Accumulation of Epigallocatechin Quinone Dimers during Tea Fermentation and Formation of Theasinensins

Takashi Tanaka,[†] Chie Mine,[†] Sayaka Watarumi,[†] Toshihiro Fujioka,[‡] Kunihide Mihashi,[‡] Ying-Jun Zhang,[†] and Isao Kouno*,[†]

Department of Molecular Medicinal Sciences, Graduate School of Biomedical Sciences, Nagasaki University, Bunkyo Machi 1-14, Nagasaki 852-8521, Japan, and Faculty of Pharmaceutical Sciences, Fukuoka University, 8-19-1 Nanakuma, Jonan-ku, Fukuoka 814-0180, Japan

Production and accumulation of catechin dimer quinones during tea fermentation were chemically confirmed for the first time by trapping as phenazine derivatives. Direct treatment of the fermented tea leaves with *o*-phenylenediamine yielded five phenazine derivatives (**8**–**12**) of *o*-quinones of an epigallo-catechin dimer and its galloyl esters (**13**–**16**), in which two flavan units were linked at the B-rings through a C–C bond. Atrop isomerism of the biphenyl bonds was shown to be the *R* configuration, suggesting that the *o*-quinone dimers were generated by stereoselective coupling of monomeric quinones. The total concentration of the phenazine derivatives in the *o*-phenylenediamine-treated tea leaves was higher than that of theaflavins. In contrast, phenazine derivatives of monomeric quinones of epigallocatechin were not isolated. When the fermented tea leaves were heated, the quinone dimers were converted to theasinensins, which are constituents of black tea, suggesting that theasinensins are generated by reduction of the quinone dimers during the heating and drying steps in black tea manufacturing.

Polyphenol oxidation during black tea manufacturing remains unclear despite many studies by a number of groups. When fresh tea leaves [*Camellia sinensis* (L.) O. Kuntze, Theaceae] are crushed at the initial stage of black tea manufacturing, four major catechins in the leaves, (−)-epicatechin (**1**), (−)-epigallocatechin (**2**), and their galloyl esters (**3** and **4**), are enzymatically oxidized, and the resulting quinones undergo complex chemical changes.[1] Theaflavins (**5**),[2–4] reddish-orange pigments of black tea, are catechin dimers having a characteristic benzotropolone moiety produced by condensation of a pair of quinones (**1a**–**4a**) derived from dihydroxy and trihydroxy B-rings of catechins (**1**–**4**) (Figure 1). In contrast, the remaining components of the color of black tea infusions, the so-called thearubigins,[1,5] are a heterogeneous mixture of compounds with large molecular size. Although they may be partly produced by further oxidation of theaflavins,[6,7] most of the chemical studies up to the present have presumed that these metabolites are oligomers and polymers of the quinones.[1] In the late 1950s, Roberts, who designated the major black tea pigments, pointed out that **2** and its gallate are important as the principal substrates in the oxidation during tea fermentation,[8] because they comprise about 70% of the green tea catechins and their trihydroxy benzene rings have the lowest oxidation–reduction potential among oxidizable polyphenols in tea leaves.[9] Furthermore, besides production of theaflavins, he postulated a mechanism by which **2** or **4** is oxidatively coupled to form dimeric quinone intermediates (**6**), and further polymerization beyond the dimer stage appears unlikely.[8,10] Following that, a few short reports supporting his hypothesis appeared within several years.[11,12] However, later studies on structural elucidation of theaflavins in the early 1960s overshadowed his pioneering work.[2–4]

In the course of a chemical study on the oxidation of catechins in plants,[6,7,13,14] we have recently succeeded in isolating the metabolite **7** (Figure 2), corresponding to a hydrated form of the Roberts' quinone (**6**).[14] Although the yield of **7** was significantly lower than that of theaflavins, we believed that it might represent only a part of unstable quinone metabolites generated during catechin oxidation, based on our previous findings which suggested the presence of various unknown quinones in the fermentation mixture.[6,7] Since formation of *o*-quinones at the initial stage of the oxidation is generally accepted, we attempted to trap the unstable quinones by addition of *o*-phenylenediamine, which condenses with *o*-quinones to form stable phenazine derivatives.

Results and Discussion

Fresh tea leaves were crushed and spread on a glass tray in a thin layer at 25 °C. After 2.5 h, an aliquot of the fermented leaves was extracted with EtOH and another aliquot was extracted with EtOH containing 1% acetic acid and 0.2% *o*-phenylenediamine, the quinone-trapping reagent,[15] and the extracts were separately analyzed by reversed-phase HPLC (Figure 3). The chromatogram of the ethanol extract (Figure 3a) showed peaks arising from caffeine, catechins (**1**–**4**), flavonol glycosides, and theaflavins. In contrast, in the chromatogram of the extract treated with *o*-phenylenediamine (Figure 3b), peaks due to many additional products appeared. Photodiode array detection indicated that the major products (**8**–**12**) showed similar UV spectra with absorption maxima at 376 nm.

Follow-up experiments with larger amounts of the leaves led to the isolation of the major products. The 80% acetone extract of the *o*-phenylenediamine-treated leaves was concentrated and partitioned between H_2O and ethyl acetate. The ethyl acetate layer was subjected to a combination of column chromatography over MCI-gel CHP20P, Sephadex LH-20, Chromatorex ODS, and Toyopearl HW-40F to give four compounds (**8**–**11**). We failed to isolate another product **12**; however, the products **8** and **12** were easily obtained, along with theaflavin, by oxidation of a mixture of **1** and **2** with banana homogenate,[6,14] followed by treatment with *o*-phenylenediamine. Banana homoge-
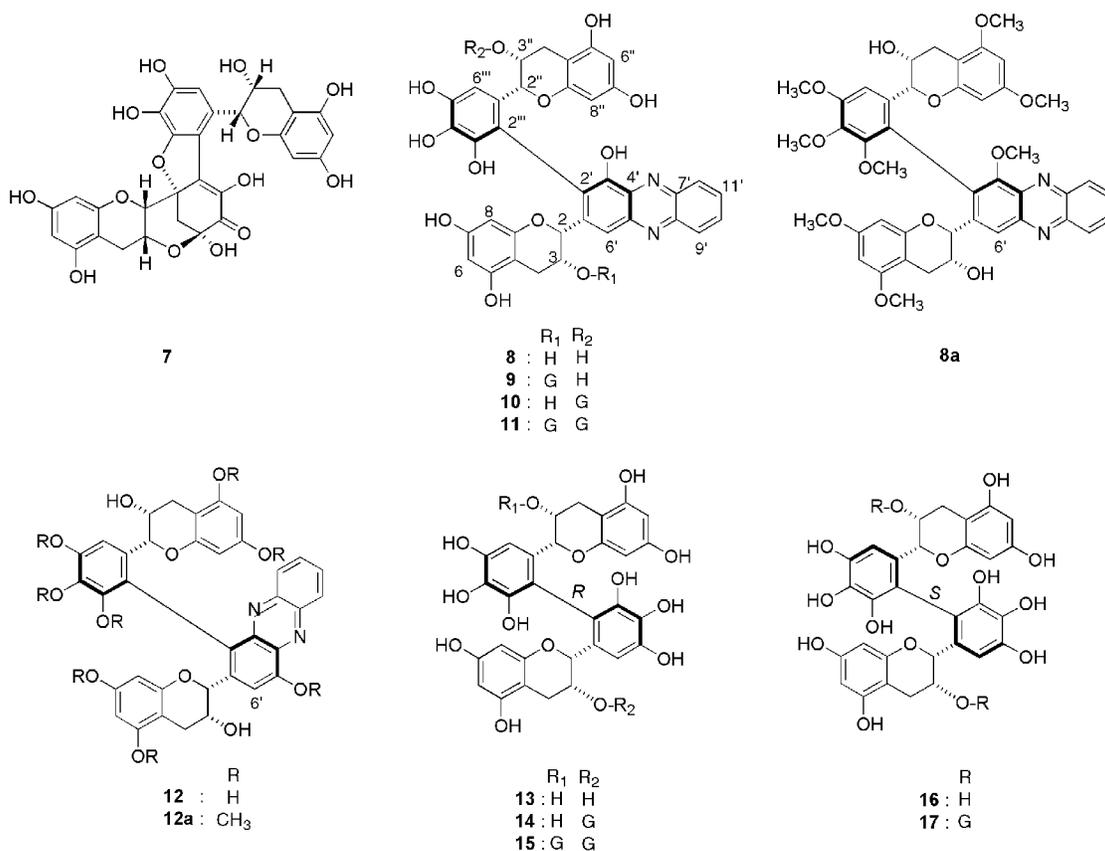
* Corresponding author. Tel: +81-95-847-1111. Fax: +81-95-848-4387. E-mail: ikouno@net.nagasaki-u.ac.jp.
† Nagasaki University.
‡ Fukuoka University.
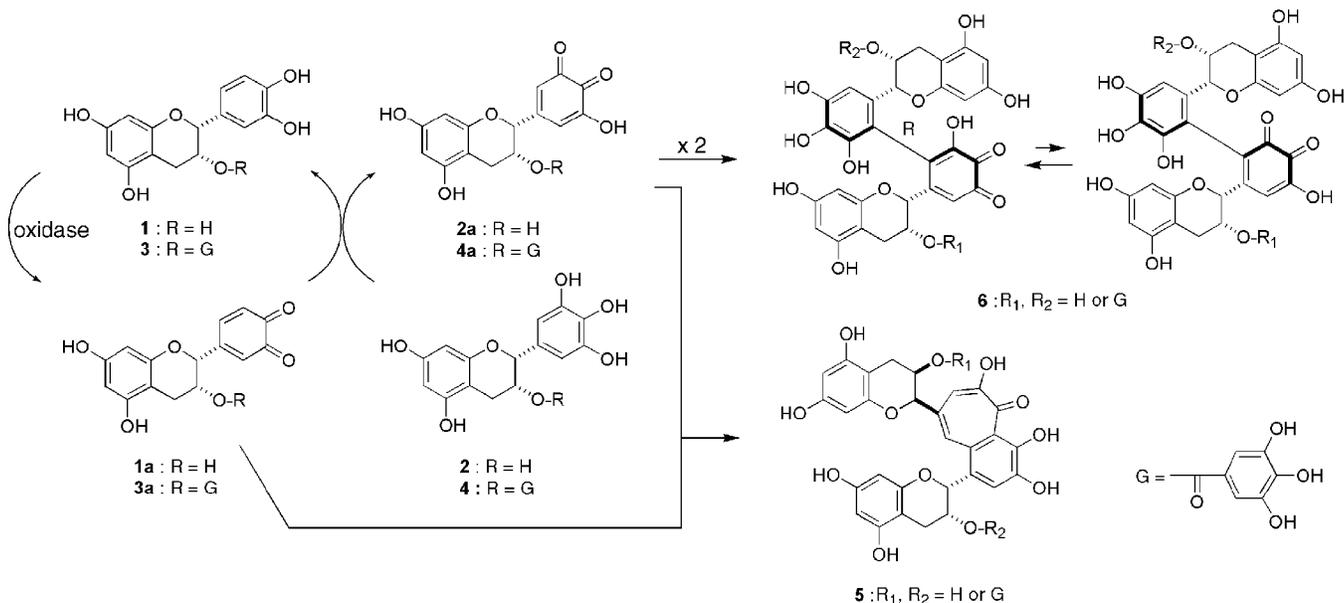
Figure 1. Proposed mechanism for formation of theaflavins and quinone dimers.


Figure 2. Structures of epigallocatechin oxidation products and phenazine derivatives.

nate was used because it efficiently catalyzes the oxidation of **1** and **2** to theaflavin without production of interfering minor products.[14]

The ^{1}H NMR spectra of **8**–**11** were related to each other, showing two sets of signals arising from A- and C-rings of 2,3-*cis*-flavan-3-ols similar to those of **1**–**4**. In addition, the [M + H][+] peak in the FABMS (*m/z* 681 for **8**, *m/z* 833 for **9** and **10**, *m/z* 985 for **11**) indicated that each of these products contains two flavan-3-ol units. The difference of their molecular weights (152 mass units) coincided with the mass of a galloyl group. The presence of the galloyl groups in **9**–**11** was also shown by the appearance of two-

proton singlet signals in the ^{1}H NMR spectra (δ 7.06 for **9**, δ 7.07 for **10**, δ 7.12 and 7.06 for **11**). Hydrolysis of the galloyl groups of **9**–**11** by treatment with tannase yielded **8** and gallic acid, confirming that the products **9**–**11** were galloyl esters of **8**. In the HMBC spectrum of **10** (Figure 4), two one-proton singlets at δ 8.24 and 7.12 were attributable to the B-ring protons H-6′ and H-6‴, respectively, because of their long-range coupling with the C-2 and C-2″ carbons. The correlations between the H-6‴, H-2″, H-3″, and galloyl protons indicated the presence of a 3-*O*-galloyl epigallocatechin unit similar to that of theasinensin A (**15**).[16] The ^{13}C NMR chemical shifts of the B-ring (C-
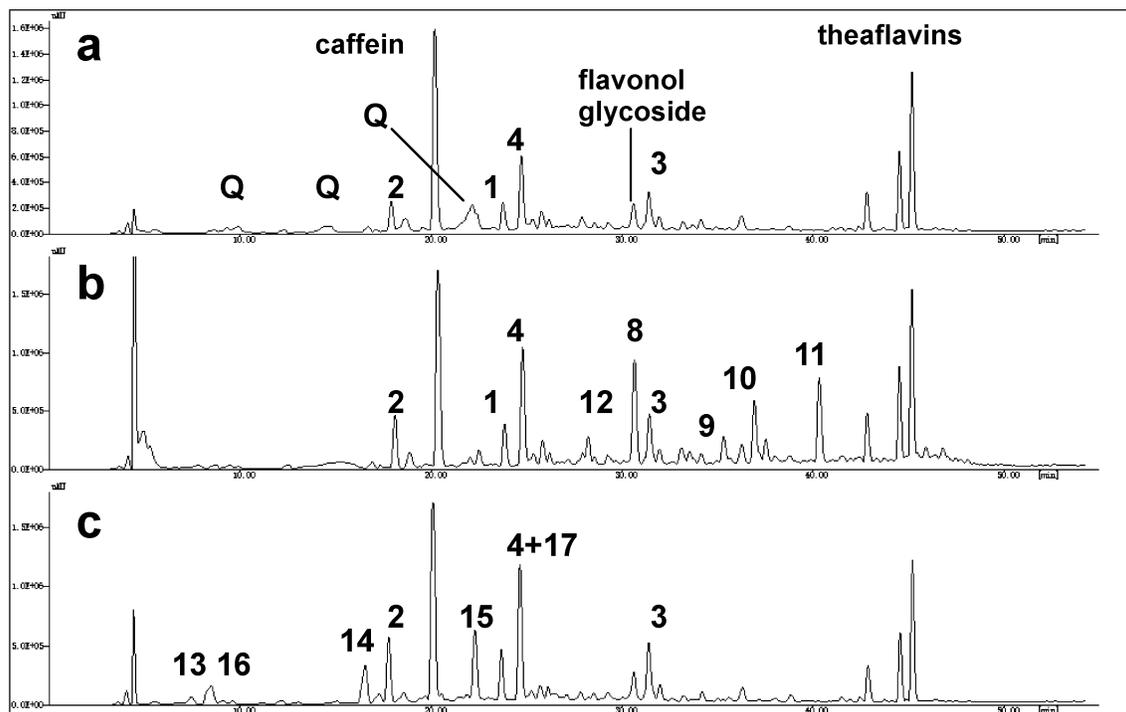
Figure 3. HPLC profiles of fermented tea leaves (UV max. absorbance obtained by photodiodearray detector). (a) Chromatogram of the ethanol extract of fermented tea leaves. (b) Chromatogram of the ethanol extract of fermented tea leaves treated with *o*-phenylenediamine. The reagent was detected as a large peak at the solvent front. (c) Chromatogram of the ethanol extract of fermented tea leaves heated at 90 °C for 10 min.
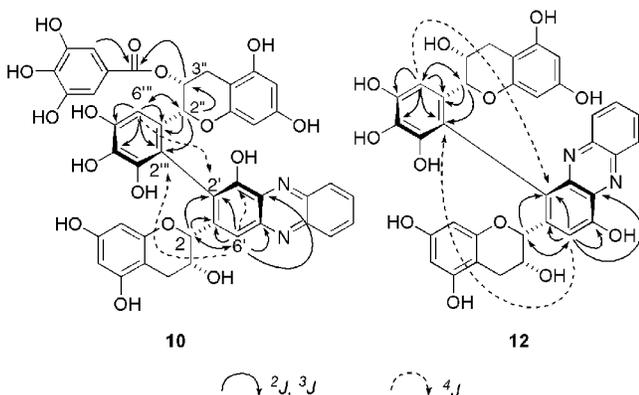
Figure 4. Important HMBC correlations of **10** and **12**.

$1''' - C-6'''$) were also consistent with those of **15**. The other B-ring proton H-6' was correlated with six aromatic carbon signals at δ 135.6 (C-4'), 116.8 (C-2'), 143.8 (C-1'), 144.2 (C-5'), 151.7 (C-3'), and 112.4 (C-2''') through ^2J, ^3J, and ^4J couplings. Since the presence of a *o*-phenylenediamine unit was apparent from the aromatic multiplets at δ 7.94 (2H, m) and 8.25 (2H, m), the aromatic ring of C-1'−C-6' and the phenylenediamine unit should form a hydroxyphenazine moiety.[15] The structure of this hydroxyphenazine moiety was confirmed as follows: compound **8** was methylated with CH_2N_2 to give octamethyl ether **8a**, and the differential NOE experiment of **8a** showed no NOE between H-6' and any methoxyl protons. Therefore, the position of the hydroxyl group was concluded to be at C-3'. On the basis of these spectroscopic findings, the structures of **8** and **10** were determined to be as shown in Figure 2. The ¹H NMR spectrum of **9** showed the low-field shift of the H-3 [δ 5.49 (br d, $J = 4.4$ Hz)] instead of H-3'' as in **10**. The location of the galloyl groups in **11** was determined to be at the C-3 and C-3'' positions because of the low-field shifts of H-3 and H-3'' (δ 5.52 and 5.44, respectively).

Hence, **9** and **10** were concluded to be 3-*O*-gallate and the 3,3''-di-*O*-gallate of **8**, respectively.

The molecular weight of **12** was the same as that of **8** by FABMS, and the ¹H and ¹³C NMR spectral comparison indicated that these products were closely related to each other. However, the chemical shifts of H-6' (δ 7.81), H-3'' (δ 3.71), and H-4'' (δ 2.32 and 1.89) of **12** were observed at higher field compared to those of **8** [δ 8.21 (H-6'), 4.10 (H-3''), 2.53 and 2.11 (H-4'')]. The HMBC spectrum of **12** (Figure 4) revealed that this product is a positional isomer of **8** by the appearance of a strong ^2J correlation between H-6' (δ 7.81) and a hydroxyl-bearing carbon (δ 152.2, C-5') and the absence of correlation between H-6' and C-3' (δ 144.4). The location of the hydroxyl group on the hydroxyphenazine moiety was further confirmed by a differential NOE experiment of the octamethyl ether **12a**, which showed a strong NOE between H-6' (δ 7.64) and a methoxyl proton (δ 4.26). The high-field shifts of H-3'' and H-4'' of **12** could be explained by the anisotropic effect of the phenazine ring, which hung over these protons. Therefore, the structure of **12** was determined to be as shown in Figure 2.

The configuration of the biphenyl bond of **8**−**12** was shown to be *R* by comparison of the CD spectrum of **8** and **12** with those of theasinensin C (**13**), having an *R* biphenyl bond, and theasinensin E (**16**), having an *S* biphenyl bond.[17,18] The CD spectra of **8** and **12** were very similar to each other and showed positive Cotton effects (CE) at 244 and 242 nm and negative ones at 211 and 216 nm, respectively. These Cotton effects were similar to those of **13** (positive CE at 240 nm and negative CE at 219 nm) and different from those of **16** (negative CE at 235 nm and positive CE at 227 nm). This result indicated that dimerization of epigallocatechin quinones (**2a** and **4a**) was highly stereoselective. It was noteworthy that the hydrated quinone dimer **7**, previously obtained from our model fermentation experiments as a minor metabolite,[14] was derived from

the quinone with opposite configuration, which corresponded to a quinone of theasinensin E (**16**).

Our results clearly confirmed that the quinone dimer (**6**) derived from **2** and **4** is present in fairly high concentrations in tea leaves during fermentation as predicted by Roberts in 1957.[10] In the case of the experiment shown in Figure 3b, the total concentration of these phenazine derivatives was estimated to be about 6.4 μmol/g of fresh leaves, which is higher than that of theaflavins (about 5.4 μmol/g). Although we have previously confirmed the generation of the monomeric quinones (**1a** and **2a**) by reaction with gluthatione, no phenazine derivatives of monomeric quinones were isolated in this experiment. The reason may be that the rate of the dimerization reaction was too fast to trap the monomeric quinones with *o*-phenylenediamine. The quinone dimer (**6**) was also generated from **2** alone on treatment with banana homogenate.[13] However, the reaction was accelerated in the presence of **1**, because **1** is oxidized by enzymes much faster than **2** and the resulting quinone **1a** readily oxidizes **2** to the quinone **2a**.[9,14] It is known that theaflavins can be chemically synthesized from **1** and **2** by treatment with potassium ferricyanide.[2] Interestingly, addition of *o*-phenylenediamine to the reaction mixture yielded **8** together with a small amount of **12**. These results indicated that stereoselective dimerization of the quinone **2a** is a nonenzymatic process.

The HPLC analysis of the fermented tea leaves showed broad peaks attributable to the Roberts' quinones (peaks Q in Figure 3a), which disappeared after treatment with *o*-phenylenediamine (Figure 3b). An attempt to isolate the quinones from the fermented tea leaves by the usual column chromatography was not successful because the quinones disappeared during separation, and theasinensins were isolated instead, which were not detected originally before the separation. The result suggested that theasinensins were produced by reduction of these unstable quinone dimers. In the fermented tea leaves, the quinone dimers were also decomposed on heating (90 °C, 10 min) to give theasinensins as shown in Figure 3c, and the phenazine derivatives were no longer generated from the fermented leaves after heating. This result explained why the commercial black tea did not produce the phenazine derivatives on treatment with *o*-phenylenediamine. The Roberts' quinones were probably converted to theasinensins in the later stage of black tea manufacturing, especially at the stage of heating and drying, where enzymes were inactivated. The oxidation products, which should be formed concomitantly with the reduction of the quinones to theasinensins, could not be isolated at this stage. A dismutation between two molecules of the quinone intermediates was possible because the intermediates could function as suitable hydrogen donors and acceptors.[5,8] Reaction with coexisting substances, including other polyphenols, might occur. Our results suggested that the mechanism of theasinensin production in fermented tea leaves was different from that of radical oxidation of epigallocatechin and its gallate.[19]

Experimental Section

General Procedures. UV spectra were obtained with a JASCO V-560 UV/vis spectrophotometer. Optical rotations were measured with a JASCO DIP-370 digital polarimeter. ^{1}H and ^{13}C NMR spectra were measured in a mixture of acetone-d_6 and D_2O (19:1, v/v) with a Varian Gemini 300 (300 MHz for ^{1}H and 75 MHz for ^{13}C) or a Varian Unity plus 500 (500 MHz for ^{1}H and 125 MHz for ^{13}C) spectrometer. The ^{1}H–^{1}H COSY, HSQC, and HMBC spectra were recorded at 27 °C with a Varian Unity plus 500 spectrometer. Coupling constants

are expressed in Hz, and chemical shifts are given on a δ (ppm) scale with TMS as an internal standard. MS were recorded on a JEOL JMS DX-303 spectrometer, and glycerol or *m*-nitrobenzyl alcohol was used as a matrix for FABMS measurement. Column chromatography was performed with MCI-gel CHP 20P (75–150 μm, Mitsubishi Chemical Co.), Sephadex LH-20 (25–100 μm, Pharmacia Fine Chemical Co. Ltd.), Toyopearl HW-40F (Tosoh Corp.), and Chromatorex ODS (Fuji Silysia Chemical Ltd.). TLC was performed on precoated Kieselgel 60 F_{254} plates (0.2 mm thick, Merck) with benzene–ethyl formate–formic acid (1:7:1, v/v) or $CHCl_3$–MeOH–H_2O (14:6:1, v/v), and spots were detected by UV illumination and by spraying with 2% ethanolic $FeCl_3$ or 10% sulfuric acid reagent followed by heating. Compounds **1** and **2** were isolated from commercial green tea according to Nonaka et al. and recrystallized from H_2O.[20] Tannase (a crude enzyme prepared from *Aspergillus* sp.) was provided from Sankyo Co., Ltd.

Plant Material. Fresh tea leaves (*Camellia sinensis* var. *sinensis*) were collected in May at Nagasaki Agricultural and Forestry Experimental Station, Higashisonogi tea branch.

HPLC Analysis of Phenazine Derivatives. Tea leaves (80 g), left withering for 12 h, were crumpled and crushed. The leaves was spread on a tray in a thin layer at 24 °C and mixed well at intervals with occasional spraying of H_2O to avoid drying. After 2.5 h, a portion (10 g) of the leaves was extracted with EtOH (50 mL), a portion (10 g) was treated with 0.2% *o*-phenylenediamine solution in 1% AcOH–EtOH (50 mL), and another portion (10 g) was heated at 90 °C for 10 min in a steamer and extracted with EtOH (50 mL). Aliquots (2 mL) of extracts were separately passed through a Sep-pak cartridge (Waters Associates) with 90% EtOH, and the volume was adjusted to 5.0 mL. Each of the solutions was analyzed by reversed-phase HPLC performed on a Cosmosil 5C_{18}-AR II (Nacalai Tesque Inc.) column (250 × 4.6 mm i.d.) with gradient elution from 4% to 30% (39 min) and 30% to 75% (15 min) of CH_3CN in 50 mM H_3PO_4 (flow rate, 0.8 mL/min; detection, JASCO photodiode array detector MD-910). Quantification of the total theaflavins and phenazine derivatives was performed by incorparting the peak area onto the calibration curve of the theaflavin (at 375 nm) and phenazine derivative **8** at 370 nm, respectively.

Isolation. Fresh tea leaves (450 g) were macerated with H_2O (200 mL) using a Waring blender and spread onto trays in thin layers at 28 °C. After 3 h, the leaves were treated with *o*-phenylenediamine (1%) in EtOH–AcOH (9:1, v/v, 500 mL) for 8 h and then extracted with 80% acetone. The extract was concentrated and partitioned between H_2O and ethyl acetate. The ethyl acetate extract (30.7 g) was subjected to column chromatography over MCI-gel CHP20P with H_2O containing increasing proportions of MeOH to give four fractions. The second fraction, eluted with 30–60% MeOH, was further separated by Sephadex LH-20 column chromatography with EtOH into four fractions (2-1–2-4). Fraction 2-2 was applied to a column of Chromatorex ODS (H_2O–MeOH) and then Toyopearl HW-40F (H_2O–MeOH) to give **8** (100.2 mg). Similar separation of the fraction 2-3 yielded **9** (28.3 mg) and **10** (45.5 mg). Fraction 2-4 was also chromatographed over Chromatorex ODS followed by Toyopearl HW-40F to give **11** (102.7 mg).

Synthesis of 8 and 12 from 1 and 2. Fresh banana fruit (50 g) was homogenized with H_2O (100 mL) in a Waring blender, and the homogenate was filtered through four layers of gauze. The filtered homogenate (40 mL) was mixed with a solution of **1** (229 mg) and **2** (245 mg) in H_2O (12 mL) and vigorously stirred for 1.5 h at room temperature. Then, an EtOH solution (200 mL) containing 0.2% *o*-phenylenediamine and 1% AcOH was added, and the resulting mixture was filtered. HPLC analysis of the filtrate showed that **2** was absent and the major products were **1**, **8**, **12**, and theaflavin. The filtrate was concentrated and separated by a combination of column chromatography over Chromatorex ODS (H_2O–MeOH) and Sephadex LH-20 (80% MeOH) to yield **1** (142 mg), **12** (25.8 mg), **8** (83.5 mg), and theaflavin (132.2 mg).

Compound 8: reddish brown amorphous powder; $[\alpha]^{26}_D$ −6.0° (*c* 0.8, MeOH); UV (EtOH) λ_{max} (log ϵ) 275 (4.79), 376

(3.89) nm; CD (EtOH) $\Delta\epsilon$ (nm) +11.5 (275), −3.6 (255), +0.82 (244), −32.2 (211); ^{1}H NMR (acetone-d_6, 300 MHz) (see Figure 2 for numbering scheme) δ 8.28 (2H, m, H-9′, H-12′), 8.21 (1H, s, H-6′), 7.96 (2H, m, H-10′, H-11′), 7.04 (1H, s, H-6′′′), 6.01, 6.00, 5.88, 5.83 (each 1H, br s, H-6, H-8, H-6′′, H-8′′), 4.98 (1H, br s, H-2), 4.55 (1H, br s, H-2′′), 4.30 (1H, br s, H-3), 4.10 (1H, br s, H-3′′), 2.76 (1H, br d, $J_{4a,4b}$ = 16.5 Hz, H-4a), 2.53 (1H, br d, $J_{4'a,4'b}$ = 16.5 Hz, H-4′a), 2.42 (1H, dd, $J_{3,4b}$ = 4.4 Hz, $J_{4a,4b}$ = 16.7 Hz, H-4b), 2.11 (1H, dd, $J_{3'',4''b}$ = 3.8 Hz, $J_{4''a,4''b}$ = 16.5 Hz, H-4′′b); ^{13}C NMR (acetone-d_6, 75.5 MHz) δ 157.6, 157.5, 157.4, 157.2, 157.1, 157.0 (C-5, C-7, C-8a, C-5′′, C-7′′, C-8a′′), 151.3 (C-3′), 146.0 (C-5′′′), 144.7, 142.0 (C-7′, C-8′), 144.5 (C-3′′′), 144.2 (C-5′), 144.0 (C-1′), 135.5 (C-4′), 133.3 (C-4′′′), 131.5, 131.1, 130.3, 130.1 (C-9′, C-10′, C-11′, C-12′), 129.2 (C-1′′′), 119.4 (C-6′), 117.3 (C-2′), 112.0 (C-2′′′), 108.1 (C-6′′′), 99.4, 99.1 (C-4a, C-4a′′), 96.2, 96.0, 95.7, 95.5 (C-6, C-8, C-6′′, C-8′′), 77.7, 77.3 (C-2, C-2′′), 64.9, 63.8 (C-3, C-3′′), 29.5−30.5 (C-4, C-4′′, overlapped with solvent signals); HRFABMS m/z 681.1720 (M + H)$^+$ (calcd for $C_{36}H_{29}N_2O_{12}$, 681.1721).

Methylation of 8. A solution of **8** (40 mg) in EtOH (2 mL) was treated with CH_2N_2 in Et$_2$O for 12 h at 0 °C. After evaporation of the solvent, the residue was separated by silica gel column chromatography with hexane−acetone (3:2, v/v) to give octamethyl ether (9.4 mg) as a yellow amorphous powder: $[\alpha]^{25}_D$ +45.1° (c 0.5, MeOH); ^{1}H NMR (CDCl$_3$, 300 MHz) δ 8.49 (1H, d, $J_{6',2}$ = 0.8 Hz, H-6′), 8.29 (2H, m, H-9′, 12′), 7.86 (2H, m, H-10′, 11′), 7.31 (1H, s, H-6′′′), 6.17, 6.08, 6.06, 5.95 (each 1H, d, J = 2 Hz, H-6, H-8, H-6′′, H-8′′), 4.88, 4.52 (each 1H, br s, H-2, H-2′′), 4.30, 3.99, 3.93, 3.91, 3.76, 3.74, 3.69, 3.62 (each 3H, s, OCH$_3$), 2.87, 2.67 (each 1H, br d, J = 17.0 Hz, H-4a, H-4′′a), 2.48, 2.25 (each 1H, dd, J = 4.4, 17.0 Hz, H-4b, H-4′′b); HRFABMS m/z 793.2972 (M + H)$^+$ (calcd for $C_{44}H_{45}N_2O_{12}$, 793.2974).

Compound 9: reddish brown amorphous powder; $[\alpha]^{26}_D$ −15.4° (c 0.6, MeOH); UV(EtOH) λ_{max} (log ϵ) 274 (4.83), 376 (3.89) nm; ^{1}H NMR (acetone-d_6, 300 MHz) δ 8.38 (1H, s, H-6′), 8.26 (2H, m, H-9′, H-12′), 7.95 (2H, m, H-10′, H-11′), 7.11 (1H, s, H-6′′′), 7.06 (2H, s, galloyl-H), 6.08, 6.04, 5.89, 5.87 (each 1H, d, J = 2.2 Hz, H-6, H-8, H-6′′, H-8′′), 5.49 (1H, br d, $J_{3'',4''b}$ = 4.4 Hz, H-3), 5.20 (1H, br s, H-2), 4.56 (1H, br s, H-2′′), 4.19 (1H, br s, H-3′′), 3.09 (1H, br d, $J_{4a,4b}$ = 17.5 Hz, H-4a), 2.57 (1H, br d, $J_{4a,4b}$ = 17.5 Hz, H-4′′a), 2.56 (1H, dd, $J_{3,4b}$ = 4.4 Hz, $J_{4a,4b}$ = 17.5 Hz, H-4b), 2.11 (1H, dd, $J_{3'',4''b}$ = 4.4 Hz, $J_{4''a,4''b}$ = 17.5 Hz, H-4′′b); ^{13}C NMR (acetone-d_6, 75.5 MHz) δ 166.5 (galloyl C-7), 157.7, 157.5, 157.4, 157.2 (2C), 156.8 (C-5, C-7, C-8a, C-5′′, C-7′′, C-8a′′), 151.6 (C-3′), 146.3 (C-5′′′), 145.8 (galloyl C-3, C-5), 144.6, 142.4 (C-7′, C-8′), 144.0 (C-5′, C-3′′′), 143.1 (C-1′), 138.8 (galloyl C-4), 135.8 (C-4′), 133.5 (C-4′′′), 131.1, 131.3, 130.3, 130.2 (C-9′, C-10′, C-11′, C-12′), 129.2 (C-1′′′), 121.2 (galloyl C-1), 118.7 (C-6′), 117.7 (C-2′), 112.0 (C-2′′′), 109.5 (galloyl C-2, C-6), 108.4 (C-6′′′), 99.1, 98.3 (C-4a, C-4a′′), 96.6, 96.5, 95.7, 95.6 (C-6, C-8, C-6′′, C-8′′), 77.2, 76.6 (C-2, C-2′′), 67.9, 64.8 (C-3′′, C-3), 28.8, 26.8 (C-4, C-4′′); HRFABMS m/z 833.1826 (M + H)$^+$ (calcd for $C_{43}H_{33}N_2O_{16}$, 833.1830).

Compound 10: reddish brown amorphous powder; $[\alpha]^{26}_D$ −32.9° (c 0.5, MeOH); UV (EtOH) λ_{max} (log ϵ) 275 (4.83), 375 (3.89) nm; ^{1}H NMR (acetone-d_6, 500 MHz) δ 8.25 (2H, m, H-9′, H-12′), 8.24 (1H, d, $J_{2,6'}$ = 0.9 Hz, H-6′), 7.94 (2H, m, H-10′, H-11′), 7.12 (1H, s, H-6′′′), 7.07 (2H, s, galloyl-H), 6.03, 6.01 (each 1H, d, J = 2.2 Hz, H-6, H-8), 5.93, 5.89 (each 1H, d, J = 2.2 Hz, H-6′′, H-8′′), 5.39 (1H, br d, $J_{3'',4''b}$ = 4.4 Hz, H-3′′), 4.99 (1H, br s, H-2), 4.80 (1H, br s, H-2′′), 4.35 (1H, br d, $J_{3,4b}$ = 4.4 Hz, H-3), 2.80 (1H, br d, $J_{4a,4b}$ = 16.7 Hz, H-4a), 2.77 (1H, br d, $J_{4''a,4''b}$ = 17.6 Hz, H-4′′a), 2.47 (1H, dd, $J_{3,4b}$ = 4.4 Hz, $J_{4a,4b}$ = 16.7 Hz, H-4b), 2.29 (1H, dd, $J_{3'',4''b}$ = 4.4 Hz, $J_{4''a,4''b}$ = 17.6 Hz, H-4′′b); ^{13}C NMR (acetone-d_6, 125.7 MHz) 166.2 (galloyl C-7), 157.7, 157.6, 157.5, 157.3, 157.2, 157.1 (C-5, C-7, C-8a, C-5′′, C-7′′, C-8a′′), 151.7 (C-3′), 146.3 (C-5′′′), 145.9 (galloyl C-3, C-5), 145.0, 142.1 (C-7′, C-8′), 144.5 (C-3′′′), 144.2 (C-5′), 143.8 (C-1′), 138.8 (galloyl C-4), 135.6 (C-4′), 133.6 (C-4′′′), 131.6, 131.2, 130.4, 130.2 (C-9′, C-10′, C-11′, C-12′), 128.6 (C-1′′′), 121.9 (galloyl C-1), 119.9 (C-6′), 116.8 (C-2′), 112.4 (C-2′′′), 110.0 (galloyl C-2, C-6), 108.1 (C-6′′′), 99.4 (C-4a), 98.6 (C-4a′′), 96.5, 96.4, 96.0, 95.8 (C-6, C-8, C-6′′, C-8′′), 77.7 (C-

2), 76.4 (C-2′′), 68.7 (C-3′′), 64.0 (C-3), 29.5 (C-4), 26.9 (C-4′′); HRFABMS m/z 833.1815 (M + H)$^+$ (calcd for $C_{43}H_{33}N_2O_{16}$, 833.1830).

Compound 11: reddish brown amorphous powder; $[\alpha]^{26}_D$ −24.3° (c 0.7, MeOH); UV (EtOH) λ_{max} (log ϵ) 274 (4.84), 376 (3.88) nm; ^{1}H NMR (acetone-d_6, 300 MHz) δ 8.41 (1H, s, H-6′), 8.27 (2H, m, H-9′, H-12′), 7.94 (2H, m, H-10′, H-11′), 7.18 (1H, s, H-6′′′), 7.12, 7.06 (each 2H, s, galloyl-H), 6.07, 6.06, 5.93, 5.91 (each 1H, d, J = 2.2 Hz, H-6, H-8, H-6′′, H-8′′), 5.52 (1H, br s, H-3), 5.44 (1H, br s, H-3′′), 5.19 (1H, br s, H-2), 4.80 (1H, br s, H-2′′), 3.12 (1H, br d, $J_{4a,4b}$ = 18.1 Hz, H-4a), 2.80 (1H, br d, $J_{4a,4b}$ = 18.1 Hz, H-4′′a), 2.60 (1H, dd, $J_{3,4b}$ = 4.4 Hz, $J_{4a,4b}$ = 18.1 Hz, H-4b), 2.35 (1H, dd, $J_{3'',4''b}$ = 4.4 Hz, $J_{4''a,4''b}$ = 18.1 Hz, H-4′′b); ^{13}C NMR (acetone-d_6, 75.5 MHz) δ 166.5, 166.3 (galloyl C-7), 157.8, 157.5 (2C), 157.2, 157.1, 156.8 (C-5, C-7, C-8a, C-5′′, C-7′′, C-8a′′), 152.0 (C-3′), 146.5 (C-5′′′), 145.9, 145.8 (galloyl C-3, C-5), 144.5, 142.4 (C-7′, C-8′), 144.2, 143.5, 143.4 (C-1′, C-5′, C-3′′′), 138.8, 138.7 (galloyl C-4), 135.8 (C-4′), 133.7 (C-4′′′), 132.1, 131.4, 130.3, 129.5 (C-9′, C-10′, C-11′, C-12′), 128.5 (C-1′′′), 121.8, 121.2 (galloyl C-1), 119.1 (C-6′), 117.0 (C-2′), 112.1 (C-2′′′), 109.9, 109.6 (galloyl C-2, C-6), 108.3 (C-6′′′), 98.4 (2C) (C-4a, C-4a′′), 96.7, 96.3, 95.8, 95.6 (C-6, C-8, C-6′′, C-8′′), 76.5, 76.3 (C-2, C-2′′), 68.7, 67.8 (C-3′′, C-3), 26.8 (2C) (C-4, C-4′′); HRFABMS m/z 985.1932 (M + H)$^+$ (calcd for $C_{50}H_{37}N_2O_{20}$, 985.1939).

Hydrolysis of 9−11. A solution of each compound (3−5 mg) in H$_2$O (0.5 mL) was treated with tannase at 35 °C for 3 h. The reaction mixture was concentrated, and the residue was treated with EtOH. The insoluble material was filtered off, and the filtrate was analyzed by HPLC. The chromatograms showed peaks corresponding to gallic acid (8.6 min) and **8** (31.1 min).

Compound 12: reddish brown amorphous powder; $[\alpha]^{26}_D$ +18.1° (c 0.2, MeOH); CD (EtOH) $\Delta\epsilon$ (nm) +14.5 (274), +0.1 (255), +1.80 (242), −22.8 (216); ^{1}H NMR (acetone-d_6, 300 MHz) δ 8.21, 8.13 (each 1H, dd, J = 7, 2 Hz, H-9′, H-12′), 7.87 (2H, m, H-10′, H-11′), 7.81 (1H, s, H-6′), 7.08 (1H, s, H-6′′′), 6.01, 5.94, 5.85, 5.81 (each 1H, d, J = 2.4 Hz, H-6, H-8, H-6′′, H-8′′), 5.11 (1H, br s, H-2), 4.54 (1H, br s, H-2′′), 4.40 (1H, br s, H-3), 3.71 (1H, br s, H-3′′), 2.78 (1H, br d, $J_{4a,4b}$ = 17.0 Hz, H-4a), 2.52 (1H, dd, $J_{3,4b}$ = 4.7 Hz, $J_{4a,4b}$ 17.0 Hz, H-4b), 2.32 (1H, br d, $J_{4''a,4''b}$ = 17.0 Hz, H-4′′a), 1.89 (1H, dd, $J_{3'',4''b}$ = 4.1 Hz, $J_{4''a,4''b}$ = 17.0 Hz, H-4′′b); ^{13}C NMR (acetone-d_6, 75.5 MHz) δ 157.6, 157.5, 157.3, 157.2, 157.1, 157.0 (C-5, C-7, C-8a, C-5′′, C-7′′, C-8a′′), 152.2 (C-5′), 145.7 (C-5′′′), 145.2 (C-3′′′), 144.6 (C-1′), 144.4 (C-3′), 144.2, 141.7 (C-7′, C-8′), 135.9 (C-4′), 133.4 (C-4′′′), 131.4, 131.3, 130.6, 129.9 (C-9′, C-10′, C-11′, C-12′), 129.3 (C-1′′′), 124.1 (C-2′), 114.8 (C-2′′′), 111.5 (C-6′), 108.5 (C-6′′′), 99.3, 99.2 (C-4a, C-4a′′), 96.4, 96.0, 95.9, 95.8 (C-6, C-8, C-6′′, C-8′′), 78.0, 77.3 (C-2, C-2′′), 65.1, 65.0 (C-3, C-3′′), 30.2, 29.0 (C-4, C-4′′); HRFABMS m/z 681.1719 (M + H)$^+$ (calcd for $C_{36}H_{29}N_2O_{12}$, 681.1721).

Methylation of 12. A solution of **12** (15 mg) in EtOH (1 mL) was treated with CH_2N_2 in Et$_2$O for 12 h at 0 °C. After evaporation of the solvent, the residue was separated by silica gel column chromatography with hexane−acetone (3:2, v/v) to give octamethyl ether **12a** (5.0 mg) as a yellow amorphous powder, $[\alpha]^{25}_D$ +12.4° (c 0.3, CHCl$_3$); ^{1}H NMR (CDCl$_3$, 300 MHz) δ 8.44, 8.49 (each 1H, dd, J = 8.2, 1.9 Hz, H-9′, 12′), 7.82 (2H, m, H-10′, 11′), 7.64 (1H, s, H-6′), 7.30 (1H, s, H-6′′′), 6.16, 6.09, 6.08, 5.98 (each 1H, d, J = 2 Hz, H-6, H-8, H-6′′, H-8′′), 4.97, 4.62 (each 1H, br s, H-2, H-2′′), 4.36 (1H, br s, H-3), 4.26, 4.02, 3.93, 3.77, 3.73, 3.70, 3.62, 3.45 (each 3H, s, OCH$_3$), 2.83 (1H, d, J = 16.8 Hz, H-4a), 2.55 (1H, dd, J = 4.4, 16.8 Hz, H-4b), 2.48 (1H, d, J = 16.8 Hz, H-4′′a), 1.91 (1H, dd, J = 4.8, 16.8 Hz, H-4′′b); HRFABMS m/z 793.2976 (M + H)$^+$ (calcd for $C_{44}H_{45}N_2O_{12}$, 793.2974).

Ferricyanide Oxidation of 1 and 2. To a solution of **1** and **2** (each 5 mg) in H$_2$O (0.5 mL) was added a solution of K$_3$[Fe(CN)$_6$] (15 μg) and NaHCO$_3$ (4 μg) in H$_2$O (0.1 mL) and left to stand at 0 °C for 15 min. The mixture was acidified with 0.1 M HCl (0.4 mL), and a portion (400 μL) of the mixture was applied to a MCI-gel CHP 20P short column (8 mm i.d. × 50 mm). After the column was washed with H$_2$O (10 mL), a solution of o-phenylenediamine (2 mg) in EtOH−acetic acid

(3:2, v/v, 0.5 mL) was added to the column, followed by elution with EtOH. The product eluted out with EtOH was collected and analyzed by HPLC. The chromatogram showed peaks arising from **2**, **1**, **12**, **8**, and theaflavin.

References and Notes

(1) Haslam, E. *Practical Polyphenolics, from Structure to Molecular Recognition and Physiological Action*; Cambridge University Press: Cambridge, 1998; pp 335−354.
(2) Takino, Y.; Imagawa, H.; Horikawa, H.; Tanaka, A. *Agric. Biol. Chem.* **1964**, *28*, 64−71.
(3) Takino, Y.; Ferretti, A.; Flanagan, M.; Gianturco, M.; Vogel, M. *Tetrahedron Lett.* **1965**, 4019−4025.
(4) Brown, A. G.; Falshaw, C. P.; Haslam, E.; Holmes, A.; Ollis, W. D. *Tetrahedron Lett.* **1996**, 1193−1204.
(5) Roberts, E. A. H. Economic importance of flavonoid substances: tea fermentation. *The Chemistry of Flavinoid Compounds*; Pergammon Press: Oxford, 1962; pp 468−512.
(6) Tanaka, T.; Betsumiya, Y.; Mine, C.; Kouno, I. *Chem. Commun.* **2000**, 1365−1366.
(7) Tanaka, T.; Inoue, K.; Betsumiya, Y.; Mine, C.; Kouno, I. *J. Agric. Food Chem.* **2001**, *49*, 5785−5789.
(8) Roberts, E. A. H. *J. Sci. Food Agric.* **1959**, *10*, 167−172.
(9) Roberts, E. A. H. *Chem. Ind.* **1957**, 1354−1355.
(10) Roberts, E. A. H. *Chem. Ind.* **1957**, 1355−1356.
(11) Imagawa, H.; Takino, Y. *Agric. Biol. Chem.* **1962**, *26*, 541−542.
(12) Takino, Y.; Imagawa, H.; Aoki, Y.; Ozawa, T. *Agric. Biol. Chem.* **1963**, *27*, 562−568.
(13) Tanaka, T.; Kondou, K.; Kouno, I. *Phytochemistry* **2000**, *53*, 311−316.
(14) Tanaka, T.; Mine, C.; Inoue, K.; Matsuda, M.; Kouno, I. *J. Agric. Food Chem.* **2002**, *50*, 2142−2148.
(15) Nonaka, G.; Hayashi, M.; Tanaka, T.; Saijo, R.; Nishioka, I. *Chem. Pharm. Bull.* **1990**, *38*, 861−865.
(16) Nonaka, G.; Kawahara, O.; Nishioka, I. *Chem. Pharm. Bull.* **1983**, *31*, 3906−3914.
(17) Hashimoto, F.; Nonaka, G.; Nishioka, I. *Chem. Pharm. Bull.* **1988**, *36*, 1676−1684.
(18) Hashimoto, F.; Nonaka, G.; Nishioka, I. *Chem. Pharm. Bull.* **1992**, *40*, 1383−1389.
(19) Zhu, N.; Wang, M.; Wei, G.-J.; Lin, J.-K.; Yang, C. S.; Ho, C.-T. *Food Chem.* **2001**, *73*, 345−349.
(20) Nonaka, G.; Kawahara, O.; Nishioka, I. *Chem. Pharm. Bull.* **1983**, *31*, 3906−3914.