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Tannins and Related Compounds. XLVI.[1] Isolation and Structures of Stenophynins A and B, Novel Tannins from *Quercus stenophylla* MAKINO

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Two novel tannins, stenophynins A and B, isolated from the bark of *Quercus stenophylla* MAKINO (Fagaceae), have been characterized on the basis of chemical and spectroscopic evidence as (+)-catechin 8-*C*-β-D-[2″,3″:4″,6″-bis-(*S*)-hexahydroxydiphenoyl]-glucopyranoside (**1**) and (+)-catechin 8-*C*-β-D-[2″,3″-(*S*)-hexahydroxydiphenoyl-6″-galloyl]-glucopyranoside (**2**), respectively.

Keywords——*Quercus stenophylla*; Fagaceae; stenophynin A; stenophynin B; tannin; *C*-glucosyl flavan-3-ol; catechin; hexahydroxydiphenic acid; gallic acid

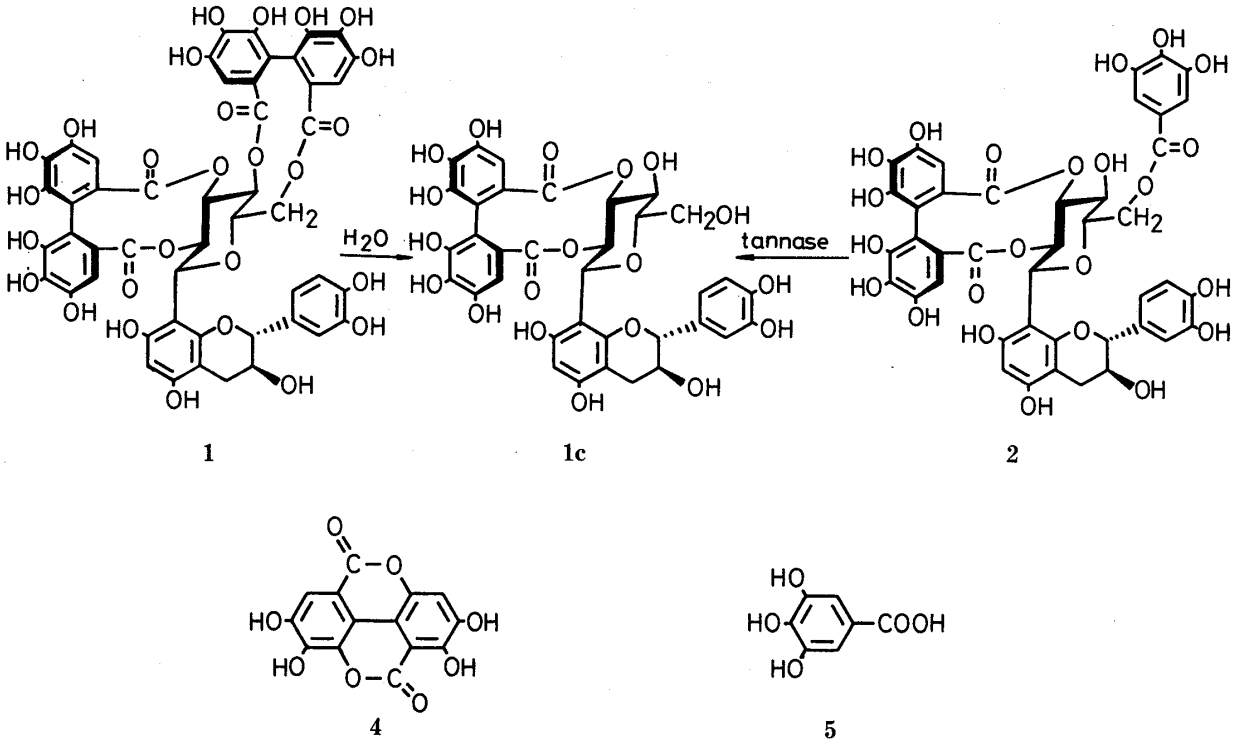
In 1966, Haslam stated in his book, "Chemistry of Vegetable Tannins," that tannins can in principle be divided on the basis of their chemical properties into two groups; the hydrolyzable and the non-hydrolyzable or condensed.[3] However, the presently known vegetable tannins may be classified into three groups based mainly on the structural features rather than on the chemical properties. The third group is one (stenophyllanins) first isolated by us from the bark of *Quercus stenophylla* MAKINO (Fagaceae) (Japanese name: Urajirogashi), and possesses structures in which hydrolyzable tannin and a flavan-3-ol (catechin), one of the component units of condensed tannins, are connected through a carbon–carbon linkage.[4] In continuing chemical examination of the polyphenolic constituents in *Q. stenophylla*, we have now isolated two new tannins, designated as stenophynins A (**1**) and B (**2**), which belong to the above third group. This paper presents details of the isolation and structure elucidation of these compounds.

Due to the complexity of the polyphenolic mixture, repeated chromatography was necessary for their isolation. Satisfactory separation was achieved by a combination of chromatographies on Sephadex LH-20 with various solvent systems (H_2O–MeOH, EtOH–H_2O, EtOH, EtOH–H_2O–acetone, *etc.*), and on MCI-gel CHP 20P and Bondapak C_{18} Porasil B with H_2O–MeOH. Stenophynin B (**2**) was isolated from the ethyl acetate-soluble portion of the aqueous acetone extract, while the aqueous layer after the extraction with ethyl acetate yielded stenophynin A (**1**).

Stenophynins A (**1**) and B (**2**) were obtained as off-white amorphous powders, and both showed similar brown colorations (typical of ellagitannins) in the sodium nitrite–acetic acid test[5] and reddish brown colorations (typical of condensed tannins) with the anisaldehyde–sulfuric acid reagent.[6]

The ^{1}H-nuclear magnetic resonance (^{1}H-NMR) spectrum of **1**, measured at room temperature, was complicated, probably by conformational isomerism, but the spectrum taken in dimethyl sulfoxide-d_6 at elevated temperature (120 °C) showed the first-order splitting pattern. The presence of two 4,4′,5,5′,6,6′-hexahydroxydiphenoyl ester groups was readily deduced from this spectrum, which showed two pairs of one-proton singlets at δ 6.19, 6.24, 6.41 and 6.51. The observation of aromatic ABX-type signals at δ 6.77 (d, J=2 Hz), 6.81

(dd, $J=8$, 2 Hz) and 6.82 (d, $J=8$ Hz), and a high-field one-proton singlet at δ 6.02, in conjunction with resonances due to two methines at δ 4.64 (d, $J=8$ Hz) and 3.95 (m) and a methylene at δ 2.41 (dd, $J=16$, 8 Hz) and 2.74 (dd, $J=16$, 6 Hz), suggested the presence of a 2,3-*trans* flavan-3-ol (catechin) moiety with a C-6 or C-8 substitution system. On the other hand, six aliphatic resonances at δ 80.2, 77.0, 75.5, 73.2, 69.7 (each d) and 63.9 (t) in the ^{13}C-nuclear magnetic resonance (^{13}C-NMR) spectrum clearly indicated the occurrence of a sugar moiety in **1**, while the absence of an anomeric proton signal was suggestive of its *C*-glycosidic nature.



1 **1c** **2**

4 **5**

Chart 1

On methylation with dimethyl sulfate and potassium carbonate in dry acetone, **1** afforded the hexadecamethyl ether (**1a**). In the ^{13}C-NMR spectrum of **1a** with proton off-resonance decoupling, an aromatic doublet signal arising from the A-ring of the flavan-3-ol moiety appeared at δ 89.5, the chemical shift being consistent with those observed in the methyl ethers of C-8 substituted catechin derivatives (for example, gambiriin A_1 nonamethyl ether[7,8] δ 88.9 and stenophyllanin A nonamethyl ether[4] δ 89.3). This finding suggested that the sugar moiety is attached through a carbon–carbon linkage to the C-8 position of the catechin moiety. Subsequent alkaline methanolysis of **1a** cleaved the ester linkages to yield dimethyl 4,4′,5,5′,6,6′-hexamethoxydiphenoate (**3**) and a hydrolysate (**1b**). The negative sign of the specific optical rotation [−86.9 (acetone)] of **3** confirmed the atropisomerism to be in the *S*-series.[9] The ^{1}H-NMR spectrum of **1b** showed the presence of four methoxyl groups and a catechin nucleus, together with a *C*-glycosyl moiety whose configuration at the C-1 position could be assigned as β on the basis of the coupling constant ($J=8$ Hz). The ^{13}C-NMR chemical shifts for the sugar carbons in **1b** were in good agreement with those found in *C*-glucosyl compounds such as mangiferin[10] and aloesin.[11] From these observations, **1b** was presumed to be tetra-*O*-methyl-(+)-catechin 8-*C*-β-D-glucopyranoside, and comparison of the physical and ^{1}H-NMR data with those of an authentic sample[1] confirmed this.

To allocate specifically the two hexahydroxydiphenoyl ester groups in **1**, the following examinations were made. When heated in boiling water, **1** liberated ellagic acid (**4**) and a

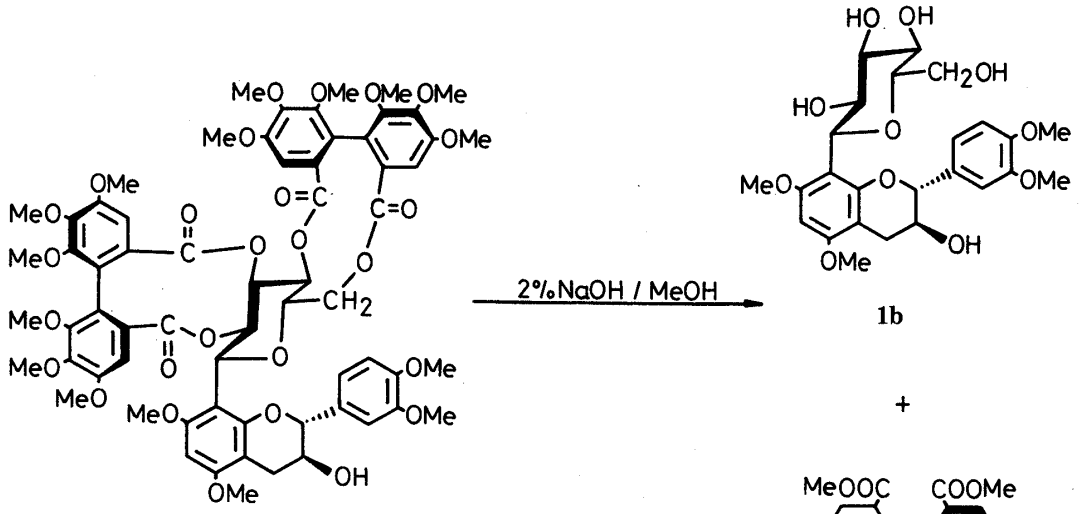


Chart 2

partial hydrolysate (**1c**). The fast atom bombardment mass spectrum (FAB-MS) of **1c** exhibited $[M+H]^+$ and $[M+Na]^+$ peaks at *m/z* 755 and 777, respectively, being consistent with the ^{1}H-NMR data, which showed the presence of one hexahydroxydiphenoyl group (δ 6.09 and 6.46, each 1H, s), as well as catechin and sugar moieties. In the ^{1}H-NMR spectrum of **1c**, signals due to protons geminal to the ester groups appeared as triplets at lower field (δ 5.56 and 4.81, each $J=10$ Hz). Irradiation of the triplet at δ 5.56 caused the change of the triplet at δ 4.81 to a doublet, and also of the C-1 proton doublet to a singlet, thus indicating that these triplets are assignable to the C-2 and C-3 protons, respectively. On the basis of these observations, the partial hydrolysate (**1c**) was concluded to be (+)-catechin 8-*C*-β-D-[2″,3″-(*S*)-hexahydroxydiphenoyl]-glucopyranoside. Since it is clear from the above chemical and spectral data that the remaining hexahydroxydipenoyl group is located in the glucose moiety, the structure of stenophynin A is formulated as **1**.

Based on the results of the coloring reactions mentioned above, stenophynin B (**2**) was speculated to be structurally related to **1**. The ^{1}H-NMR spectrum of **2**, measured at room temperature, showed a complex signal pattern similar to that observed in **1**, and first-order analysis was possible by measuring it at 90 °C. From this spectrum, one galloyl (δ 6.98, 2H, s) and one hexahydroxydiphenoyl ester group (δ 6.59 and 6.69, each 1H, s) were clearly seen, together with catechin and sugar moieties. The ^{13}C-NMR spectrum of **2** was closely related to that of **1c** except for the presence of one additional galloyl group. Enzymatic hydrolysis of **2** with tannase yielded gallic acid (**5**) and a hydrolysate, which was found to be identical with **1c** by comparison of their physical and ^{1}H-NMR data. The location of the galloyl group in **2** was concluded to be at the C-6 position of the glucose moiety on the basis of the low-field shifts of the C-6 carbon (δ 63.9) and the C-6 methylene protons (δ 4.56, m) in its ^{13}C- and ^{1}H-NMR spectra. From these chemical and spectroscopic findings, stenophynin B was characterized as (+)-catechin 8-*C*-β-D-[2″,3″-(*S*)-hexahydroxydiphenoyl-6″-galloyl]-glucopyranoside (**2**).

Stenophynins A and B differ structurally from previously reported stenophyllanins[4] in having a pyranose form of the glucose moiety.

Experimental

Details of the instruments and chromatographic procedures used in this work were essentially the same as

described in our previous paper.[12]

Isolation of Stenophynins A (1) and B (2)——The air-dried, ground bark (4.74 kg) of *Quercus stenophylla*, which was collected in Tokushima Prefecture, Japan, was extracted with 80% aqueous acetone at room temperature. Concentration of the extract under reduced pressure afforded a dark brown precipitate, which was removed by filtration. The filtrate was successively extracted with ether and ethyl acetate. The ethyl acetate-soluble portion (162 g) thus obtained was chromatographed on Sephadex LH-20 with a solvent system of ethanol–water–acetone[13] to yield six fractions; frs. I (94.7 g), II (25.8 g), III (5.7 g), IV (9.5 g), V (4.4 g) and VI (4.2 g). Among these fractions, fr. IV was rechromatographed over Sephadex LH-20. Elution with methanol–water (3 : 2) gave a further five fractions (frs. IV-1—IV-5). Repeated chromatography of fr. IV-2 on Sephadex LH-20 with ethanol and ethanol–water, and on MCI-gel CHP 20P with water–methanol (7 : 3) yielded crude stenophynin B (**2**). Purification by Bondapak C_{18} Porasil B chromatography in water containing increasing amounts of methanol gave stenophynin B (**2**) (yield: 0.0009%). The above aqueous layer after the extraction with ethyl acetate was subjected to Sephadex LH-20 chromatography. Elution with water containing increasing proportions of methanol afforded six fractions; frs. I′ (296 g), II′ (122 g), III′ (198 g), IV′ (41 g), V′ (138 g) and VI′ (68 g). Fraction IV′ was repeatedly chromatographed on MCI-gel CHP 20P with water–methanol (7 : 3) and on Sephadex LH-20 with water–methanol (1 : 4) and water–ethanol (4 : 1) to give crude stenophynin A (**1**), which was finally purified by chromatography on Bondapak C_{18} Porasil B with water–methanol (yield: 0.0068%).

Stenophynin A (1)——An off-white amorphous powder, $[\alpha]_D^{23}$ +71.3° (c=0.5, MeOH). *Anal.* Calcd for $C_{49}H_{36}O_{27}\cdot 2H_2O$: C, 53.85; H, 3.69. Found: C, 53.55; H, 3.95. FAB-MS m/z: 1057 $[M+H]^+$, 1079 $[M+Na]^+$, 1095 $[M+K]^+$. ^{1}H-NMR (dimethyl sulfoxide-d_6, at 120 °C) ppm: 2.41 (1H, dd, J=16, 8 Hz, H-4), 2.74 (1H, dd, J=16, 6 Hz, H-4), 3.79 (1H, d, J=16 Hz, H-6″), 3.95 (1H, m, H-3), 4.64 (1H, d, J=8 Hz, H-2), 4.9—5.3 (4H, H-1″, H-2″, H-4″ and H-6″), 5.85 (1H, t, J=8 Hz, H-3″), 6.02 (1H, s, H-6), 6.19, 6.24, 6.41, 6.51 (each 1H, s, HHDP[14]-H), 6.77 (1H, d, J=2 Hz, H-2′), 6.81 (1H, dd, J=8, 2 Hz, H-6′), 6.82 (1H, d, J=8 Hz, H-5′). ^{13}C-NMR (acetone-d_6 + D_2O) ppm: 29.7 (t, C-4), 63.9 (t, C-6″), 68.3 (d, C-3), 69.7, 73.2, 75.5, 77.0, 80.2 (each d, C-1″, C-2″, C-3″, C-4″ and C-5″), 81.4 (d, C-2), 97.1 (d, C-6), 100.6 (s, C-8), 102.0 (s, C-4a), 107.2, 107.6, 108.2 (each d, HHDP-C), 114.4, 116.1 (each d, C-2′ and C-5′), 116.3 (s, HHDP-C), 119.9 (d, C-6′), 126.3, 126.7 (each s, HHDP-C), 131.6 (s, C-1′), 136.0 (s, HHDP-C), 144.3, 144.4, 145.0, 145.3, 145.7 (each s, C-3′, C-4′ and HHDP-C), 155.0, 156.0, 157.2 (each s, C-5, C-7 and C-8a), 168.3, 168.4, 169.0, 169.9 (each s, –COO–).

Methylation of 1——A mixture of **1** (200 mg), dimethyl sulfate (2 ml) and potassium carbonate (3 g) in dry acetone (15 ml) was heated under reflux for 3 h. After removal of the inorganic salts by filtration, the solution was concentrated under reduced pressure. The oily residue was subjected to silica gel chromatography. Elution with benzene–acetone (4 : 1) yielded the hexadecamethyl ether (**1a**) (90 mg) as a white amorphous powder, $[\alpha]_D^{18}$ −13.3° (c=0.75, $CHCl_3$). *Anal.* Calcd for $C_{65}H_{68}O_{27}$: C, 60.08; H, 5.43. Found: C, 60.54; H, 5.17. FD-MS m/z: 1280 $[M]^+$. ^{1}H-NMR ($CDCl_3$) ppm: 2.60, 3.20 (each 1H, m, H-4), 3.51—3.95 (OMe), 4.71 (1H, d, J=9 Hz, H-2), 5.1—5.4 (4H, m, H-1″, H-2″, H-4″, H-5″ and H-6″), 5.98 (1H, m, H-3″), 6.1—6.8 (4H, H-6 and HMDP[14]-H), 6.9—7.1 (3H, m, H-2′, H-5′ and H-6′). ^{13}C-NMR ($CDCl_3$) ppm: 24.5 (C-4), 55.5, 55.9, 56.8, 60.6, 60.9, 61.0 (OMe), 63.9 (C-6″), 66.0, 68.9, 71.7, 72.0, 74.2, 74.4, 76.3, 76.7, 79.3, 79.7, 81.5 (C-1″, C-2″, C-3″, C-4″, C-5″ and C-3), 88.0 (C-2), 89.5 (C-6), 101.4 (C-4a), 109.0, 109.6 (C-2′ and C-5′), 111.3 (C-8), 167.0—168.7 (–COO–).

Acetylation of 1a——The methyl ether (**1a**) (20 mg) was kept overnight in a mixture of pyridine and acetic anhydride (1 : 1, 2 ml) at room temperature. Usual work-up yielded the monoacetate (12 mg) as a white amorphous powder, $[\alpha]_D^{18}$ −15.4° (c=0.6, $CHCl_3$). *Anal.* Calcd for $C_{67}H_{70}O_{28}$: C, 60.81; H, 5.33. Found: C, 60.56; H, 5.53. FD-MS m/z: 1322 $[M]^+$. ^{1}H-NMR ($CDCl_3$) ppm: 1.96 (3H, s, OCOMe), 2.58, 3.16 (each 1H, m, H-4), 3.52—3.96 (OMe), 4.9—5.6 (7H, H-1″, H-2″, H-4″, H-6″ and H-3), 5.96 (1H, m, H-3″), 6.10—7.14 (8H, H-6, H-2′, H-5′ and H-6′).

Alkaline Methanolysis of 1d——A solution of **1b** (30 mg) in 2% sodium hydroxide/methanol (2 ml) was left at room temperature for 1 h. The reaction mixture was neutralized with Dowex 50 W-X8 (H^+ form) resins, and the solvent was evaporated off under reduced pressure. The residue was chromatographed on silica gel. Elution with ethyl acetate–acetone–water (10 : 3 : 0.3) yielded dimethyl (*S*)-hexamethoxydiphenoate (**3**) (16 mg), $[\alpha]_D^{23}$ −25.6° (c=1.2, $CHCl_3$), and the hydrolysate (**1b**) (6 mg) as a white amorphous powder, $[\alpha]_D^{26}$ −86.9° (c=0.18, acetone). FAB-MS m/z: 508 $[M]^+$. ^{1}H-NMR (acetone-d_6) ppm: 2.52 (1H, dd, J=17, 8 Hz, H-4), 2.87 (1H, dd, J=17, 6 Hz, H-4), 3.2—4.4 (7H, m, H-2″, H-3″, H-4″, H-5″, H-6″ and H-3), 3.80 (9H, s, 3 × OMe), 3.85 (3H, s, OMe), 4.73 (1H, d, J=9 Hz, H-2), 4.81 (1H, d, J=8 Hz, H-1″), 6.31 (1H, s, H-6), 6.89 (1H, d, J=8 Hz, H-5′), 7.07 (1H, dd, J=8, 2 Hz, H-6′), 7.08 (1H, d, J=2 Hz, H-2′). The hydrolysate (**1b**) was identified as (+)-catechin 8-*C*-β-D-glucopyranoside by $[\alpha]_D$ and ^{1}H-NMR comparisons with an authentic sample.[1]

Partial Hydrolysis of 1——A solution of **1** (50 mg) in water was heated on a boiling water bath for 13 h. After cooling, the resulting precipitates were collected by filtration. Recrystallization from pyridine afforded ellagic acid (**4**) as pale brown needles. The mother liquor was evaporated to dryness, and the residue was applied to a column of Bondapak C_{18} Porasil B. Elution with water–methanol (3 : 2) furnished the partial hydrolysate (**1c**) (7 mg) as an off-white amorphous powder, $[\alpha]_D^{17}$ +37.4° (c=0.19, MeOH). *Anal.* Calcd for $C_{35}H_{30}O_{19}\cdot 3/2H_2O$: C, 53.78; H, 4.26. Found: C, 53.85; H, 4.37. FAB-MS m/z: 755 $[M+H]^+$, 777 $[M+Na]^+$. ^{1}H-NMR (dimethyl sulfoxide-d_6, at 90 °C) ppm: 4.42 (1H, d, J=8 Hz, H-2), 4.81 (1H, t, J=10 Hz, H-3″), 4.93 (1H, d, J=10 Hz, H-1″), 5.56 (1H, t, J=10 Hz,

H-2″), 6.01 (1H, s, H-6), 6.09, 6.46 (each 1H, s, HHDP-H), 6.68 (1H, d, J=8 Hz, H-5′), 6.80 (1H, dd, J=8, 2 Hz, H-6′), 6.80 (1H, d, J=2 Hz, H-2′).

Stenophynin B (2)——An off-white amorphous powder, $[\alpha]_D^{17}$ +51.8° (c=0.12, acetone). *Anal.* Calcd for $C_{42}H_{34}O_{23} \cdot 1/2H_2O$: C, 55.08; H, 3.85. Found: C, 55.14; H, 3.85. FAB-MS m/z: 907 $[M+H]^+$. ^{1}H-NMR (dimethyl sulfoxide-d_6, at 90 °C) ppm: 4.40 (1H, d, J=8 Hz, H-2), 4.56 (2H, m, H-6″), 4.87 (1H, t, J=10 Hz, H-3″), 4.97 (1H, d, J=10 Hz, H-1″), 5.62 (1H, t, J=10 Hz, H-2″), 6.01 (1H, s, H-6), 6.12, 6.51 (each 1H, s, HHDP-H), 6.72 (1H, d, J=8 Hz, H-5′), 6.81 (1H, dd, J=8, 2 Hz, H-6′), 6.85 (1H, d, J=2 Hz, H-2′), 6.98 (2H, s, galloyl H). ^{13}C-NMR (acetone-d_6 + D_2O) ppm: 29.7 (t, C-4), 63.9 (t, C-6″), 68.2 (d, C-3), 67.9, 73.0, 75.2, 79.0, 82.9 (each d, C-1″, C-2″, C-3″, C-4″ and C-5″), 81.8 (d, C-2), 97.3 (d, C-6), 101.2 (s, C-8), 102.0 (s, C-4a), 107.4 (d, HHDP-H), 110.0 (d, galloyl C-2 and C-6), 114.4, 115.0 (each d, C-2′ and C-5′), 113.5, 116.4 (each s, HHDP-C), 120.1 (d, C-6′), 120.7 (s, galloyl C-1), 126.5, 126.8 (each s, HHDP-C), 131.1 (s, C-1′), 136.1 (s, HHDP-C), 139.2 (s, galloyl C-4), 144.3, 145.0, 145.1, 145.8 (each s, C-3′, C-4′, HHDP-C and galloyl C-3 and C-5), 154.5, 156.3, 157.0 (each s, C-5, C-7 and C-8a), 167.5, 169.4, 170.4 (each s, –COO–).

Tannase Hydrolysis of 2——A solution of **2** (29 mg) in water (5 ml) was incubated with tannase at 37 °C for 1 h. The reaction mixture was concentrated under reduced pressure to give a residue, which was treated with methanol. The methanol-soluble portion was chromatographed on Sephadex LH-20 with ethanol to yield gallic acid (**5**) (6 mg) and the hydrolysate (9 mg), which was found to be identical with **1c** by physical and spectral comparisons.

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