Note

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Biogenetic Studies on Natural Products. VII.[1] Biosynthesis of Isoflavone in Red Clover.

Hiroshi Mitsuhashi, Ko Kaneko, and Marekichi Sasaki :

(*Faculty of Pharmaceutical Sciences, School of Medicine, Hokkaido University*[*1])

For the past several years experiments on the biosynthesis of phenylpropanoide have provided some evidences of biosynthetic pathways of lignin and flavone. Neish, *et al.*[2,3] reported the results on the biosynthesis of quercetin in buckwheat, and concluded that the C_6–C_3 type of precursor was incorporated in quercetin for the B ring and C-2, 3 and 4 without rearrangement. Nord, *et al.*[4] described the biochemical pathway of lignin from phenylpyruvate in the sugar plant. Kaneko[1,5,6] established the enzymatic pathway of the biosynthesis of anethole which was formed from phenylalanine without rearrangement of its side chain.

Geissman and Hinreiner suggested that the origin of the B ring and C-2, 3 and 4 of isoflavone may be formed in plants from a C_6-C-C-C fragment by Wagner-Meerwein rearrangement.[7~9]

Recently, Grisebach[10] demonstrated that in clover, phenylalanine was incorporated into formononetin with the retention of the carboxyl group and with a migration of the aryl group within a C_6-C-C-C fragment. Furthermore in chana plant, mechanism of biosynthesis of formononetin and biochanin-A was shown to be identical with that in clover[11] using phenylalanine. Moreover, it was recognized that the synthetic 4,4′,6′-trihydroxychalcone-4′-glucoside(β-^{14}C) was incorporated into formononetin in clover.[12] From these results, Grisebach suggested that isoflavone might be formed with a migration of the aryl Group of phenylalanine in plant. Present work was therefore undertaken to reexamine this problem, employing as the plant material red clover, *Trifolium pratense* sp., which contains genistein and formononetin.

Experimental

Plant Material——*Trifolium pratense* (1.5 kg.) was harvested by severing the stem just above the ground level immediately before the flowering, and was cultivated by the same method as described previously.[5] The culture solution contained 100 mg. of DL-phenylalanine which contained 100 mc. of phenylalanine[2-^{14}C]. The cultivation was continued for 6 days at 15~20°.

Experiments 1 and 2 were carried out in October and June, and the plant weights increased to 1.6 and 1.65 kg., respectively. The treated plant material was dried below 60°.

Extraction and Purification of Isoflavone——The modified Curnow's method[13] was employed :

*1 Kita-12-jo, Nishi 5-chome, Sapporo, Hokkaido (三橋 博, 金子 光, 佐々木希吉).

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The dried material was extracted 5 times with 800 cc. of EtOH. These EtOH extracts were combined and evaporated *in vacuo* to a volume of 1.5 L. H_2O was added to make the total volume 2.5 L. and then the mixture was extracted 4 times with 300 cc. benzene. The benzene extracts were washed 4 times with 100 cc. of 60% EtOH. These EtOH solutions were added to the original EtOH extract. The resulting pale-yellow solution was evaporated *in vacuo* to 50 cc. and extracted 3 times with 300 cc. of Et_2O.

The Et_2O phase was washed 5 times with 100 cc. of saturated $NaHCO_3$ solution and 100 cc. of H_2O and evaporated, yielding 1.6 g. of a yellow solid. For the separation of formononetin and genistein, 1.6 g. of the above material was chromatographed on 600 g. alumina, and eluted with BuOH-saturated H_2O. The ultraviolet absorption was measured for each 10 cc. of the eluate to locate formononetin and genistein. Formononetin was first eluted, and then the mixture of both compounds was eluted out. When this mixture was rechromatographed on silica gel, formononetin was eluted out with Et_2O. Finally genistein was eluted out with Et_2O-EtOH (9 : 1). Thus 43 mg. of formononetin and 40 mg. of genistein were obtained, which after recrystallization from aq. EtOH showed m.p. 256~257° and 298~299°, respectively. The purity of each compound was determined by the mixed melting point, IR spectra, micro analysis and chromatostrip as described previously.[5]

Degradation of Formononetin——The scheme of degradation is shown in Chart 1. The solution of 40 mg. of formononetin dissolved in 4 cc. of 0.4*N* NaOH was heated for 15 min. in H_2 gas on a boiling bath. After acidification by the addition of 10% H_3PO_4, the crude crystals of 2-(*p*-methoxyphenyl)-2′,4′-dihydroxyacetophenone were collected by filtration and recrystallized from aq. EtOH to crystals, m.p. 158°. Yield, 34 mg. (85%).

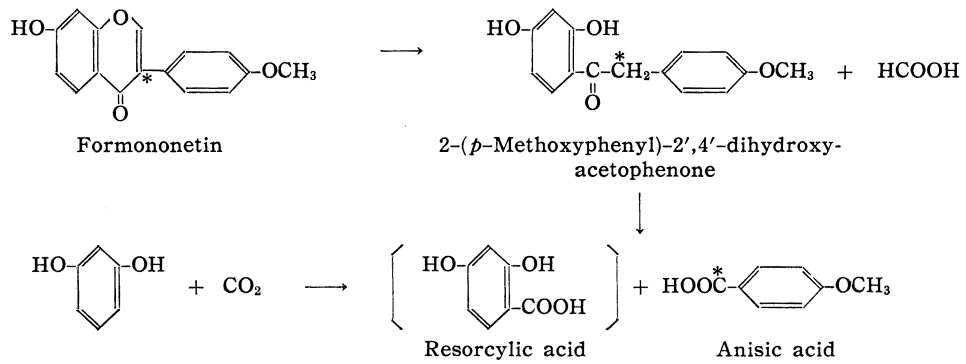
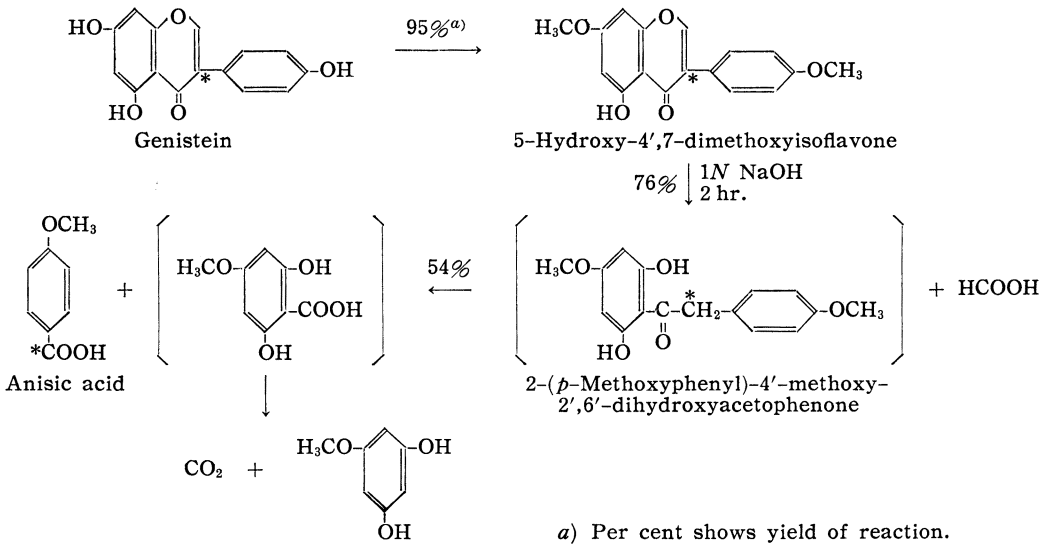


Chart 1. Degradation of Formononetin



a) Per cent shows yield of reaction.

Chart 2. Degradation of Genistein

The filtrate was distilled and the distillate was titrated with 0.01*N* NaOH to determine the amount of HCOOH generated. This neutral distillate was lyophilized and 10 mg. of HCOONa was obtained. A solution of 30 mg. of 2-(*p*-methoxyphenyl)-2′,4′-dihydroxyacetophenone dissolved in 3 cc. of 12.5% NaOH and 2 cc. of 14% of $KMnO_4$ was heated for 6 hr. in H_2 gas on a boiling bath. After acidification by the addition of dil. H_3PO_4, CO_2 evolved was washed with 5% $KMnO_4$ and absorbed in 0.2*N* NaOH solution (CO_2-free). $BaCl_2$ was added to this alkaline solution with exclusion of air and the resulting $BaCO_3$ was collected for radioactivity assay. The trapped CO_2 was originated from the carboxyl carbon of resorcylic acid. After cool, 13.5 mg. of anisic acid obtained from the reaction mixture, was recrystallized from H_2O, m.p. 182°. Yield 50%.

Degradation of Genistein——The degradation of genistein was carried out by essentially the same method as that for formononetin, except that before the degradation genistein was methylated with CH_2N_2. The results are shown in Chart 2.

Measurement of Radioactivty——The radioactivity of the samples was measured as described previously.[5]

Results and Discussion

It is evident that, as shown in Table I, red clover metabolized phenylalanine and the relative distribution of ^{14}C was 2.72% in the whole plant, 0.5% in the Et_2O extracted fraction, and 38.2% in respiratory carbon dioxide.

Table I.

Culture medium	Radioactivity (c. p. m. × 10^5)	Culture medium	Radioactivity (c. p. m. × 10^5)
Initial	2568	Whole plant	55.4
Final	575.5	Et_2O extract	1.3
Net	1993.5	Respiratory carbon dioxide	760.9

In the case of formononetin, it was found that the radioactivity of phenylalanine-[2-^{14}C] was distributed neither in formic acid nor in carbon dioxide, but concentrated upon anisic acid. The ratio of radioactivity of anisic acid for formononetin was 55~52% as shown in Experiments 1 and 2 in Table II. The experiment on genistein gave identical results with that on formononetin, as shown in Table III. Therefore, it may

Table II.

Formononetin	Number of carbon	Radioactivity (c. p. m. × 10^4/mmole $BaCO_3$) Found Exp. 1	Found Exp. 2	Calcd. Exp. 1	Calcd. Exp. 2	Result (%) Exp. 1	Result (%) Exp. 2
Formononetin	16	9.032	3.850	—	—	100.0	100.0
2-(*p*-Methoxyphenyl)-2′,4′-dihydroxyacetophenone	15	9.328	2.984	9.635	4.107	96.8	72.7
Formic acid	1	0	0	144.512	61.60	0	0
CO_2	1	0.023	0	144.512	61.60	0	0
Anisic acid	8	10.023	4.025	18.064	7.70	55.0	52.3

a) Calculated from radioactivity of formononetin.

Table III.

	Number of carbon	Radioactivity (c.p.m. × 10^4/mmole $BaCO_3$) Found	Calcd.	Result (%)
5-Hydroxy-4′,7-dimethoxyisoflavone	17	2.578	—	100.0
2-(*p*-Methoxyphenyl)-4′-methoxy-2′,6′-dihydroxyacetophenone	16			
Formic acid	1	0	43.826	0
CO_2	1	0.059	43.826	0.13
Anisic acid	8	2.80	5.477	51.9

a) Calculated from radioactivity of 5-hydroxy-7,4′-dimethoxyisoflavone.

be concluded that the biosynthesis of isoflavone in red clover was carried out through migration of the aryl group within the molecule of precursor's phenylpropanoides, as reported by Grisebach.

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Summary

It was shown that DL-phenylalanine[2-^{14}C] was incorporated into C-3 of two kinds of isoflavone, formononetin and genistein, by *Trifolium pratense* sp., *in vivo*.

These results indicate that the aryl group undergoes a migration within the C_6-C-C-C fragment, and this observation agrees with Grisebach's experimental data.

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