Fluorescent Glycosides Accumulated in *Taphrina wiesneri*-infected Cherry Stems

Part I.[†] Contents of Glycosides, and the Isolation and Identification of Gentisic Acid-5- β -D-glucoside

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It was observed that three kinds of fluorescent glycosides (FG-1, FG-2, and FG-3) were more abundantly accumulated in *Taphrina wiesneri*-infected cherry (*Prunus yedoensis*) stems than in healthy ones. The contents of FG-1, FG-2, and FG-3 in infected stems were, respectively, 2.7, 7.2, and 4.3 times those in healthy stems. In dried infected stems, FG-1, FG-2, and FG-3, amounted respectively to 0.005, 0.18, and 0.12% in the bark part; and to 0.003, 0.048, and 0.008% in the wood part.

FG-1 was isolated in crystalline form, and was identified with gentisic acid-5- β -D-glucopyranoside by the comparison of its chemical and physical properties with those of synthesized gentisic acid-5- or 2- β -D-glucopyranoside.

When the Japanese flowering cherry is infected by *Taphrina wiesneri* various symptoms appear. In previous papers,^{1,2)} we dealt with the chemical components of infected cherry leaves and found that the melilotic acid content in infected leaves was higher than that in healthy ones. Next we examined the chemical components of infected stems.

In a preliminary experiment, paperchromatographic comparisons between components of infected cherry stems and those of healthy ones were carried out according to method described in a previous paper.¹⁾ Chromatograms of components extracted by organic

solvents (petroleum ether, diethyl ether, chloroform or ethanol) showed no remarkable differences between infected stems and healthy ones. However, in water extracts, some fluorescent compounds were found which were more abundantly accumulated in infected stems. In ether extracts, coumarin, *o*- and *p*-coumaric acid, vanillic acid, and *p*-hydroxybenzoic acid as well as gentisic acid were contained as common components of infected and healthy stems. In addition, the substance named F-1 in a previous paper,¹⁾ a component of leaves, could be identified with gentisic acid using paper chromatography.

Fluorescent compounds were of three types, named FG-1, FG-2, and FG-3, (R_f =0.42, 0.39 and 0.35 on paper chromatograms. Solvent system: *n*-BuOH-AcOH-H₂O=6:1:1), respectively. Since these compounds were hydrolyzed by hydrochloric acid to give reducing sugars and a phenolic aglycon (paper chromato-

[†] This paper constitutes Part IV of "Studies on the Hypertrophic Disease of Cherry (Genus *Prunus*) So-called "Witch's Broom" Caused by *Taphrina wiesneri*."

1) S. Fujii, H. Aoki, M. Kômoto and K. Munakata, *Agr. Biol. Chem.*, **32**, 816 (1968).

2) *idem, ibid.*, **35**, 1133 (1971).

graphy), it was suggested that they were similar glycosides.

This paper is concerned with the estimation of their relative amounts and with the identification of FG-1.

EXPERIMENTAL AND RESULTS

Mps are uncorrected. IR spectra were measured with a Hitachi EPI-S2 infrared spectrophotometer. UV spectra were recorded with a Hitachi EPS-2 recording spectrophotometer. NMR spectra were recorded at 100 MHz with a Japan Electron Optics Lab. JNM-4H-100 spectrometer. Optical rotations were measured with a Yanagimoto OR-10 photomagnetic direct reading polarimeter.

I. Estimation of fluorescent glycosides

Infected and healthy cherry stems (*Prunus yedoensis*) were collected at the campus of The Hyogo University of Agriculture (Sasayama, Hyogo) in June, 1967. Both were air-dried and separated in two parts, the bark part (outer bark, inner bark, and cambium) and the wood part (sap wood and heart wood). Each chipped sample (50 g) was decocted with 200 ml of water by heating at 95°C for 2 hr, then the mixture was filtered. A similar process for the residue was repeated with 200 ml of water, then with 150 ml. The combined filtrates and washings were evaporated *in vacuo* under a nitrogen stream to a final volume of about 50 ml. The concentrated solution was shaken with ethyl acetate until the organic layer showed no more color. To the aqueous layer was added 5.5 g of active carbon. The mixture was stirred for 1 hr at 60~70°C, then filtered. The filtrate was treated again with an additional 2.3 g of active carbon. The combined carbons were washed thoroughly with water, then were desorbed three times with 50 ml portions of a methanol-28% aq. ammonia mixture (40:1.5) and filtered. The combined filtrates were concentrated *in vacuo* under nitrogen to a volume of 5 ml. A one

milliliter aliquot of the solution was applied to a thin-layer plate (20×20×0.075 cm) of Wako Gel B-5 and developed with a chloroform-ethanol mixture (4:1). Locations of the fluorescent glycosides were detected under an ultraviolet lamp. Each band which corresponded to the glycosides, was gathered by suction and the glycoside was eluted with methanol. The volume of the eluate was brought to 10 ml with methanol. The resulting turbidity which was attributed to a silica gel contaminant was removed by centrifugation. Thus clear supernatants were obtained which all fluoresced maximally at 443 m μ when activated with 365 m μ light, according to their glycosides which had a common fluorescent aglycon moiety. The amount of glycoside in each supernatant was fluorometrically determined on a Hitachi EPU-2 recording spectrophotometer equipped with a Hitachi L-3 attachment. The amount of FG-1 was estimated as gentisic acid-5- β -D-glucoside (see below), and FG-2 and FG-3, as gentisic acid-5-rhamnosylglucoside.*¹ Results are shown in Table I. Amounts of these glycosides in

TABLE I. CONTENTS OF FLUORESCENT GLYCOSIDE IN CHERRY STEMS

Contents of 50 g samples are given.

		FG-1 (mg)	FG-2 (mg)	FG-3 (mg)
Infected	Bark part	2.4	90.2	6.2
	Wood part	1.7	24.2	4.2
Healthy	Bark part	0.8	14.3	1.6
	Wood part	0.7	1.6	0.8

infected stems were larger than those in healthy stems. Amounts of FG-1, FG-2, FG-3 in the infected part were, respectively, 2.7, 7.2, and 4.3 times those in the healthy part. The bark part contained generally large

*¹ FG-2 is identified with this compound. FG-3 also gives gentisic acid, glucose and rhamnose on hydrolysis. Details will be reported in subsequent papers.

FIG. 1. Isolation Procedure for Fluorescent Glycosides.

amounts of the glycosides as compared with the wood part.

II. Isolation of fluorescent glycosides

Air-dried stems of the infected cherry plant (*Prunus yedoensis*), collected at the campus of The Hyogo University of Agriculture (Sasayama, Hyogo) in June, 1966, were chipped and ground in an iron mortar. Fluorescent compounds were isolated from the ground stems according to Fig. 1. Ground stems (4 kg) were

decocted with 8 liters of water by heating them at 95°C for 2 hr. The water layer was decanted and the residue was treated again as specified above. The combined water layers and washings were evaporated to a volume of about 1.6 liters *in vacuo* under a nitrogen stream, then centrifuged (-3°C , 13,000 G). The supernatant was shaken with ethyl acetate until the solvent layer showed no more color. The aqueous layer was treated with 50 g of active carbon at 60°C . The active carbon

was filtered and the filtrate was treated again with 50 g of additional carbon. The combined carbons were washed thoroughly with water and sucked until they were almost dried. The dried carbon was suspended in 2 liters of methanol-aq. ammonia mixture (40:1.5). The suspension was stirred for 2 hr at 60°C, then filtered. This procedure was repeated twice. The combined filtrates were evaporated *in vacuo* under a nitrogen stream to a thick syrup. About seventy milliliters of absolute methanol was added to the syrup, and the resulting

amorphous mass was filtered off. The filtrate was re-evaporated, and 580 mg of a colorless crystal (C-1) was obtained. C-1 (mp 175~178°C) was filtered off and the filtrate was evaporated *in vacuo* under a nitrogen stream to a syrup. To the syrup 1N hydrochloric acid was added dropwise until its blue fluorescence changed into yellow under an ultraviolet lamp. The mixture was allowed to stand for several days in a refrigerator, and 730 mg of a crystalline mass (FG-2) was deposited. FG-2 was filtered off, mp 152~155°C. The filtrate

FIG. 2. Infrared Spectra of Gentisic Acid-glucosides (KBr).

- A: FG-1 (mp 124~126°C)
- B: Gentisic Acid-5- β -D-glucoside (mp 124~126°C)
- C: Gentisic Acid-5- β -D-glucoside (mp 128~129°C)

was applied to thin-layer plates ($20 \times 20 \times 0.075$ cm) of Wako Gel B-5 and developed with a chloroform-ethanol mixture (4:1). Each band corresponding to a fluorescent glycoside was gathered by suction, and eluted with methanol. The eluate was concentrated *in vacuo* under a nitrogen stream to a thick syrup. Yields: FG-1, 20 mg; additional FG-2, 65 mg; FG-3, 40 mg.

The syrup (20 mg) of FG-1 was dissolved in water and treated with active carbon as described above. The carbon was subjected to the desorption procedure with the methanol-aq. ammonia mixture as above. After filtering the mixture the filtrate was evaporated to a thick syrup, and dil. hydrochloric acid was added. The solution was allowed to stand for several weeks, and FG-1 crystallized out. Recrystallization from water gave 5.4 mg of colorless prisms, mp $124 \sim 126^\circ\text{C}$. $[\alpha]_D^{25} -62.5^\circ$ ($c=0.07$, methanol). Its IR spectrum is shown in Fig. 2. UV $\lambda_{\text{max}}^{\text{MeOH}}$ $m\mu$ (ϵ): 235 (4200), 324 (3110). Fluorescent peak: 443 $m\mu$ (in methanol, activated at 365 $m\mu$). Color reaction: ferric chloride, violet; methyl red, red; diazotized sulfanilic acid, faint brown. For Fehling's solution, gave a negative test, after hydrolysis, a positive test.

III. Chemical structure of FG-1

Acid hydrolysis. Syrupy FG-1 (ca. 20 mg) was dissolved in 0.2N sulfuric acid and heated on a boiling water bath for 2 hr. The paper chromatogram of the mixture (Fig. 3) exhibited new two spots, R_f 0.89 corresponding to gentisic acid, and R_f 0.30 corresponding to glucose. The mixture was shaken with ether. This ethereal solution was washed with water, then dried over anhydrous sodium sulfate and evaporated. The residue was crystallized out. Sublimation of the crystals under reduced pressure ($110^\circ\text{C}/18$ mmHg) gave 5.5 mg of colorless needles, mp $199 \sim 202^\circ\text{C}$ (in a sealed tube). MS m/e : 154 (M^+). Anal. Found: C, 54.57; H, 3.57. Calcd. for $\text{C}_7\text{H}_6\text{O}_4$: C, 54.55; H, 3.92%. UV absorption maxima (237 and

FIG. 3. Paper Chromatograms of the Hydrolyzate of FG-1. Solvent: *n*-BuOH-AcOH- H_2O (6:1:1).

○: Fluorescence under UV lamp

⊙: Positive to Aniline Hydrogen Phthalate

337 $m\mu$, in ethanol), the IR spectrum, and paper chromatographic behavior were in good agreement with those of authentic gentisic acid. Thus, the aglycon in FG-1 is identified with gentisic acid.

The aqueous layer was neutralized by addition of barium carbonate, then filtered. The filtrate was treated with Glucostat (Washington Biochemical Corp.), after which it turned dark blue. The paper chromatogram of the mixture did not exhibit any spot corresponding to sugar. Consequently, the sugar moiety of FG-1 was considered to be glucose.

Mol ratio determination of glucose and gentisic acid in FG-1. FG-1 (1.5 mg) was hydrolyzed with 0.5N hydrochloric acid as above. After evaporation of the hydrolyzate solution, the dry residue was trimethylsilylated according to the method reported by Kagan and Mobry,³¹

³¹ J. Kagan and T. J. Mobry, *Anal. Chem.*, **37**, 288 (1965).

then gas-chromatographed on a Hitachi K-53 gas chromatograph equipped with FID. A stainless steel column (1 m×3 mm) packed with 10% Silicone SE-52 on 60/80 Chromosorb W was used. The carrier gas was nitrogen at a flow rate of 30 ml per min. The temperature of the oven was 200°C, and that of the injection block was 280°C. As a standard, an equimolar mixture of glucose and gentisic acid was treated with hydrochloric acid then trimethylsilylated, and gaschromatographed, as

was FG-1. Both chromatograms showed three peaks; corresponding to α -glucose, β -glucose, and gentisic acid. In order to evaluate the relative amounts of glucose and gentisic acid, the peak area was cut off and weighed. With FG-1, the ratio of the peak area of gentisic acid to that of α -glucose plus β -glucose was 1 : 1.42; with the standard it was 1 : 1.36. Thus the mol ratio of gentisic acid to glucose in FG-1 is 1 : 1.

FIG. 4. Schema for Syntheses of Gentisic Acid-glucoside.

Hydrolysis of FG-1 with emulsin. To a solution of FG-1 (ca. 2 mg) in 2 ml of 0.2 M acetate buffer (pH 7.0) was added 5 mg of emulsin. This mixture was kept at 30°C for 20 hr, then paperchromatographed. FG-1 was hydrolyzed to give glucose and gentisic acid (Fig. 3). From this result a β -glucosidic linkage in FG-1 was suggested.

IV. Synthesis of gentisic acid-5- and 2- β -D-glucopyranoside

Previously reported methods^{4,5)} were used with a slight modification. The process is outlined in Fig. 4.

A. Gentisic acid-5- β -D-glucopyranoside (I). 2-O-Acetyl-gentisic acid methyl ester (II), mp 99.5~100°C, NMR $\delta_{\text{Me}_4\text{Si}}^{\text{CDCl}_3}$: 2.33 (3H, s., 2-OCOCH₃), 3.83 (3H, s., COOCH₃), 6.90 (2H aryl H-3 and H-4), 7.53 (1H, t., $J=1$ Hz, aryl H-6), was prepared⁴⁾ from gentisic-acid *via* 2,5-di-O-acetylgentisic acid, and then 2-O-acetylgentisic acid,⁶⁾ α -Acetobromoglucose (III) was prepared according to Lemieux,⁷⁾ mp 88~89°C. One gram of II, 2 g of III, and 0.56 g of freshly prepared silver oxide⁸⁾ were kneaded with 1 ml of freshly prepared quinoline. The slurry was allowed to stand in a desiccator in which air was replaced by nitrogen. After 3 hr the slurry was triturated with chloroform, and centrifuged. The process was repeated twice. The combined supernatants were washed in the following order: twice with 10% hydrochloric acid, twice with water, four times with 10% sodium bicarbonate solution, and four times with water. The chloroform layer was dried over calcium chloride and evaporated to a thick syrup. The

syrup was dissolved in hot methanol, and allowed to stand for several days, which gave 700 mg of a crystalline mass. Recrystallization from methanol gave colorless needles of 2-O-acetylgentisic acid methyl ester-5-(tetra-O-acetyl- β -D-glucopyranoside) (IV), mp 121~122°C, NMR $\delta_{\text{Me}_4\text{Si}}^{\text{CDCl}_3}$: 2.04, 2.07 (12H, glucose-OCOCH₃); 2.28 (3H, s., aryl 2-OCOCH₃), 3.85 (3H, s., COOCH₃), 4.22~5.30 (glucose-CH-), 7.20 (2H, aryl H-3 and H-4), 7.53 (1H, t., $J=1$, aryl H-6). IV (300 mg) was dissolved in 15 ml of 0.5 N sodium methylate in methanol and refluxed for 30 min. The mixture was neutralized with sulfuric acid and centrifuged to remove resulting sodium sulfate. The supernatant was evaporated under reduced pressure to a thick syrup. The syrup was dissolved in 7 ml of saturated barium hydroxide solution and allowed to stand for 5 hr at room temperature. The mixture was neutralized by bubbling carbon dioxide through it, then it was centrifuged. The supernatant was concentrated *in vacuo* under a nitrogen stream to a thick syrup. To the syrup was added drop by drop 1 N hydrochloric acid under an ultraviolet lamp until the blue fluorescence turned yellow, then the mixture was allowed to stand in a refrigerator. After several days 92.3 mg of colorless prisms were deposited. Recrystallization from water gave pure I which melted at 124~126°C. *Anal.*^{*)} Found: C, 49.40; H, 4.98. Calcd. for C₁₃H₁₆O₉: C, 49.37; H, 5.10%. $[\alpha]_D^{25} -65.0^\circ$ ($c=0.1$, methanol).

B. Gentisic acid-2- β -D-glucopyranoside (V)

5-O-Acetylgentisic acid methyl ester (VI), mp 88~89°C, NMR $\delta_{\text{Me}_4\text{Si}}^{\text{CDCl}_3}$: 2.33 (3H, s., 5-OCOCH₃), 3.88 (3H, s., COOCH₃), 6.99 (1H, d., $J=8$, aryl H-3), 7.23 (1H, q., aryl H-4, $J=3$, and 8), 7.58 (1H, d., $J=3$, aryl H-6),

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6) M. Bergmann and D. Dangschat, *Ber.*, **52B**, 371 (1919).

7) R. U. Lemieux, "Method in Carbohydrate Chemistry," Vol. II, Academic Press, **1963**, p. 221.

8) E. L. Hirst and E. Percival, *ibid.*, p. 146.

*) Elementary analysis was carried out after drying a sample for 15 hr over P₂O₅ at 80°C *in vacuo*. Zane and Wender⁵⁾ obtained a dihydrate crystal (mp 98~100°C) from hot water and a sesquihydrate form (mp 126~128°C) after dryness (6 hr, over P₂O₅ 110°C, *in vacuo*).

10.58 (1H, s., 2-OH), was prepared⁴⁾ from gentisic acid *via* 5-O-acetylgentisic acid,⁹⁾ mp 88~89°C. One gram of VI and 2 g of III were treated with silver oxide as specified in A. The resulting 5-O-acetylgentisic acid methyl ester-2-(tetra-O-acetyl- β -D-glucoside) (VII), mp 135~136°C, NMR $\delta_{\text{Me}_4\text{Si}}^{\text{CDCl}_3}$: 2.04, 2.08 (12H, glucose-OCOCH₃), 2.33 (3H, s., aryl 5-OCOCH₃), 3.88 (3H, s., COOCH₃), 6.99 (1H, d., $J=8$, aryl H-3), 7.23 (1H, q., $J=3$, and 8, aryl H-4), 7.65 (1H, d., $J=3$, aryl H-6), was de-esterified first with sodium methylate, then by barium hydroxide. Crystallization of the de-esterified product from dil. hydrochloric acid gave 15 mg of fine needles of V. mp 128~129°C. (lit.,⁶⁾ mp 129~131°C for monohydrate), $[\alpha]_D^{24} -67.8^\circ$ ($c=0.1$, methanol).

DISCUSSION

Gentisic acid is extensively distributed in the plant kingdom.^{10,11)} Gentisic acid-glycoside, however, was scarcely found in boron-deficient sunflower plants, as reported by Watanabe and his co-workers.¹²⁾ In cherry (*Prunus yedoensis*) stems, we found three new kinds of gentisic acid-glycosides (FG-1, FG-2, and FG-3). The contents of these glycosides were generally higher in the dark part than in the wood part; and they, especially FG-2, were remarkably increased when stems were infected by *Taphrina wiesneri*. The amount of FG-2 in the infected bark part reached 0.18% in dry matter. On the other hand, the bark of some other *Prunus* species with resistances to "Witch's Broom" contain large amounts of these glycosides as compared with the bark of healthy *Prunus yedoensis*.¹³⁾

Antifungal activities of gentisic acid¹⁴⁾ or its esters¹⁵⁾ were reported. However, in the test against *T. wiesneri*,¹³⁾ the activity was not so strong. (Threshold concentrations were 1/100 mol for gentisic acid, and 6/100 mol for the gentisic acid-glycosides.) Plant hormone and growth regulating tests of the glycosides were also negative (*Avena* test, rice lamina-joint test and germination test of rice and rape seeds).¹³⁾ As yet, no reasonable explanation for the large accumulation of these compounds in the infected stems of *Prunus yedoensis* can be offered.

FG-1 which amounted to 0.005% of the dry infected bark and 0.003% of the dry infected wood, was a gentisic acid-monoglucoside. In gentisic acid-monoglucoside, there must be three kinds of position isomers; gentisic acid-2- and 5-glucoside, and 1-O-gentisoyl glucose. Each isomer can be further classified into two anomers, α - and β -anomer, even if configuration of the glucose moiety is restricted to D-form. Gentisic acid methyl ester resembles 1-O-gentisoyl glucose at the point where the two hydroxyls of the hydroquinone type are liberated in both molecules. The ester exhibits dark blue fluorescence under an ultraviolet lamp, and reacts readily with alkaline silver nitrate at room temperature. On the other hand, FG-1 did not exhibit these properties. Hence, 1-O-gentisoyl glucose is tentatively excluded from our considerations. The other two glucosides are composed of two anomers, as mentioned above. To decide the probable linkage of the glucose moiety in FG-1, the optical rotation was calculated according to Hudson's rule.¹⁶⁾ The values¹⁷⁾ of rotations of ethyl α - and β -D-glucoside (I), or of phenyl

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TABLE II. OPTICAL ROTATIONS OF GENTISIC ACID-GLUCOSIDES

	Calculated				Observed	
	from I		from II		[α] _D	M _D
	[α] _D	M _D	[α] _D	M _D		
Gentisic acid- α -D-glucoside	100	31,360	146	46,330		
Gentisic acid- β -D-glucoside	-22	-6,950	-57	-18,190		
FG-1					-62.5	-19,750
Gentisic acid-5- β -D-glucoside					-65.0	-20,540
Gentisic acid-2- β -D-glucoside					-67.8	-21,420

I: Data for ethyl glucosides

II: Data for phenyl glucosides

TABLE III. PROPERTIES OF FG-1, AND GENTISIC ACID-2- AND 5- β -D-GLUCOPYRANOSIDE

	UV $\lambda_{\text{max}}^{\text{MeOH}}(\text{m}\mu)$		Color of fluorescence (under UV lamp)	R _f Value (TLC) ^{a)}
FG-1	235	324	blue	0.10
Gentisic acid-2- β -D-glucopyranoside	294		dark blue	0.08
Gentisic acid-5- β -D-glucopyranoside	235	324	blue	0.10

^{a)} The solvent used in TLC was CHCl₃/EtOH (4:1).

α - and β -D-glucoside (II) were used as the basis of the calculation. The A value calculated from the data of I is 19,155, and from the data of II, 32,260. The B value calculated from the data of I is 12,205, and from the data of II, 14,070. Results obtained from these values are given in Table II. The observed specific rotation (-62.5) was considerably larger than the value (-22) calculated from the data of I, but was similar to that (-57) from the data of II. Furthermore, FG-1 was hydrolyzed with emulsin. Thus, the glucosidic linkage in FG-1 was tentatively assigned to β form.

As a next step it was necessary to determine the position of the attachment of the glucose moiety to gentisic acid in FG-1. Although the expected 5- or 2- β -D-glucoside had synthesized by Zane and Wender,⁵⁾ the described melting point of both glucosides approximated each other. IR spectra and specific rotations were not reported. To identify FG-1 the two isomers were synthesized and their properties were compared with those of FG-1. The IR spectrum (Fig. 1), UV absorption maxima

(Table III), the thin-layer chromatogram (Table III), the melting point, and specific rotation (Table II) of FG-1 were all in good agreement with those of gentisic acid-5- β -D-glucoside.

The compound in crystalline form was first isolated by us from the *T. wiesneri*-infected cherry plant. Syrupy gentisic acid-5- β -D-glucopyranoside had already been isolated from boron-deficient sunflower plants as stated and was later identified by Zane and Wender¹⁸⁾ on the basis of its UV spectrum and its paperchromatographic behavior.

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