each labelled compound.

Administration of Sodium [1-^{14}C]*Acetate.*—*G. echinata* callus M-1-3 strain was cultured for 4 weeks in suspension in six Erlenmeyer flasks each containing liquid medium (125 ml). At the 3 week period during this culture cycle, 0.1 mCi of sodium[1-^{14}C]acetate in 70% ethanol (3 ml) was distributed equally into the flasks, and the callus was incubated under dark at 27 °C for a further week.

Extraction and Purification Procedures.—The culture cells fed with radiolabelled precursors were collected by filtration through nylon cloth and were thoroughly washed with water. The cells were extracted successively with cold and hot methanol (at reflux) two or three times. The methanol extract, after concentration to a small volume (*ca.* 50 ml), was distributed between ethyl acetate and water. The aqueous layer was extracted with ethyl acetate a further 5—10 times. The organic layer afforded a deep brown gum (from the parent strain and M-1-3) or a yellowish gum (from M-2-9) on evaporation, which was then separated by column chromatography. Data for the freshly harvested cells, the ethyl acetate extracts, and the isolated compounds in each of the experiments are summarized in Table 6.

Echinatin (1). Column chromatography of the ethyl acetate extract on silica gel (100—200 equiv. weights of the extracts) with the eluants C_6H_6–EtOAc (19 : 1→17 : 3) afforded echinatin-rich fractions. In Expts. 1 and 2 (cinnamate feeding) these fractions were concentrated and rechromatographed on silica gel (5 g and 8 g in Expts. 1 and 2, respectively) with the eluant $CHCl_3$–MeOH (49 : 1→19 : 1). Compound (1), comprising fractions from the first and second column chromatography, was further purified by preparative t.l.c. ($CHCl_3$–MeOH, 9 : 1), and final recrystallization from ethanol–water gave yellow needles. In Expt. 5 (licodione feeding), preparative t.l.c. (BEMP) of compound (1), contaminated with minor other substances, was carried out. Column fractions and the material yielded on preparative t.l.c. were combined and recrystallized from ethanol–water. In Expts. 6 and 7 (isoliquiritigenin and 7,4′-dihydroxyflavone feeding), fractions containing compound (1) were combined and evaporated to dryness and recrystallized from ethanol–water. In Expt. 3 (acetate feeding), echinatin (1) was purified only by repeated preparative t.l.c. [(i) BEMP, (ii) $CHCl_3$–MeOH, 9 : 1] of the column fractions. The yield of compound (1) in Expt. 3 was determined by u.v. spectroscopy of an ethanol solution of the material afforded by the final preparative t.l.c. [λ310 (ε18 100) and 370 nm (ε33 900)].

Portions of echinatin isolated from the cells were diluted with suitable amounts (20—50 mg) of radioinactive carrier and recrystallized repeatedly (4—5 times) until constant specific radioactivities were obtained (*cf.* Tables 1 and 2).

7,4′-Dihydroxyflavone (6). Column chromatography of ethyl acetate extracts of the cultured cells in Expts. 5 and 6 yielded fractions that contained compound (6); these fractions were eluted more slowly than those that contained compound (1). Concentrated fractions were submitted to preparative t.l.c. [Expt. 5 BEMP; Expt. 6 (i) BEMP, (ii) $CHCl_3$–MeOH, 9 : 1] to give minute amounts of a pale yellow powder. The yield was determined from the absorbances at λ252 (ε10 600) and λ340 nm (18 000) in the u.v. spectrum of an ethanol solution. The flavone (6) thus obtained was added to radioinactive carrier (*ca.* 30 mg), and the diluted material was acetylated with acetic anhydride (*ca.* 0.3 ml) in dry pyridine (*ca.* 1 ml). Work-up as usual and recrystallization from methanol afforded 7,4′-diacetoxyflavone as needles, which were recrystallized from MeOH a further 3 times to constant specific radioactivity (*cf.* Table 2). The radioinactive sample gave m.p. 196—197.5 °C (lit.,[29] m.p. 182—183 °C); $\nu_{max.}$ (KBr) 1 765 and 1 350 cm^{-1}; *m/e* 338 (M^+, 8%), 296 (32), 254(100), and 226(21).

Formononetin (5). The ethyl acetate extracts of the M-2-9 strain fed with ^{14}C-labelled-(11) (Expt. 8) and compound (9) (Expt. 9) were chromatographed on silica gel (60—70 g) and eluted with $CHCl_3$–MeOH (99 : 1→49 : 1). The fractions that contained mainly compound (5) were collected, evaporated, and the residues recrystallized from methanol 4—5 times to give needles with constant specific radioactivity (*cf.* Table 3). The radioinactive sample gave m.p. 264.5—266 °C (lit.,[30] m.p. 260—261 °C).

Degradation of Radiolabelled Echinatin (1).—In general, radioactive echinatin (1) (*ca.* 15 mg) (after dilution with carrier to constant specific radioactivity) was dissolved in 30% KOH (5 ml) and heated on an oil-bath for 4 h (bath temperature 130—140 °C). When at room temperature, the mixture was made acidic with 6M hydrochloric acid and extracted with ethyl acetate. The products were separated by preparative t.l.c. [light petroleum (b.p. 35—50 °C)–Et_2O, 1 : 1]. Isolated *p*-hydroxyacetophenone (12) and 4-hydroxy-2-methoxybenzaldehyde (13) were recrystallized from $CHCl_3$–hexane (1 : 1) and 5% ethanol, respectively (two or three times), to constant specific radioactivity (*cf.* Tables 1 and 2).

The amounts of radioactive echinatin that were submitted to the degradation reaction and the yields of the products are also included in Table 6.

Feeding Experiment with Sodium [1,2-$^{13}C_2$]*Acetate.*—The *G.echinata* callus M-1-3 strain (4 weeks old, fresh weight 542.4 g) was suspended in liquid medium (2 l) divided in eight flasks to which were added equal portions of sodium [1,2-$^{13}C_2$]acetate (90% enriched, Merck Sharp and Dohme, Montreal) (total 77.2 mg), and the flasks were shaken (*ca.* 85 r.p.m.) at 27 °C in the dark. After ten days 1 ml of a 50% ethanol solution of sodium [1,2-$^{13}C_2$]acetate (87.9 mg in 8 ml) was added through a filter (Millex-GS, 0.22 μm filter unit, Millipore Corp.) to each flask, and the incubation was continued under the same conditions for a further 8 days.

The callus was separated from the medium by filtration through nylon cloth and extracted with methanol. The condensed methanol extract was fractionated between ethyl acetate and water, as described above. The concentrated ethyl acetate extract (0.91 g) was subjected to column chromatography on silica gel (300 g) with benzene–ethyl acetate (9 : 1→17 : 3) as eluant. The fractions that contained echinatin (1) (90 : 15 mg, after evaporation) were rechromatographed on silica gel (90 g) with $CHCl_3$–MeOH (197 : 3) as eluant to yield fractions that gave one spot of compound (1) on t.l.c. Recrystallization from ethanol–water gave ^{13}C-enriched compound (1) (56.60 mg) as yellow needles. The fractions from the first column chromatography that eluted first and that contained formononetin (5) were combined, concentrated, and submitted to silica gel (30 g) column chromatography ($CHCl_3$–MeOH, 197 : 3) to afford ^{13}C-enriched compound (5) (15.5 mg) as needles (recrystallized from methanol).

^{13}C N.m.r. (25 MHz) spectra of ^{13}C-enriched echinatin (1) and formononetin (5) were recorded at room temperature, pulse width 12 μs, repetition 1.5 s, frequency range 5 000 Hz,

data points 8 K, number of scans 4 000 [for compound (1)] and 108 000 [for compound (5)]. The spectrum (50 MHz) of ^{13}C-enriched compound (1) was recorded at room temperature, pulse width 2.5 μs, repetition 2.0 s, frequency range 10 000 Hz, data points 16 K, number of scans 4000.

Grateful acknowledgement is made to Professor S. Shibata (Meiji College of Pharmacy), Professor T. Saitoh (Teikyo University), and Professor U. Sankawa (University of Tokyo) for biosynthetic experiments with tritium-labelled isoliquiritigenin and for useful discussion; also to Dr. T. Yoshikawa (Kitasato University) for derivation and culture of mutant strains of *G. echinata* callus, and to the members of the Pharmaceutical Development Laboratories (Meiji Seika Kaisha Ltd.) for measurement of 50 MHz ^{13}C n.m.r. spectra.

[2/288 *Received*, 16*th February*, 1982]

REFERENCES

[1] Part 35, S. Ayabe and T. Furuya, *Tetrahedron Lett.*, 1981, **22**, 2097.
[2] For preliminary papers, see (*a*) T. Saitoh, S. Shibata, U. Sankawa, T. Furuya, and S. Ayabe, *Tetrahedron Lett.*, 1975, **4463**; (*b*) S. Ayabe and T. Furuya, ref. 1.
[3] (*a*) F. Kreuzaler and K. Hahlbrock, *Eur. J. Biochem.*, 1975, **56**, 205; (*b*) W. Heller and K. Hahlbrock, *Arch. Biochem. Biophys.*, 1980, **200**, 617; *cf.* (*c*) K. Hahlbrock and H. Grisebach, in ' The Flavonoids,' eds. J. B. Harborne, T. J. Mabry, and H. Mabry, Chapman and Hall, London, 1975, p. 866.
[4] T. Furuya, M. Hikichi, and K. Matsumoto, *Tetrahedron Lett.*, 1971, 2567.
[5] S. Ayabe, M. Kobayashi, M. Hikichi, K. Matsumoto, and T. Furuya, *Phytochemistry*, 1980, **19**, 2179.
[6] T. Saitoh and S. Shibata, *Tetrahedron Lett.*, 1975, 4461.
[7] M. Kaneda, T. Saitoh, Y. Iitaka, and S. Shibata, *Chem. Pharm. Bull.*, 1973, **21**, 1338.
[8] T. Furuya, S. Ayabe, and M. Kobayashi, *Tetrahedron Lett.*, 1976, 2539.
[9] P. R. White, ' The Cultivation of Animal and Plant Cells,' Ronald Press, New York, 2nd edn., 1963, p. 59.
[10] A. Nishi, A. Yoshida, M. Mori, and N. Sugano, *Phytochemistry*, 1974, **13**, 1653.
[11] *cf.* P. Maliga, in ' Frontiers of Plant Tissue Culture,' ed. T. A. Thorpe, Univ. of Calgary, Calgary, 1978, p. 381.
[12] T. Murashige and F. Skoog, *Physiol. Plant.*, 1962, **15**, 473.
[13] S. Ayabe, T. Yoshikawa, M. Kobayashi, and T. Furuya, *Phytochemistry*, 1980, **19**, 2331.
[14] D. J. Dewar, Ph.D. Thesis, Univ. of Saskachewan, 1973 (*Chem. Abstr.*, 1974, **80**, 105120u; *Diss. Abstr. Int. B*, 1973, **34**, 1919).
[15] (*a*) W. Hosel and W. Barz, *Biochem. Biophys. Acta*, 1972, **261**, 294; (*b*) W. Hosel, G. Frey, and W. Barz, *Phytochemistry*, 1975, **14**, 417.
[16] G. Frey-Schröder and W. Barz, *Z. Naturforsch. Teil C*, 1979, **34**, 200.
[17] (*a*) A. H. Williams, *Phytochemistry*, 1979, **18**, 1897; (*b*) G. Camele, F. D. Monache, and G. B. M. Bettolo, *ibid.*, 1980, **19**, 707; *cf.* (*c*) K. Venkataraman, ref. 3*c*, p. 279.
[18] G. Stotz and G. Forkman, *Z. Naturforsch. Teil C*, 1981, **36**, 737.
[19] L. Britsch, W. Heller, and H. Grisebach, *Z. Naturforsch. Teil C*, 1981, **36**, 742.
[20] A. Pelter, R. S. Ward, and T. I. Gray, *J. Chem. Soc., Perkin Trans. 1*, 1976, 2475.
[21] (*a*) B. Ternai and K. R. Markham, *Tetrahedron*, 1976, **32**, 565; (*b*) G. C. Levy and G. L. Nelson, ' Carbon-13 Nuclear Magnetic Resonance for Organic Chemists,' Wiley-Interscience, New York, 1972, p. 79.
[22] R. J. Light and K. Hahlbrock, *Z. Naturforsch. Teil C*, 1980, **35**, 717.
[23] P. Dimroth, H. Walter, and F. Lynen, *Eur. J. Biochem.*, 1970, **13**, 98.
[24] A. Pelter, R. S. Ward, and R. J. Bass, *J. Chem. Soc., Perkin Trans. 1*, 1978, 666.
[25] A. Stoessl and J. B. Stothers, *Z. Naturforsch. Teil C*, 1979, **34**, 87.
[26] (*a*) F. Bohlmann, P. K. Mahanta, and C. Zdero, *Phytochemistry*, 1978, **17**, 1935; (*b*) G. D. Manners and L. Jurd, *ibid.*, 1979, **18**, 1037.
[27] L. R. Pohl, R. Haddock, W. A. Garland, and W. F. Trager, *J. Med. Chem.*, 1975, **18**, 513.
[28] D. R. Nadkarni and T. S. Wheeler, *J. Chem. Soc.*, 1938, 1320.
[29] St. V. Kostanecki and F. W. Osius, *Chem. Ber.*, 1899, **32**, 321.
[30] E. C. Bate-Smith, T. Swain, and G. S. Pope, *Chem. Ind. (London)*, 1953, 1127.