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Biosynthesis of Puerarin in Pueraria Root[1]

TAKAO INOUE and MASAO FUJITA

Hoshi College of Pharmacy[2]

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^{14}C-Labelled isoliquiritigenin was efficiently incorporated into puerarin of *Pueraria lobata* but ^{14}C-labelled daidzein was almost not into it. These results support that the C-glycosylation occurs at the stage of chalcone or flavanone. Further competitive feeding experiments with T- or ^{14}C-labelled isoliquiritigenin and liquiritigenin suggest that C-glycosylchalcone would be a possible intermediate for the biosynthesis of puerarin.

Keywords——*Pueraria lobata* (WILLD.) OHWI; biosynthesis; C-glycosylation of isoflavone; competitive feeding experiment; puerarin; daidzin; isoliquiritigenin-α-^{14}C, -(carbonyl-^{14}C) and -3,5-T_2; liquiritigenin-4-^{14}C and -3′,5′-T_2; daidzein-4-^{14}C

Various flavonoid C-glycosides as well as a number of the O-glycosides have been found in plants.

Wallace, *et al.*[3] observed after administration of ^{14}C-labelled flavones to *Spirodela* and *Lemna* species that these aglycones were incorporated into the O-glycoside but almost not into the C-glycosides. Furthermore, Wallace and Grisebach[4] reported that C-glycosylation of flavone occurs at the flavanone level in the same species of plants. We have studied on the biosynthesis of a isoflavone C-glycoside, puerarin in *Pueraria* root and propose that C-glycosylation of isoflavone probably takes place rather at the stage of chalcone than that of flavanone.

Results and Discussion

Pueraria lobata (WILLD.) OHWI[5] was used as the plant material, the root of which contains daidzein, daidzin (daidzein 7-O-glucoside) and puerarin (daidzein 8-C-glucoside) as the main isoflavonoids.[6]

Feeding Experiments with ^{14}C-Labelled Compounds

The labelled compounds were prepared as follows. Isoliquiritigenin (2′,4′,4-trihydroxychalcone)-α-^{14}C (I) and -(carbonyl-^{14}C) (II) were synthesized *via* the corresponding resacetophenone-^{14}C from sodium acetate-2-^{14}C and -1-^{14}C, respectively.[7,8] (±)-Liquiritigenin (7,4′-dihydroxyflavanone)-4-^{14}C (III) was obtained by isomerization of II with acid.[8] Daidzein-4-^{14}C (IV) was synthesized *via* 2,4-dihydroxyphenyl-4′-methoxybenzylketone-(carbonyl-^{14}C) from

1) A part of this study has been reported as a preliminary communication: T. Inoue and M. Fujita, *Chem. Pharm. Bull.* (Tokyo), **22**, 1422 (1974).
2) Location: *Ebara 2-4-41, Shinagawa-ku, Tokyo.*
3) J.W. Wallace, T.J. Mabry, and R.E. Alston, *Phytochemistry*, **8**, 93 (1969); J.W. Wallace, *ibid.*, **14**, 1765 (1975).
4) J.W. Wallace and H. Grisebach, *Biochim. Biophys. Acta*, **304**, 837 (1973).
5) *P. thunbergiana* (SIEB. et ZUCC.) BENTH. (synonym) was described as the scientific name in the preliminary communication.
6) S. Shibata, T. Murakami, and Y. Nishikawa, *Yakugaku Zasshi*, **79**, 757 (1959); T. Murakami, Y. Nishikawa, and T. Ando, *Chem. Pharm. Bull.* (Tokyo), **8**, 688 (1960).
7) S.R. Cooper, "Organic Syntheses," Vol. 21, ed. by N.L. Drake, John Wiley and Sons, Inc., New York, N.Y., 1941, p. 103.
8) T. Kubota and T. Hase, *Nippon Kagaku Zasshi*, **87**, 1201 (1966).

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potassium cyanide-^{14}C according to the method by Barz, *et al.*[9] and daidzin-^{14}C was prepared biosynthetically by feeding phenylalanine-3-^{14}C to the plant.

Various labelled compounds shown in Table I were fed to the intact plants with the cotton thread method. Phenylalanine was administered as an aqueous solution of pH 7.0 and 9.0 in order to examine the influence of alkaline medium on the incorporation, and flavonoid compounds as sodium salts in an aqueous solution. After 80 hr feeding, radioactive daidzin and puerarin were isolated from the roots by the procedure described in the experimental part.

Table I. Incorporation of ^{14}C-Labelled Precursors into Daidzin and Puerarin in *Pueraria* Roots

Expt.	Precursors (sp. act.; amount fed)	Daidzin			Puerarin		
		Yield (mg)	Sp. act. (dpm/mM)	Incorporation (%)	Yield (mg)	Sp. act. (dpm/mM)	Incorporation (%)
1.	Phenylalanine-3-^{14}C[a)]	38	1.71×10^7	1.35	75	5.67×10^6	0.92
2.	Phenylalanine-3-^{14}C[a,b)]	278	2.54×10^6	1.47	610	4.64×10^5	0.61
3.	Phenylalanine-3-^{14}C[a)] +isoliquiritigenin 4′-glucoside (5 mg)	236	7.06×10^4	0.034	446	1.95×10^4	0.018
4.	Isoliquiritigenin-α-^{14}C (I) (3.18×10^7 dpm/mM; 5 mg)	70	2.70×10^4	0.75	176	4.37×10^3	0.32
5.	Isoliquiritigenin-(carbonyl-^{14}C) (II) (3.46×10^8 dpm/mM; 5 mg)	193	9.80×10^4	0.69	421	1.57×10^4	0.25
6.	Daidzein-4-^{14}C (IV) (1.42×10^9 dpm/mM; 5.3 mg)	170	1.45×10^5	0.20	228	5.75×10^3	0.011
7.	Daidzin-^{14}C (1.71×10^7 dpm/mM; 5 mg)	64	1.38×10^4	1.03[c)]	102	1.39×10^2	0.017

Weight of dried roots: 1. 4.7 g, 2. 35.5 g, 3. 31.0 g, 4. 11.4 g, 5. 22.8 g, 6. 17.1 g, 7. 7.5 g.
a) Total act.: 50 μCi, 1.9 mg.
b) The same amount of 0.1N NaOH as in the case of Expt. 3 was added to an aqueous solution.
c) Recovery (%).

As shown in Table I, phenylalanine-3-^{14}C in neutral or week alkaline medium was incorporated into daidzin and puerarin in high ratios, but when it was fed together with isoliquiritigenin 4′-glucoside, the incorporation was clearly suppressed (Expt. 1—3). In addition to these findings, two chalcones (I) and (II) were efficiently incorporated into daidzin and puerarin (Expt. 4 and 5). In order to confirm the presence of ^{14}C at the expected position of the isoflavone nucleus, daidzin and puerarin obtained in Expt. 4 were directly oxidized with hydrogen peroxide in alkaline solution to give *p*-hydroxybenzoic acid. From the comparison of the specific activities of two glucosides and their degradation products, more than 96% of radioactivities of daidzin and puerarin were found to be present in *p*-hydroxybenzoic acid including the expected labelled carbon (C-3) of both glucosides. Consequently, the chalcone was indicated to be incorporated into daidzin and puerarin without randomization. These results reveal that the chalcone is not only a good precursor for daidzin but also puerarin. It has been reported that flavonoid is O-glycosylated after the formation of aglycone.[3,10] Daidzein-4-^{14}C (IV) was incorporated into daidzin as expected, but IV and daidzin-^{14}C were also slightly incorporated into puerarin. Their incorporation into puerarin were found to be much lower than that of IV into daidzin (Expt. 6 and 7). The fact suggests that daidzein is not a direct precursor for puerarin and the slight incorporation of daidzein into puerarin seems to be due to the utilization of some radioactive degradation products of

9) *a*) W. Barz, Ch. Adamek, and J. Berlin, *Phytochemistry*, **9**, 1737 (1970); *b*) P.M. Dewick, W. Barz, and H. Grisebach, *ibid.*, **9**, 775 (1970).
10) A. Sutter, R. Ortmann, and H. Grisebach, *Biochim. Biophys. Acta*, **258**, 71 (1972).

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daidzein, as it was observed by Barz, *et al.*[9a] that daidzein was relatively rapidly metabolized in mung been plants.

These results indicate that C-glycosylation in puerarin occurs during the biosynthesis of isoflavone nucleus from the chalcone, whereas O-glycosylation in daidzin occurs after the formation of aglycone.

Competitive Feeding Experiments with ^{14}C- and T-Labelled Compounds

Wong[11] demonstrated from the results of parallel competitive feeding experiments with ^{14}C-chalcone or (−)-flavanone that chalcone and (−)-flavanone are biochemically interconvertible but chalcone is a more immediate precursor than flavanone for the biosynthesis of flavonoid, and this conclusion has been supported by further studies of Wong and Grisebach.[12] Recently it was reported that flavanone is a more immediate precursor with regard to the biosynthesis of flavone by enzymatic studies.[13]

It would be necessary to comfirm whether C-glycosylation of isoflavone occurs at the stage of chalcone or flavanone, although flavone was reported to be C-glycosylated at the flavanone stage.[4] Thus competitive experiments were carried out by application of the method described in the above reports.[11,12]

Isoliquiritigenin-3,5-T_2 (V) was prepared from *p*-hydroxybenzaldehyde-3,5-T_2 (VI) which was obtained according to the method of Wong and Grisebach[12] by exchange reaction with tritiated water, and (±)-liquiritigenin-3′,5′-T_2 (VII) was prepared by isomerization of V with acid.

In the first series of experiments, (a) a mixture of isoliquiritigenin-(carbonyl-^{14}C)(II) (3 mg) and non-radioactive (±)-liquiritigenin (3 mg) or (b) a mixture of (±)-liquiritigenin-4-^{14}C (III) (3 mg) of the same specific activity and non-radioactive isoliquiritigenin (3 mg) was fed to the plants. Each mixture was administered as sodium salt in an aqueous solution to the intact plants by the cotton thread method. After 80 hr feeding, daidzin and puerarin were isolated from the roots and the incorporation ratios of II and III were compared. As

Table II. Incorporation of ^{14}C-Labelled Chalcone or Flavanone into Daidzin and Puerarin in *Pueraria* Roots

Expt.	Precursors[a]	Daidzin Yield (mg)	Daidzin Sp. act. (dpm/mM)	Daidzin Incorporation (%)	Puerarin Yield (mg)	Puerarin Sp. act. (dpm/mM)	Puerarin Incorporation (%)
8. (a)	Isoliquiritigenin-(carbonyl-^{14}C) (II) + (±)-liquiritigenin	537	2.49×10^4	0.81	1256	4.17×10^3	0.33
(b)	(±)-Liquiritigenin-4-^{14}C (III) + isoliquiritigenin	476	4.44×10^3	0.13[b]	998	1.12×10^3	0.07[b]

Weight of dried roots: (a) 57.2 g, (b) 56.5 g.
a) Sp. act.: 3.46×10^8 dpm/mM, compounds fed: 3 mg each.
b) Calculated as incorporation of (±)-flavanone.

shown in Table II, the incorporation into puerarin was found to be higher in the case of (a) than (b), even if (+)-flavanone was almost not to be utilized as a precursor. The results suggest that isoliquiritigenin is probably more efficient precursor for puerarin than liquiritigenin. In order to obtain more conclusive evidence for this suggestion, the second series of experiments were carried out, in which (a) a mixture of II (3 mg) and VII (3 mg), (b) a mixture of III (3 mg) and V (3 mg) or (c) a mixture of III (4 mg) and V (2 mg) was fed to the

11) E. Wong, *Phytochemistry*, **7**, 1751 (1968).
12) E. Wong and H. Grisebach, *Phytochemistry*, **8**, 1419 (1969).
13) A. Sutter, J. Poulton, and H. Grisebach, *Arch. Biochem. Biophys.*, **170**, 547 (1975).

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plants. After working in a similar manner as in the first series of experiments, the incorporation ratios into daidzin and puerarin were compared. The results are summarized in Table III.

Table III. Ratio of T and ^{14}C Activities of Labelled Precursors, and Daidzin and Puerarin in *Pueraria* Roots

Expt.	Precursors[a] and products	Yield (mg)	Sp. act. (dpm/mM) T	Sp. act. (dpm/mM) ^{14}C	Ratio T/^{14}C	Incorporation (%) T	Incorporation (%) ^{14}C
9.	Isoliquiritigenin-(carbonyl-^{14}C) (II) + (±)-liquiritigenin-3′,5′-T_2 (VII)		1.24×10^9	2.05×10^8			
	(a) Daidzin	226	5.65×10^4	2.04×10^4	2.77	0.24	0.56
	Puerarin	443	1.76×10^4	8.49×10^3	2.07	0.14	0.40
	Isoliquiritigenin-3,5-T_2 (V) + (±)-liquiritigenin-4-^{14}C (III)		1.89×10^9	1.62×10^8			
	(b) Daidzin	304	1.34×10^5	5.30×10^3	25.28	0.45	0.21
	Puerarin	582	3.73×10^4	1.38×10^3	27.03	0.25	0.11
	(c) Daidzin	102	2.37×10^5	1.81×10^4	13.09	0.40	0.18
	Puerarin	169	9.18×10^4	6.84×10^3	13.42	0.27	0.12

Weight of dried roots: (a) 29.6 g, (b) 25.2 g, (c) 7.1 g.
a) Compound fed; T/^{14}C of precursor mixtures: (a) 3 mg each; 6.05, (b) 3 mg each; 11.67, (c) chalcone-T 2 mg, flavanone-^{14}C 4 mg; 5.84.

On the other hand, daidzin and puerarin obtained in Expt. 9-(a) were degraded to confirm the presence of T and ^{14}C at the expected position of the isolavone nucleus. Daidzin was hydrolized to obtain daidzein, which was degraded by alkali to 2,4-dihydroxyphenyl-4′-hydroxybenzylketone.[14] As shown in Table IV, most of T of daidzin and puerarin was found to be localized in *p*-hydroxybenzoic acid obtained by the degradation of them. ^{14}C of daidzin was mostly distributed in the above ketone but not in *p*-hydroxybenzoic acid. Accordingly, it was demonstrated that T is almost located at C-3′ and -5′ of isoflavone nucleus, and ^{14}C probably at the carbonyl carbon.

Table IV. Distribution of T and ^{14}C of Daidzin and Puerarin in *Pueraria* Roots after Feeding of Chalcone-^{14}C and Flavanone-T

Degradation products	Sp. act. (dpm/mM) T	Sp. act. (dpm/mM) ^{14}C	Ratio (%) T	Ratio (%) ^{14}C
Daidzin	5.65×10^4	2.04×10^4	100	100
Daidzein	5.03×10^4	2.01×10^4	89.0	98.5
2,4-Dihydroxyphenyl-4′-hydroxybenzylketone	5.11×10^4	1.99×10^4	90.4	97.5
p-Hydroxybenzoic acid	5.59×10^4	0	98.9	0
Puerarin	1.76×10^4	8.49×10^3	100	100
p-Hydroxybenzoic acid	1.78×10^4	0	100.1	0

In the case of Expt. 9-(a) the T/^{14}C ratio of puerarin was found to be obviously smaller than half of that of the mixture fed, while in (b) and (c) the ratios in puerarin were shown to be more than two times of that of the mixture fed. These results support the suggestion obtained from the first series of experiments that isoliquiritigenin is more immediate precursor for puerarin than liquiritigenin.

14) E. Walz, *Ann. Chem.*, **489**, 118 (1931).

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These competitive feeding experiments indicate that C-glycosylation in puerarin occurs rather at the stage of chalcone than that of flavanone. Although any C-glycosylchalcone has not been recorded in literature, occurrence of C-glycosyldihydrochalcones has been recognized in other species.[15] Consequently, we propose that puerarin is biosynthesized from isoliquiritigenin *via* C-glycosylchalcone as a possible intermediate. Probable biosynthetic pathway for daidzin and puerarin in *Pueraria* root is shown in Chart 1.

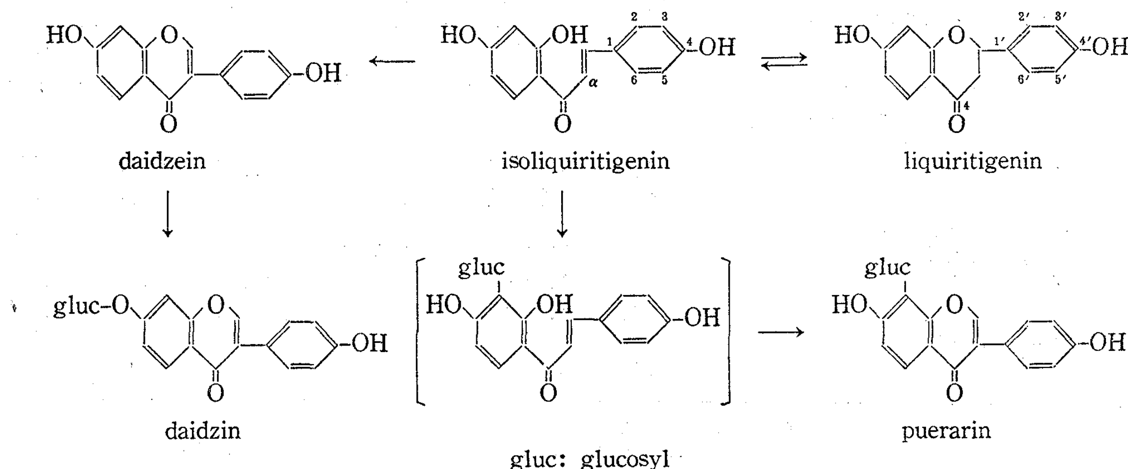

Chart 1

Experimental

Sodium acetate-1-^{14}C and -2-^{14}C were purchased from Daiichi Pure Chemicals Co., Ltd., phenylalanine-3-^{14}C from New England Nuclear Corp. and potassium cyanide-^{14}C from the Radiochemical Centre, Amersham. All radioactive samples were purified to constant specific activity and counted with an Aloka LSC-602 liquid scintillation counter, in a POP-POPOP-naphthalene-dioxane scintillator solution.

Isoliquiritigenin-*α*-^{14}C (I)[7,8]——Anhydrous $ZnCl_2$ (3.0 g) was dissolved in a mixture of $^{14}CH_3COONa$ (1 mCi) and AcOH (4.0 ml) by heating and resorcinol (5.7 g) was added to this hot solution at 140° with stirring. The solution was heated at 150° for 20 min and poured into a mixture of HCl–H_2O (1 : 1). After cooling at 5°, the resulting precipitate was collected and washed with HCl–H_2O (1 : 3). Crude resacetophenone was recrystallized from dilute HCl to give light yellowish brown needles. Yield 1.37 g, mp 143—144°. The solution of resacetophenone (0.5 g) and *p*-hydroxybenzaldehyde (0.4 g) in EtOH (1 ml) was added to a solution of KOH (4 g) and H_2O (3 ml) under cooling. The mixture was kept in N_2 stream at room temperature for 4 days, then diluted with H_2O and acidified with dilute HCl. The resulting crystals were recrystallized from 50% EtOH to give yellow needles. Yield 208 mg, mp 200—202°, sp. act. 3.18×10^7 dpm/mM.

Isoliquiritigenin-(carbonyl-^{14}C) (II)——Resacetophenone was synthesized from $CH_3{}^{14}COONa$ (3 mCi), AcOH (1.3 ml) and resorcinol (1.9 g) in a smaller scale than the above method, and II was prepared from resacetophenone-(carbonyl-^{14}C) (0.4 g) and *p*-hydroxybenzaldehyde (0.32 g). Crude crystals of II were purified by column chromatography on silica gel (Merck), using AcOEt as eluent. Yield 112 mg, mp 198—200°, sp. act. 3.46×10^8 dpm/mM.

(±)-Liquiritigenin-4-^{14}C (III)[8]——A solution of II (70 mg) in EtOH (2 ml) was refluxed with 3.5% HCl (5 ml) for 15 hr. After cooling, the reaction mixture was diluted with H_2O and extracted with ether. The ether extract was concentrated and separated by preparative thin-layer chromatography (TLC) (Merck, Silica Gel GF_{254}), using isopropyl ether and then isopropyl ether–toluene (1 : 5) as the developing solvents. Crude crystals of III were chromatographed on polyamide (Woelm) and elution with 50% MeOH gave colorless needles, which were recrystallized from dilute EtOH. Yield 40 mg, mp 200—203°.

Daidzein-4-^{14}C (IV)——2,4-Dihydroxyphenyl-4′-methoxybenzylketone-(carbonyl-^{14}C) was prepared from resorcinol (400 mg) and *p*-methoxyphenylacetonitrile-1-^{14}C which was synthesized from anisyl chloride (400 mg), $K^{14}CN$ (2.4 mg, 2 mCi) and KCN (250 mg) according to the method by Dewick, *et al.*[9b] The crude ketone was recrystallized from $CHCl_3$ to give almost colorless needles. Yield 313 mg, mp 154—156°. The above ketone (290 mg) was treated with pyridine, piperidine and ethyl orthoformate to give formononetin-

15) J. Chopin and M.L. Bouillant, "The Flavonoids," ed. by J.B. Harborne, T.J. Mabry, and H. Mabry, Chapman and Hall Ltd., London, 1975, pp. 632—691.

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4-^{14}C as described by Barz, *et al.*[9a)] Crude formononetin-4-^{14}C was chromatographed on silica gel and elution with C_6H_6–AcOEt (4 : 1), followed by recrystallization from EtOH afforded colorless needles. Yield 49.2 mg, mp 254—256°. Formononetin-4-^{14}C was demethylated with HI by the usual method and IV obtained was recrystallized from 50% EtOH to give colorless needles. Yield 29 mg, mp 315—317°. Sp. act. 1.42×10^9 dpm/mM.

***p*-Hydroxybenzaldehyde-3,5-T_2 (VI)**——Labelling[16)] of *p*-hydroxybenzaldehyde was carried out by exchange with tritiated water under the condition of Wong and Grisebach.[12)] After an addition of appropriate amount of carrier, VI was purified by column chromatography on silica gel using C_6H_6–ether (1 : 1) and recrystallized from H_2O. Sp. act. 1.87×10^9 dpm/mM.

Isoliquiritigenin-3,5-T_2 (V) and (±)-Liquiritigenin-3′,5′-T_2 (VII)——These were prepared according to the synthetic method of I and III. V was obtained from VI (0.3 g) and resacetophenone (0.38 g). Yield 167 mg, sp. act. 1.89×10^9 dpm/mM. VII was obtained from V (110 mg) by heating with dilute HCl. Yield 71 mg, sp. act. 1.24×10^9 dpm/mM.

Feeding Experiments——Phenylalanine-3-^{14}C dissolved in H_2O (0.5 ml) or any flavonoid precursor dissolved in a minimum amount of 0.1 N NaOH and H_2O (0.5 ml) was administered by the cotton thread method to *Pueraria lobata* plants which were cultivated for 1—2 years before use. The solution was absorbed into the plants for 15—20 hr and then a little amount of H_2O was frequently supplied in order to make the remaining precursors to be absorbed. After 80 hr feeding, the roots containing a part of stem were cut into small bulk and dried at 60°.

Isolation of Daidzin and Puerarin——Dried roots were cut into pieces and extracted repeatedly with MeOH. The MeOH extract was concentrated to a small volume and stored overnight in a refrigerator. The resulting precipitate was filtered off and the filtrate was chromatographed on polyamide. Elution with 10—30% MeOH afforded daidzin and then puerarin. Daidzin was recrystallized from MeOH to give colorless needles, mp 215—217°. Puerarin was recrystallized from 80% AcOH to give colorless prisms, mp 185°. Yields and sp. acts. of them are summarized in Table I, II, and III.

Oxidation of Daidzin and Puerarin with Hydrogen Peroxide——Daidzin obtained in Expt. 4 and 9-(a), and puerarin in Expt. 4 were diluted to about 2—10 times with carrier and degraded as follows. To a solution of daidzin (100 mg) in 10% KOH (10 ml) was added 6% H_2O_2 (24 ml) and the mixture was allowed to stand overnight at room temperature. After decomposition of excess H_2O_2 with MnO_2, the reaction mixture was acidified with 10% H_2SO_4 and extracted with ether. Removal of ether afforded *p*-hydroxybenzoic acid, which was recrystallized from H_2O to give colorless needles. Yield 5 mg, mp 212—213°. Puerarin (200 mg) was degraded by the same method to *p*-hydroxybenzoic acid. Yield 7 mg, mp 212—214°.

Hydrolysis of Daidzin——Daidzin (80 mg) was refluxed with 5% HCl in MeOH for 3 hr. Removal of MeOH afforded crude daidzein, which was recrystallized from 50% MeOH to give colorless needles. Yield 43 mg, mp 313° (dec.).

Alkali Fission of Daidzein——A mixture of daidzein (40 mg) and 0.4 N NaOH (4 ml) was heated at 90° for 20 min. After cooling, the reaction mixture was acidified with dilute HCl and the resulting precipitate was purified by preparative TLC using ether as solvent. Crude 2,4-dihydroxyphenyl-4′-hydroxybenzylketone was recrystallized from dilute MeOH to give colorless needles. Yield 12 mg, mp 186—187°.

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16) The labelling operation was carried out by request in the Radiochemical Centre, Amersham.

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