TWO NEW ELLAGITANNIN METABOLITES, CARPINUSIN AND CARPINUSNIN FROM CARPINUS LAXIFLORA[1]

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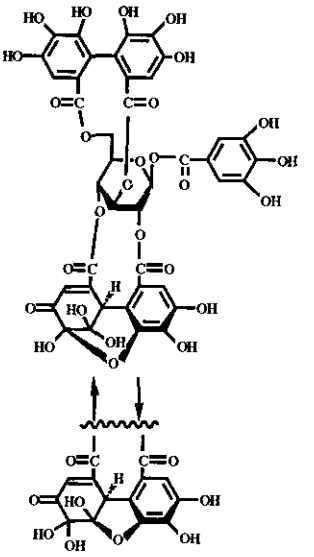
Abstract——From the fresh leaves of Carpinus laxiflora (Betulaceae), two new hydrolyzable tannins, carpinusin (**2**) and carpinusnin (**3**), have been isolated, together with thirteen known hydrolyzable tannins and related compounds, and their structures were elucidated on the basis of physico-chemical evidence.

The metabolism of gallic acid through an oxidative carbon to carbon coupling to form 3,3',4,4',5,5'-hexahydroxydiphenic acid is found to predominate in Nature,[2] whereas the mechanism of the coupling mode, as well as the further metabolic fate of this diphenic acid, is not fully understood. In the course of our continuous search for the metabolites of gallic acid esters in higher plants, we have encountered in the Betulaceous plant, Carpinus laxiflora (Sieb. et Zucc.) Blume, large accumulations of a series of highly oxidized hydrolyzable tannins, particularly of geraniin (**1**)[3](1.5% based on the fresh material) which is regarded as one of the major metabolic pools of ellagitannins.[4] Detailed chemical examination has now resulted in the isolation of two new ellagitannin

metabolites named carpinusin (**2**) and carpinusnin (**3**), together with thirteen known hydrolyzable tannins and related compounds. This paper describes the structure elucidation of these compounds.



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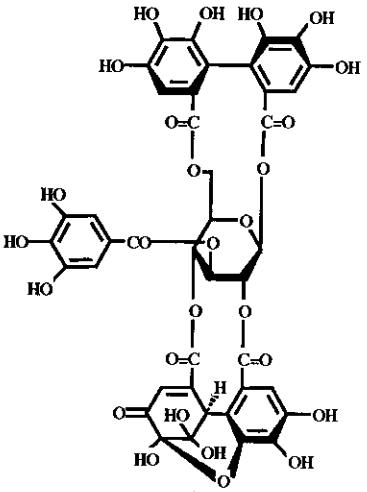
The water-soluble portion of the aqueous acetone extract of the fresh leaves was subjected to chromatography over Sephadex LH-20 dextran gels,[5] and repeated chromatography of the tannin fractions over a variety of reverse-phase gels, such as MCI-gel CHP-20P, Fuji-gel ODS-G3, Prep-PAK 500/C_{18} and Bondapak C_{18}/Porasil B,[6] yielded fifteen compounds. Of these, thirteen were identified as chlorogenic acid, glucogallin,[7] 6-*O*-galloylglucose,[5] 2,3,4,6-tetra-*O*-[8] and 1,2,3,4,6-penta-*O*-galloylglucoses,[9] corilagin,[10] furosin,[11] geraniin(**1**),[10] casuarinin,[12] pedunculagin,[13] punicafolin,[14] elaeocarpusin[10] and repandusinic acid monopotassium salt,[15] by comparisons of their physical and spectral data with those of authentic specimens.

Carpinusin (**2**) was obtained as an off-white powder (H_2O), mp 238-239°C (decomp), $[\alpha]_D$ +34.4° (MeOH), and showed the positive coloration of ellagitannins with the $NaNO_2$-AcOH reagent.[16] The ^1H nmr spectrum of **2**, measured immediately after dissolution, exhibited a two-proton singlet at δ 7.18 due to a galloyl group and a pair of one-proton singlets at δ 6.89 and 6.91 attributable to a hexahydroxydiphenoyl (HHDP) group. The appearance of a couple of a benzylic methine signal at δ 5.01 (d, *J*=1.5 Hz) and an olefinic proton signal at δ 6.27 (d, *J*=1.5 Hz) suggested the presence of a dehydrohexahydroxydiphenoyl (DHHDP) group.[10] This was supported by ^{13}C nmr spectroscopy (measured after standing for 24 h), which showed the characteristic duplication of signals [*e.g.* δ 191.8, 194.6 (C=O); δ 125.5, 128.8 (-*C*H=C<); δ 46.2, 51.8 (-*C*H<)] owing to the equilibration between the five- and six-membered hemiacetal rings in the DHHDP group.

When treated with *o*-phenylenediamine in the presence of AcOH,[17] **2** yielded the phenazine



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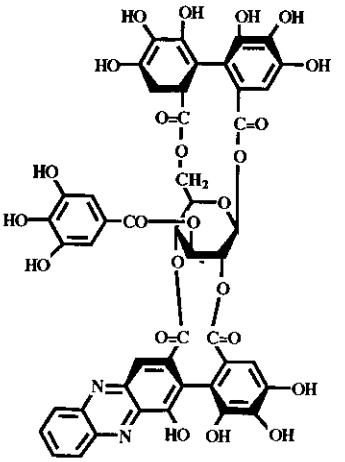
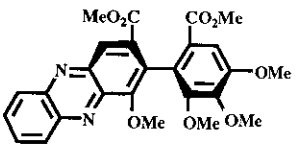
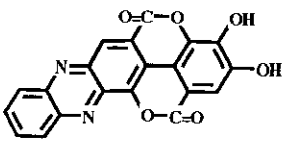


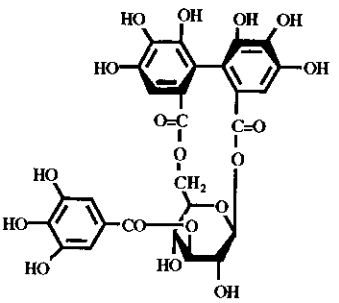
2a



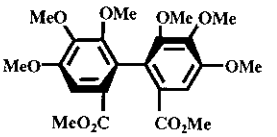
2d



2b



2c



2e

derivative (**2a**)[positive fab ms m/z: 1006 $(M)^+$]. The ^1H nmr spectrum of **2a** was straightforward, and showed well-defined signal patterns (see Experimental). The appearance of the sugar H-1, H-2, H-3 and H-4 signals as singlets implied the presence of a glucopyranose ring adopting the $^1C_4$-conformation. In addition, the lowfield shifts of all the aliphatic signals indicated that the hydroxyl groups in the sugar moiety are

completely acylated.

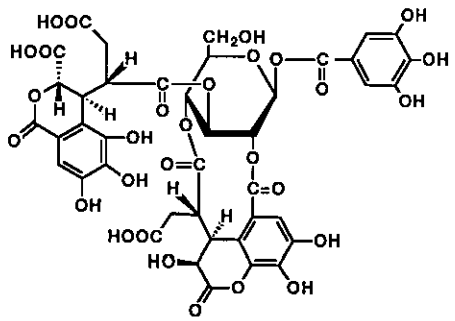
The allocations and the chiralities of the phenolcarboxylic acid esters were determined as follows. Heating of **2a** in water, followed by repeated chromatography over Sephadex LH-20 and MCI-gel CHP 20P, afforded the phenazine bislactone (**2b**),[17] 3-O-galloyl-D-glucose[18] and 1,6-(S)-HHDP-3-O-galloyl-β-D-glucose (**2c**).[18] The formation of **2c** thus establihsed unequivocally, as well as the atropisomerism of the HHDP group, the locations of each acyl group including that of the DHHDP group at the glucose C-2 and C-4 positions. On the other hand, alkaline methanolysis of the tridecamethyl ether of **2a** yielded the dextrorotatory tetramethoxylphenazine dimethyl ester (**2d**) $[[\alpha]_D$ +33.0° ($CHCl_3$)],[18] together with methyl trimethoxybenzoic acid and dimethyl (S)-hexamethoxydiphenoate (**2e**) $[[\alpha]_D$ -29.3° ($CHCl_3$)],[19] thus confirming the chirality of the DHHDP group to be in the R-series.

The determination of the orientation of the DHHDP group at the glucose C-2 and C-4 positions was achieved by examination of the ^1H-^{13}C long-range COSY spectrum (J=5 Hz) of **2**, which, *although duplicated by existence of a mixture of the five- and six-membered* hemiacetal forms, was amenable to first-order analysis, showing that the five-membered hemiacetal form is predominant. Among the glucose signals, a broad singlet at δ 5.49 arising from the major tautomer was assignable to H-2 based on the ^1H-^1H COSY *examination*, and this signal was found to be correlated with the carboxyl carbon signal at δ165.4. Similar correlation was observed between this carboxyl carbon signal and the aromatic proton signal at δ 7.29. Since the signal at δ 7.29 was attributed to the aromatic proton in the DHHDP group by consideration of the chemical shift, the aromatic ring of the DHHDP group could be concluded to be located at the glucose C-2 position.

On the basis of these findings, carpinusin was characterized as 1,6-(S)-HHDP-2,4-(R)-DHHDP-3-O-galloyl-β-D-glucose (**2**).

Carpinusnin (**3**) was isolated as a white powder (H_2O), mp 215-217°C, $[\alpha]_D$ +44.0° (MeOH). The ^1H nmr spectrum exhibited signals due to a galloyl group at δ 7.24 (2H, s) and two isolated aromatic protons at δ 7.16 and 7.53 (each 1H, s). The sugar signals, except

for the H-6 signal centered at ca. δ 4.10, appeared downfield (see Experimental), and the relatively small coupling constants (J=1-4 Hz) of the H-1 - H-4 signals suggested the presence of a glucopyranose ring with a $^1C_4$ or a related skew boat conformation. Besides sugar signals, the aliphatic signals were found by ^1H-^1H COSY to be composed of two independent spin systems; one was a series of signals at δ 2.23 (d-like, J=8 Hz, H-5), 3.90 (t-like, J=8 Hz, H-4), 4.91 (d, J=7 Hz, H-2) and 5.12 (d, J=7 Hz, H-3), whose chemical shifts and coupling patterns are in good accord with those of a chebulic acid diester,[15] while the other spin system consisting of the signals at δ 2.63 (dd, J=4.5, 17 Hz, H-5), 2.85 (dd, J=10, 17 Hz, H-5), 3.43 (m, H-4), 4.12 (dd, J=1, 7 Hz, H-3) and 5.34 (d, J=1 Hz, H-2) suggested the presence of a neochebuloyl group.[15]
When hydrolyzed in 2N sulfuric acid, **3** furnished D-glucose, gallic acid and chebulic acid [$[\alpha]_D$ +19.6° (MeOH)],[15] whereas enzymatic hydrolysis of **3** with tannase yielded a



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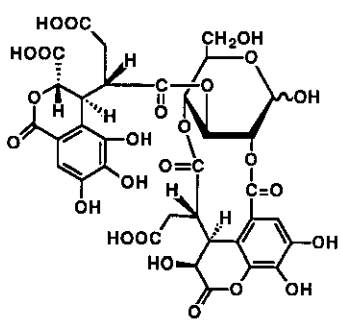
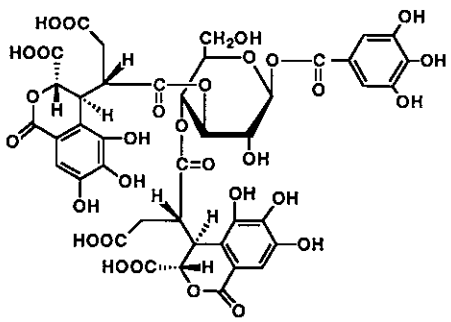


3a



3b

partial hydrolysate (**3a**), together with gallic acid. The ^{1}H nmr spectrum of **3a** revealed the absence of the gallic acid ester and showed complex signal patterns due to α- and β-glucopyranose protons, thus indicating that the galloyl group is located at the glucose C-1 position. On heating in water, **3** afforded, as the major product, another partial hydrolysate (**3b**), whose ^{1}H nmr spectrum revealed the presence of a galloyl and two neochebuloyl groups in the molecule. The lowfield shifts of the glucose H-1, H-3 and H-4 signals (δ 5.91, 5.50 and 5.28, respectively) indicated that these positions are esterified. In addition, the large coupling constants (J=7, 8 Hz) of these signals showed the conformational change of the glucopyranose ring into $^4C_1$. These findings are fully consistent with **3b** being 1-*O*-galloyl-3,4-bis-neochebuloyl-β-D-glucose. Since as mentioned before, the glucose C-6 hydroxyl was found to be not acylated originally, the chebuloyl group should bridge the 2,4-positions of the glucopyranose ring to adopt the $^1C_4$ or skew boat conformation. From these chemical and spectroscopic evidence, the structure of carpinusnin was determined to be 1-*O*-galloyl-2,4-chebuloyl-3-neochebuloyl-β-D-glucose (**3**).

Very recently, we have found that some of the *Euphorbia* species (*E. helioscopia*, *E. jolkini*, *E. adenochlora*) are rich sources of geraniin (**1**) and ellagitannins having the HHDP at the relatively uncommon 1,6-positions in the glucopyranose moiety.[20] From this point of view, the metabolism of gallic acid esters in *C. laxiflora* is considered to be very similar to that in *Euphorbia* plants. In addition, although carpinusnin (**3**) is regarded as one of the metabolites derived from the common intermediate, 1,2,3,4,6-Penta-*O*-galloyl-β-D-glucose or 1-*O*-galloyl-2,4;3,6-bis-HHDP-β-D-glucose through the oxidation of the aromatic ring(s),[2] followed by partial hydrolysis, its biosynthetic relationship with co-occurring geraniin (**1**) is not clear. Finally, it should be noted that to the best of our knowledge, this is the first isolation of hydrolyzable tannins from *Carpinus* species.

EXPERIMENTAL

Melting points were determined with a Yanaco micro-melting point apparatus and are uncorrected. Optical rotations were measured with a JASCO DIP-4 digital polarimeter. Fab ms were taken with a JEOL JMS-HX 100/JMA 3500 data system using MeOH-glycerol as a matrix. ^{1}H and ^{13}C nmr spectra were recorded on JEOL FX-100, GX-270 and GX-400 spectrometers using TMS as an internal standard. Column chromatography was carried out with Sephadex LH-20 (25-100 μ, Pharmacia Fine Chemical Co., Ltd.), MCI-gel CHP 20P (75-150 μ, Mitsubishi Chemical Industries, Ltd.), Fuji gel ODS G3 (43-65 μ, Fuji gel Hanbai Co., Ltd.), Prep-PAK 500/C_{18} (37-75 μ, Waters Associates, Inc.) and Bondapak C_{18}/Porasil B (37-75 μ, Waters Associates, Inc.).

Isolation of Tannins

The fresh leaves (20.1 Kg) of C. laxiflora, collected in the medicinal plant garden of Kyushu University, Fukuoka, Japan, were extracted five times with 80% aqueous acetone at room temperature. The combined extracts were concentrated *in vacuo* below 40°C to give precipitates consisting of chlorophylls, waxes, *etc*. After removal of the precipitates by filtration with the aid of Celite 545, the filtrate was applied to a Sephadex LH-20 column. Elution first with H_2O afforded non-phenolic compounds, and successive elution with H_2O containing increasing proportions of MeOH yielded four fractions. The first fraction consisting of lower-molecular-weight phenolic compounds was chromatographed over MCI-gel CHP 20P with a mixture of H_2O-MeOH (9:1) to give chlorogenic acid (49.8 g), glucogallin (0.13 g) and 6-*O*-galloylglucose (1.9 g). The second fraction was repeatedly chromatographed over Sephadex LH-20 with EtOH and MCI-gel CHP 20P and Fuji gel ODS G3 with H_2O containing an increasing amount of MeOH to yield corilagin (1.0 g) and carpinusin (**2**)(2.2 g). Repeated chromatography of the third fraction over Sephadex LH-20 with EtOH and Fuji gel ODS G3, Prep-PAK 500/C_{18} and Bondapak C_{18}/Porasil B with H_2O-MeOH furnished furosin (1.1 g), geraniin (*ca*. 300 g), repandusinic acid monopotassium salt (32 mg), elaeocarpusin (41 mg), casuarinin (0.12 g), pedunculagin (0.25 g), carpinusnin (**3**)(0.18 g) and punicafolin (1.1 g). The final fraction was found to contain gallotannins by the negative coloration with the $NaNO_2$-AcOH reagent, and was separated by MCI gel CHP 20P with H_2O-MeOH to give 2,3,4,6-tetra-*O*- and 1,2,3,4,6-penta-*O*-galloyl-glucoses (0.31 g and 1.2 g, respectively).

Carpinusin (2)

An off-white powder (H_2O), mp 238-239°C (decomp), $[\alpha]_D^{20}$ +34.4° (*c*=1.0, MeOH), Anal. Calcd for $C_{41}H_{28}O_{27}\cdot 5/2H_2O$: C, 49.36; H, 3.33. Found: C, 48.94; H, 3.35. Negative fab ms *m/z*: 951 $(M-H)^-$. ^{1}H Nmr (measured after 1 h, 100 MHz, acetone-d_6): 4.06, 4.12 (1H in total, each d, *J*=12 Hz, glc. H-6), 4.50-4.80 (1H in total, m, glc. H-5), 5.01 (5/6H, d, *J*=1.5 Hz, DHHDP-H-1), 5.24 (1/6H, s, DHHDP-H-1), 5.00-5.15 (1H in total, m, glc. H-

6), 5.30–5.60 (2H in total, m, glc. H-2, 4), 6.08 (1H, m, glc. H-3), 6.26 (1H, br s, glc. H-1), 6.27 (5/6H, d, J=1.5 Hz, DHHDP-H-3), 6.57 (1/6H, s, DHHDP-H-3), 6.89, 6.91 (each 1H, s, HHDP-H), 7.18 (2H, s, galloyl H), 7.29 (1H, s, DHHDP-H-3'). ^{13}C Nmr (25 MHz, acetone-d_6): 46.2, 51.8 (1C in total, DHHDP-C-1), 60.2-96.2 (6C in total, glc.C), 90.4, 92.6 (1C in total, DHHDP-C-5,6), 191.8, 194.6 (1C in total, DHHDP-C-4).

Formation of Phenazine (**2a**)

A mixture of **2** (200 mg) and o-phenylenediamine (60 mg) in 20% ethanolic AcOH (6 ml) was stirred at room temperature for 2 h. The reaction mixture was subjected to chromatography over Sephadex LH-20 with EtOH to give the phenazine (**2a**)(193 mg) as a pale brown amorphous powder, $[\alpha]_D^{20}$ -40.4° (c=0.7, MeOH). Positive fab ms m/z: 1006 $(M)^+$. ^{1}H Nmr (270 MHz, acetone-d_6): 4.24 (1H, dd, J=5, 13 Hz, glc. H-6), 4.79 (1H, dd, J=5, 13 Hz, glc. H-5), 5.19 (1H, s, glc. H-2), 5.20 (1H, s, glc. H-4), 5.26 (1H, t, J=13 Hz, glc. H-6), 5.67 (1H, br s, glc. H-3), 6.09 (1H, s, glc. H-1), 6.59, 6.86 (each 1H, s, HHDP-H), 7.12 (2H, s, galloyl H), 7.14 (1H, s, ph. H-3'), 7.97 (1H, s, ph. H-3), 8.00–8.40 (4H, m, ph.-H).

Partial Hydrolysis of **2a**

An aqueous solution of **2a** (150 mg) in H_2O (8 ml) was heated at 95°C for 6 h. The products were separated by chromatography over MCI gel CHP 20P with H_2O containing increasing amounts of MeOH to afford the phenazine bislactone (**2b**) (8 mg), 3-O-galloyl-D-glucose (5 mg) and 1,6-(S)-HHDP-3-O-galloyl-β-D-glucose (**2c**)(35 mg), which were identified by spectral comparisons with those of authentic samples.

Methylation of **2a**, Followed by Alkaline Methanolysis

A solution of **2a** (25 mg) in MeOH (3 ml) was treated with ethereal CH_2N_2 at room temperature for 1 h. After evaporation of the solvent, the residue was chromatographed over silica gel with benzene containing increasing amounts of acetone to give the tridecamethyl ether (16 mg) as a white amorphous powder, $[\alpha]_D^{20}$ -7.7° (c=0.7, MeOH). ^{1}H Nmr (270 MHz, acetone-d_6): 6.78, 6.95 [each 1H, s, hexamethoxydiphenoyl H], 7.28(2H, s, galloyl H), 7.50 (1H, s, ph. H-3'), 8.00–8.30 (4H in total, m, ph.-H), 8.81 (1H, s, ph. H-3). A solution of the methyl ether (119 mg) in 2% NaOMe/MeOH (10 ml) was kept at room temperature overnight. After neutralization with Amberlite IRA-120B (H^+ form), the reaction products were separated by silica gel chromatography with benzene-acetone to give methyl trimethoxybenzoic acid (32 mg), dimethyl (S)-hexamethoxydiphenoate (**2e**)(18 mg), $[\alpha]_D^{22}$ +29.3° (c=0.2, $CHCl_3$) and the tetramethoxyphenazine dimethyl ester (**2d**)(12 mg), $[\alpha]_D^{22}$ +33.0° (c=0.3, $CHCl_3$).

Carpinusnin (**3**)

A white powder (H_2O), mp 215–217°C, $[\alpha]_D^{20}$ +44.0° (c=0.6, MeOH). Anal. Calcd for $C_{41}H_{34}O_{29}\cdot 3H_2O$: C, 47.14; H, 3.86. Found: C, 47.22; H, 3.78. Negative fab ms m/z: 989 $(M-H)^-$. ^{1}H Nmr (270 MHz, acetone-d_6+D_2O): 2.23 (2H, d-like, J=8 Hz, che. H-5), 2.63 (1H,

dd, J=4.5, 17 Hz, neo. H-5), 2.85 (1H, dd, J=10, 17 Hz, neo. H-5), 3.43 (1H, m, neo. H-4), 3.90 (1H, t-like, J=8 Hz, che. H-4), 4.03 (1H, dd, J=7, 12 Hz, glc. H-6), 4.12 (1H, dd, J=1, 7 Hz, neo. H-3), 4.13 (1H, dd, J=7, 12 Hz, glc. H-6), 4.49 (1H, t, J=7 Hz, glc. H-5), 4.91 (1H, d, J=7 Hz, che. H-2), 4.99 (1H, d, J=3.5 Hz, glc. H-4), 5.12 (1H, d, J=7 Hz, che. H-3), 5.34 (1H, d, J=1 Hz, neo. H-2), 5.43 (1H, br s, glc. H-3), 5.34 (1H, d, J=1 Hz, neo. H-2), 5.43 (1H, br s, glc. H-2), 6.23 (1H, br s, glc. H-3), 6.44 (1H, d, J=2.5 Hz, glc. H-1), 7.16 (1H, s, neo. H-3'), 7.53 (1H, s, che. H-3'), 7.24 (2H, s, galloyl H). ^{13}C Nmr (25 MHz, acetone-d_6+D_2O): 30.3 (che. C-5), 34.3 (neo. C-5), 36.2 (neo. C-3), 39.3 (che. C-4), 41.1 (che. C-3), 45.3 (neo. C-4), 62.4 (glc. C-6), 62.7 (glc. C-3), 66.6 (che. C-2), 69.0 (glc. C-4), 70.6 (glc. C-2), 78.5 (neo. C-2), 79.0 (glc. C-5), 92.6 (glc. C-1), 165.0, 165.3, 165.7, 170.0, 172.1, 172.3, 173.5, 173.8, 174.0 (-COO-).

Acid Hydrolysis of 3

A solution of **3** (30 mg) in 2N H_2SO_4 (3 ml) was heated on a water bath for 6 h. The reaction mixture was directly chromatographed over Sephadex LH-20, and elution with H_2O yielded gallic acid (6 mg), chebulic acid (5 mg), $[\alpha]_D^{22}$ +19.6° (c=0.3, MeOH), and glucose, the latter being identified by cellulose tlc [Rf 0.39. solvent: n-BuOH-pyridine-H_2O (6:4:3)].

Tannase Hydrolysis of 3

A solution of **3** (30 mg) in H_2O (4 ml) was incubated with tannase at room temperature for 1 h. The reaction mixture was concentrated in vacuo to dryness. The residue was treated with EtOH, and the EtOH-soluble portion was subjected to chromatography over Sephadex LH-20 with EtOH to furnish gallic acid (4 mg) and the hydrolysate (**3a**)(6 mg) as a white amorphous powder. Negative fab ms m/z: 837 $(M-H)^-$. ^{1}H Nmr (100 MHz, acetone-d_6+D_2O): 7.12, 7.18 (1H in total, each s, neo. H-3'), 7.46, 7.56 (1H in total, each s, che. H-3').

Hydrolysis of 3 in Hot Water

A solution of **3** (30 mg) in H_2O (3 ml) was heated at 95°C for 2 h. After cooling, the product was separated by MCI-gel CHP 20P chromatography with H_2O-MeOH to afford the partial hydrolysate (**3b**) as a white amorphous powder (8 mg). Negative fab ms m/z: 1007$(M-H)^-$. ^{1}H Nmr (100 MHz, acetone-d_6+D_2O): 2.50-3.00 (4H, neo. H-5), 3.30 (2H, m, neo. H-4), 5.33 (2H, m, neo. H-2), 5.28 (1H, t, J=8 Hz, glc. H-4), 5.50 (1H, t, J=8 Hz, glc. H-3), 5.91 (1H, d, J=7 Hz, glc. H-1), 7.12 (2H, s, neo. H-3'), 7.22 (2H, s, galloyl H).

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