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METABOLISM OF PHENYLPROPANOIDS IN *HYDRANGEA SERRATA* VAR. *THUNBERGII* AND THE BIOSYNTHESIS OF PHYLLODULCIN

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Key Word Index—*Hydrangea serrata* var. *thunbergii*; Saxifragaceae; metabolism; DL-phenylalanine-[3-^{14}C]; cinnamic acid-[3-^{14}C]; biosynthesis of phyllodulcin.

Abstract—DL-Phenylalanine-[3-^{14}C] and cinnamic acid-[3-^{14}C] were fed to this plant and the label from cinnamic acid was incorporated into gallic acid, phyllodulcin and quercetin. By feeding *p*-coumaric acid-[U-^{3}H], caffeic acid-[U-^{3}H] and hydrangea glucoside A-[U-^{3}H], it was possible to show that hydroxylation at C-3′ in phyllodulcin occurs after the ring closure of dihydroisocoumarin. The biosynthetic pathway of phyllodulcin in this plant is thus: phenylalanine → cinnamic acid → *p*-coumaric acid → hydrangenol → phyllodulcin.

INTRODUCTION

In a preceding paper [1] we described the identification of *p*-hydroxybenzoic acid, protocatechuic acid, gallic acid, chlorogenic acid, methyl chlorogenate, kaempferol, quercetin, isoquercitrin, rutin, umbelliferone, hydrangenol, phyllodulcin, hydrangenol glucoside, hydrangea glucosides A, B and C, and phyllodulcin glucoside from the leaves of *Hydrangea serrata* var. *thunbergii*. From the point of view of phyllodulcin biosynthesis, it is of significance that hydrangea glucosides A and B (prototypes of dihydroisocoumarin glucoside) were isolated from this plant. This paper deals with the metabolism of DL-phenylalanine-[3-^{14}C] and cinnamic acid-[3-^{14}C] and biosynthetic studies of phyllodulcin.

RESULTS AND DISCUSSION

Metabolism of DL-*phenylalanine-[3-^{14}C] and cinnamic acid-[3-^{14}C]*

As shown in Table 1, the incorporation of label into C_6-C_1 carboxylic acids [2] occurred. High incorporation in gallic acid reveals the presence of the pathway via cinnamic acid from phenylalanine [3, 4], in addition to an usual route from shikimate [5]. The pathway to umbelliferone [6] through the glucoside is present in this plant. From this set of feeding experiments, it appears that cinnamic acid-[3-^{14}C] is effectively incorporated into phyllodulcin.

Biosynthesis of phyllodulcin

In hydrangenol biosynthesis, it has been shown that a hydroxylation at C-4′ in hydrangenol precedes the condensation of the phenylpropanoid molecule with polyketide [7]. Our evidence that *p*-coumaric acid is well incorporated into dihydroisocoumarins agrees with these earlier results. The stage at which hydroxylation at C-3′ in phyllodulcin occurs was studied by means of parallel feeding experiments with *p*-coumaric acid-[U-^{3}H], caffeic acid-[U-^{3}H] and hydrangea glucoside A-[U-^{3}H]. *p*-Coumaric acid appears to be a better precursor than caffeic acid for dihydroisocoumarins and hydrangea glucoside A is well incorporated into phyllodulcin. These findings suggest that the hydroxylation at C-3′ in phyllodulcin occurs after formation of the dihydroisocoumarin ring. Comparisons of incorporation ratio of *p*-coumaric acid-[U-^{3}H], caffeic acid-[U-^{3}H] and hydrangea glucoside A-[U-^{3}H] indicate that C-3′ hydroxy group in phyllodulcin is not introduced until after the formation of hydrangenol. Therefore, it is concluded that phyllodulcin is derived from hydrangenol by C-3′ hydroxylation and C-4′*O*-methylation. The biosynthetic pathways from phenylalanine to phyllodulcin is thus: phenylalanine → cinnamic acid → *p*-coumaric acid → hydrangenol → phyllodulcin.

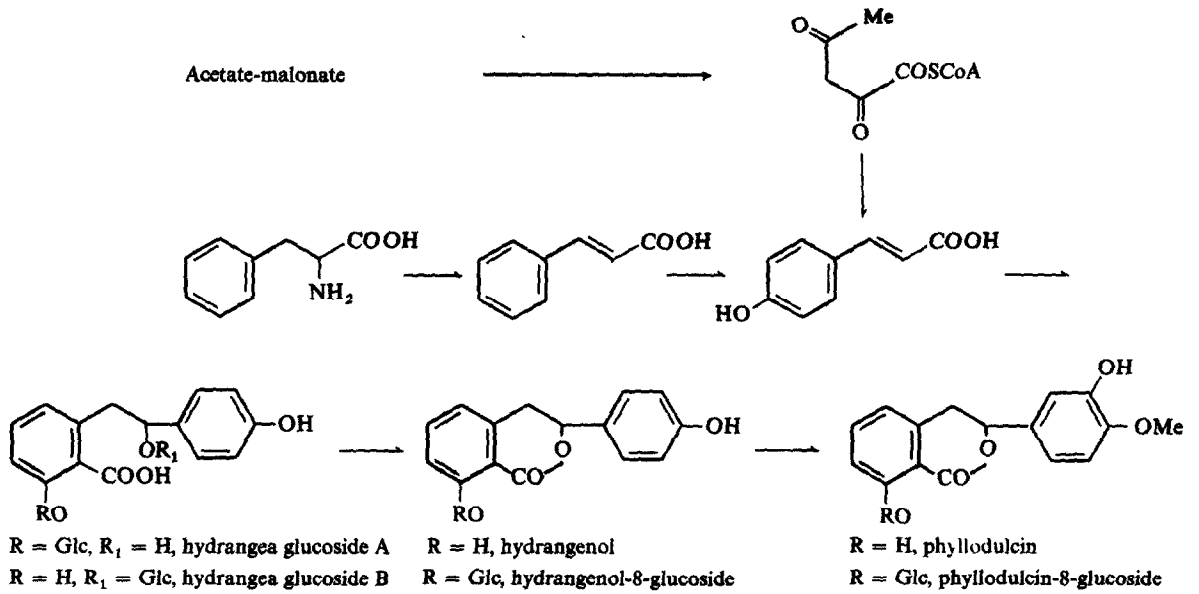
EXPERIMENTAL

General procedure. For the separation of phenolic acids, glucosides, coumarins and dihydroisocoumarins the following TLC systems were employed: TLC-1:C_6H_6–MeOH–HOAc (45:8:4); . TLC-2:C_6H_6–*iso*-PrOH–AcOH (45:8:4); TLC-3:$CHCl_3$–MeOH–H_2O (7:3:1, lower layer); TLC-4: C_6H_6–*iso*-PrOH (5:2 or 3:2). For the separation of *p*-hydroxybenzoic acid, protocatechuic acid and gallic acid: TLC-1, -2, -4 and TLC-5:EtOAc–MeCOEt–HCO_2H–H_2O–C_6H_6 (4:3:1:1:2). For the separation of umbelliferone, hydrangenol and phyllodulcin: TLC-4, TLC-6:$CHCl_3$ and TLC-7:C_6H_6–EtOAc (10:1). For the separation of gallic acid, kaempferol and quercetin: TLC-8: 30% AcOH on cellulose plate, TLC-9: BuOH–AcOH–H_2O (4:1:5, upper layer) on cellulose plate. Si gel (Merck) was used for TLC. As detecting reagents 1% $FeCl_3$ and diazotized benzidine solutions were used and as a monitor for hydrangenol, phyllodulcin and umbelliferone, a UV lamp was employed.

Preparation of radioactive compounds. DL-Phenylalanine-[3-^{14}C] and cinnamic acid-[3-^{14}C] were purchased from Daiichi Chemical Company (Osaka, Japan). *p*-Coumaric acid-[U-^{3}H], caffeic acid-[U-^{3}H] and hydrangea glucoside A-[U-^{3}H] were obtained by Wiltzbach method and unstable tritium was removed by the repeated evaporation under reduced pressure. The purity of radioactive compounds was monitored by radiochromatogram (Aloka TRN-1B) on TLC. The radioactivity

Table 1. Incorporation results from DL-phenylalanine-[3-^{14}C] and cinnamic acid-[3-^{14}C]

Metabolites	DL-Phenylalanine-[3-^{14}C] Incorpn(%)	Dilution	Cinnamic acid-[3-^{14}C] Incorpn(%)	Dilution
Free.				
p-Hydroxybenzoic acid	0.017	3.39×10^4	0.032	2.75×10^4
Protocatechuic acid	0.008	1.64×10^5	0.024	4.23×10^4
Gallic acid	0.035	2.93×10^4	0.237	7.60×10^3
Quercetin	0.005	8.18×10^5	0.192	4.30×10^3
Umbelliferone	0.234	6.04×10^3	0.099	4.05×10^3
Hydrangenol	0.015	6.06×10^4	0.051	4.93×10^3
Phyllodulcin	0.084	1.95×10^4	0.143	2.18×10^3
Conjugated:				
Umbelliferone	0.487	1.07×10^3	0.729	1.60×10^3
Kaempferol	0.006	8.29×10^5	0.099	1.93×10^4
Quercetin	0.011	3.12×10^4	0.196	4.20×10^3
Hydrangenol	0.024	3.50×10^4	0.024	1.48×10^4
Phyllodulcin	0.059	5.87×10^4	0.069	7.07×10^3



R = Glc, R_1 = H, hydrangea glucoside A
R = H, R_1 = Glc, hydrangea glucoside B
R = H, hydrangenol
R = Glc, hydrangenol-8-glucoside
R = H, phyllodulcin
R = Glc, phyllodulcin-8-glucoside

Scheme 1. The biosynthesis of phyllodulcin in *Hydrangea serrata* var. *thunbergii*.

Table 2. Incorporation results from *p*-coumaric acid-[U-^{3}H], caffeic acid-[U-^{3}H] and hydrangea glucoside A-[U-^{3}H]

Metabolites	*p*-Coumaric acid-[U-^{3}H] Incorpn(%)	Dilution	Caffeic acid-[U-^{3}H] Incorpn(%)	Dilution	Hydrangea glucoside A-[U-^{3}H] Incorpn(%)	Dilution
Free:						
Hydrangenol	0.273	32	0.008	1990		
Phyllodulcin	0.113	2650	0.015	37300	0.126 (0.226)*	217 (121)
Conjugated:						
Hydrangenol	1.127	917	0.187	1970		
Phyllodulcin	0.016	487	0.003	5020	0.479 (0.857)	77 (43)

* The values in parenthesis were calculated from the percentage (55.9%) of an aglycone, hydrangenol, obtained by acid hydrolysis of hydrangea glucoside A-[U-^{3}H].

was measured by a liquid scintillation soln consisting of PPO (0.4 g), POPOP (0.06 g) in toluene (100 ml) or of naphthalene (10 g), PPO (0.7 g) and POPOP (0.05 g) in dioxane (100 ml).

Feeding experiment. 1) An aq. soln of DL-phenylalanine-[3-^{14}C] (50 μCi, 1.46 × 10^{10} dpm/mM) was administered to the plant (60–70 cm, high) by cotton wick method and after the feeding for 48 hr leaves were cut off, washed, chipped and exhaustively extracted with MeOH. MeOH extract (3.96 × 10^7 dpm) suspended in Et_2O was separated into Et_2O soluble (Fr. 1) and $NaHCO_3$ soluble (Fr. 2) fractions. The Et_2O fraction (Fr. 1) was saponified with 5% KOH–EtOH for 0.5 hr refluxing and after the removal of an unsaponifiable fraction aq. layer was acidified with 10% HCl and extracted with Et_2O. The Et_2O extract was purified by preparative TLC to afford phyllodulcin, hydrangenol and umbelliferone. An alkaline fraction (Fr. 2) was acidified with 10% HCl and extracted with Et_2O to give *p*-hydroxybenzoic acid, protocatechuic acid and gallic acid. The H_2O layer was extracted with BuOH and after the removal of non-glycosidic substances, such as gallic acid, by preparative TLC, the glycosidic fraction was hydrolyzed with 5% H_2SO_4 for 1 hr and the hydrolysate was extracted with Et_2O to give aglycones. Aglycones were purified by preparative TLC to afford phyllodulcin, hydrangenol, umbelliferone, kaempferol and quercetin. Parallel feeding experiment using cinnamic acid-[3-^{14}C] (25 μCi, 1.01 × 10^{10} dpm/mM) was carried out. The MeOH extract (2.03 × 10^7 dpm) was separated to Fr. 1 and Fr. 2, and was carried out in the same way. The metabolites obtained from each feeding experiment were identified by dilution method and the results are summarized in Table 1. Also an aq. soln of *p*-coumaric acid-[U-^{3}H] (9.90 × 10^6 dpm, 2.64 × 10^9 dpm/mM), caffeic acid-[U-^{3}H] (1.13 × 10^7 dpm, 3.73 × 10^9 dpm/mM) or hydrangea glucoside A-[U-^{3}H] (2.95 × 10^6 dpm, 5.87 × 10^8 dpm/mM) was separately fed to cut plants. After feeding for 29 hr, each plant was extracted with MeOH and the MeOH extract [*p*-coumaric acid-[U-^{3}H] (5.98 × 10^6 dpm); caffeic acid-[U-^{3}H] (4.81 × 10^6 dpm); hydrangea glucoside A-[U-^{3}H] (2.85 × 10^6 dpm)] was chromatographed on polyamide by elution with MeOH–H_2O (4:1). The phenolic metabolites were separated by preparative TLC into acid-flavonoid, glycoside and dihydroisocoumarin fractions. The acid-flavonoid fraction was separated by preparative TLC to give *p*-hydroxybenzoic acid, protocatechuic acid, gallic acid, quercetin and rutin. Gylcosidic fraction was hydrolyzed with β-glucosidase and the aglycones extracted with Et_2O were separated by preparative TLC to afford hydrangenol and phyllodulcin. The fraction consisting of dihydroisocoumarins was separated by preparative TLC to give umbelliferone, hydrangenol and phyllodulcin. The metabolites obtained from each feeding experiment were identified by dilution method (see Table 2).

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ANTITUMOR AGENT FROM *JUNIPERUS BERMUDIANA* (PINACEAE): DEOXYPODOPHYLLOTOXIN

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As a result of the continuing search for plants having tumor-inhibitory constituents, it was found that the EtOH extract of the twigs and leaves of *Juniperus bermudiana* L. (Pinaceae)† showed inhibitory activity toward the P-388 lymphocytic leukemia (PS) and cytotoxic activity toward the human epidermoid carcinoma of the nasopharynx (KB) test systems of the Division of Cancer Treatment, National Cancer Institute, N.I.H., Bethesda, MD. *Juniperus bermudiana* L. has not been previously examined but other *Juniperus* species have been shown to contain lignans [1], including deoxypodophyllotoxin [2]. We now wish to report that deoxypodophyllotoxin has been isolated from *J. bermudiana* and indeed was the agent solely responsible for the cytotoxic activity.

The ethanol extract of the defatted, dried and ground twigs and leaves of *Juniperus bermudiana* was partitioned between $CHCl_3$ and H_2O (1:1). The $CHCl_3$ phase, after evaporation of the solvent, was treated with hexane. The hexane-soluble portion was extracted with 5% $NaHCO_3$ and the neutral portion was chromatographed on a Si gel 60 (E. Merck) column. The separation was monitored at all stages by testing the fractions obtained in the KB test system. Thus the

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† The plant was collected in Hawaii in February, 1972. Identification was confirmed by Dr. Robert E. Perdue, Chief, Medicinal Plant Resources Laboratory, U.S.D.A., Beltsville, MD. A reference specimen is maintained by the U.S.D.A.