Tannins of Tamaricaceous Plants. III.[1] New Dimeric Hydrolyzable Tannins from *Reaumuria hirtella*

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Four new dimeric hydrolyzable tannins, hirtellins C, D, E and F, besides previously reported hirtellins A and B, have been isolated from the leaf extract of *Reaumuria hirtella* JAUB. *et* SP. (Tamaricaceae). Macrocyclic structures, 1 and 12, having both dehydrodigalloyl (DHDG) and isodehydrodigalloyl (isoDHDG) groups as the connecting units between monomers, were respectively assigned for hirtellin C and F, based on chemical methods, two-dimensional nuclear magnetic resonance measurement and other spectroscopic analyses. Hirtellin D (7) was characterized as an isomer of hirtellin F (12). Hirtellin E (15), having a dehydrotrigalloyl (hellinoyl) group, was characterized as an analog of hirtellin B.

Keywords ellagitannin; *Reaumuria hirtella*; Tamaricaceae; hirtellin C; hirtellin D; hirtellin E

Some tannin-rich species of Tamaricaceous plants have been used as folk medicines for various diseases in East and Southeast Asia.[2] Although the occurrence of flavonoids, gallic acid, dehydrodigallic acid and ellagic acid derivatives in the plants of this family was reported,[3] little was known concerning their tannin constituents. We recently reported the isolation and characterization of four new ellagitannins, remurins A (**11**) and B (**10**) (monomers), and hirtellins A and B (**13**) (dimers) from *Reaumuria hirtella* JAUB. *et* SP., a Tamaricaceous plant indigenous to Egypt.[1,4] We also isolated the latter two dimers and tamarixinin A (**14**) from *Tamarix pakistanica* QUAISER.[1] Further investigation on the tannin constituents of *R. hirtella* has led to the isolation of four additional new ellagitannins, named hirtellins C (**1**), D (**7**), E (**15**) and F (**12**), from the EtOAc- and *n*-BuOH-soluble portions of the 70% aqueous acetone homogenate.[1b]

The new compounds were obtained as off-white amorphous powders. Their positive coloration with the $FeCl_3$ and $NaNO_2$–AcOH reagents,[5] and fast-atom bombardment mass spectra (FAB-MS), which showed molecular ion species larger than 1500 mass units (m.u.), indicated that they are dimeric ellagitannins.

Structure of Hirtellin C (1) Hirtellin C (**1**) showed the $(M+Na)^+$ ion peak at *m/z* 1895 in the FAB-MS. Methylation of **1** with dimethyl sulfate and potassium carbonate in acetone gave an octacosamethyl derivative (**1a**), which upon methanolysis afforded methyl tri-*O*-methylgallate (**2**), dimethyl hexamethoxydiphenate (**3**), dimethyl penta-*O*-methyldehydrodigallate (**4**) and a hepta-methyl derivative (**5**). The electron-impact mass spectrum (EI-MS) of **5** exhibited the molecular ion (M^+) peak at *m/z* 436, which is the same as that of **4**. The proton nuclear magnetic resonance (^{1}H-NMR) spectrum showed a 2H singlet (δ 7.30) and a 1H singlet (δ 7.13) in the aromatic region, which respectively showed clear nuclear Overhauser effects (NOE's) with the methoxyl signals at δ 3.73 (6H, s) and 3.85 (3H, s), in the NOE enhancement two-dimensional spectroscopy (NOESY) spectra. Compound **5** was thus characterized as permethylated isodehydrodigallic acid. The sugar component liberated by acid hydrolysis of hirtellin C was identified as glucose.

The ^{1}H-NMR spectrum of **1** showed the proton signals of two glucose cores, which were clearly distinguished from each other by the ^{1}H–^{1}H shift correlation spectrum (COSY) and assigned as shown in Table I. Some (H-1, 2, 1′, 2′) of these glucose proton signals are broadened and/or multiplied, suggesting the presence of a flexible large ring formed by the linkages among C-1, 2, 1′, 2′, with slow conformational interconversion in the NMR time scale. This observation is analogous to that for macrocyclic dimers, oenothein B and its analogs.[6] However, the coupling pattern of the other proton signals indicated that both glucose cores adopt the *C1* conformation. The spectrum also exhibited two 2H singlets (δ 6.95, 6.96) due to two galloyl groups and four 1H singlets (δ 6.45, 6.46, 6.59, 6.60) which are assignable to two hexahydroxydiphenoyl (HHDP) groups. The aromatic proton signals, attributable to dehydrodigalloyl (DHDG) and isoDHDG groups, were observed at δ 7.17, 6.86 (each 1H, br s), 7.06, 6.44 (each 1H, br d, $J=2$ Hz), and 6.90 (2H, br s). The signal at δ 6.90 was shown by ^{1}H–^{13}C COSY to be an incidental overlapping signal of two nonequivalent protons. The two HHDP groups in **1** are located at O-4/O-6 and O-4′/O-6′ of each glucose core in **1**, as revealed by the large difference ($\Delta\delta$ *ca.* 1.5 ppm) between the chemical shifts of the C-6 *geminal* protons of each *C1* glucopyranose (see Table I).[1] The glucose carbon resonances in the ^{13}C-NMR spectrum of **1** were in agreement with those of tellimagrandin II.[7] Based on these findings, hirtellin C (**1**) was assumed to be a dimer composed of two moles of tellimagrandin II (TG-II) connected with each other through DHDG and isoDHDG groups, which are biogenetically producible by C–O oxidative coupling between two galloyl groups. The remaining two galloyl groups, in addition to the DHDG and isoDHDG groups, should thus be at O-1—O-3 and O-1′—O-3′. The anomeric proton (H-1′) of one of the glucose cores resonates at a higher field (δ 6.07) than that of tellimagrandin II (δ 6.20). Similar upfield shift of the H-1′ signal was also observed in the permethylated derivative (**1a**) (δ 5.90). This anomaly is analogous to that of hirtellin A,[1] which has a DHDG group at O-2′ and O-1. In addition to these findings, the ^{1}H-NMR spectrum of compound **6**, which is obtained by the treatment of **1** with hot water, inducing isomerization of the isoDHDG group in **1** to the DHDG group (described later), showed a

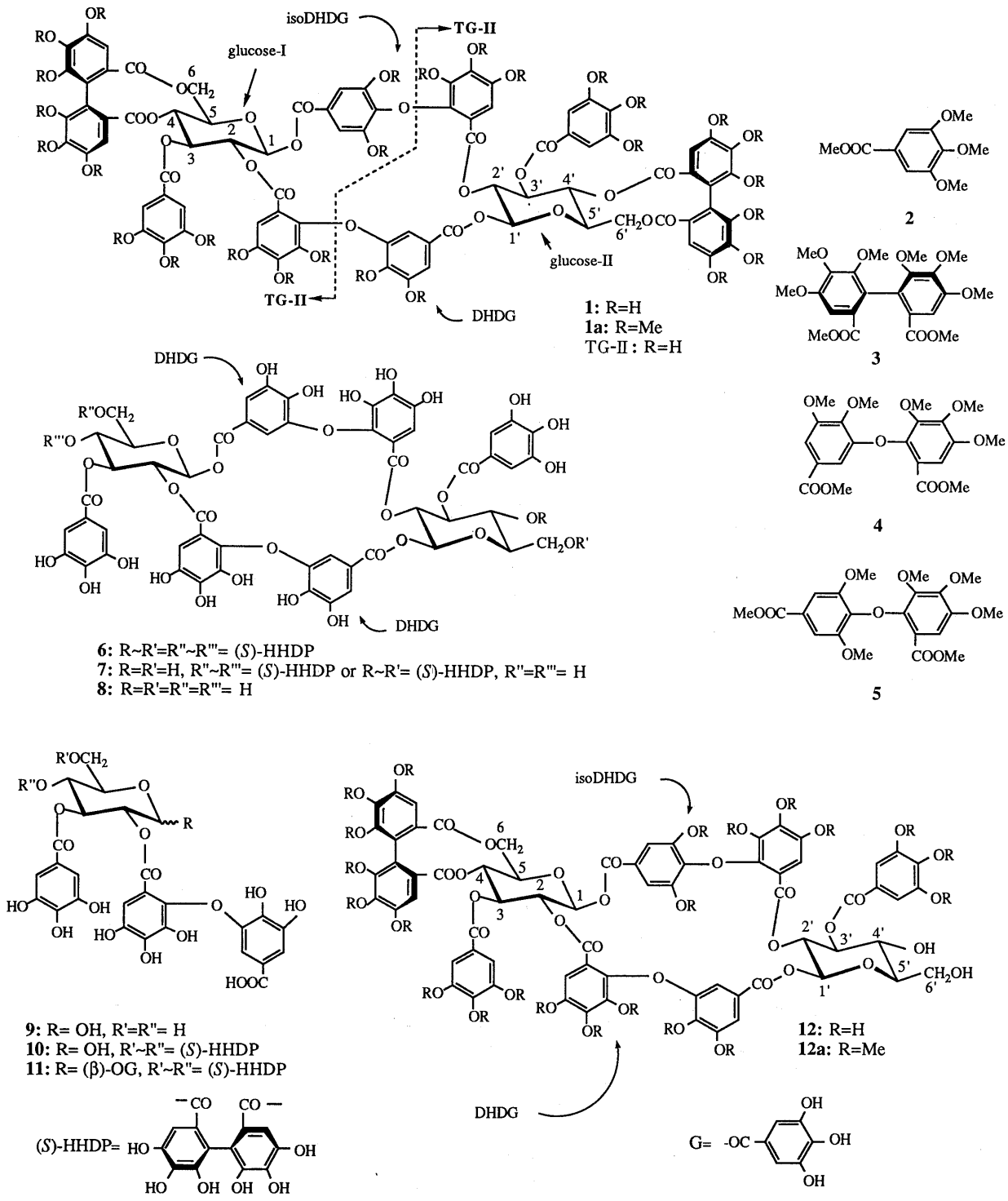


Chart 1

significant upfield shift ($\Delta\delta$ 1.16 ppm) of anomeric proton signals of both glucose cores from those of tellimagrandin II. This indicates that the DHDG and isoDHDG units are at O-2′—O-1 and O-2—O-1′ of the glucose cores.[1] The two galloyl groups should thus be at O-3 and O-3′ of **1**.

The location of each acyl group on the glucose cores in **1** was further substantiated by the ^{1}H–^{13}C long-range COSY spectrum of the octacosamethyl ether (**1a**), which shows no multiplication of signals in the NMR spectrum. The measurements set at $J=7$ Hz for two- or three-bond coupling, revealed all connectivities among glucose proton–ester carbonyl carbon–aromatic proton through three-bond couplings (Fig. 1), except for that of the glucose H-6 (H-6′) and HHDP proton. The *meta*-coupled doublets of ring A protons of DHDG at δ 7.38 and 6.47 were correlated with H-1′ (δ 5.90) of glucose-II through a common ester carbonyl carbon signal at δ 163.72, while the singlet of the ring B proton (δ 7.27) was correlated with H-2 (δ 5.69) of glucose-I through the ester carbonyl carbon signal at δ 162.61. On the other hand, the H-1 signal (δ 6.33) of glucose-I showed a cross peak with the ester carbonyl signal at δ 164.18, which was also correlated with two *meta*-coupled proton

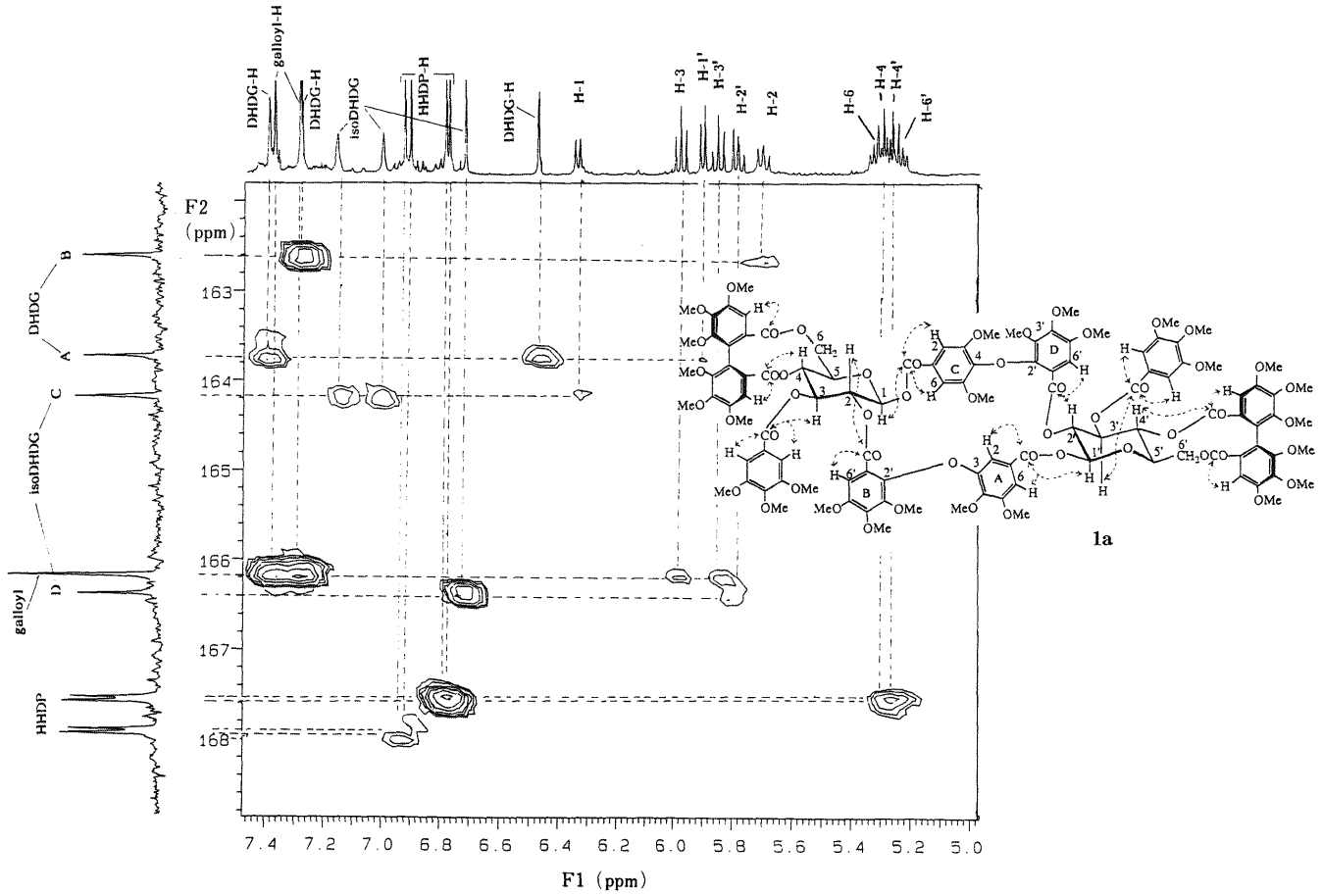


Fig. 1. ^{1}H–^{13}C Long-Range Shift Correlation Spectrum of **1a** in Acetone-d_6
J_{CH} = 7 Hz. ^{13}C-NMR: δ 162.61—167.93.

TABLE I. ^{1}H-NMR Spectral Data for Glucose Moieties of **1**, **12**, **6**, **7** and **8**

	1	**12**	**6**	**7**	**8**
H-1	6.10—6.20 br	6.18—6.22 br	5.04 d (J=8.5)	4.96 d (J=8)	4.96 d (J=8.5)
H-2	5.48 dd (J=8.5, 10)	5.48 dd (J=8.5, 10)	5.26 dd (J=8.5, 10)	5.26 dd (J=8, 10)	5.02 dd (J=8.5, 10)
H-3	5.86 t (J=10)	5.85 t (J=10)	5.51 t (J=10)	5.54 t (J=10)	5.37 t (J=10)
H-4	5.13 t (J=10)	5.11 t (J=10)	5.01 t (J=10)	5.05 t (J=10)	3.83 t (J=10)
H-5	4.49 dd (J=6.5, 10)	4.48 dd (J=6.5, 10)	4.23 ddd (J=1, 6.5, 10)	4.19 ddd (J=1, 6.5, 10)	3.51 ddd (J=2.2, 4.5, 10)
H-6	5.33 dd (J=6.5, 13)	5.30 dd (J=6.5, 13)	5.21 dd (J=6.5, 13)	5.25 dd (J=6.5, 13.5)	3.68 dd (J=4.5, 12.2)
	3.80 d (J=13)	3.79 d (J=1, 13)	3.74 dd (J=1, 13)	3.74 dd (J=1, 13.5)	3.78 dd (J=2.2, 12.2)
H-1′	6.07 br d (J=8)	5.83—6.06 m. d (J=8.5)	5.04 d (J=8.5)	4.99 d (J=8)	4.96 d (J=8.5)
H-2′	5.57 br dd (J=8, 10)	5.34 m	5.26 dd (J=8.5, 10)	5.03 dd (J=8, 10)	5.02 dd (J=8.5, 10)
H-3′	5.74 br t (J=10)	5.55 br t (J=10)	5.51 t (J=10)	5.35 t (J=10)	5.37 t (J=10)
H-4′	5.12 t (J=10)	3.86 t (J=10)	5.01 t (J=10)	3.83 t (J=10)	3.83 t (J=10)
H-5′	4.28 dd (J=6.5, 10)	3.52 ddd (J=2.5, 4.5, 10)	4.23 ddd (J=1, 6.5, 10)	3.52 ddd (J=2, 4.5, 10)	3.51 ddd (J=2.2, 4.5, 10)
H-6′	5.27 dd (J=6.5, 13.5)	3.69 dd (J=4.5, 12.5)	5.21 dd (J=6.5, 13)	3.69 dd (J=4.5, 12)	3.68 dd (J=4.5, 12.2)
	3.75 d (J=13.5)	3.78 dd (J=2.5, 12.5)	3.74 dd (J=1, 13)	3.79 dd (J=2, 12)	3.78 dd (J=2.2, 12.2)

500 MHz, acetone-d_6 + D_2O, J in Hz.

signals (δ 7.15, 7.07) of ring C of the isoDHDG group. Similarly, the H-2′ signal (δ 5.78) of glucose II was correlated with the ring D proton signal (δ 6.72) through the carbonyl carbon signal at δ 166.38. These data clearly indicated that the DHDG and isoDHDG groups respectively bridge O-1′—O-2 and O-1—O-2′ of the two glucose moieties, with the orientations shown in Fig. 1.

Chemical evidence for the structure of hirtellin C (**1**) was obtained as follows. In a hot water-bath at 95 °C, **1** was first converted into compound **6**, which was gradually degraded to three other hydrolyzates **7**, **8** and **9** upon prolonged reaction, as shown by monitoring of the time course of the reaction by HPLC. Compound **6**, named isohirtellin C, was proved to be an isomer of hirtellin C, in which the isoDHDG unit was isomerized to a DHDG unit as follows. Its FAB-MS showed the $[M+Na]^+$ ion peak at m/z 1895 which is the same as that of hirtellin C (**1**). Methanolysis of its methylated derivative afforded methyl tri-*O*-methylgallate (**2**), dimethyl hexamethoxydiphenate (**3**) and dimethyl penta-*O*-methyldehydrodigallate (**4**), without

affording methylated isodehydrodigallic acid. In spite of its dimeric nature, isohirtellin C (**6**) exhibited a ^{1}H-NMR spectrum which looks like that of a monomeric tannin composed of a fully acylated *C1* glucose core, a galloyl, an HHDP and a DHDG unit (Table I). This spectrum could be easily interpreted in terms of the isomerization of the isoDHDG group to a DHDG group through Smiles rearrangement,[8] resulting in the formation of a dimer in which each glucose core is acylated with the above-mentioned groups at the same positions. The dimeric nature of isohirtellin C was further confirmed by the production of the hydrolyzate **7** (*m/z* 1593 $[M+Na]^+$ in FAB-MS) upon continuation of hydrolysis of **6** in hot water. The ^{1}H-NMR spectrum of **7** again showed the fourteen proton signals due to two glucose moieties, among which H-4 (δ 3.83) and one of the C-6 methylene protons (δ 3.69) of a glucose core (Table II) are shifted significantly upfield. The hydrolyzate **7** was thus regarded as a dimer lacking identical acyl groups on each glucose core, owing to the elimination of only one of the two HHDP groups. The hydrolyzate **8**, which again showed the monomer-like ^{1}H-NMR spectrum, was found to be a dimer having two glucoses identically substituted at C-1(1′), 2(2′) and 3(3′). Elimination of two HHDP groups O-4/O-6 (O-4′/O-6′) of both glucose cores from **1** was indicated by the large upfield shifts of the H-4(4′) and H-6(6′) proton signals relative to those of isohirtellin C (**6**) (Table I). The ^{1}H-NMR spectrum of the hydrolyzate (**9**) showed duplication of each signal due to formation of an anomer mixture. The chemical shifts of the glucose protons of both α- and β-anomer are similar to those of remurin B (**10**),[1] except for significant upfield shifts for H-4 and H-6 ($\Delta\delta$; 1.23, 1.27 ppm for H-4; 1.47, 1.54 ppm for H-6, respectively). The structure (**9**) of this hydrolyzate was substantiated by partial hydrolysis of remurin A (**11**), yielding **9**. Upon treatment with tannase at 37 °C for 9 d, 70% of hirtellin C (**1**) was isomerized into isohirtellin C (**6**), which suffered subsequent partial hydrolysis producing **7** as judged by monitoring with HPLC. This reaction is similar to that of partial hydrolysis of **1** in hot water as described above, and may thus be interpreted in terms of isomerization of isoDHDG in the hirtellin C molecule to DHDG, occurring in the aqueous solution in the absence of tannase. This observation of the isomerization of **1** under such mild condition prompted us to examine the stability of **1** under various conditions as follows. As shown in Fig. 2, hirtellin C (**1**) is quite stable in acetone, where 18% of **1** was converted into **6** after two weeks at room temperature, whereas it is fairly unstable in water, in which 50% of **1** was isomerized after 3 d. Since the Smiles rearrangement can proceed in boiling alkaline solution,[9] the behavior of hirtellin C in aqueous solution of various pH values (1.0—7.4) was then examined by using HPLC analysis. The results showed that the rate of formation of **6** from **1** was accelerated at higher pH (0.1 M phosphate buffer) and elevated temperature (40 °C). As illustrated in Fig. 3, which shows the results at 40 °C, hirtellin C (**1**) at pH below 3.0 was almost unchanged after 10 h. However, it was completely converted to **6** at pH 7.4 (0.1 M phosphate buffer), with subsequent degradation to **7**, demonstrating that the Smiles rearrangement occurs for **1** in a solution of low temperature and moderate pH. It has been reported that in this type of intramolecular nucleophilic aromatic substitution reaction,[10] the steric effect (from the adjacent hydroxyl group) rather than the electronic stress accelerates the *SN*2 reaction, through the restricted rotation of the ether bond, leading to a favorable conformation (Chart 2). The occurrence of Smiles rearrangement at low temperature for hirtellin C (**1**) and other related tannins isolated from Tamaricaceous plants[11] may be due to such a steric effect.

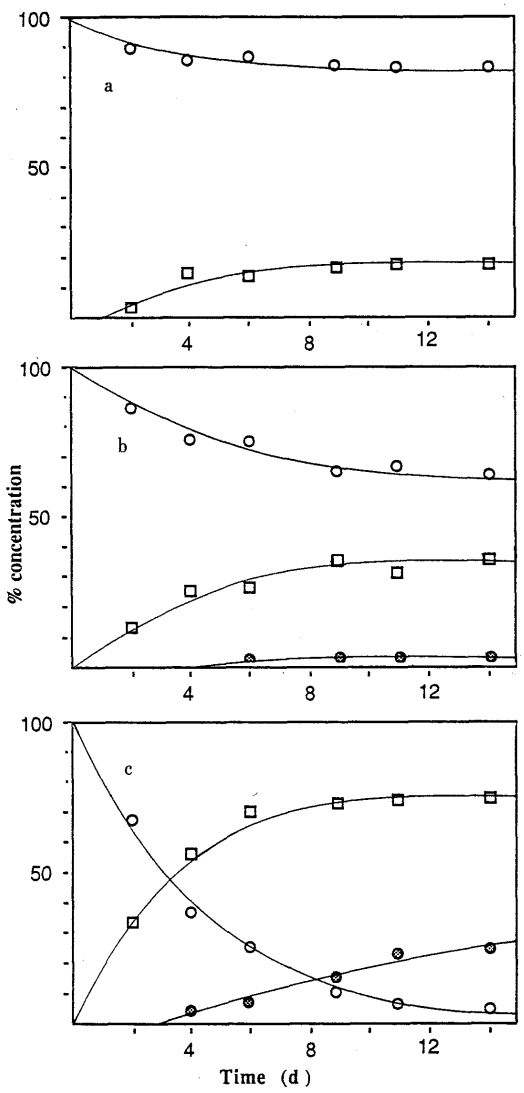


Fig. 2. Stability of Hirtellin C in (a) Acetone, (b) Acetone–Water, (c) Water at Room Temperature

Hirtellin C (**1**) [○], isohirtellin C (**6**) [□], hirtellin D (**7**) [◎]. Concentrations (%) of hirtellin C, isohirtellin C and hirtellin D were calculated based on the peak areas.

Structure of Hirtellin F (12) Hirtellin F (**12**), $[\alpha]_D$ +33° (MeOH), was characterized as a congener of hirtellin C (**1**) lacking an HHDP group as follows. Its FAB-MS exhibited the $[M+Na]^+$ ion peak at *m/z* 1593, which is 302 m.u. (corresponding to an HHDP group) less than that of **1**. Methylation of **12** followed by methanolysis afforded **2**, **3**, **4** and **5**, which were the same products as those obtained from **1**. The presence of two galloyl, an HHDP, a DHDG and an isoDHDG groups in **12** was indicated by the ^{1}H-NMR spectrum (see Experimental). The (*S*)-configuration of the HHDP group in **12** was shown by the positive Cotton effect in the circular dichroism (CD) spectrum at 228 nm ($[\theta]+7\times10^4$), whose amplitude is smaller than that

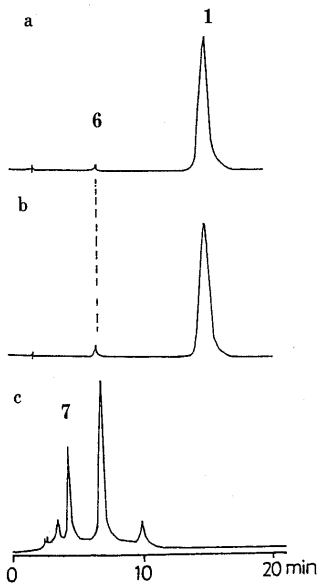


Fig. 3. Stability of Hirtellin C (**1**) in Solutions of Various pH Values

a: pH=1.0, after 10 h. b: pH=3.0, after 10 h. c: pH=7.4, after 30 min. Reversed-phase HPLC conditions: column, LiChrospher RP-18 (Merck); temperature, 40 °C; mobile phase, 0.01 M KH_2PO_4–0.01 M H_3PO_4–EtOH–EtOAc (42.5:42.5:15:5); flow rate, 1.0 ml·min^{-1}; UV detection at 280 nm.

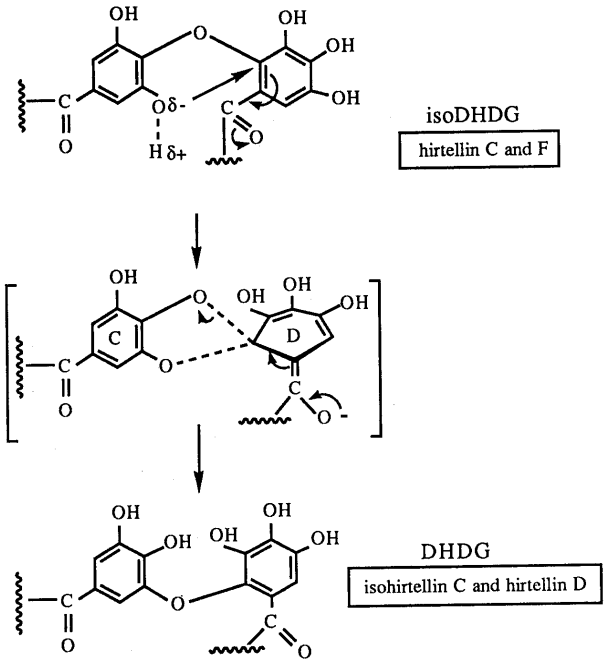


Chart 2. Smiles Rearrangement

of hirtellin C. The broadening and multiplication of some glucose proton signals (H-1, 1′, 2′, 3′) are similar to those of **1** (see Experimental), indicating that **12** is a dimer in which monomers are linked through the DHDG and isoDHDG groups, forming a flexible ring structure like that of **1**. The ^{1}H-NMR spectral comparison between **1** and **12** (Table I) revealed that the signals (H-1—6) of a glucose core are similar in these compounds, while the H-4′—6′ signals (δ 3.69—3.86) of the other glucose core in **12** show large upfield shifts from those of **1**. These upfield shifts indicate the presence of free hydroxyl groups at C-4′ and C-6′ in **12**. The structure of **12** was confirmed by partial

Table II. ^{1}H-NMR Spectral Data for Glucose Moieties of **13** and **15**

	13	**15**
H-1	6.16 d (J=8)	6.08 d (J=8)
H-2	5.66 t (J=8, 10)	5.47 dd (J=8, 9.5)
H-3	5.74 t (J=10)	5.56 t (J=9.5)
H-4	5.14 t (J=10)	3.89 t (J=9.5)
H-5	4.51 dd (J=6, 10)	3.82 ddd (J=2, 4.5, 9.5)
H-6	5.35 dd (J=6, 13)	3.91 dd (J=2, 12)
	3.86 dd (J=13)	3.74 dd (J=4.5, 12)
H-1′	5.60 d (J=8.5)	5.64 d (J=8.5)
H-2′	5.36 dd (J=8.5, 10)	5.37 dd (J=8.5, 9.5)
H-3′	5.69 t (J=10)	5.71 t (J=10)
H-4′	5.19 t (J=10)	5.20 t (J=10)
H-5′	4.35 dd (J=1.5, 7, 10)	4.37 ddd (J=1.5, 6.5, 10)
H-6′	5.31 dd (J=7, 13)	5.34 dd (J=6.5, 13)
	4.13 dd (J=1.5, 13)	4.13 dd (J=1.5, 13)

500 MHz, acetone-d_6 + D_2O, J in Hz.

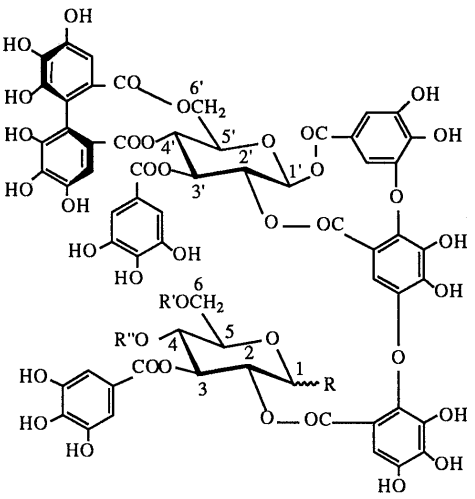
acid hydrolysis of hirtellin C, producing **6**, **7** and **12**. The partial hydrolysis of **12** in hot water at 95 °C yielded **7**, **8** and **9**, through Smiles rearrangement.

Hirtellins C (**1**) and F (**12**) are the first examples of dimeric hydrolyzable tannins having both isoDHDG and DHDG groups as linking units between monomers in a molecule. The Smiles rearrangement of the isoDHDG group into a DHDG group in a tannin molecule has been demonstrated for the first time in the present study.

Structure of Hirtellin D (7) Hirtellin D (**7**), $[\alpha]_D$ +135° (MeOH), is a dimeric ellagitannin composed of glucose, galloyl, DHDG and HHDP moieties. Its FAB-MS showed the $[M+Na]^+$ ion peak at m/z 1593 which is 302 m.u. (HHDP) less than that of **6**. Its methylation followed by methanolysis gave **2**, **3** and **4**. The presence of two galloyl, two DHDG and an HHDP groups in **7** was indicated by the ^{1}H-NMR spectrum, which showed two 2H-singlets, four 1H-singlets and four *meta*-coupled doublets (see Experimental). The presence of two *C1* glucose cores was also revealed by the ^{1}H–^{1}H COSY spectrum (Table I). These spectral data of hirtellin D are in agreement with those of the hydrolyzate **7** obtained from **1**, and their identity was confirmed by direct comparison.

Structure of Hirtellin E (15) Hirtellin E (**15**), $[\alpha]_D$ +117° (MeOH), showed the $[M+Na]^+$ ion peak at m/z 1593 in FAB-MS. It was characterized as a dimeric hydrolyzable tannin structurally correlated with hirtellin B (**13**),[4] based on the result of methanolysis of the permethylated derivative, giving **2**, **3** and trimethyl hepta-*O*-methylhellinate (**16**), and the ^{1}H-NMR spectrum, which indicated the presence of three galloyl, a hellinoyl and an HHDP group (see Experimental). The sugar proton signals of **15** in the ^{1}H-NMR spectrum (Table II) showed a close resemblance to those of **13**, except for significant upfield shifts (δ 3.78—3.89) of the H-4—H-6 signals of the glucose-I. From these findings, the structure of hirtellin E was formulated as **15**, which was confirmed by partial hydrolysis of **13** in hot water, giving **15**.

Among the compounds isolated in the present study, hirtellin D (**7**) has a structure which may be producible by the Smiles rearrangement of hirtellin F (**12**) or hirtellin C (**1**) followed by hydrolysis, during the extraction procedure. However, the possibility that hirtellin D is an artifact is ruled out because of the acidity of the total tannin extract



13: R= (β)-OG, R'~R"= (*S*)-HHDP
14: R= OH, R'~R"= (*S*)-HHDP
15:R= (β)-OG, R'=R"=H

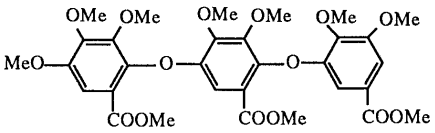


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Chart 3

(below pH 4), which is unfavorable for the occurrence of the Smiles rearrangement of hirtellin F (**12**) to D (**7**), or hirtellin C (**1**) to isohirtellin C (**6**) (the precursor of hirtellin D). This view is supported by the absence of isohirtellin C in the extract and also by the fact that not even a trace amount of hirtellin F was detected in the extract of *Tamarix pakistanica*, in which hirtellins C and D were found.[11]

It is noteworthy that the structural features of hirtellins, having DHDG and/or isoDHDG groups between glucose O-1 of a monomer and glucose O-2 of the other monomer, are characteristic of a series of hydrolyzable tannin dimers in Tamaricaceous plants. These dimers may thus be useful as chemotaxonomic markers.

Upon the screening of the tannins presented in this paper and those previously isolated from Tamaricaceae,[1] along with many other tannins from plant sources, for ability to stimulate of iodination (incorporation of radioactive iodine into an acid-insoluble fraction) of human peripheral blood monocytes, remurins A and B[1] were found to be the most potent compounds among the screened monomers, and hirtellins C, D and E were also significantly active.[12]

Experimental

General Normal-phase HPLC was conducted on a Superspher Si60 cartridge column using solvent systems of *n*-hexane–MeOH–THF–HCOOH, 55:33:11:1 containing 450 mg/l oxalic acid (N1), and 60:45:15:1 containing 450 mg/l oxalic acid (N2). Reversed-phase HPLC was carried out on a LiChrospher RP-18 cartridge column using the solvent system 0.01 M KH_2PO_4–0.01 M H_3PO_4–EtOH–EtOAc, 42.5:42.5:15:5 (R1). The other chromatographic methods, the material (*R. hirtella*) and instruments used in this work were the same as those described in the preceding paper[1].

Isolation of Tannins The eluate with MeOH–H_2O–acetone (5:2:3) in the column chromatography of a part (28.0 g) of the EtOAc extract (65.7 g) over Toyopearl HW-40 (coarse)[1] gave hirtellin C (**1**) (1.35 g). The earliest fraction eluted with MeOH–H_2O–acetone (6:2:2) gave a mixture (227 mg) containing hirtellin F, which was further purified by rechromatography over MCI-gel CHP-20P with H_2O and aqueous MeOH, to yield hirtellin F (**12**) (37.4 mg). A part (35 g) of the *n*-BuOH extract (130 g) was chromatographed in a similar way to that reported previously,[4] to afford gallic acid (100 mg) and hirtellin B (**13**) (1.77 g), in addition to remurin B (**10**). The eluate from MeOH–H_2O–acetone (6:2:2) gave a mixture of two major dimers, which was subjected to repeated chromatography over MCI-gel CHP-20P column to give hirtellin D (**7**) (16 mg) and hirtellin E (**15**) (16 mg).

Hirtellin C (1) An off-white amorphous powder, $[\alpha]_D$ +45° (c=1, MeOH). *Anal.* Calcd for $C_{82}H_{56}O_{52}\cdot 12H_2O$: C, 47.14; H, 3.86. Found: C, 47.22; H, 3.82. UV λ_{max} (MeOH) nm (log ε): 224 (5.18), 275 (4.94). FAB-MS m/z: 1895 $(M+Na)^+$. CD (MeOH) [θ] (nm): $+2.3\times10^5$ (233), -6.4×10^4 (263), $+3.8\times10^4$ (287). ^{1}H-NMR (acetone-d_6+D_2O), see Table I and the text. ^{13}C-NMR (acetone-d_6+D_2O): δ 62.89 (2C) [glucose (Gluc) C-6,6'], 70.40 (2C) (Gluc C-4, 4'), 70.74 (Gluc C-2), 71.31, 71.50 (br, 1C in total, Gluc C-2'), 72.79 (2C) (Gluc C-5, 5'), 73.04, 73.42 (br 1C in total, Gluc C-3'), 73.25 (Gluc C-3), 93.45 (2C) (Gluc C-1, 1').

Methylation of Hirtellin C A mixture of hirtellin C (**1**) (100 mg), anhydrous K_2CO_3 (1 g) and dimethyl sulfate (1 ml) in dry acetone (25 ml) was stirred overnight at room temperature, and refluxed for 4 h. After removal of the inorganic material by centrifugation, the supernatant was concentrated and submitted to preparative thin layer chromatography (TLC) (Kieselgel PF_{254}, benzene–acetone, 5:1). The methylation was repeated twice to yield the octacosamethyl derivative (**1a**) as a white amorphous powder (108 mg). FAB-MS m/z: 2265 $(M+H)^+$. *Anal.* Calcd for $C_{110}H_{112}O_{52}\cdot 3H_2O$: C, 56.95; H, 5.13. Found: C, 57.25; H, 5.31. ^{1}H-NMR (acetone-d_6) δ: 7.36, 7.28 [each 2H, s, galloyl (Gal)], 7.38, 6.47 (each 1H, d, J=2 Hz, DHDG), 7.27 (1H, s, DHDG), 7.15, 7.07 (each 1H, br s, isoDHDG), 6.72 (1H, s, isoDHDG), 6.92, 6.90, 6.77, 6.76 (each 1H, s, HHDP), 6.33 (1H, d, J=8 Hz, Gluc H-1), 5.69 (1H, dd, J=10, 8 Hz, Gluc H-2), 5.97 (1H, t, J=10 Hz, Gluc H-3), 5.29 (1H, t, J=10 Hz, Gluc H-4), 4.64 (1H, dd, J=10, 6 Hz, Gluc H-5), 5.36 (1H, dd, J=13.5, 6 Hz, Gluc H-6), 4.02 (1H, d, J=13.5 Hz, Gluc H-6), 5.90 (1H, d, J=8 Hz, Gluc H-1'), 5.78 (1H, dd, J=10, 8 Hz, Gluc H-2'), 5.84 (1H, t, J=10 Hz, Gluc H-3'), 5.26 (1H, t, J=10 Hz, Gluc H-4'), 4.55 (1H, dd, J=10, 6.5 Hz, Gluc H-5'), 5.24 (1H, dd, J=13.5, 6.5 Hz, Gluc H-6'), 3.85 (overlapped by OMe signals, H-6'), 3.94, 3.90, 3.88, 3.83, 3.72, 3.71, 3.69, 3.65, 3.62, 3.53 (3H each, s, OMe×10), 3.75, 3.63 (6H each, s, OMe×4), 3.79 (12H, diffused s, OMe×4), 3.83—3.85 (30H, diffused s, OMe×10). ^{13}C-NMR (acetone-d_6) δ: 63.56 (Gluc C-6'), 63.67 (Gluc C-6), 71.09 (Gluc C-4), 71.15 (Gluc C-4'), 71.99 (Gluc C-2), 72.18 (Gluc C-2'), 72.49 (Gluc C-5'), 72.91 (Gluc C-5), 73.91 (2C) (Gluc C-3, 3'), 93.38 (Gluc C-1), 93.71 (Gluc C-1'), 106.35, 106.38, 106.63 (2C) (HHDP C-3, 3'), 107.09 (isoDHDG C-6'), 107.33 (isoDHDG C-6 or 2), 108.02 (2C) (Gal C-2, 6), 108.10 (3C) (Gal C-2, 6, isoDHDG C-2 or 6), 109.23, 109.39, 110.03 (DHDG C-6, 6', 2), 118.66 (DHDG C-1'), 120.33 (isoDHDG C-1'), 122.81, 122.86, 123.32, 123.38 (HHDP C-1, 1'), 123.29 (isoDHDG C-1), 123.69 (DHDG C-1), 124.81, 124.86 (Gal C-1), 129.03 (2C), 129.60 (2C) (HHDP C-2, 2'), 141.76 (isoDHDG C-4), 143.62 (DHDG C-4), 143.71, 143.79 (Gal C-4), 143.83 (DHDG C-4'), 144.28 (2C), 145.19 (2C) (HHDP C-5, 5'), 145.22 (isoDHDG C-2'), 146.78 (isoDHDG C-5'), 148.63 (DHDG C-2'), 149.85 (isoDHDG C-4'), 151.14 (DHDG C-2'), 152.49 (DHDG C-3), 153.19, 153.20, 153.36 (2C) (HHDP C-6, 6'), 154.04—154.12 (8C) (HHDP C-4, 4' and Gal C-3, 5), 154.29 (DHDG C-5), 162.61, 163.72 (DHDG C-7', 7), 164.18 (isoDHDG C-7), 166.17 (2C) (Gal C-7), 166.38 (isoDHDG C-7'), 167.53, 167.57, 167.89, 167.93 (HHDP C-7, 7'), 145.97, 147.46, 151.51, 152.01 (isoDHDG C-3, 5, 3' and DHDG C-3).

Methanolysis of the Octacosamethyl Derivative (1a) A mixture of **1a** (20 mg) and 1% NaOMe (0.6 ml) in absolute MeOH (2 ml) was left standing overnight at room temperature. After neutralization with AcOH, the reaction mixture was evaporated under an N_2 stream, and treated overnight with an excess of ethereal CH_2N_2. The residue obtained after removal of the solvent was submitted to preparative TLC [Kieselgel PF_{254}, benzene–acetone (16:1)] to yield methyl tri-*O*-methylgallate (**2**) (3.0 mg), dimethyl hexamethoxydiphenate (**3**) (5.0 mg), dimethyl penta-*O*-methyldehydrodigallate (**4**) (2.8 mg) and dimethyl penta-*O*-methylisodehydrodigallate (**5**) (3.0 mg).

Dimethyl Penta-*O*-methyldehydrodigallate (**4**): EI-MS m/z (%): 436 (M^+), 405 (12), 373 (20), 346 (12), 303 (10), 239 (8), 167 (7), 149 (8), 119 (6), 59 (40). ^{1}H-NMR (acetone-d_6) δ: 7.31 (1H, s, H-6'), 7.29, 6.76 (1H, each d, J=2 Hz, H-2, 6), 3.94, 3.93, 3.92, 3.91, 3.74, 3.71, 3.69 (3H each, s, OMe×7).

Dimethyl Penta-*O*-methylisodehydrodigallate (**5**): EI-MS m/z (%): 436 (M^+), 405 (10), 373 (4), 303 (2), 210 (8), 209 (7), 167 (7), 59 (5). ^{1}H-NMR

(acetone-d_6) δ: 7.30 (2H, s, H-2, 6), 7.13 (1H, s, H-6'), 3.86, 3.85, 3.82, 3.69, 3.52 (3H each, s, OMe × 5), 3.73 (6H, s, OMe × 2).

Acid Hydrolysis of Hirtellin C (1) A solution of **1** (30 mg) in 2 N HCl (2 ml) in a sealed ampule was heated on a water bath at 70 °C for 7 h. After cooling and filtration, the filtrate was extracted with EtOAc. HCl in the aqueous layer was removed by adding AgO followed by filtration. The co-chromatography of the filtrate with an authentic sample on TLC [Kieselgel PF_{254}, *n*-butanol–AcOH–water (4 : 1 : 2); detection, 2-amino-biphenylhydrogen oxalate reagent] showed the presence of glucose.

Partial Hydrolysis of Hirtellin C (1) A solution of **1** (100 mg) in H_2O (30 ml) was heated at 90 °C for 11 h. After cooling, the precipitate deposited was removed by centrifugation. The supernatant was concentrated to dryness, the residue was dissolved in 10% MeOH and the solution was subjected to column chromatography over MCI-gel CHP-20P (1.1 cm i.d. × 23 cm) developing with 10% MeOH→20% MeOH→30% MeOH→40% MeOH in a stepwise gradient mode. The 10% MeOH eluate gave gallic acid (1.8 mg) and hydrolyzate **9** (1.5 mg). The 20% MeOH eluate gave hydrolyzate **8** (5.0 mg). The 30% MeOH eluate gave hydrolyzate **7** (13.6 mg) and crude isohirtellin C (**6**) which was further purified over MCI-gel CHP-20P (1.1 cm i.d. × 9 cm) with development in the same mode as described above. The 30% and 40% MeOH eluates afforded **6** (9.0 mg).

Isohirtellin C (**6**): An off-white amorphous powder. FAB-MS *m/z*: 1895 $(M+Na)^+$, 1911 $(M+K)^+$. Normal phase HPLC, (N2), t_R, 7.8 min. ^{1}H-NMR (acetone-d_6 + D_2O) δ: 7.19 (1H, s), 7.09, 6.10 (1H each, d, J=2 Hz) (DHDG), 6.91 (2H each, s, Gal), 6.58, 6.48 (1H each, s, HHDP), glucose protons, see Table I.

Hydrolyzate **8**: An off-white amorphous powder. FAB-MS *m/z*: 1291 $(M+Na)^+$. Normal-phase HPLC (N2), t_R 4.4 min. ^{1}H-NMR (acetone-d_6 + D_2O) δ: 6.98 (2H, s, Gal), 7.15 (1H, s, DHDG), 7.09, 6.08 (1H each, d, J=2 Hz, DHDG), and glucose protons, see Table I.

Hydrolyzate **9**: A buff amorphous powder, normal-phase HPLC (N2), t_R 2.1 min. ^{1}H-NMR (acetone-d_6 + D_2O) δ: 7.10, 7.07 (each s, 1H in total, DHDG), 7.08, 7.04 (each s, 2H in total, Gal), 7.18, 7.20, 7.68, 6.67 (each, d, J=2 Hz, 2H in total, DHDG), α-anomer; 5.26 (d, J=3.5 Hz, Gluc H-1), 4.80 (dd, J=3.5, 10 Hz, Gluc H-2), 5.71 (t, J=10 Hz, Gluc H-3), 3.83 (t, J=10 Hz, Gluc H-4), 3.94 (ddd, J=2.5, 4.5, 10 Hz, Gluc H-5), 3.74 (dd, J=4.5, 12 Hz, Gluc H-6), 3.81 (dd, J=2.5, 12 Hz, Gluc H-6), β-anomer; 4.31 (d, J=8 Hz, Gluc H-1), 4.96 (dd, J=8, 10 Hz, Gluc H-2), 5.29 (t, J=10 Hz, Gluc H-3), 3.75 (t, J=10 Hz, Gluc H-4), 3.36 (ddd, J=2.5, 5, 10 Hz, Gluc H-5), 3.68 (dd, J=5, 12 Hz, Gluc H-6), 3.77 (dd, J=2.5, 12 Hz, Gluc H-6).

Treatment of Hirtellin C (1) with Tannase A solution of hirtellin C (**1**) (50 mg) in H_2O (10 ml) was incubated with tannase (15 drops) at 37 °C for 9 d. The reaction mixture was evaporated and the residue was dissolved in 10% MeOH (1 ml) and subjected to column chromatography over MCI-gel CHP-20P, developing with 10% MeOH→20% MeOH→30% MeOH→40% MeOH in a stepwise gradient mode. The 10% MeOH eluate gave gallic acid (0.5 mg) and the last portion of the 30% MeOH eluate gave isohirtellin C (**6**) (10 mg). The earliest portion of the 30% MeOH eluate was proved by HPLC (reversed phase and normal phase mode) to contain a mixture of hirtellin D (**7**) and isohirtellin C (**6**). Isohirtellin C and unchanged starting material were also obtained from the 40% MeOH eluate. Isohirtellin C (**6**) (15 mg) was identified by co-chromatography on HPLC and by ^{1}H-NMR spectral comparison with the product obtained by partial hydrolysis of hirtellin C (**1**).

Partial Hydrolysis of Remurin A (11) A solution of **11** (1 mg) in H_2O (1 ml) was heated on a boiling water bath for 7 h and then the reaction mixture was analyzed by normal and reversed phase HPLC to show the formation of hydrolyzate **9** [normal phase HPLC, t_R 3.0 min (N1) and t_R 2.1 min, (N2); reversed phase HPLC, t_R 2.5 and 2.9 min, (R1). 1,3-Di-*O*-galloyl-4,6-*O*-(*S*)-hexahydroxydiphenoyl-β-D-glucose, remurin B (**10**) and gallic acid were also detected.

Hirtellin D (7) An off-white amorphous powder, $[\alpha]_D$ +135° (*c*=1, MeOH). FAB-MS *m/z*: 1593 $(M+Na)^+$. Normal phase HPLC (N2), t_R 5.8 min. UV λ_{max} (MeOH) nm (log ε): 220 (5.05), 276 (4.91). ^{1}H-NMR (acetone-d_6 + D_2O) δ: 6.99, 6.90 (2H each, s, Gal), 6.59, 6.46 (1H each, s, HHDP), 7.16, 7.14 (1H, each s, DHDG), 7.11, 7.09, 6.10, 6.08 (1H each, d, J=2 Hz, DHDG) and glucose protons, see Table I.

Methylation of Hirtellin D (7) Followed by Methanolysis Hirtellin D (**7**) (1.3 mg) in MeOH (0.5 ml) was methylated with ethereal CH_2N_2 at room temperature for 5 h. The residue obtained after removal of the solvent was directly methanolyzed with 1% NaOMe (50 μl) in MeOH (0.2 ml) at room temperature overnight to give methyl tri-*O*-methylgallate (**2**), dimethyl hexamethoxydiphenate (**3**) and dimethyl penta-*O*-methyldehydrodigallate (**4**), which were identified by co-chromatography with authentic samples on TLC [Kieselgel PF_{254}, benzene–acetone (15 : 1)] and by normal phase HPLC [*n*-hexane–EtOAc (2 : 1)].

Preparation of Hirtellin D (7) from Hirtellin F (12) and Isohirtellin C (6)

a) Partial Hydrolysis of Hirtellin F (**12**): An aqueous solution of **12** (1 mg/1 ml) in a sealed tube was heated in a boiling-water bath (95 °C) for 2 h. Normal phase HPLC (N2) of the reaction mixture showed the presence of hirtellin D (**7**), **8** and **9**, along with gallic acid.

b) Partial Hydrolysis of Isohirtellin C (**6**): An aqueous solution of **6** (1 mg/1 ml) in a sealed tube was heated in a boiling water bath (95 °C) for 6 h, and the course of the reaction was monitored by normal phase HPLC (N2). The formation of hirtellin D (**7**) along with **8** was seen after 1.5 h, and they were degraded to **9** 5 h later.

Measurement of Stability of Hirtellin C (1) in Solvents Solutions (0.1%, 1 ml) of **1** (1 mg) in acetone, H_2O, and 50% aqueous acetone were each left to stand for 2 weeks at room temperature. An aliquot of each reaction mixture was analyzed by HPLC after 2, 4, 6, 9, 11 and 14 d. Changes in the concentrations of hirtellin C (**1**) and the products, **6** and **7**, in each experiment were determined from the peak areas in the HPLC (R1) relative to those of authentic samples (**1**, 19.3 min; **6**, 8.3 min; **7**, 4.8 min); see Fig. 2.

Measurement of Stability of Hirtellin C (1) at Various pH Values Hirtellin C (**1**) (1 mg) was dissolved in aqueous trifluoroacetic acid under pH control with trifluoroacetic acid, and also in a 0.1 M phosphate buffer pH 7.4. Each solution was gently shaken in an incubator at 40 °C for 10 h and the stability of hirtellin C was examined by HPLC (reversed-phase mode, R1) after 30 min and 1.5, 3, 5, 8 and 10 h.

Hirtellin E (15) An off-white amorphous powder, $[\alpha]_D$ +117° (*c*=1.0, MeOH). FAB-MS *m/z*: 1593 $(M+Na)^+$. UV λ_{max} (MeOH) nm (log ε): 222 (5.11), 276 (4.91). ^{1}H-NMR (acetone-d_6 + D_2O) δ: 7.56, 6.72 (1H each, s, hellinoyl), 6.80, 5.78 (1H each, d, J=2 Hz, hellinoyl), 7.03, 6.97, 6.92 (2H each, s, Gal), 6.68, 6.49 (1H each, s, HHDP) and glucose protons, see Table I.

Methylation of Hirtellin E (15) Followed by Methanolysis A solution of **15** (1.3 mg) in MeOH (0.5 ml) was methylated with ethereal CH_2N_2 at room temperature for 5 h. The residue after removal of the solvent was directly methanolyzed with 1% NaOMe (50 μl) in MeOH (0.2 ml) and was worked up in a similar way to that described for methanolysis of hirtellin D, to give methyl tri-*O*-methylgallate (**2**), dimethyl hexamethoxydiphenate (**3**), and trimethyl hepta-*O*-methylhellinate (**16**), which were found to be identical with respective authentic samples by TLC and normal-phase HPLC in the same ways as used for the methylation and methanolysis of **7**.

Partial Hydrolysis of Hirtellin B (13) A solution of **13** (100 mg) in H_2O (30 ml) was heated at 95 °C for 10 h. After cooling, the precipitate was removed by centrifugation, and the concentrated supernatant was subjected to column chromatography over MCI-gel CHP-20P (1.1 cm i.d. × 30 cm) developing with H_2O→10% MeOH→20% MeOH→30% MeOH→40% MeOH in a stepwise gradient mode. The H_2O eluate gave gallic acid (5.6 mg), and the 30% MeOH eluate gave crude hirtellin E, which was further purified on MCI-gel CHP 20P (1.1 cm i.d. × 18 cm) to give hirtellin E (**15**). The starting material (17.7 mg) was recovered from the 40% MeOH eluate.

Hirtellin F (12) An off-white amorphous powder, FAB-MS *m/z*: 1593 $(M+Na)^+$, $[\alpha]_D$ +33° (*c*=1, MeOH). UV λ_{max} (MeOH) nm (log ε): 217 (5.15), 275 (4.79), CD (MeOH) $[\theta]$ (nm): $+16.2\times10^4$ (228), -5.9×10^4 (264), $+2.7\times10^4$ (281). ^{1}H-NMR (acetone-d_6 + D_2O) δ: 7.04, 6.94 (2H each, s, Gal), 6.59, 6.47 (1H each, s, HHDP), 7.15, 7.14, 7.12 (each s, 1H in total), 7.00, 6.95 (each s, 1H in total), 6.88 (2H, broad diffused singlet), 6.77 (1H, s), 6.42 (1H, d, J=2 Hz).

Methylation of Hirtellin F (12) Followed by Methanolysis A mixture of hirtellin F (**12**) (10 mg), anhydrous K_2CO_3 (100 mg) and dimethyl sulfate (0.1 ml) in dry acetone (3 ml) was stirred overnight at room temperature, and then refluxed for 6 h. The reaction mixture was worked up in a way similar to that described for methylation of hirtellin C, to give permethylated hirtellin F (**12a**) (3.2 mg) as a white amorphous powder. ^{1}H-NMR (acetone-d_6) δ: 7.49, 7.29 (2H each, s, Gal), 7.34, 6.43 (1H each, d, J=2 Hz, DHDG), 7.24 (1H, s, DHDG), 7.08, 6.99 (1H each, br d, J=2 Hz, isoDHDG), 6.78 (1H, s, isoDHDG), 6.93, 6.78 (1H each, s, HHDP), 6.31 (1H, d, J=8 Hz, Gluc H-1), 5.69 (1H, t, J=9 Hz, Gluc H-2), 5.97 (1H, t, J=10 Hz, Gluc H-3), 5.29 (1H, t, J=10 Hz, Gluc H-4), 4.64 (1H, dd, J=10, 6 Hz, Gluc H-5), 5.32 (1H, dd, J=12.5, 6 Hz, Gluc H-6), the other H-6 is overlapped by OMe signals, 5.78 (1H, d, J=8.5 Hz, Gluc H-1'), 5.49 (1H, dd, J=10, 8.5 Hz, Gluc H-2'), 5.73 (1H, t, J=10 Hz, Gluc H-3'), H-4', H-5' and the two H-6' protons are overlapped by OMe signals.

Methanolysis of the Permethylated Hirtellin F (12a) Compound **12a** (3.2 mg) was methanolyzed with 1% NaOMe (0.1 ml) in MeOH (0.5 ml) and then purified by preparative TLC to give methyl tri-*O*-methylgallate (**2**), dimethyl hexamethoxydiphenate (**3**), dimethyl penta-*O*-methyldehydrodigallate (**4**) and dimethyl penta-*O*-methylisodehydrodigallate (**5**).

Partial Hydrolysis of Hirtellin F (12) in Hot Water An aqueous solution (1 ml) of **12** (1 mg) in a sealed tube was heated in a boiling water bath for 2 h. Normal- and reversed-phase HPLC of the reaction mixture using the solvent systems N1 and R1 showed the production of hirtellin D (**7**), **8** (after 1 h) and **9**, and of ellagic acid.

Partial Acid Hydrolysis of Hirtellin C (1) A mixture of an aqueous solution (pH 1.0) (1 ml) of **1** (1 mg) and trifluoroacetic acid (0.5 ml) in a sealed tube was heated in a hot-water bath (60 °C) for 5 h. HPLC analyses (normal and reversed modes) of the reaction mixture showed peaks identical with those of authentic hirtellin F (**12**), isohirtellin C (**6**), hirtellin D (**7**) and ellagic acid.

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