CAMELLIATANNIN D, A NEW INHIBITOR OF BONE RESORPTION, FROM *CAMELLIA JAPONICA*

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Camelliatannin D (**1**), a new complex tannin which inhibits Ca release from mouse calvaria, was isolated from the leaves and fruits of *Camellia japonica* L. (Theaceae). This tannin is the first example of complex tannin composed of a dimeric hydrolyzable tannin and a flavan-3-ol.

KEY WORDS camelliatannin D; bone resorption; osteoporosis; *Camellia japonica*; Theaceae; tannin

Since the imbalance of formation and resorption of bones causes osteoporosis, substances inhibiting resorption or stimulating bone formation are expected to be candidates as the remedy for osteoporosis. The screening of inhibitors against bone resorption among natural materials using cultured mouse calvaria[1] revealed that a new compound isolated from *Camellia japonica* has a potent inhibitory effect on Ca release from the calvaria. This compound has been found to be the first example of complex tannin[2] composed of a dimeric hydrolyzable tannin and a flavan-3-ol.

The new tannin, named camelliatannin D (**1**),[3] was obtained from the fresh leaves and fruits of *C. japonica*, together with several tannins reported previously.[4-6] The $[M+H]^+$ ion peak at *m/z* 1859 (positive-ion mode) and the $[M-H]^-$ ion peak at *m/z* 1857 (negative-ion mode) in the FAB-MS of **1** correspond to the molecular formula $C_{83}H_{62}O_{50}$. Although the ^{1}H-NMR spectrum of **1** is complicated by duplication of each signal, signals of fourteen aliphatic protons in the spectrum were assigned to those of an open-chain glucose (Glucose I in formula **1**) and a $^4C_1$ glucopyranose residue (Glucose II) (Table 1) with the aid of the ^{1}H *J*-resolved spectrum and the ^{1}H-^{1}H correlation spectroscopy (COSY), and the duplication was attributed to anomerization of glucose II (α-anomer:β-anomer=1: 2). The spectrum also indicated the presence of a flavan-3-ol residue in **1** (Table 1). The small coupling constant between H-2 and H-3 (< 2 Hz) is that of the 2,3-*cis* structure of epicatechin (**2**), and the chemical shifts (δ 5.71 and 5.70) of the A-ring proton are close to that of 6-substituted epicatechin in camelliatannin C (**3**) (δ 5.84),[6] rather than 8-substituted epicatechin in camelliatannin E (δ 6.03).[6]

The remaining nine pairs of aromatic singlets in the spectrum of **1** suggested the presence of a galloyl group [δ 7.02 (α-anomer); δ 6.97 (β-anomer); 2H in total], two hexahydroxydiphenoyl (HHDP) groups [δ 6.65, 6.63, 6.53 and 6.51 (α-anomer); δ 6.67, 6.62, 6.52 and 6.49 (β-anomer); 4H in total] and a valoneoyl group [δ 7.16, 6.70 and 6.38 (α-anomer); δ 7.05, 6.70 and 6.30 (β-anomer); 3H in total]. The presence of these acyl groups was substantiated by methanolysis of methylated **1**, which gave methyl tri-*O*-methylgallate (**4**), dimethyl hexamethoxydiphenate (**5**) and trimethyl octa-*O*-methyl-valonate (**6**) in a molar ratio of 1:2:1.[7]

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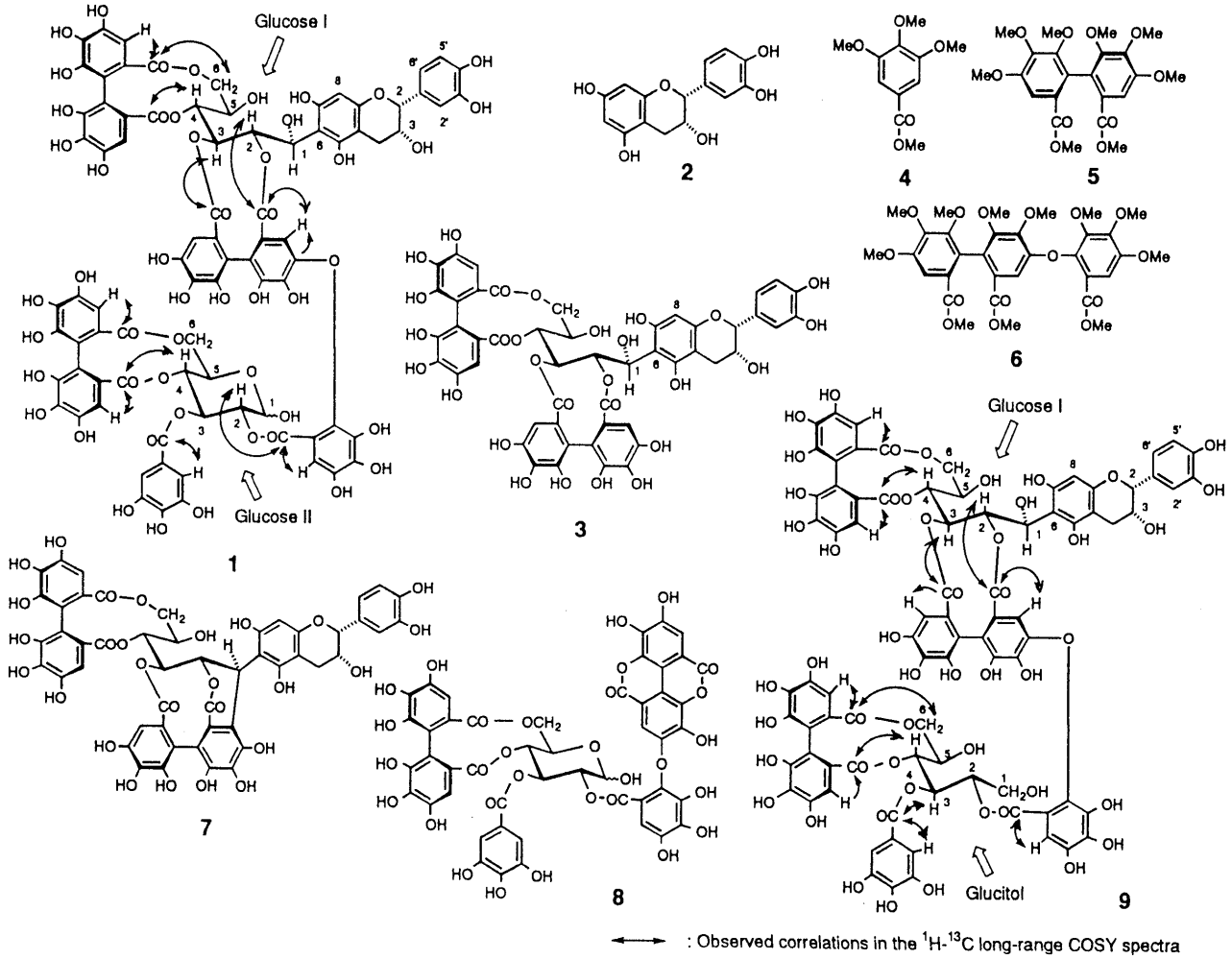
Table 1. ^{1}H-NMR Spectral Data for the Glucose (or Glucitol) and Flavan-3-ol Residues in **1** and **9** (500 MHz, in acetone-d_6 + D_2O)

		α-Anomer of **1**	β-Anomer of **1**	Compound **9**
Glucose I	H-1	5.67 (d, J=1.5 Hz)	5.65 (d, J=1.5 Hz)	5.61 (d, J=1.5 Hz)
	H-2	5.33 (dd, J=1.5, 9 Hz)	5.31 (dd, J=1.5, 9 Hz)	5.27 (dd, J=1.5, 9 Hz)
	H-3	5.82 (dd, J=2, 9 Hz)	5.83 (dd, J=2, 9 Hz)	5.79 (dd, J=2, 9 Hz)
	H-4	5.52 (dd, J=2, 8 Hz)	5.53 (dd, J=2, 8 Hz)	5.48 (dd, J=2, 8 Hz)
	H-5	4.33 (br d, J=8 Hz)	4.33 (br d, J=8 Hz)	4.30 (br d, J=8 Hz)
	H-6	4.58 (dd, J=2.5, 12 Hz)	4.55 (dd, J=2.5, 12 Hz)	4.56 (dd, J=2.5, 12 Hz)
		3.99 (dd, J=1, 12 Hz)	3.99 (dd, J=1, 12 Hz)	3.96 (dd, J=1, 12 Hz)
Glucose II (Glucitol)[a]	H-1	5.43 (d, J=3.5 Hz)	4.53 (d, J=8 Hz)	3.75 (dd, J=3.5, 12 Hz)
				3.72 (dd, J=5, 12 Hz)
	H-2	5.11 (dd, J=3.5, 10 Hz)	5.15 (dd, J=8, 10 Hz)	5.32 (ddd, J=3.5, 5, 8.5 Hz)
	H-3	5.80 (t, J=10 Hz)	5.58 (t, J=10 Hz)	5.84 (dd, J=2, 8.5 Hz)
	H-4	5.07 (t, J=10 Hz)	5.04 (t, J=10 Hz)	5.16 (dd, J=2, 8.5 Hz)
	H-5	4.61 (br dd, J=6.5, 10 Hz)	4.13 (br dd, J=6.5, 10 Hz)	4.08 (br dd, J=2.5, 8.5 Hz)
	H-6	5.22 (dd, J=6.5, 13 Hz)	5.26 (dd, J=6.5, 13 Hz)	4.64 (dd, J=2.5, 12 Hz)
		3.75 (br d, J=13 Hz)	3.88 (br d, J=13 Hz)	3.85 (br d, J=12 Hz)
Epicatechin	H-2	4.61 (br s)	4.61 (br s)	4.61 (br s)
	H-3	4.25 (m)	4.23 (m)	4.25 (m)
	H-4	2.56 (dd, J=2.5, 17 Hz)	2.56 (dd, J=2.5, 17 Hz)	2.56 (dd, J=2.5, 17 Hz)
		2.25 (dd, J=3.5, 17 Hz)	2.16 (dd, J=3.5, 17 Hz)	2.15 (dd, J=3.5, 17 Hz)
	H-8	5.71 (s)	5.70 (s)	5.71 (s)
	H-2'	6.97 (d, J=1.5 Hz)	6.97 (d, J=1.5 Hz)	6.97 (d, J=1.5 Hz)
	H-5'	6.75 (d, J=8.5 Hz)	6.74 (d, J=8.5 Hz)	6.75 (d, J=8.5 Hz)
	H-6'	6.78 (dd, J=1.5, 8.5 Hz)	6.78 (dd, J=1.5, 8.5 Hz)	6.78 (dd, J=1.5, 8.5 Hz)

a) Reduction of Glucose II in **1** with $NaBH_4$ gave the glucitol residue in **9**.



The chemical shifts and coupling constants of the glucose I protons are similar to those of **3**, suggesting further similarity in the substitution pattern on the glucose residues, and also in the configuration of glucose C-1. In fact, formation of **3**[8] and camelliatannin B (**7**)[4] was observed upon the treatment of an aqueous solution of **1** in a boiling-water bath for 4.7 h. Production of (-)-epicatechin (**2**) and cornusiin B (**8**)[9] were also observed upon the treatment. The two carboxyl groups of the biphenyl moiety of the valoneoyl group in **8** should thus be esterified with hydroxyl groups at C-2/C-3 or C-4/C-6 of glucose I in **1**. Locations of acyl groups on the two glucose cores were further evidenced by the ^{1}H-^{13}C long-range COSY spectrum of **1**. Observed correlations concerning the ester carbonyl carbons are shown in formula **1**, and the correlations for the valoneoyl group indicated that the HHDP moiety at O-2/O-3 of glucose I and the galloyl moiety at O-2 of glucose II form the valoneoyl group, and the orientation of the valoneoyl group is as shown in formula **1**. The locations of the remaining acyl groups were further confirmed by the ^{1}H-^{13}C long-range COSY spectrum of **9** (see the formula, ^{1}H-NMR data: Table 1), which was obtained by the treatment of **1** with $NaBH_4$.[10]

The CD spectrum of **1** showed a positive Cotton effect in the short wave-length region ($[\theta]_{234}$ +2.3 x 10^5), indicating[11] that the configuration of all of the biphenyl moieties of the valoneoyl and HHDP groups is *S*. Thus the structure of camelliatannin D was assigned to be **1**.

Camelliatannin D inhibited elevation of Ca concentration induced by PTHrp(1-34),[12] with the IC_{50} value of 2.4 x 10^{-7} M, while phenols of low molecular weight such as liquiritin showed generally weak inhibition (IC_{50} value of liquiritin, 2.5 x 10^{-5} M). Although the mechanism of inhibition is not yet clear, further investigation on the effects of various polyphenols on the PTHrp-induced Ca release is now in progress.

REFERENCES AND NOTES

1) The calvaria from ICR mouse of 4-6 days old were used for the experiment after pre-incubation for 24 h, and PTHrp(1-34), a synthetic peptide structurally related to parathyroid hormone, was added for induction of the Ca release from the calvaria. After incubation of the calvaria for 2 days in the presence of PTHrp and tested compound, Ca concentration was measured for the estimation of the inhibitory activity.
2) Okuda T., Yoshida T., Hatano T., "Economic and Medicinal Plant," Vol. 5, ed. by Wagner H. and Farnsworth N. R., Academic Press, New York, 1991, pp. 129-165.
3) An off-white powder, $[\alpha]_D$ +46° (*c*=0.9, MeOH). *Anal.* Calcd for $C_{83}H_{62}O_{50}\cdot 12H_2O$: C, 48.02; H, 4.15. Found: C, 48.10; H, 4.17. UV λ_{max} (MeOH) nm (log ε): 207 (5.32), 280 (sh., 4.83). IR ν_{max} (KBr) cm^{-1}: 1730 (ester carbonyl), 1620. CD (MeOH) [θ] x 10^{-4} (nm): 2.7 (285), -6.0 (262), +23.0 (234), -12.6 (206).
4) Hatano T., Shida S., Han L., Okuda T., *Chem. Pharm. Bull.*, **39**, 876-880 (1991).
5) Han L., Hatano T., Yoshida T., Okuda T., *Chem. Pharm. Bull.*, **42**, 1399-1409 (1994).
6) Hatano T., Han L., Taniguchi S., Shingu T., Okuda T., Yoshida T., *Chem. Pharm. Bull.*, **43**, 1629-1633 (1995).
7) The quantitation was performed by HPLC using a Sumipax Zorbax BP Sil column (4.6 mm i.d. x 250 mm; Sumika) and a mixture of *n*-hexane and EtOAc (2:1, v/v) at room temperature.
8) Cleavage of the ether linkage of valoneoyl group is often observed upon partial degradation of oligomeric hydrolyzable tannins. See, Yoshida T., Hatano T., Kuwajima T., Okuda T., *Heterocycles*, **33**, 463-482 (1992).
9) Hatano T., Ogawa N., Kira R., Yasuhara T., Okuda T., *Chem. Pharm. Bull.*, **37**, 2083-2090 (1989).
10) Hatano T., Yoshida, T., Okuda, T., *J. Chromatogr.*, **435**, 285-295 (1988).
11) Okuda T., Yoshida T., Hatano T., Koga T., Toh N., Kuriyama K., *Tetrahedron Lett.*, **23**, 3937-3940 (1982).
12) Ca concentrations with and without PTHrp were 13.56±0.36 mg/dl and 10.46±0.18 mg/dl, respectively. The values are expressed as the means±S.E. from 5 experiments.

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