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Camptothins A and B, New Dimeric Hydrolyzable Tannins from *Camptotheca acuminata* DECNE.

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Two new hydrolyzable tannins, named camptothins A (**1**) and B (**4**), have been isolated from the leaf of *Camptotheca acuminata* (Nyssaceae) along with cornusiin A (**2**), gemin D (**3**), tellimagrandin I (**5**), tellimagrandin II (**6**), 1,2,6-tri-*O*-galloyl-β-D-glucose (**7**) and 1,2,3,6-tetra-*O*-galloyl-β-D-glucose (**8**). Pedunculagin (**9**) was isolated from the fruit of *C. acuminata*. The dimeric structures of camptothins A and B have been established based on chemical and spectroscopic evidence.

Keywords——tannin; hydrolyzable tannin; ellagitannin; dimeric hydrolyzable tannin; *Camptotheca acuminata*; Nyssaceae; camptothin A; camptothin B; cornusiin A; centrifugal partition chromatography

Camptotheca acuminata DECNE. (Nyssaceae), has been used as a medicine in China.[1] Its fruit is used as a remedy for leukemia, and the leaf has been used to treat some skin diseases. Our preliminary research on tannins of medicinal plants using the *RMB* (relative affinity to methylene blue) determination[2] revealed that the leaf is rich in tannins (*RMB* value of the extract from the leaf: 0.20), and therefore we investigated the tannins of this plant.

Results and Discussion

A concentrated filtrate obtained from the aqueous acetone homogenate of fresh leaves of *C. acuminata* was extracted with ether, ethyl acetate and *n*-butanol, successively. A new dimeric hydrolyzable tannin, named camptothin A (**1**), was isolated from the aqueous mother liquor by centrifugal partition chromatography (CPC)[3] followed by column chromatography over Sephadex LH-20. Cornusiin A (**2**)[4] and gemin D (**3**)[5] were also isolated from the mother liquor. Another new tannin, named camptothin B (**4**), was isolated from the *n*-butanol extract using CPC followed by column chromatography over Toyopearl HW-40 and Sephadex LH-20. The ethyl acetate extract afforded tellimagrandin I (**5**)[6] and tellimagrandin II (**6**),[6,7] along with 1,2,6-tri-*O*-galloyl-β-D-glucose (**7**)[4] and 1,2,3,6-tetra-*O*-galloyl-β-D-glucose (**8**),[4] upon column chromatography over Sephadex LH-20.

Although the *RMB* value of the crude extract from the fruit was lower (0.08) than the value for the leaf, pedunculagin (**9**)[6] was isolated from the ethyl acetate extract of the fruit by column chromatography over Sephadex LH-20.

Camptothin A (**1**), $C_{61}H_{46}O_{40}\cdot 6H_2O$, $[\alpha]_D$ +46° ($c=1$, MeOH), was obtained as an off-white amorphous powder. The fast-atom bombardment mass spectrum (FAB-MS) of **1** shows the $[M+Na]^+$ ion at m/z 1441 and the $[M+K]^+$ ion at m/z 1457. Although the ^1H nuclear magnetic responance (^1H-NMR) spectrum (500 MHz, in acetone-d_6+D_2O) of camptothin A

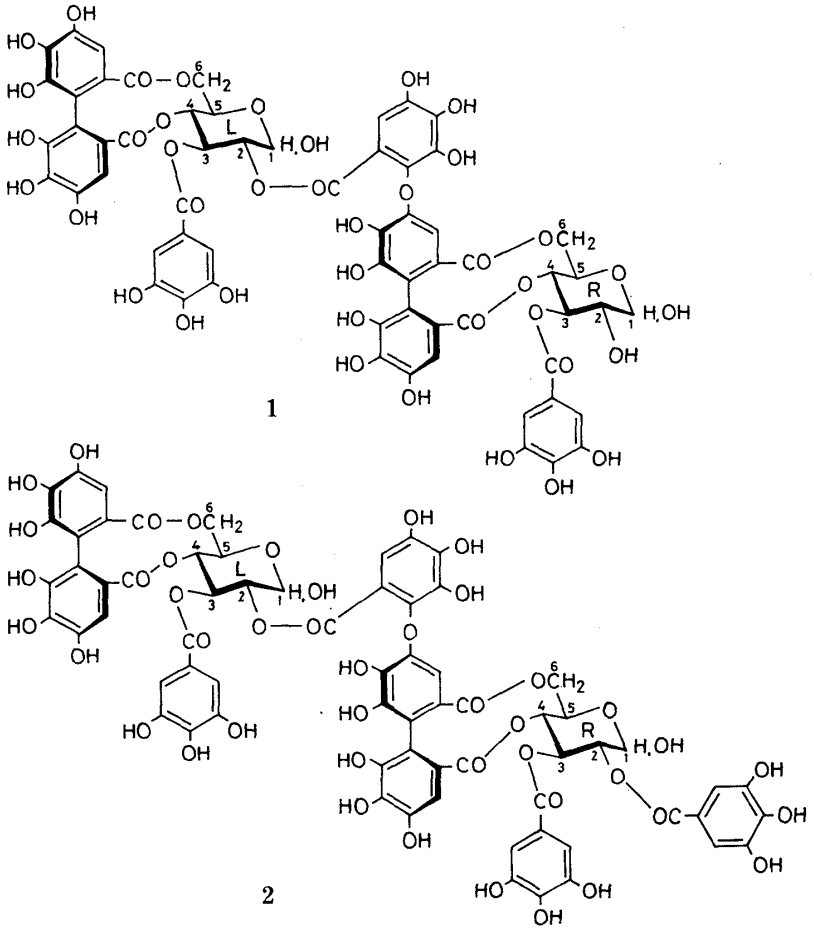


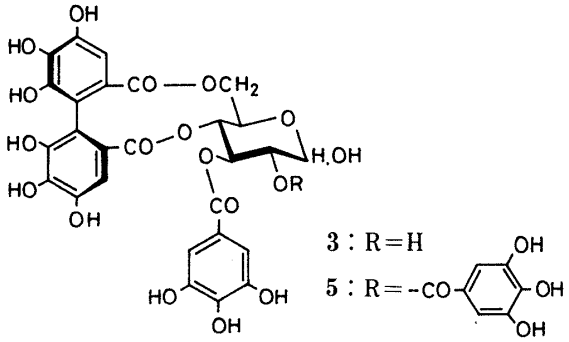
Chart 1

shows complicated peaks, the signals in the aromatic region indicate that this tannin has two galloyl groups [δ 7.00, 7.00, 6.96, 6.95 (each s, 2H in total), 6.93, 6.91, 6.91, 6.90 (each s, 2H in total)], a valoneoyl group and a hexahydroxydiphenoyl (HHDP) group [δ 7.08, 7.07, 7.02, 7.01 (each s, 1H in total), 6.65, 6.63, 6.63, 6.62 (each s, 1H in total), 6.62, 6.62, 6.61, 6.60 (each s, 1H in total), 6.50, 6.50, 6.47, 6.44 (each s, 1H in total), 6.23, 6.23, 6.20, 6.20 (each s, 1H in total)], and that the tannin exists as a mixture of four isomeric structures (in the ratio of 4 : 5 : 6 : 8), induced by anomerization at the two glucose cores, which are not acylated at O-1. Indeed, signals of the anomeric protons were not observed at lower field than 5.4 ppm.

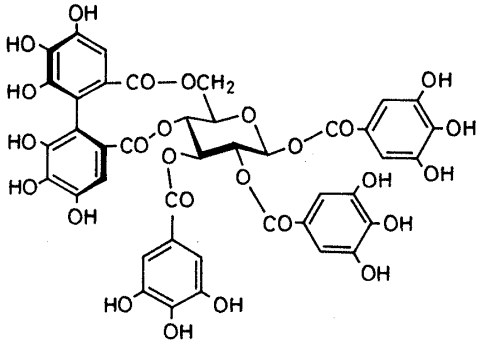
Enzymatic partial hydrolysis of cornusiin A (**2**), which also exists as an anomeric mixture of four forms,[4] afforded camptothin A. Therefore, camptothin A has a structure in which one of the three galloyl groups in **2** is lacking.

Hydrolysis of camptothin A in boiling water yielded cornusiin B (**10**),[4] gemin D (**3**)[5] and 3-*O*-galloyl-D-glucose (**11**),[4] which were identified by comparison with authentic samples, while analogous treatment of cornusiin A[4] gave 2,3-di-*O*-galloyl-D-glucose (**12**) together with **10** and **3** (Chart 4).

The H-1 signals of the two glucose cores forming the β-anomer were observed at δ 4.83, 4.69, 4.46 and 4.42 as doublets (J = 8 Hz) in the ^{1}H-NMR spectrum of camptothin A. The upfield shifts of two of them (δ 4.46 and 4.42) can be explained by the anisotropic effect of the HHDP part of the valoneoyl group (Chart 5) on the α-proton of the anomeric center of the β-anomer of the glucose core L (the left glucose core in formula **1** in Chart 1). The doublets at δ 4.83 and at δ 4.69 are therefore due to H-1 of the β-anomer of the glucose core R (the right glucose core in the formula **1** in Chart 1). Decoupling experiments on the H-1 signals revealed



3 : R=H
5 : R=-CO-



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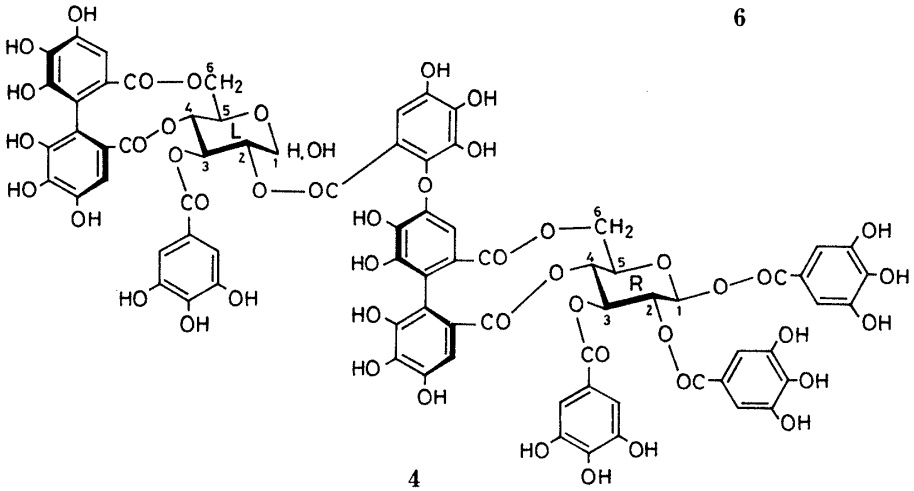

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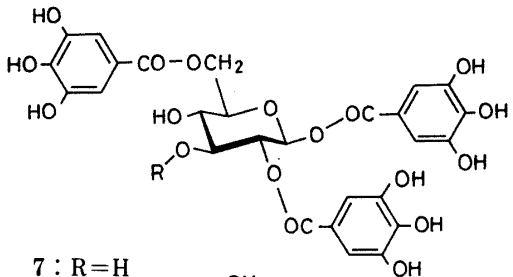
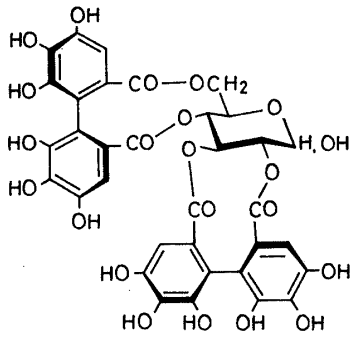
Chart 2



7 : R=H
8 : R=-CO-



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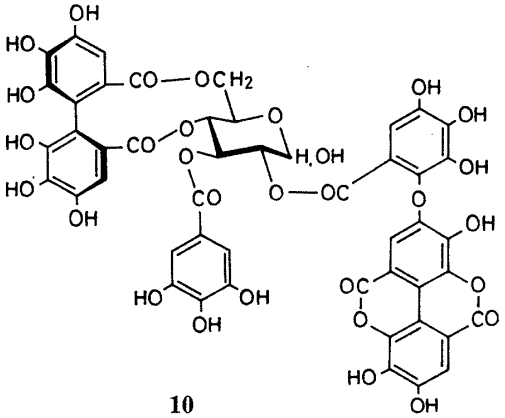

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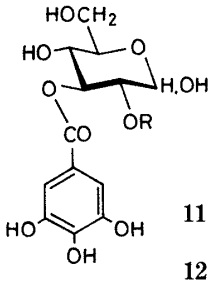

11 : R=H
12 : R=-CO-

Chart 3

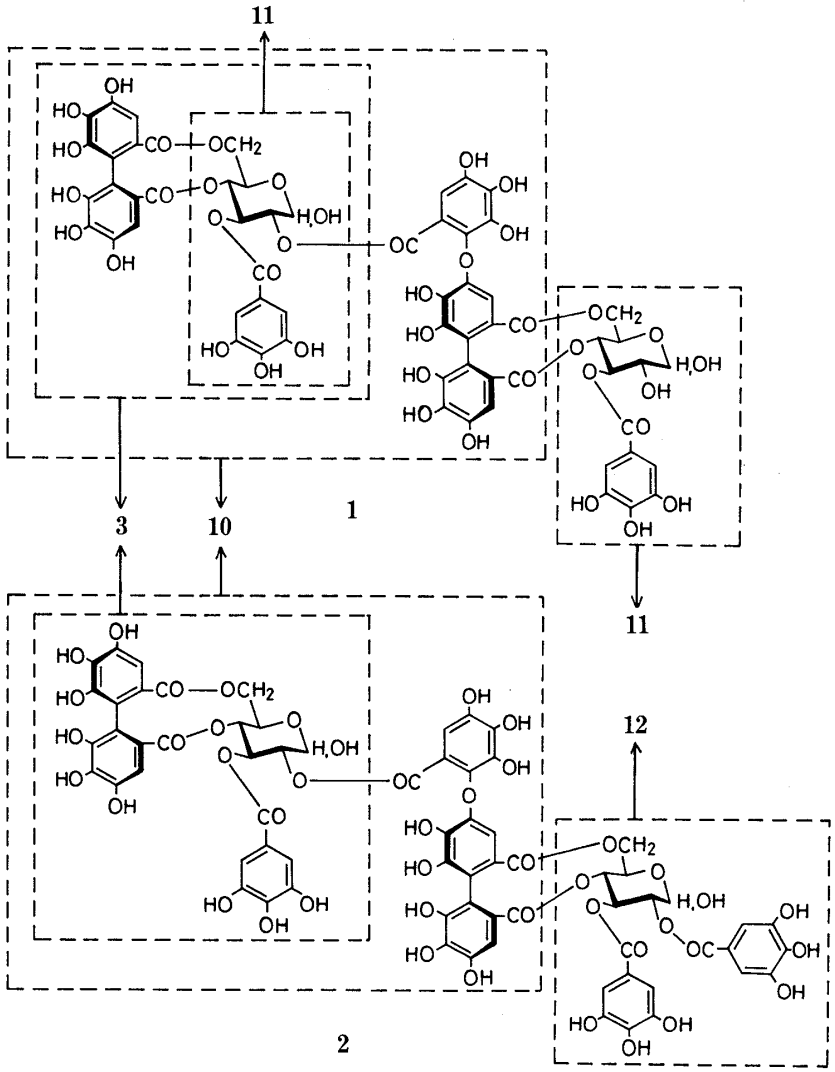

Chart 4

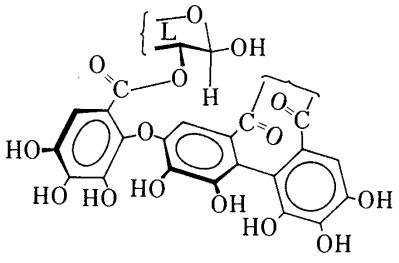

Chart 5. Stereostructure Inducing Anisotropic Effect on the Anomeric Proton of the Glucose Core L in Camptothin A (**1**)

that the H-2 signals of the β-anomer of the glucose core R are at high field [two double doublets ($J=8$, 10 Hz) at δ 3.56 and δ 3.51]. Therefore, the structure of camptothin A was established as formula **1**, in which the hydroxyl group at C-2 of the glucose core R is unacylated.

Camptothin B (**4**), $C_{75}H_{54}O_{48}\cdot 6H_2O$, $[\alpha]_D$ +48° ($c=0.5$, MeOH), was obtained as an off-white amorphous powder. The ^{1}H-NMR spectrum of camptothin B (400 MHz, in acetone-d_6+D_2O) indicates that this tannin exists as a mixture of two anomers in the ratio of 1 : 2, and that it has four galloyl groups [δ 7.13, 7.03, 7.02 and 6.88 (4/3H each, s, major form), 7.07, 7.05, 7.02 and 6.95 (2/3H each, s, minor form)], a valoneoyl group and an HHDP group [δ7.12, 6.70, 6.65, 6.55 and 6.21 (2/3H each, s, major form), 7.15, 6.69, 6.67, 6.55 and 6.23 (1/3H each, s, minor form)], and two glucopyranose cores in which one of the anomeric

centers is acylated [δ 6.23 (2/3H, d, J=8 Hz) and 6.18 (1/3H, d, J=8 Hz); H-1 of the acylated glucose core] and the other is unacylated [δ 5.41 (1/3H, d, J=3.5 Hz) and 4.53 (2/3H, d, J=8 Hz); H-1 of the unacylated glucose core]. The peak areas of the H-1 signals of the unacylated glucose cores indicate that the major form of captothin B is the β-anomer, and the minor one is the α-anomer.

Partial hydrolysis of camptothin B with tannase afforded cornusiin A (**2**). The structure of camptothin B therefore should have an additional galloyl group on one of the anomeric centers of cornusiin A. The chemical shift (δ 4.53) of H-1 of the unacylated glucose core of the β-anomer of camptothin B is identical with that of H-1 (δ 4.53) of the glucose core L (the left glucose core in formula **2** in Chart 1) of the β_L–β_R form (in which both of the glucose cores take β-form) of cornusiin A. This fact indicates that the unacylated glucose core of camptothin B is the glucose core L, and that the acylated glucose core is the glucose core R. Accordingly, the structure of camptothin B should be formulated as **4**.

Experimental

Optical rotations were measured on a JASCO DIP-4 polarimeter. Ultraviolet (UV) spectra were recorded on a Hitachi 200-10 spectrophotometer and infrared (IR) spectra on a JASCO A-102 spectrometer. FAB-MS were measured on a JEOL JMS D-300 spectrometer. ^{1}H-NMR spectra were measured on a Varian VXR-500 instrument (500 MHz) in the SC-NMR Laboratory of Okayama University, a Bruker AM-400 spectrometer (400 MHz) and a Hitachi R22-FTS spectrometer (90 MHz). Chemical shifts are given in δ values (ppm) from tetramethylsilane. CPC[3] was performed on a Sanki L-90 machine equipped with twelve cartridges, developing with *n*-butanol–*n*-propanol–water (4 : 1 : 5) at 1000 rpm. Thin layer chromatography (TLC) was performed on Avicel SF (Funakoshi) cellulose plates developing with 7% acetic acid. The spots were visualized by spraying the $FeCl_3$ reagent. Normal-phase high-performance liquid chromatography (HPLC) was run on a column (4 × 150 mm) packed with Develosil 60-5, developing with hexane–methanol–tetrahydrofuran–formic acid (55 : 33 : 11 : 1, v/v) containing oxalic acid (450 mg/l). An Atto SF-1205A detector (280 nm) was used and the flow rate was set at 2.5 ml/min. Reversed-phase HPLC was conducted on a YMC A312 (6 × 150 mm) column with 0.1 M H_3PO_4–0.1 M KH_2PO_4–ethanol–ethyl acetate (10 : 10 : 2 : 1, v/v) as a developer. A Shimadzu UVD-1 detector (254 nm) was used and the flow rate was set at 2.2 ml/min. A column oven was used (set at 40 °C) for the reversed-phase HPLC. Gas chromatography (GC) was performed on a Hitachi 163 gas chromatograph equipped with a glass column (3 mm × 1 m) packed with 3% OV-1 on Chromosorb W. The injection temperature and column temperature were 280 and 240 °C, respectively. The flow rate of N_2 was 50 ml/min. For GC-MS, a Shimadzu LKB-9000 instrument was used.

Isolation of Tannins from the Leaf and Fruit of *Camptotheca acuminata* ——Fresh leaves (1.75 kg) of *C. acuminata* were homogenized in 70% acetone, and the homogenate was filtered. After concentration, the resulting aqueous solution was extracted with ether, ethyl acetate and *n*-butanol, successively. The aqueous mother liquor was evaporated, and a portion (3 g) of the residue (131 g) was subjected to CPC (normal-phase development); 12-g portions of the eluate were collected. Further purification of combined fractions 46—55 (36 mg) by column chromatography over Sephadex LH-20 with 70% ethanol gave camptothin A (**1**, 8 mg). Another portion (4 g) of the residue from the aqueous solution was subjected to chromatography over Amberlite XAD-2. Elution was carried out with water and then with methanol, and the residue (1.5 g) obtained by removal of the solvent from the methanol eluate was further chromatographed over Sephadex LH-20 with 70% ethanol to give gemin D (**3**)[5] (55 mg) and cornusiin A (**2**)[4] (124 mg). A portion (18 g) of the *n*-butanol extract (77 g) was subjected to chromatography over Toyopearl HW-40 (coarse grade); elution was done with 70% ethanol and then 70% acetone. The solvent of the 70% acetone eluate was removed, and the residue (3 g) was subjected to CPC (reversed-phase development). Combined fractions 12—34 (5-g fractions) afforded cornusiin A (1.2 g). Camptothin B (**4**, 37 mg) was isolated from combined fractions 40—65 (0.24 g) by further purification on a Sephadex LH-20 column. A part (2 g) of the ethyl acetate extract (10 g) was subjected to column chromatography over Sephadex LH-20 (2.2 × 34 cm). Elution was carried out with ethanol (for fractions 1—420) and then 70% ethanol (for fractions 421—500), and 500-drop fractions were collected. Upon rechromatography of the combined fractions 76—120 over Sephadex LH-20, 1,2,6-tri-*O*-galloyl-β-D-glucose (**7**)[4] (99 mg) was obtained. 1,2,3,6-Tetra-*O*-galloyl-β-D-glucose (**8**)[4] (39 mg, from fractions 170—200), tellimagrandin I (**5**)[6] (217 mg, from fractions 230—300) and tellimagrandin II (**6**)[6,7] (69 mg, from fractions 455—470) were also isolated in analogous ways.

The fresh fruit (1.3 kg) was homogenized in 70% acetone, and the homogenate was filtered. After concentration, the resulting precipitate was filtered off, and the filtrate was extracted with benzene and then with ethyl acetate. A portion (2 g) of the ethyl acetate extract (5.9 g) was subjected to column chromatography over Sephadex LH-20 (2.2 × 32 cm) with 70% ethanol as a developer; 10-g fractions were collected. After removal of the solvent from the combined fractions 81—97, the residue was dissolved in water and treated with a SEP-PAK C_{18} cartridge (Waters)

using water and then methanol as eluants. The methanol eluate afforded pedunculagin (**9**)[6] (50 mg).

Camptothin A (1)——An off-white amorphous powder, $[\alpha]_D$ +46° (c=1, MeOH). TLC, Rf 0.51. *Anal.* Calcd for $C_{61}H_{46}O_{40}\cdot 6H_2O$: C, 47.98; H, 3.83. Found: C, 47.80; H, 3.97. UV λ_{max}^{MeOH} nm (log ε): 223 (4.94), 267 (4.73). IR ν_{max}^{KBr} cm^{-1}: 1725 (ester carbonyl), 1620. FAB-MS m/z: 1441 ([M+Na]$^+$), 1457 ([M+K]$^+$). ^{1}H-NMR (500 MHz, in acetone-d_6 + D_2O) δ: 7.00, 7.00, 6.96, 6.95 (each s, 2H in total), 6.93, 6.91, 6.91, 6.90 (each s, 2H in total) (2 × galloyl); 7.08, 7.07, 7.02, 7.01 (each s, 1H in total), 6.65, 6.63, 6.63, 6.62 (each s, 1H in total), 6.62, 6.62, 6.61, 6.60 (each s, 1H in total), 6.50, 6.50, 6.47, 6.44 (each s, 1H in total), 6.23, 6.23, 6.20, 6.20 (each s, 1H in total) (valoneoyl and HHDP); 5.77, 5.76 [each t, J=10 Hz, H-3$_L$ (glucose core L) signals of α_L–α_R form (α-form of glucose core L and α-form of glucose core R) and α_L–β_R form]; 5.44, 5.40 (each t, J=10 Hz, H-3$_L$ signals of β_L–α_R form and β_L–β_R form); 5.37—5.04 (complicated peaks); 5.06, 5.06, 5.01, 5.01 (each t, J=10 Hz, H-4$_L$ signals of the four forms); 4.90, 4.86, 4.85, 4.83 (each t, J=10 Hz, H-4$_R$ signals of the four forms); 4.83, 4.69 (each d, J=8 Hz, H-1$_R$ signals of β_L–β_R form and α_L–β_R form); 4.71, 4.57, 4.56, 4.48, 4.37, 4.19, 4.16, 4.03 (each dd, J=6, 10 Hz, H-5$_L$ and H-5$_R$ signals of the four forms); 4.46, 4.42 (each d, J=8 Hz, H-1$_L$ signals of β_L–α_R form and β_L–β_R form); 3.83—3.65 (complicated peaks); 3.56, 3.51 (each dd, J=8, 10 Hz, H-2$_R$ signals of β_L–β_R form and α_L–β_R form).

Enzymatic Transformation of Cornusiin A (2) into Camptothin A (1)——A tannase solution was added to an aqueous solution (30 ml) of cornusiin A (120 mg), and the mixture was kept at 37 °C for 43 h. The solvent was evaporated off, and the residue was subjected to column chromatography over Sephadex LH-20 (1.1 × 44 cm) with 70% ethanol as a developer to afford camptothin A (44 mg), which was identified by comparisons of the ^{1}H-NMR spectrum and $[\alpha]_D$ with those of an authentic sample.

Partial Hydrolysis of Camptothin A in Hot Water——1) An aqueous solution (1 ml) of camptothin A (1 mg) in a sealed tube was kept in a boiling water-bath for 17 h. The solvent was removed, and the residue was analyzed by normal-phase HPLC (N) and reversed-phase HPLC (R). The peaks of cornusiin B (**10**) [retention time (t_R): 3.61 min (N); 4.09 and5.24 min[8] (R)], gemin D (**3**) [t_R: 2.81 min (N); 1.69 and 1.87 min (R)] and 3-*O*-galloyl-D-glucose (**11**) [t_R: 2.06 min (N); 1.37 min (R)] were identified by co-chromatography with authentic samples.

2) An aqueous solution (1 ml) of camptothin A (10 mg) was treated in a boiling water-bath for 60 h. The solvent was removed, and the residue was subjected to chromatography over Sephadex LH-20 (1.1 × 40 cm) with 70% ethanol as an eluant; 100-drop fractions were collected. 3-*O*-Galloyl-D-glucose in combined fractions 19—26 was identified by GC (t_R: 4.2 and 5.6 min) after trimethylsilylation. The identity of the two trimethylsilyl derivatives (α-anomer and β-anomer) was further verified by GC-MS [m/z 836 (M$^+$)].

Camptothin B (4)——An off-white amorphous powder, $[\alpha]_D$ +48° (c=0.5, MeOH). TLC, Rf 0.42. *Anal.* Calcd for $C_{75}H_{54}O_{48}\cdot 6H_2O$: C, 49.19; H, 3.63. Found: C, 49.06; H, 3.90. UV λ_{max}^{MeOH} nm (log ε): 218 (5.11), 277 (4.80). IR ν_{max}^{KBr} cm^{-1}: 1725 (ester carbonyl), 1620. ^{1}H-NMR (400 MHz, in acetone-d_6+D_2O) δ: 7.13, 7.03, 7.02, 6.88 (4/3H each, s, galloyl, β-form); 7.07, 7.05, 7.02, 6.95 (2/3H each, s, galloyl, α-form); 7.12, 6.70, 6.65, 6.55, 6.21 (2/3H each, s, valoneoyl and HHDP, β-form); 7.15, 6.69, 6.67, 6.55, 6.23 (1/3H each, s, valoneoyl and HHDP, α-form); 6.23 (2/3H, d, J=8 Hz, H-1$_R$, β-form); 6.18 (1/3H, d, J=8 Hz, H-1$_R$, α-form); 5.85 (1/3H, d, J=10 Hz, H-3$_L$, α-form); 5.70—5.45 (complicated peaks); 5.41 (1/3H, d, J=3.5 Hz, H-1$_L$, α-form); 5.30—5.02 (complicated peaks); 4.66 (1/3H, dd, J=6, 10 Hz, H-5$_R$, α-form); 4.62 (2/3H, dd, J=6, 10 Hz, H-5$_R$, β-form), 4.53 (2/3H, d, J=8 Hz, H-1$_L$, β-form), 4.50 (1/3H, dd, J=6, 10 Hz, H-5$_L$, α-form), 4.18 (2/3H, dd, J=6, 10 Hz, H-5$_L$, β-form). The H-6 signals at δ 4.0—3.8 overlap the signal of water.

Enzymatic Partial Hydrolysis of Camptothin B (4)——Camptothin B (10 mg) in aqueous solution was treated with tannase at 37 °C for 30 min. Then the solution was acidified to pH 2 with 1% H_2SO_4 and subjected to SEP-PAK C_{18} cartridge treatment with water and then methanol as eluants. The methanol eluate afforded cornusiin A (**2**, 8 mg), which was identified by comparisons of the ^{1}H-NMR spectrum and $[\alpha]_D$ with those of an authentic sample.

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