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ALNUSNINS A AND B FROM THE LEAVES OF *ALNUS SIEBOLDIANA**

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Key Word Index—*Alnus sieboldiana*; Betulaceae; hydrolysable tannin; ellagitannin; alnusnin A; alnusnin B; tegallic acid; (*S*)-tergalloyl group.

Abstract—Two new ellagitannins, alnusnins A and B, have been isolated from the leaves of *Alnus sieboldiana* (Betulaceae) and characterized as 1-*O*-galloyl-2,3-(*S*)-hexahydroxydiphenoyl-4,6-(*S*)-tergalloyl-β-D-glucose and 2,3-(*S*)-hexahydroxydiphenoyl-4,6-(*S*)-tergalloyl-D-glucose, respectively, by chemical and spectral evidence. The occurrence of eight known tannins, 1,2,6-tri-*O*-galloyl-β-D-glucose, strictinin, casuarinin, stachyurin, pedunculagin, 1(β)-*O*-galloylpedunculagin and stenophyllanin A, and two flavonoids, pinocembrin and quercetin 3-*O*-β-D-glucuronide, has also been demonstrated.

INTRODUCTION

Most of the component phenolcarboxylic acid moieties in hydrolysable tannins are biosynthetically formed by oxidative C–C and/or C–O coupling(s) of appropriately positioned galloyl groups attached to a polyalcohol core (in most cases, D-glucose). For example, the 4,4′,5,5′,6,6′-hexahydroxydiphenoyl (HHDP) group, the commonest acid ester group in hydrolysable tannins, is considered to be derived by an oxidative C–C coupling of two galloyl groups [2, 3], and various triphenoyl groups [4–6] are regarded as being formed through further oxidative coupling of HHDP and galloyl groups. In a previous paper, we elucidated the structure of a hydrolysable tannin, tergallin (**1**) [7], which contains a novel triphenoic acid ester group, i.e. tergalloyl group, in an optically inactive form. Further chemical examination of tannins in the leaves of *Alnus sieboldiana* (Betulaceae) has resulted in the isolation and characterization of alnusnins A and B which possess the optically active tergalloyl moiety, together with eight known hydrolysable tannins and two flavonoids. We now report herein the isolation and characterization of these compounds.

RESULTS AND DISCUSSION

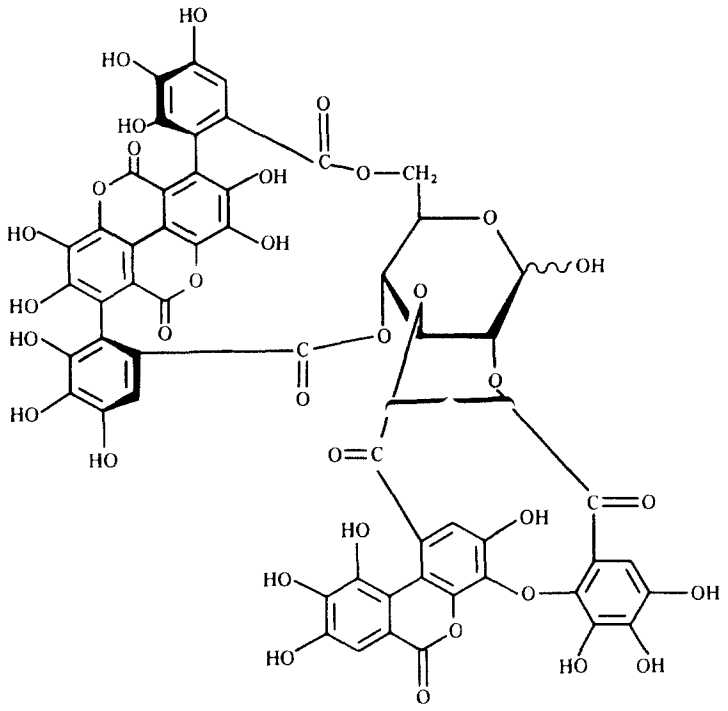
The fresh leaves of *A. sieboldiana* were extracted with aq. acetone and the extract was subjected to a combination of Sephadex LH-20, MCI-gel CHP 20P and Bondapak C_{18}/Porasil B chromatography to yield two flavonoids and 10 hydrolysable tannins. Of these compounds, the flavonoids and eight tannins were found to be identical with pinocembrin (**2**) [8], quercetin 3-*O*-β-D-glucuronide (**3**) [9], 1,2,6-tri-*O*-galloyl-β-D-glucose (**4**) [10], strictinin (**5**), casuarinin (**6**), stachyurin (**7**) [11], 1-desgalloyleugeniin (**8**) [12], pedunculagin (**9**) [13], 1(β)-*O*-galloylpedunculagin (**10**) [3] and stenophyllanin A (**11**) [14], by comparison of their physical and spectral data with those of authentic samples.

The new tannins alnusnins A (**12**) and B (**13**) were characterized as ellagitannins by their giving blue and reddish brown colours with ferric chloride and sodium nitrite–acetic acid [15] reagents, respectively. The ^{1}H NMR spectrum of **12** showed a two-proton singlet at δ7.10 arising from a galloyl group and five one-proton aromatic singlets at δ6.43, 6.47, 6.60, 6.93 and 6.98, two of which suggested the presence of an HHDP group. The remaining three singlet resonances implied the existence of a triphenoyl group in the molecule. The ^{13}C NMR spectrum of **12** showed the presence of six aromatic rings and six carboxylic acid esters (see Experimental) in the molecule. In the ^{1}H and ^{13}C NMR spectra, the chemical shifts and coupling patterns of sugar signals were in good agreement with those found in **10**, indicating that **12** has a β-D-glucopyranose core with $^4C_1$ conformation [3].

On the other hand, the appearance of a complex signal pattern in the sugar region and of a upfield shift of the anomeric signal in the ^{1}H NMR spectrum of **13** indicated that the sugar anomeric position is not acylated. The observation of ^{1}H NMR aromatic signals [δ6.30 (1H), 6.54 (0.4H), 6.59 (1.6H), 6.92 (1H), 6.96 (0.4H) and 6.98 (0.6H)] corresponding to five protons and five carboxylic carbon signals [δ163.2, 167.3, 168.0, 168.8 and 169.4] suggested the presence of one HHDP group and one triphenoyl group in **13**. Furthermore, the ^{1}H and ^{13}C NMR sugar signal patterns similar to those found in **9**, suggested that the HHDP and the triphenoyl groups were attached to the 2,3 and 4,6-positions of the glucose moiety. The desgalloyl structure of **13** was consistent with the negative ion FABMS with the prominent $[M-H]^-$ ion peak at *m/z* 951, which was found to be 152 mass units (corresponding to the loss of one galloyl group) less than that (*m/z* 1103 $[M-H]^-$) of **12**. Further support for this was obtained by partial enzymatic hydrolysis of **12** with tannase, which afforded gallic acid and compound **13**.

Methylation of **13** with dimethyl sulphate and potassium carbonate in dry acetone and subsequent alkaline

*Part 78 in the series 'Tannins and Related Compounds'. For Part 77 see ref [1].



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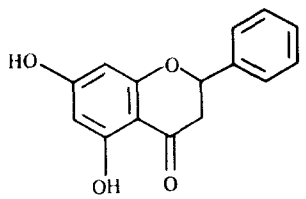

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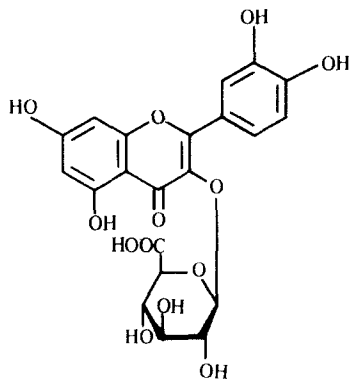

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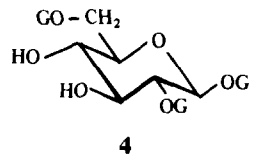
methanolysis yielded dimethyl hexamethoxydiphenoate (**14**) and a methyl triphenoate (**15**). The atropisomerism of **14** was confirmed to be in the *S*-series [16] by the negative sign of the specific optical rotation ($-26.5°$, $CHCl_3$). The ^1H NMR spectrum of **15** showed three aromatic singlets [δ7.18, 7.34 and 7.39 (each 1H, *s*)] and 11 methoxyl signals, the chemical shifts being found to be identical with those of **15'** which had been obtained from tergallin (**1**) [7], except for the value of the specific optical rotation ($-19.8°$, $CHCl_3$) in **15**. The chirality of the biphenyl bond in **15** was established to be in the *S*-series based on the similarity of the CD spectra of **15** and (*S*)-dimethyl hexamethoxydiphenoate (**14**).

The location of (*S*)-hexahydroxydiphenoyl group in the glucose moiety was determined to be at the 2,3-positions from the fact that partial hydrolysis of **13** in hot water yielded 2,3-(*S*)-hexahydroxydiphenoyl-D-glucose (**16**) [17], together with tergallic acid dilactone (**17**) [7]. Thus, the (*S*)-tergalloyl group could be concluded to be located at the 4,6-positions.

Based on these results, the structures of alnusnins A and B were established to be 1-*O*-galloyl-2,3-(*S*)-hexahydroxydiphenoyl-4,6-(*S*)-tergalloyl-β-D-glucose (**12**) and 2,3-(*S*)-hexahydroxydiphenoyl-4,6-(*S*)-tergalloyl-D-glucose (**13**), respectively. The orientation of the (*S*)-tergalloyl group still remains to be determined.

Alnusnins A and B represent the first hydrolysable tannins possessing the optically active (*S*)-tergalloyl group. In addition, all the tannins in this plant, except for alnusnins A and B, have also been isolated from various



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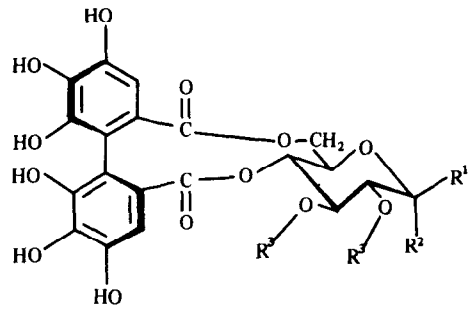
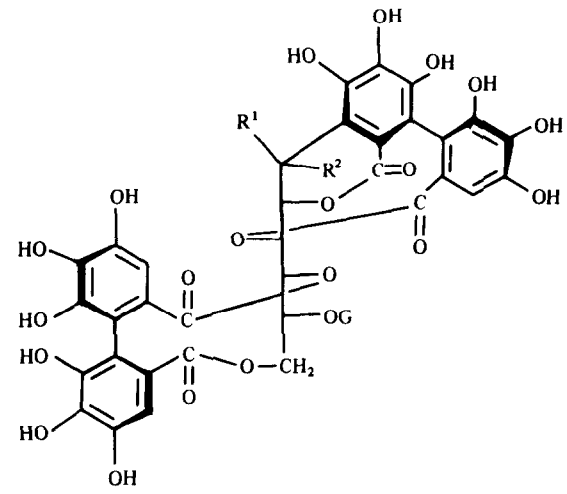


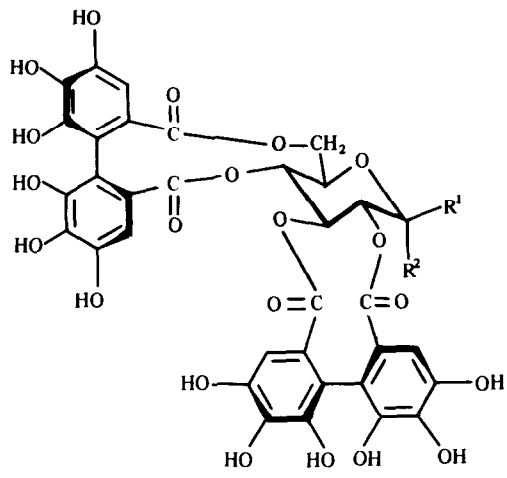
5 R^1 = OG, R^2 = R^3 = H

8 R^1, R^2 = H,OH, R^3 = G



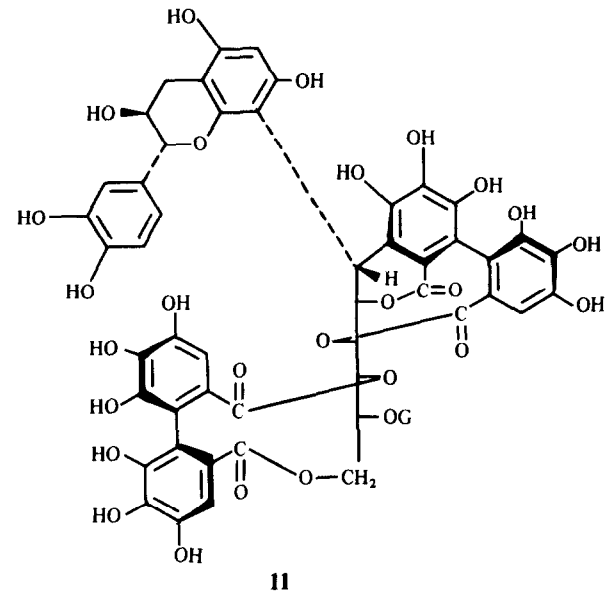
6 R^1 = H, R^2 = OH

7 R^1 = OH, R^2 = H



9 R^1 = R^2 = H, OH

10 R^1 = OG, R^2 = H



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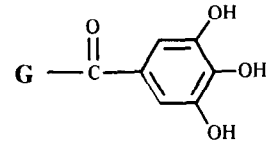
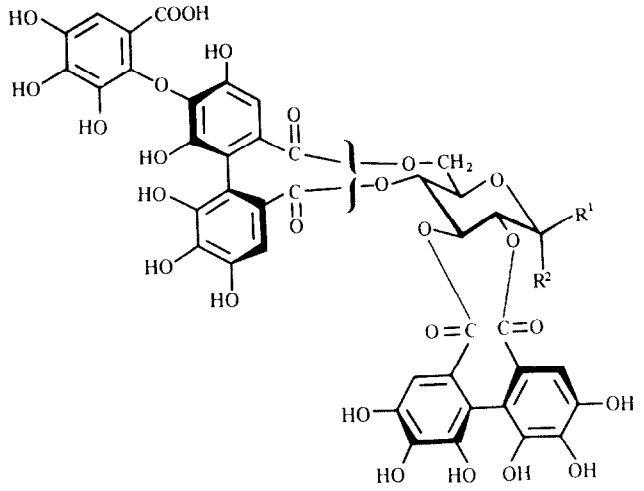


member of the Fagaceae. This suggests that there is a close taxonomic relationship between the Fagaceae and the Betulaceae.

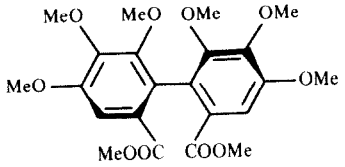
EXPERIMENTAL

General. Mps: uncorr. ^1H and ^{13}C NMR spectra were recorded at 100 and 25.05 MHz, respectively, and chemical shifts are given in δ(ppm) scale with TMS as int. standard. Negative FABMS were measured at 1.5 kV (accelerating voltage) with Me_2CO–glycerol as matrix, while EIMS at 30 eV (ionizing voltage). CC was carried out with Sephadex LH-20 (25–100 μ), MCI-gel CHP-20P (75–150 μ, Mitsubishi Chemical Industry Co.), Bondapak C_{18}/Porasil B (37–75 μ) and Kieselgel 60 (70–230 mesh, Merck). TLC was conducted on precoated silica gel 60 F_{254} plates (Merck) and precoated cellulose F_{254} plates.



12 R^1 = OG, R^2 = H

13 R^1 = R^2 = H, OH



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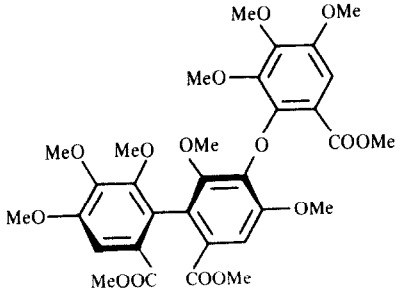

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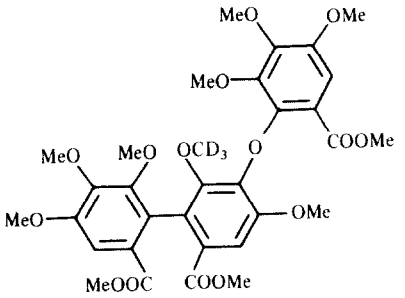
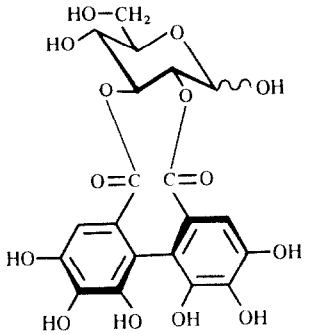


15'



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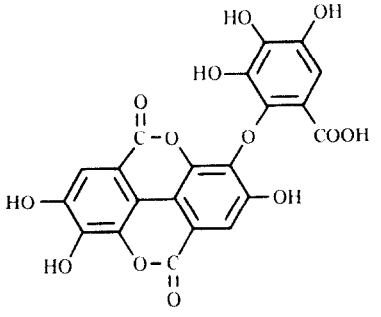

17

Spots were visualized under UV and by spraying $FeCl_3$ (for phenolics), dil. H_2SO_4 (for phenolics and Me ethers). HPLC was conducted with a Toyo Soda CCPM solvent delivery system and UV-8000 spectrophotometer over a Cosmosil 5PH column (4.6 i.d. × 250 mm) Nakarai Chemicals, Ltd.) using MeCN–H_2O (50 mM H_3PO_4).

Plant material. The leaves of *Alnus sieboldiana* Matsumura were collected in Kasuya Forestry Experimental station of Kysushu University. A voucher specimen is deposited at the Herbarium, Faculty of Pharmaceutical Sciences, Kyushu University.

Extraction and isolation. Fresh leaves (7.5 kg) were extracted ×3 with 80% aq. Me_2CO at room temp. After removal of Me_2CO *in vacuo*, the aq. soln was filtered to remove chlorophylls, etc. The filtrate was concd and them subjected to a column of Sephadex LH-20. Elution with a solvent system of H_2O containing increasing proportions of MeOH afforded five fractions; I (89 g), II (65.3 g), III (49.2 g), IV (37.9 g) and V (15.0 g). CC of a part (5 g) of fr. I on Sephadex LH-20 with *n*-PrOH gave quercetin 3-*O*-*β*-D-glucuronide (**3**) (124 mg). Separation of fr. II by repeated CC on Sephadex LH-20 with EtOH and MCI-gel CHP 20P with an H_2O–MeOH gradient solvent system to yield pinocembrin (**2**) (440 mg), 1,2,6-tri-*O*-galloyl-*β*-D-glucose (**4**) (448 mg), strictinin (**5**) (149 mg), stachyurin (**7**) (598 mg) and pedunculagin (**9**) (1.10 g). Fr. III gave casuarinin (**6**) (484 mg), 1-desgalloyleugeniin (**8**) (532 mg), and stenophyllanin A (**11**) (68 mg) on Sephadex LH-20 (H_2O–MeOH (1:4), MCI-gel and Bondapak C_{18}/Porasil B (H_2O–MeOH 3:7) chromatographies. On similar separation, fr. IV afforded 1 (*β*)-*O*-galloylpedunculagin (**10**) (893 mg) and alnusnin B (**13**) (82 mg). The CC of fr. V over Sephadex LH-20 with EtOH–H_2O–Me_2CO and then Bondapak C_{18} with H_2O–MeOH furnished alnusnin A (**12**) (43 mg).

Pinocembrin (**2**). Colourless needles (H_2O–MeOH), mp 203–205°, ^{1}H NMR (Me_2CO-d_6): *δ* 2.79 (1H, *dd*, *J* = 4, 16 Hz, H-3), 3.16 (1H, *dd*, *J* = 12, 16 Hz, H-3), 5.54 (1H, *dd*, *J* = 4, 12 Hz, H-2), 6.60 (2H, *s*-like, H-6 and H-8), 7.32–7.52 (5H, *m*, B-ring H).

Quercetin 3-O-*β*-D-*glucuronide* (**3**). Pale yellow crystals (H_2O–MeOH), mp 188–190°, $[\alpha]_D^{24}$ −49.4° (H_2O; *c* 1.0). ^{1}H NMR (DMSO-d_6): *δ* 3.44–4.15 (5H, *m*, sugar-H), 5.40 (1H, *d*, *J* = 7 Hz, anomeric-H), 6.15, 6.33 (each 1H, *d*, *J* = 2 Hz, H-6 and 8), 6.85 (1H, *d*, *J* = 8 Hz, H-2′), 7.43 (1H, *dd*, *J* = 2, 8 Hz, H-6′, 7.97 (1H, *d*, *J* = 2 Hz, H-5′). ^{13}C NMR (DMSO-d_6): *δ* 71.5, 73.8, 74.4, 76.2 (sugar C-2, 3, 4, 5), 93.8 (C-8), 98.6 (C-6), 101.7 (sugar C-1), 103.5 (C-8a), 115.2 (C-2′), 117.0 (C-5′), 120.8, 121.0 (C-1′ and 6′), 133.4 (C-3), 144.3 (C-3′), 148.1 (C-4′), 156.3, 156.7 (C-2 and 4a), 160.6 (C-5), 164.3 (C-7), 172.2 (sugar C-6), 177.2 (C-4). Enzymatic hydrolysis of **3** with crude hesperidinase gave quercetin and glucuronic acid.

1,2,6-*Tri*-O-*galloyl*-*β*-D-*glucose* (**4**). Colourless needles (H_2O), mp 194–196°, $[\alpha]_D^{25}$ −62.9° (Me_2CO; *c* 1.1), ^{1}H NMR (Me_2CO-d_6): *δ* 3.6–4.1 (3H, *m*, H-3, 4, 5), 4.42 (1H, *dd*, *J* = 4, 13 Hz, H-6), 4.68 (1H, *d*, *J* = 13 Hz, H-6), 5.28 (1H, *t*, *J* = 8 Hz, H-2), 5.96 (1H, *d*, *J* = 8 Hz, H-1), 7.07, 7.09, 7.15 (each 2H, *s*, galloyl-H).

Strictinin (**5**). An off-white amorphous powder, $[\alpha]_D^{27}$ −10.9° (Me_2CO; *c* 1.0). ^{1}H NMR (Me_2CO-d_6): *δ* 3.7–4.2 (4H, *m*, H-2, 3, 5 and 6), 4.90 (1H, *t*, *J* = 9 Hz, H-4), 5.21 (1H, *dd*, *J* = 7, 13 Hz, H-6), 5.76 (1H, *d*, *J* = 8 Hz, H-1), 6.60, 6.71 (each 1H, *s*, HHDP-H), 7.18 (2H, *s*, galloyl-H).

Casuarinin (**6**). An off-white amorphous powder, $[\alpha]_D^{27}$ +44.8° (Me_2CO; *c* 1.1). ^{1}H NMR (Me_2CO-d_6): *δ* 4.06 (1H, *d*, *J* = 13 Hz, H-6), 4.46 (1H, *dd*, *J* = 2, 4 Hz, H-2), 4.89 (1H, *dd*, *J* = 3, 13 Hz, H-6), 5.28–5.56 (3H, *m*, H-3, 4, 5), 5.67 (1H, *d*, *J* = 4 Hz, H-1), 6.48, 6.55, 6.74 (each 1H, *s*, arom.-H), 7.10 (2H, *s*, galloyl-H).

Stachyurin (**7**). An off-white amorphous powder, $[\alpha]_D^{27}$ +39.8° (Me_2CO; *c* 1.3). ^{1}H NMR (Me_2CO-d_6): *δ* 4.02 (1H, *d*, *J* = 13 Hz, H-6), 4.86 (1H, *dd*, *J* = 3, 13 Hz, H-6), 4.8–5.1 (3H, *m*, H-1, 2, 3), 5.37 (1H, *dd*, *J* = 3, 9 Hz, H-5), 5.67 (1H, *dd*, *J* = 3, 9 Hz, H-4), 6.49, 6.59, 6.81 (each 1H, *s*, arom.-H), 7.12 (2H, *s*, galloyl-H).

1-*Desgalloyleugeniin* (**8**). An off-white amorphous powder, $[\alpha]_D^{27}$ −123.0° (Me_2CO; *c* 1.3). ^{1}H NMR (Me_2CO-d_6): *δ* 3.78, 3.86 (1H, each *d*, *J* = 13 Hz, H-6), 4.26, 4.67, (1H, each *dd*, *J* = 6, 10 Hz, H-5), 5.0–5.9 (5H in total, *m*, glucose-H), 6.51, 6.52, 6.66 (2H in total, each *s*, HHDP-H), 6.96, 7.00, 7.08 (4H in total, each *s*, galloyl-H).

Pedunculagin (**9**). An off-white amorphous powder, $[\alpha]_D^{25}$ +95.1° (Me_2CO; *c* 1.2). ^{1}H NMR (Me_2CO-d_6): *δ* 3.6–5.7 (7H in total, *m*, glucose-H), 6.35, 6.52, 6.56, 6.61, 6.67 (4H in total, each *s*, HHDP-H).

1(*β*)-O-*Galloylpedunculagin* (**10**). An off-white amorphous powder, $[\alpha]_D^{27}$ +40.3° (Me_2CO; *c* 1.2). ^{1}H NMR (Me_2CO-d_6): *δ* 3.88 (1H, *d*, *J* = 13 Hz, H-6), 4.49 (1H, *dd*, *J* = 7, 10 Hz, H-5), 5.19 (2H, *t*, *J* = 9 Hz, H-2 and 4), 5.28 (1H, *dd*, *J* = 7, 13 Hz, H-6), 5.48 (1H, *t*, *J* = 9 Hz, H-3), 6.22 (1H, *d*, *J* = 9 Hz, H-1), 6.40, 6.48, 6.58, 6.68 (each 1H, *s*, HHDP-H), 7.17 (2H, *s*, galloyl-H).

Stenophyllanin A (**11**). An off-white amorphous powder, $[\alpha]_D^{25}$ +50.1° (MeOH; *c* 1.1). ^{1}H NMR (Me_2CO-d_6): *δ* 2.3–3.2 (2H, *m*, cat H-4), 4.41 (1H, *br s*, H-1), 5.92 (1H, *br s*, cat H-6), 6.5–7.1 (*m*, arom.H).

Alnusnin A (**12**). An off-white amorphous powder, $[\alpha]_D^{31}$ −168.4° (Me_2CO; *c* 0.4). Negative ion FABMS *m/z*: 1103 $[M-H]^-$. ^{1}H NMR (Me_2CO-d_6): *δ* 3.93 (1H, *d*, *J* = 13 Hz, H-6), 4.52 (1H, *dd*, *J* = 6, 9 Hz, H-5), 5.19 (2H, *J* = 9 Hz, H-2 and 4), 5.28 (1H, *dd*, *J* = 6, 9 Hz, H-6), 5.50 (1H, *t*, *J* = 9 Hz, H-3), 6.24 (1H, *d*, *J* = 9 Hz, H-1), 6.43, 6.47, 6.60, 6.93, 6.98 (each 1H, *s*, HHDP- and tergalloyl-H), 7.10 (2H, *s*, galloyl-H). ^{13}C NMR (Me_2CO-d_6): *δ* 63.9 (glc C-6), 69.1 (glc C-4), 73.1 (glc C-3), 76.1 (glc C-2), 77.0 (glc C-5), 92.2 (glc C-1), 106.8, 107.3, 109.8, 110.3, 112.2, 112.7, 114.0, 114.5, 114.9, 119.3, 120.8, 120.9, 124.7, 125.7, 125.9, 132.0, 136.4, 136.5, 136.6, 140.2, 140.8, 142.9, 143.6, 143.9, 144.5, 145.2, 146.0, 146.2, 148.4 (arom. C), 164.0, 165.4, 167.4, 168.1, 168.9, 169.7 (COO). (Found: C, 48.92; H, 3.48. $C_{48}H_{32}O_{32}\cdot 3H_2O$ requires: C, 49.07; H, 3.26%).

Enzymatic hydrolysis of **12**. A soln of **12** (20 mg) in H_2O (3 ml) was incubated with tannase at room temp. for 45 min. The reaction mixture was subjected to an MCI-gel column with H_2O–MeOH (gradient) to yield gallic acid and a hydrolysate (7.8 mg), which was shown to be identical with **13** by comparison of the physical and ^{1}H NMR data.

Alnusnin B (**13**). An off-white amorphous powder, $[\alpha]_D^{27}$ −36.2° (Me_2CO; *c* 1.0). Negative ions FABMS *m/z*: 951 $[M-H]^-$, 933, 917, 783, 767. ^{1}H NMR (Me_2CO-d_6): *δ* 3.78 (1H, *m*, H-6), 4.22 (1H, *m*, H-5), 4.9–5.6 (5H, *m*, H-1, 2, 3, 4, 6), 6.30 (1H), 6.54 (0.4H), 6.59 (1.6H), 6.92 (1H), 6.96 (0.4H), 6.98 (0.6H) (each *s*, HHDP- and tergalloyl-H). ^{13}C NMR (Me_2CO-d_6): *δ* 64.6 (glc C-6), 67.6, 69.6, 72.3, 75.5, 75.6, 77.2, 78.3, (glc C-2, 3, 4, 5), 91.8 (glc C-1*α*), 95.5 (glc C-1*β*), 106.6, 107.2, 107.4, 107.8, 109.9, 112.6, 112.7, 114.5, 115.0, 120.9, 125.1, 126.4, 126.5, 128.7, 132.4, 136.0, 136.3, 136.5, 140.5, 142.9, 143.6, 144.1, 144.3, 145.0, 145.4, 145.8, 146.1, 148.3 (arom. C), 163.2, 167.3, 168.0, 168.8, 169.4 (COO). (Found: C, 46.43; H, 3.81, $C_{41}H_{28}O_{27}\cdot 6H_2O$ requires: C, 46.42; H, 3.80%).

Methylation of **13**, *followed by alkaline methanolysis.* A mixture of **13** (24 mg), Me_2SO_4 (0.25 ml) and K_2CO_3 (350 mg) in dry Me_2CO (5 ml) was heated under reflux for 2 hr. After removal of the inorganic ppt, the soln was concd to a syrup, which was subjected to silica gel CC. Elution with C_6H_6–Me_2CO (9:1) yielded a mixture (23.4 mg) consisting of *α*- and *β*-anomers. The methyl ethers thus obtained were further methylated with Me_2SO_4 in an alkaline soln (5% NaOH in H_2O–MeOH) under reflux for 2.5 hr. After removal of MeOH *in vacuo*, the aq. soln was acidified with 0.5 M HCl and then extracted with Et_2O. The

organic layer was treated with ethereal CH_2N_2 for 1 hr. The reaction mixture was concd and applied to a column of silica gel. Elution with C_6H_6–Me_2CO (15:1) furnished (*S*)-dimethyl hexamethoxydiphenoate (**14**) (6.7 mg) as a colourless syrup, $[\alpha]_D^{27}$ −25.9° ($CHCl_3$; *c* 0.5), CD (MeOH; *c* 8.4×10^{-5}): $[\theta]_{227} + 7.57 \times 10^4$, $[\theta]_{251} - 5.70 \times 10^4$, $[\theta]_{311} + 9.5 \times 10^3$, and (*S*)-trimethyl octa-*O*-methyl tergallate (**15**) (10.8 mg) as a colourless syrup, $[\alpha]_D^{27}$ −19.8° ($CHCl_3$; *c* 1.0), EIMS *m/z*: 660 (M^+, 100), 450 (6.2), 449 (2.4), 330 (1/2M^+, 6.1), 239 (27). CD (MeOH; *c* 1.6×10^{-4}): $[\theta]_{228} + 4.51 \times 10^4$, $[\theta]_{255} - 4.68 \times 10^4$, $[\theta]_{315} + 7.81 \times 10^3$. ^1H NMR ($CDCl_3$): δ3.30, 3.60, 3.62, 3.63 (× 2), 3.72, 3.81, 3.88 (× 2), 3.91, 3.93 (each 3H, s, OMe), 7.18, 7.34, 7.39 (each 1H, s, arom.H).

Partial hydrolysis of **13**. A soln of **13** (2 mg) in H_2O (1 ml) was heated at 80° for 12 hr. After cooling, the reaction mixture was examined by TLC [silica gel: C_6H_6–HCOOEt–HCOOH (2:10:3)] and HPLC [mobile phase: MeCN–H_2O (50 mM H_3PO_4); a: (1:99) and b: (3:17)] to detect 2,3-(*S*)-hexahydroxydiphenoyl-D-glucose (**16**) (R_f 0.37, solv. a: R_t 5.30 and 6.50 min) and tergallic acid dilactone (**17**) (solv.b: R_t 15.0 min).

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