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Synthesis of Theaflavin from Epicatechin and Epigallocatechin by Plant Homogenates and Role of Epicatechin Quinone in the Synthesis and Degradation of Theaflavin

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Oxidation products of (−)-epicatechin and (−)-epigallocatechin by treatment with homogenates of 62 plants belonging to 49 families were compared. Forty-six plants were capable of synthesizing theaflavin, a black tea pigment, regardless of whether they contained catechins. Loquat, Japanese pear, and blueberry had activities higher than that of fresh tea leaves after 5 h of treatment; furthermore, these plants oxidized theaflavin to theanaphthoquinone. An additional new metabolite, dehydrotheasinensin, was generated on treatment with fresh tea leaves, eggplant, and unripened Japanese orange. Evidence for the oxidation of epigallocatechin and theaflavin by electron transfer to epicatechin quinone was demonstrated in a time course study using bananas and trapping the quinone intermediates as glutathione conjugates.

KEYWORDS: Catechin; oxidation; theaflavin; dehydrotheasinensin; black tea; polyphenol

INTRODUCTION

Enzymatic polyphenol oxidation, usually accompanied by reddish coloration, is often observed in damaged fruit or vegetables. This phenomenon is important from the viewpoints of food manufacturing and the plant defense system. However, chemical details of the reaction remain ambiguous because of the complexity of the oxidation products. In black tea manufacturing, the oxidation mechanism of four of the major catechins, (−)-epicatechin (**1**), (−)-epigallocatechin (**2**), and their 3-*O*-gallates, in tea leaves has been well studied, and the co-occurrence of these catechol (**1** and its 3-*O*-gallate) and pyrogallol (**2** and its 3-*O*-gallate) types makes the oxidation characteristic (*1*). Theaflavin (**3**) and its 3- and 3′-*O*-gallates, characteristic reddish orange pigments of black tea having a benzotropolone structure, are produced by oxidative condensation between **1** and **2**. In the present study, we examined the production of **3** from a mixture of **1** and **2** by treatment with various plant homogenates and found that a number of plants are capable of synthesizing **3** regardless of whether the plant contains catechins. Further oxidation of **3** to theanaphthoquinone (**4**) (*2*) and generation of a new metabolite named dehydrotheasinensin (**6**) were also observed. Catechin oxidation by various plants and the structure determinations of the new metabolites has been investigated in this study. Evidence supporting the oxidation of **2** and **3** by epicatechin quinone (**1a**) is also presented.

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MATERIALS AND METHODS

Materials and General Procedures. UV spectra were obtained with a V-560 UV−vis spectrophotometer, and optical rotations were measured with a DIP-370 digital polarimeter (JASCO Co.). ^{1}H and ^{13}C NMR, ^{1}H−^{1}H COSY, NOESY, HSQC, and HMBC spectra were recorded in a mixture of acetone-d_6 and D_2O (19:1, v/v) with a Unity plus 500 spectrometer (Varian Inc.) operating at 500 MHz for ^{1}H and at 125 MHz for ^{13}C, respectively. Coupling constants are expressed in hertz, and chemical shifts given on a δ (parts per million) scale with tetramethylsilane as an internal standard. MS were recorded on a JMS DX-303 spectrometer (JEOL Ltd.), and glycerol or *m*-nitrobenzyl alcohol was used as a matrix for FAB-MS measurement. Elemental analysis was obtained with a MT-3 analyzer (Yanaco Analytical Instruments Co.). Column chromatography was done on MCI gel CHP 20P, 75−150 μm (Mitsubishi Chemical Co.), Chromatorex ODS (Fuji Silysia Chemical Ltd.), and Sephadex LH-20, 25−100 μm (Pharmacia Fine Chemical Co. Ltd.). Thin-layer chromatography (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 chloroform/MeOH/H_2O (14:6:1, v/v), and spots were detected by UV illumination and spraying with 2% ethanolic $FeCl_3$ or 10% sulfuric acid reagent followed by heating. Analytical high-pressure liquid chromotography (HPLC) was performed on a Cosmosil 5C_{18}-AR II, 250 × 4.6 mm i.d. column (Nacalai Tesque Inc.) with gradient elution from 10 to 30% (30 min) and from 30 to 75% (15 min) of CH_3CN in 50 mM H_3PO_4 at a flow rate of 0.8 mL/min, and detection was performed with an MD-910 photodiode array detector (JASCO Co.). Epicatechin and epigallocatechin were isolated from commercial green tea according to the method of Nonaka et al. (*3*) and recrystallized from H_2O.

Oxidation of Epicatechin by Banana. Banana fruit (*Musa acuminata* Colla cv. Giant Cavendish) purchased in a local market (100 g) was homogenized with H_2O (200 mL) in a Waring blender, and the homogenate was filtered through four layers of gauze. An aqueous

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solution (25 mL) of **1** (0.5 g) was mixed with the banana homogenate (100 mL) at room temperature and stirred vigorously for 5 h. Acetone (500 mL) was added and the mixture filtered. The filtrate was concentrated in vacuo to ~100 mL and extracted three times with ethyl acetate (100 mL). The organic layer was concentrated and subjected to Sephadex LH-20 column chromatography (20 × 2.0 cm) with ethanol to give **1** (162 mg) and a fraction containing oxidation products. The products were further purified by MCI gel CHP20P column chromatography (15 × 1.5 cm) with H_2O containing increasing proportions of MeOH (0−40%) to afford compound **5** (8.8 mg) as a yellow amorphous powder: $[\alpha]_D$ −140° (*c* 0.3, MeOH); negative FAB-MS *m*/*z* 863 $(M - H)^-$; UV λ_{max}^{EtOH}(log ϵ) 256sh (5.2), 282 (5.2), 384 (5.4). ^{1}H NMR (500 MHz, in acetone-d_6) δ 7.92 (1H, s, OH), 7.10 (1H, d, J = 2.1 Hz, H-2), 6.95 (1H, d, J = 0.5 Hz, B-2), 6.93 (1H, dd, J = 2.1, 8.5 Hz, H-6), 6.79 (1H, d, J = 8 Hz, H-5), 6.79 (1H, s, D-7 OH), 6.36 (1H, s, B-5), 6.21 (1H, d, J = 2.3 Hz, A-8), 6.19 (1H, s, G-6), 6.10 (1H, s, D-6), 6.04 (1H, s, E−5), 5.99 (1H, d, J = 2.3 Hz, A-6), 5.51 (1H, s, E−3 OH), 5.15 (1h, br s, I-2), 5.09 (1H, dt, J = 2.5, 9.2 Hz, F-3), 4.46 (1H, br s, C-2), 4.24 (1H, br s, I-3), 4.20 (1H, d, J = 2.8 Hz, F-2), 3.83 (1H, br s, C-3), 3.60, 3.31 (each 1H, d, J = 5.3 and 4.4 Hz, respectively, C-3 OH and I-3 OH), 3.31 (1H, dd, J = 4.9, 16.3 Hz, I-4), 3.21 (1H, dd, J = 9.2, 17.6 Hz, F-4), 3.14 (1H, d, J = 12.6 Hz, E−2), 2.82 (1H, dd, J = 1.8, 16.3 Hz, I-4), 2.60 (1H, br d, J = 16.0 Hz, C-4), 2.53 (1H, dd, J = 2.5, 17.6 Hz, F-4), 2.29 (1H, dd, J = 4.6, 16.0 Hz, C-4), 2.23 (1H, d, J = 12.6 Hz, E-2); ^{13}C NMR (125 MHz, in acetone-d_6) δ 190.50 (E-4), 164.30, 164.23 (E-6, G-5), 163.65 (G-7), 157.62, 157.24 (A-5, A-7), 155.84 (D-5), 154.70 (A-8a), 154.67 (D-7), 154.25 (G-8a), 152.24 (D-8a), 145.35 (2C) (H-3, 4), 144.38, 143.86 (B-3, 4), 132.16, 131.95 (B-1, H-1), 122.59 (B-6), 119.53 (H-6), 118.83 (B-5), 115.78 (B-2), 115.40 (2C) (H-2, H-5), 110.05 (E-5), 108.19 (D-8), 105.49 (G-8), 102.70 (G-4a), 99.93 (D-4a), 99.67 (A-4a), 96.93 (D-6), 96.39 (A-8), 95.79 (A-6), 94.32 (E-3), 91.96 (G-6), 90.74 (E-1), 80.26 (F-2), 79.79 (I-2), 77.19 (C-2), 68.76 (F-3), 66.18 (I-3), 64.75 (C-3), 39.74 (E-2), 28.96 (I-4), 28.53 (C-4), 24.18 (F-4). Anal. Calcd for $C_{45}H_{36}O_{18}$·2.5H_2O: C, 59.41%; H, 4.54%. Found: C, 59.65%; H, 4.80%.

Oxidation of a Mixture of Catechins by Plant Homogenates. Fresh plant (50 g) was homogenized with H_2O (100 mL) in a Waring blender and filtered through four layers of gauze. The plant homogenate (1.5 mL) was mixed with an aqueous solution (0.25 mL) of **1** (5 mg) and **2** (5 mg) and stirred vigorously for 5 h at room temperature. After addition of acetone (5 mL), the reaction mixture was filtered, and the filtrate was concentrated in vacuo. The residue was redissolved in 50% aqueous MeOH (5.0 mL), passed through a membrane filter (0.45 μm), and analyzed by HPLC with a photodiode array detector. Concurrently, a blank solution was treated in the same manner. Quantification of compounds **3**, **4**, and **6** in **Table 1** was performed by reporting the peak area into the calibration curve of the corresponding compound (**3** at 375 nm, **4** at 440 nm, and **6** at 390 nm). For the time course experiment, the scale of the reaction was twice as much and aliquots (0.1 mL) of the reaction mixture were withdrawn at the times indicated, diluted with EtOH (0.4 mL), and analyzed by HPLC.

Isolation of Dehydrotheasinensin (6). Fresh tea leaves (*Camellia sinensis* var. *assamica*) (720 g) were homogenized with H_2O (1800 mL) and Polyclar AT, polyvinylpolypyrrolidone (GAF Corp., Linden, NJ) (240 g), and filtered through four layers of gauze. The filtrate (1.2 L) was mixed with an aqueous solution (100 mL) containing **1** (2.0 g) and **2** (6.0 g) and stirred vigorously for 14 h at room temperature. The mixture was poured into acetone (1.5 L) and filtered through filter paper. After concentration, the filtrate was extracted five times with ethyl acetate to yield an ethyl acetate extract (3.2 g). The extract was subjected to Sephadex LH-20 column chromatography (25 × 2.5 cm) with ethanol containing increasing proportions of H_2O (0−20%) and then with 50% acetone. The fractions containing polyphenols were combined and applied to an MCI gel CHP20P column (15 × 2.0 cm) with H_2O containing increasing proportions of MeOH (0−80%) to yield unchanged **1** (829 mg), **6** (246.6 mg), and a mixture of theaflavin (**3**) and theanaphthoquinone (**4**). The separation of **3** (314.3 mg) and **4** (6.5 mg) was achieved by Chromatorex ODS column chromatography (0−40% MeOH). In this experiment, **2** was completely consumed.

*Dehydrotheasinensin (**6**):* yellow amorphous powder; $[\alpha]_D$ +21.1° (*c* 0.12, MeOH); FABMS (negative mode), *m*/*z* 607 $[M - H]^-$; UV λ_{max}^{EtOH} (log ϵ) 269 (4.11), 397 (4.59); ^{1}H NMR (500 MHz, in acetone-d_6) δ 8.48 (1H, s, B-3-OH), 8.37, 8.23, 8.18, 8.09 (each 1H, s, A-ring OH), 7.036 (1H, d, J = 1.0 Hz, E−6), 6.11 (2H, br s, B-5-OH, F-2), 6.03, 6.02, 6.00, 5.98 (each 1H, d, J = 2.5, 2.2, 2.5, 2.2 Hz, respectively, A-6, A-8, D-6 and D-8), 4.45 (1H, ddd, J = 1.3, 1.8, 5.0 Hz, C-3), 4.26 (1H, br s, F-3), 4.17 (1H, br s, C-2), 3.60 (1H, br s, F-3-OH), 2.97 (1H, d, J = 11.4 Hz, B-6), 2.94 (1H, dd, J = 4.4, 16.5 Hz, F-4), 2.85 (1H, dd, J = 1.8, 17.6 Hz, C-4), 2.71 (1H, dd, J = 2.5, 16.5 Hz, F-4), 2.71 (1H, dd, J = 5.0, 17.6 Hz, C-4), 2.33 (1H, dd, J = 1.1, 11.4 Hz, B-6); ^{13}C NMR (125 MHz, in acetone-d_6) δ 189.56 (B-4), 157.61, 157.55, 157.39, 157.05, 156.89 (A-5, A-7, D-5, D-7, F-8a), 155.54 (A-8a), 152.17 (E-3), 150.95 (E-5), 139.73 (B-3), 134.25 (B-2), 130.84 (E-1), 129.40 (E-4), 112.40 (E-2), 110.21 (E-6), 100.16 (D-4a), 98.47 (A-4a), 96.51, 96.17, 95.66, 95.35 (A-6, A-8, D-6, D-8), 94.67 (B-5), 89.57 (B-1), 76.80 (F-2), 72.18 (C-2), 66.43 (F-3), 65.00 (C-3), 40.67 (B-6), 28.28 (F-4), 25.02 (C-4). Anal. Calcd for $C_{30}H_{24}O_{14}$·6.5H_2O: C, 49.66%; H, 5.14%. Found: C, 49.85%; H, 5.04%.

Oxidation in the Presence of Glutathione. A mixture of **1** (0.5 g), **2** (0.5 g), and glutathione (reduced form, 1.0 g) in H_2O (25 mL) was mixed with the banana homogenate (100 mL), prepared as described above, and stirred vigorously for 3.5 h at room temperature. After addition of acetone (500 mL) and filtration, the filtrate was concentrated and applied to a Sephadex LH-20 column (25 × 3.0 cm) with H_2O containing increasing proportions of MeOH to give **3** (32.1 mg), unchanged **2** (361.9 mg), and a mixture of **7**, **8**, and **9**. The mixture of glutathione conjugates was further separated by MCI gel CHP20P chromatography (25 × 2.0 cm) with H_2O/MeOH to yield **7** (64.9 mg), **8** (111.5 mg), **9** (498.9 mg), and a mixture of **7** and **8** (195.1 mg).

*2′-Glutathionyl-(−)-epigallocatechin (**7**):* white amorphous powder; $[\alpha]_D$ −60.9° (*c* 0.3, MeOH); FABMS, *m*/*z* 612 $[M + H]^+$, 634 $[M + Na]^+$; ^{1}H NMR (300 MHz, in acetone-d_6 + D_2O) δ 6.96 (1H, s, H-6′), 6.07, 5.99 (each 1H, d, J = 2.1 Hz, H-6, H-8), 5.47 (1H, s, H-2), 4.54 (1H, dd, J = 5.8, 6.3 Hz, Cys-α), 4.29 (1H, br s, H-3), 3.80−3.93 (3H, m, Gly-α, Glu-α), 3.15 (2H, d like, Cys-β), 2.92 (1H, dd, J = 3.8, 16.5 Hz, H-4), 2.81 (1H, br d, J = 16.5 Hz, H-4), 2.56 (2H, m, Glu-γ), 2.19 (2H, m, Glu-β); ^{13}C NMR (75 MHz, in acetone-d_6 + D_2O) δ 174.80, 174.01, 173.42, 172.07 (COO), 157.33, 156.90, 156.87 (C-5, C-7, C-8a), 148.02, 147.33 (C-3′, 5′), 134.90, 132.85 (C-1′, C-4′), 109.02, 108.15 (C-2′, C-6′), 99.67 (C-4a), 96.34, 95.64 (C-6, C-8), 77.15 (C-2), 65.70 (C-3), 54.71, 54.25 (Glu-α, Cys-α), 42.08 (Gly-α), 37.94 (Cys-β), 32.27 (Glu-γ), 29.39 (C-4), 26.87 (Glu-β). Anal. Calcd for $C_{25}H_{29}N_3O_{13}S$·2H_2O: C, 46.37%; H, 5.14%; N, 6.49%. Found: C, 46.11%; H, 5.24%; N, 6.15%.

*2′-Glutathionyl-(−)-epicatechin (**8**):* white amorphous powder; $[\alpha]_D$ −30.3° (*c* 0.4, MeOH); FABMS, *m*/*z* 596 $[M + H]^+$, 618 $[M + Na]^+$, 634 $[M + K]^+$; ^{1}H NMR (300 MHz, in acetone-d_6 + D_2O) δ 7.19, 6.95 (each 1H, d, J = 8.2 Hz, H-5′, H-6′), 6.07, 5.97 (each 1H, d, J = 2.2 Hz, H-6, H-8), 5.48 (1H, s, H-2), 4.56 (1H, dd, J = 5.0, 8.0 Hz, Cys-α), 4.28 (1H, br s, H-3), 3.80−3.95 (3H, m, Gly-α, Glu-α), 3.24 (2H, br s, Cys-β), 2.92 (1H, dd, J = 3.3, 17.3 Hz, H-4), 2.80 (1H, br d, J = 17.3 Hz, H-4), 2.55 (2H, m, Glu-γ), 2.21 (2H, m, Glu-β); ^{13}C NMR (75 MHz, in acetone-d_6 + D_2O) δ 174.63, 173.47, 172.84, 171.89 (COO), 157.34, 156.97(2C) (C-5, C-7, C-8a), 146.99, 145.12 (C-3′,4′), 134.20 (C-1′), 120.48, 118.00, 116.41 (C-2′, C-5′, C-6′), 99.61 (C-4a), 96.24, 95.49 (C-6, C-8), 77.22 (C-2), 65.51 (C-3), 54.60, 54.28 (Glu-α, Cys-α), 41.67 (Gly-α), 37.06 (Cys-β), 32.23 (Glu-γ), 29.28 (C-4), 26.81 (Glu-β). Anal. Calcd for $C_{25}H_{29}N_3O_{12}S$·1.5H_2O: C, 48.23%; H, 5.18%; N, 6.75%. Found: C, 48.43%; H, 5.19%; N, 6.66%.

*5′-Glutathionyl-(−)-epicatechin (**9**):* white amorphous powder; $[\alpha]_D$ −66.5° (*c* 0.3, MeOH), FABMS, *m*/*z* 596 $[M + H]^+$, 618 $[M + Na]^+$, 634 $[M + K]^+$; ^{1}H NMR (300 MHz, in acetone-d_6 + D_2O) δ 7.12, 7.03 (each 1H, br s, H-2′, H-6′), 6.06, 5.99 (each 1H, br s, H-6, H-8), 4.88 (1H, s, H-2), 4.53 (1H, br t, J = 7.4 Hz, Cys-α), 4.30 (1H, br s, H-3), 3.86 (overlapped with HOD signal, m, Gly-α, Glu-α), 3.28 (2H, br s, Cys-β), 2.87, 2.76 (each 1H, br d, J = 14.3 Hz, H-4), 2.53 (2H, m, Glu-γ), 2.18 (2H, m, Glu-β); ^{13}C NMR (75 MHz, in acetone-d_6 + D_2O) δ 174.61, 173.81, 173.16, 172.17 (COO), 157.19, 157.01, 156.39 (C-5, C-7, C-8a), 145.41 (2C) (C-3′,4′), 131.98 (C-1′), 123.62, 119.81, 115.18 (C-2′, C-5′, C-6′), 99.51 (C-4a), 96.33, 94.20 (C-6, C-8), 78.64

Table 1. Formation of Theaflavin (**3**), Theanaphthoquinone (**4**), and Dehydrotheasinensin (**6**) from **1** and **2** (5 h of Reaction unless Otherwise Noted)

family name		plant name	part	**3**[a]	**4**[a]	**6**[a]
Asteridae	Asteraceae	*Artemisia princeps* (wormwood)	leaves	5.4	14.2	
		Arctium lappa (edible burdock)	root	3.1		1.1
		Petasites japonicus (Japanese butterbur)	stem	*b*		0.1
	Caprifoliaceae	*Viburnum odoratissimum* (sweet viburnum)	leaves	27.0		
	Rubiaceae	*Gardenia jasminoides* (cape jasmine)	leaves	3.7	25.5	
	Oleaceae	*Ligustrum japonicum* (Japanese privet)	leaves	*b*		
	Lamiaceae	*Mentha spicata* (spearmint)	leaves	*b*		
		Perilla frutescens (perilla)	leaves	*b*		1.7
	Verbenaceae	*Clerodendrum trichotomum*	leaves	0.5		
	Boraginaceae	*Borago officinalis* (common borage)	leaves	2.5		3.0
	Convolvulaceae	*Ipomoea batatas* (sweet potato)	root	1.0		0.2
	Solanaceae	*Capsicum annuum* var. *grossum* (bell pepper)	fruit	0.7		
		Solanum melongena (eggplant)	fruit	24.9		0.5
		Solanum tuberosum (potato)	rhizome	6.9		0.8
	Apocynaceae	*Nerium oleander* var. *indicum* (oleander)	leaves	14.5		0.5
Rosidae	Apiaceae	*Daucus carota* (carrot)	root	2.0		0.3
	Rutaceae	*Citrus paradisi* (grapefruit)	fruit	*d*		
		Citrus unshiu (Satsuma mandarin)	fruit (unripe)	2.8		2.8
			fruit (ripe)	*d*		
	Anacardiaceae	*Mangifera indica* (mango)	fruit	1.0		
	Sapindaceae	*Litchi chinensis* (litchi)	fruit	7.7		0.7
			peel	2.9	7.4	1.5
	Vitaceae	*Vitis vinifera* (grape)	fruit	18.2		
	Euphorbiaceae	*Phyllanthus urinaria*	leaves	1.9	5.2	
	Punicaceae	*Punica granatum* (pomegranate)	fruit	*d*		
	Fabaceae	*Phaseolus vulgaris* (common bean)	fruit, seed	0.7		1.8
	Rosaceae	*Eriobotrya japonica* (loquat)	fruit (unripe)	55.7	8.6	
			fruit (ripe)	15.9	19.9	0.8
		Malus pumila (apple)	fruit	22.3		
		Prunus mume (Japanese apricot)	fruit	17.4		
		Pyrus pyrifolia (Japanese pear)	fruit	55.6	26.4	
	Hydrangeaceae	*Hydrangea macrophylla* (hydrrangea)	leaves	6.9	1.7	
	Pittosporaceae	*Pitttosporum tobira* (tobira)	leaves	10.8		1.7
Dilleniidae						
	Ebenaceae	*Diospyros kaki* (persimmon)	fruit	1.1		
	Ericaceae	*Vaccinium myrtillus* (blueberry)	fruit	33.2	2.3	
	Brassicaeae	*Brassica oleracea* var. *capitata* (cabbage)	leaves	*d*		
		Eutrema wasabi (wasabi)	root	*d*		
	Cucurbitaceae	*Cucurbita moschata* (pumpkin)	fruit	1.4		2.1
		Momordica charantia (balsam pear)	fruit	*c*		
	Caricaceae	*Carica papaya* (papaya)	fruit	*d*		
	Malvaceae	*Abelmoschus esculentus* (okura)	fruit	0.7		
	Elaeocarpaceae	*Eleaocarpus sylvestris*	leaves	*d*		
	Actinidiaceae	*Actinidia chinensis* (kiwi fruit)	fruit	0.4		
	Theaceae	*Camellia japonica* (common camellia)	leaves	16.8	7.5	
		Camellia sasanqua (sasanqua)	leaves	23.6	2.3	
		Camellia sinensis (tea) (5 h of reaction)	leaves	29.7[e]	2.5	
		(8 h of reaction)	leaves	14.7[e]	2.0	3.5
Caryophyllidae	Polygonaceae	*Polygonum cuspidatum* (Japanese knotweed)	leaves	*d*		
	Amaranthaceae	*Achyranthes fauriei*	leaves	*b*		
	Chenopodiaceae	*Spinacia oleracea* (spinach)	leaves	4.0		0.6
Hamamelidae	Fagaceae	*Quercus dentata* (Daimio oak)	leaves	1.8		
	Myricaceae	*Myrica rubra* (Chinese strawberry tree)	fruit	*d*		
	Moraceae	*Ficus carica* (common fig)	fruit	2.8		
	Hamamelidaceae	*Distylium racemosum*	leaves	*d*		
Magniliidae	Nymphaeaceae	*Nelumbo nucifera* (Indian lotus)	rhizome	2.1		0.4
	Saururaceae	*Houttuynia cordata* (Houttuynia herb)	leaves	21.5	9.9	
	Lauraceae	*Persea americana* (avocado)	fruit	*b*		
Liliidae	Dioscoreaceae	*Dioscorea opposita* (Chinese yam)	root	2.9	7.2	1.4
	Liliaceae	*Allium fistulosum* (Welsh onion)	leaves	0.4		1.1
Zingiberidae	Zingiberaceae	*Zingiber mioga* (Mioga ginger)	seedling	0.3		
	Musaceae	*Musa acuminata* (banana)	fruit	2.4	15.8	
	Blomeliaceae	*Ananas comosus* (pineapple)	fruit	0.1		
Commelinidae	Poaceae	*Zea mays* (maize)	seed	0.6		0.7
Arecidae	Araceae	*Colocasia esculenta* (taro)	rhizome	6.3		2.4
Gymnospermae	Ginkgoaceae	*Ginkgo biloba* (ginkgo)	fruit	*d*		
Basidiomycetes	Tricholomataceae	*Lentinus edodes* (shiitake mashuroom)	fruit body	2.8		

[a] Yield (mol %) from *2* (16.3 mmol). [b] Catechins and theaflavin completely disappeared, and large broad peaks due to polymeric products were observed. [c] Yields were <0.05%. [d] No reaction. [e] Blank values were subtracted.

(C-2), 66.50 (C-3), 54.60, 53.99 (Glu-α, Cys-α), 41.94 (Gly-α), 36.22 (Cys-β), 32.27 (Glu-γ), 28.53 (C-4), 26.95 (Glu-β). Anal. Calcd for $C_{25}H_{29}N_3O_{12}S\cdot H_2O$: C, 48.94%; H, 5.09%; N, 6.85%. Found: C, 49.27%; H, 5.15%; N, 6.54%.

Oxidation of Theaflavin in the Presence of Epicatechin. Fresh banana (50 g) was homogenized with H_2O (100 mL) in a Waring blender and filtered through four layers of gauze. The homogenate (0.45 mL) was mixed with an aqueous solution (0.45 mL) containing **3** (1.5

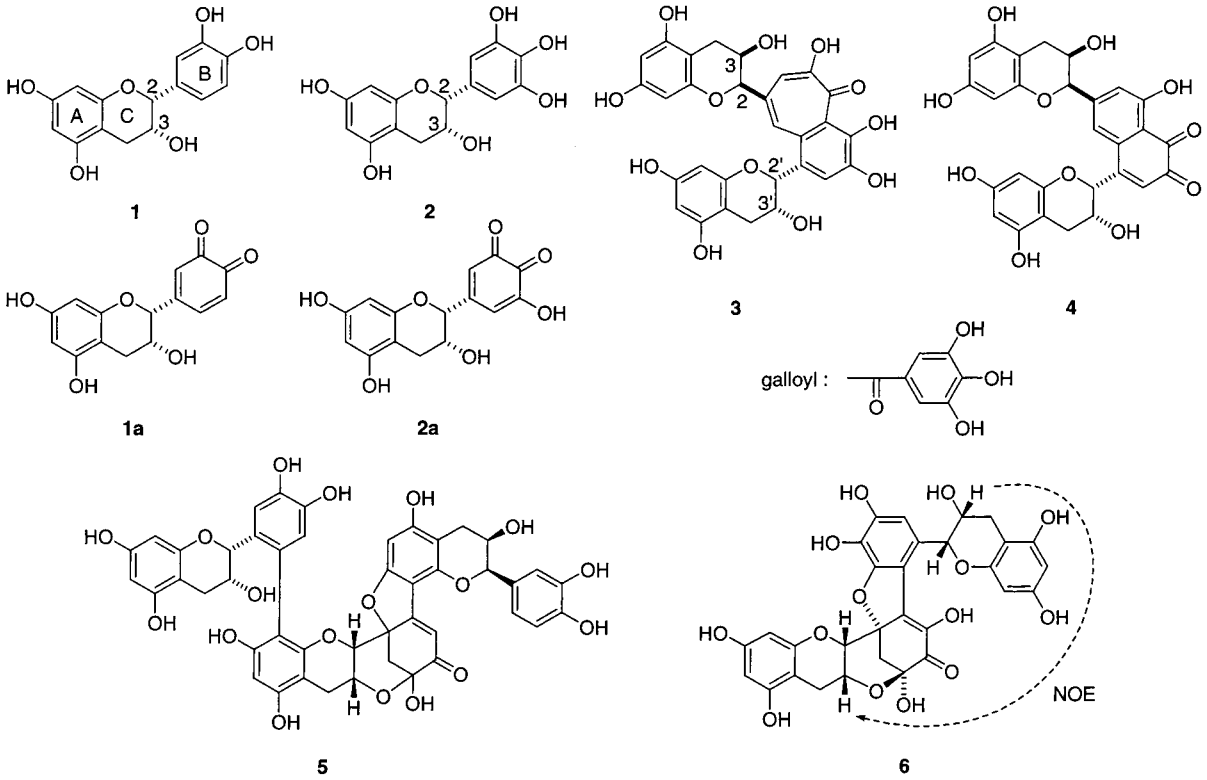


Figure 1. Structures of compounds **1**–**6** and the NOESY correlation observed for **6**.

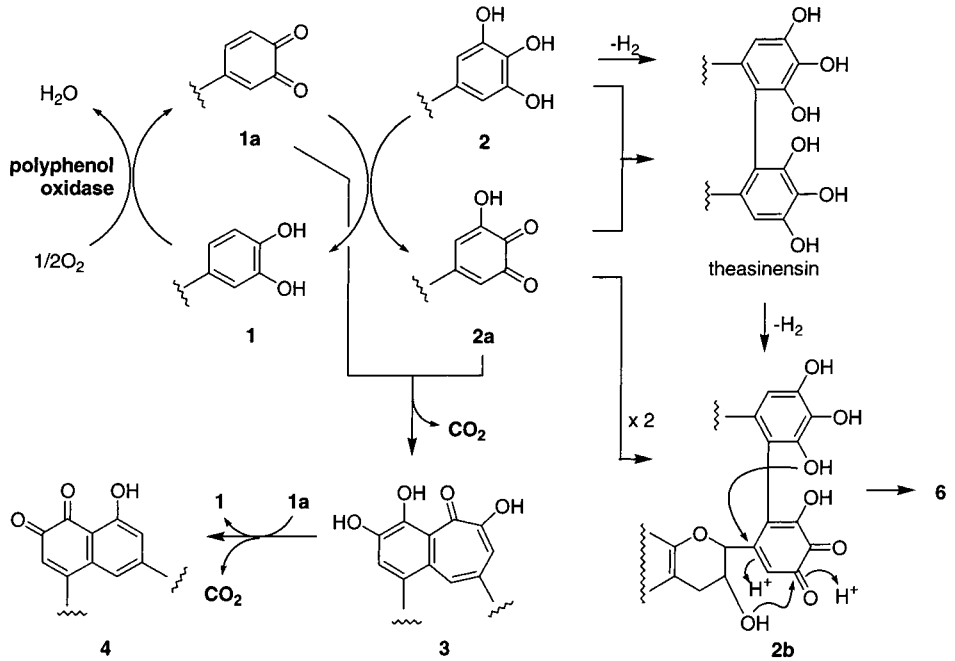
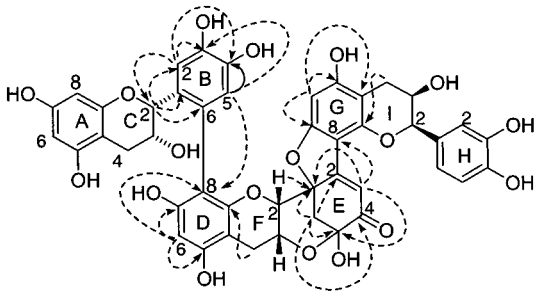


Figure 2. Possible mechanism for production of **3**, **4**, and **6** from **1** and **2**.

mg) and **1** (0.7 mg) and vigorously stirred for 30 min at room temperature. The mixture was mixed with EtOH (4.1 mL), filtered through a membrane filter (0.45 μm), and analyzed by HPLC. Concurrently, a solution containing only **3** was treated in the same manner.

RESULTS AND DISCUSSION

Oxidation of Catechin by Plant Homogenates. Previously, we reported the oxidation of (−)-epigallocatechin (**2**) on treatment with a banana homogenate (*4*). Although most of **2** was polymerized, the presence of monomeric (**2a**; **Figure 1**) and dimeric (**2b**; **Figure 2**) quinone intermediates could be demonstrated by isolation of their phenazine derivatives. The structure of the dimer (**2b**) suggested that the coupling of the B rings is important in the oxidation of **2**. In contrast, oxidation of (−)-epicatechin (**1**) under similar conditions was different, and a new trimeric metabolite (**5**) was isolated from a complex mixture of products including polymeric substances. The negative-ion FABMS of **5** exhibited an $[M - H]^-$ peak at *m*/*z* 863, indicating that this compound is an epicatechin trimer. The ^1H and ^{13}C NMR spectra were similar to those of two known oxidation products of (+)-catechin, that is, dehydrodicatechin



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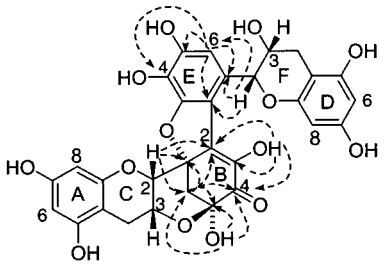

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Figure 3. Selected HMBC correlations observed for products **5** and **6**.

A (*5*, *6*) and catechin-(6′−8)-catechin (*7*). Comparison of the ^{13}C NMR spectral data of **5** with those of dehydrodicatechin A suggested the presence of an oxidized dibenzofuran moiety (E and G rings; **Figure 3**) and a hemiketal ring at the E-5 position. The appearance of isolated aromatic proton signals at δ 6.95 (1H, d, J = 0.5 Hz, B-2) and 6.36 (1H, s, B-5) was similar to those of the B′ ring proton singlets of catechin-(6′−8)-catechin. A long-range ^{1}H−^{1}H coupling of the aromatic proton signal at δ 6.95 (B-2) with the C-2 benzylic proton was observed in the ^{1}H−^{1}H COSY spectrum. Another aromatic proton signal at δ 6.36 (B-5) was correlated with the D-8 carbon in the HMBC spectrum (**Figure 3**). A careful analysis of the HMBC correlations (**Figure 3**) indicated that this product was formed by C−C bond formation between the B-6 of epicatechin and the D-8 of the isomer of dehydrodicatechin A with 2,3-cis configurations. These spectroscopic analyses could not unambiguously determine the positions of the two interflavan bonds on the D and G rings, that is, the 6 or 8 position; however, the D-8 and G-8 positions were more relevant, because electrophilic substitution on catechin A rings preferentially occurs at the 8 position rather than the 6 position (*8*). The result suggested that the nucleophilic addition of the phloroglucinol A ring to the B ring *o*-quinone prevailed in oxidation of epicatechin.

Oxidation of a Mixture of Catechins by Various Plants. In contrast with the above-mentioned oxidation of individual catechins, it is known that the coexistence of **1** and **2** results in the formation of a different oxidation product, theaflavin (**3**) (*1*). Previously, we have demonstrated that **3** and its oxidation metabolite, theanaphthoquinone (**4**), were formed by treatment of a mixture of **1** and **2** with a homogenate of fresh tea leaves or banana (*2*). The reactions using these two plants resembled each other, and their products with banana homogenate were much simpler and easier to analyze compared to the reaction with tea leaves, which additionally produced many minor and complex products. Therefore, study on the mechanism for catechin oxidation by treatment with banana was suggested as being able to provide useful information for understanding the complex oxidation during black tea manufacturing. As an extension of the study, the oxidation of a mixture of **1** and **2** by treatment with various plant homogenates was compared.

Among 62 plants tested, 46 species were capable of synthesizing theaflavin regardless of whether the plant contained catechins or not (**Table 1**). In all experiments, blank solutions were treated concurrently in the same manner, and no theaflavin peaks were detected in these tests, except for experiments using tea leaves. The peak areas of the blank solutions using tea leaves (5 and 8 h) were subtracted from those observed in the reaction mixtures. Loquat, Japanese pear, and blueberry showed higher activity than fresh tea leaves after 5 h of reaction. Plants with high activity also tended to generate **4** from **3**. Six plants, Petasites, Ligustrum, Mentha, and Perilla being four, completely oxidized not only **1** and **2** but also **3** after 5 h of reaction, and the HPLC chromatograms of their reaction mixtures exhibited only large broad peaks, indicating the production of polymeric substances.

In addition to **3** and **4**, a characteristic pigment **6** was detected in the reaction mixture with fresh tea leaves after a prolonged reaction time of 8 h. Compound **6** was also generated by reactions with unripe orange, pumpkin, and eggplant (**Table 1**). In all blank tests, peaks arising from **4** and **6** were lower than detection limits. The large scale treatment of **1** and **2** with fresh tea leaves allowed the isolation of compound **6**, and the structure was spectroscopically characterized as an oxidation product of an isomer of theasinensin, a constituent of oolong and black teas (*3*, *9*). Product **6** showed a strong UV absorption at 397 nm, and its dimeric nature was indicated by a molecular mass determined to be 608 on the basis of the result of FABMS [m/z 607 (M − H)$^-$]. Analysis of the ^{13}C NMR, HSQC, and HMBC spectra suggested the presence of a conjugated cyclohexenone moiety [δ 189.56 (B-4), 139.73 (B-3), 134.25 (B-2), 94.67 (B-5), 89.57 (B-1), 40.67 (B-6)] similar to that of **5**. The upfield shift ($\Delta\delta \sim 3.0$) of the C-4 carbon (δ 25.02) also resembled that of **5** [δ 24.18 (F-4)], suggesting a hemiketal ring formation of the adjacent C-3 hydroxyl group with the hemiketal carbon (B-5) of the cyclohexenone ring. The most valuable information was obtained from the HMBC spectrum (**Figure 3**), and the ^{1}H−^{13}C correlations observed in the spectrum led us to construct the oxidized dibenzofuran moiety related to that of **5**. The ^{1}H-^{1}H COSY spectrum exhibited long-range couplings of C-2 (δ 4.17, br s) with B-6 (δ 2.33, dd, J = 1.1, 11.4 Hz) (W-coupling) and F-2 (δ 6.11, br s) with H_{E-6} (δ 7.04, d, J = 1.0 Hz) (benzylic coupling), both of which were consistent with the structure of **6**. Furthermore, the NOESY spectrum revealed strong NOE correlation between C-3 and F-3, which unequivocally determined the configuration of the cyclohexenone ring (**Figure 1**). On the basis of the above spectroscopic evidence, the structure of **6** was established and named dehydrotheasinensin. There are two possible pathways to form **6** from **2** (**Figure 2**). In the first pathway, **6** is synthesized by oxidation of theasinensin, which is generated by a radical coupling of two molecules of **2** or by condensation between **2** and its *o*-quinone **2a**. In the alternative pathway, the quinone precursor (**2b**) is directly formed by coupling of the two B ring *o*-quinones (**2a**). The configuration of the cyclohexenone moiety of **6** suggested that if **6** was produced via theasinensin, the atropisomer of the biphenyl bond of the precursor is the *S* configuration (theasinensin E), which is the opposite of the major isomer (theasinensin C) isolated from oolong and black teas (*9*).

As mentioned previously, **4** was produced on treatment with 16 plant homogenates (**Table 1**), all of which showed high capabilities to synthesize **3**. The pH of the reaction mixture producing **4** was in the range of 3.5−5.5 and was the same as that producing **3**. On the other hand, the pH range of the reaction mixture with the plants producing **6** was 4.5−7.2, which is

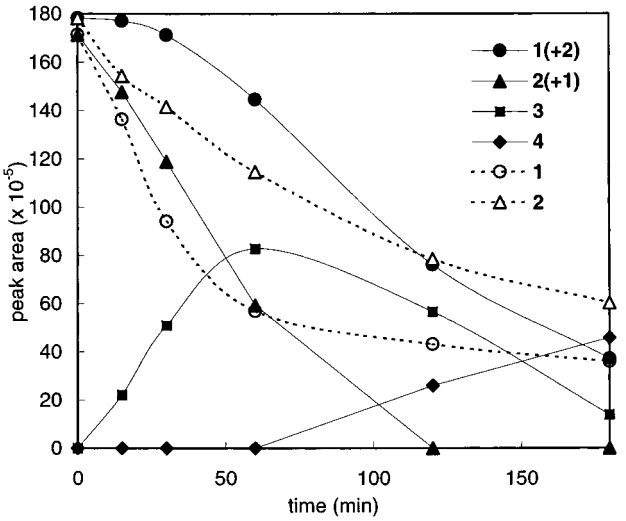

Figure 4. Time course of catechin oxidation with banana homogenate: (solid symbols) oxidation of a mixture of **1** and **2**; (open symbols) separate oxidation of **1** and **2**.

somewhat higher than that for theaflavin synthesis. This difference seemed to be related to the difference of the pH optima of the partially purified polyphenol oxidase prepared from tea leaves in the formation of theaflavin (pH 5) and thearubigin (pH 6) (*10*). In addition to the similarity of the pH optima, because it was supposed that oxidation products of **2** participate in the formation of thearubigin components (*11*), production of **6** was possibly related to thearubigin formation.

Oxidation of Epigallocatechin and Theaflavin by Epicatechin Quinone. When **1** and **2** were treated separately with banana homogenate, the rate of decrease of **1** was faster than that of **2** (**Figure 4**, open symbols). However, when the mixture of **1** and **2** was treated in the same manner, **2** more decreased rapidly compared to **1** (**Figure 4**, closed symbols). This was because **2** was oxidized by epicatechin quinone (**1a**) accompanied by regeneration of **1** (*10*). In the mechanism, molecules of **1** acted as electron carriers; that is, electrons transferred from **2** to epicatechin quinones (**1a**), formed by enzymatic oxidation of **1**, afforded epigallocatechin quinone (**2a**) and **1** (**Figure 2**). This mechanism was supported by an acceleration of the rate of decrease of **2** at a higher concentration of **1** and a prolongation of the lag time of the decrease of **1** at a higher concentration of **2** (data not shown). The mechanism was chemically confirmed by the following experiment. When a mixture of **1** and **2** was treated with banana homogenate in the presence of glutathione, **1** was completely converted into 2′-glutathionyl-(−)-epicatechin (**8**) and 5′-glutathionyl-(−)-epicatechin (**9**). By contrast, 72% of **2** remained unchanged and only small amounts of 2′-glutathionyl-(−)-epigallocatechin (**7**) were isolated (**Figure 5**). When **1** and **2** were treated separately in a similar manner, the same products were obtained in almost the same ratios. These results revealed that epicatechin was rapidly oxidized to *o*-quinone (**1a**) by polyphenol oxidase and it reacted rapidly with glutathione to give **8** and **9** (*12*). In contrast, the oxidation of epigallocatechin is very slow in the absence of epicatechin and only small amounts of quinone (**2a**) were produced (*4*).

The time course of the oxidation of a mixture of **1** and **2** with a banana homogenate (**Figure 4**) showed that the concentration of **3** began to decrease when **2** was exhausted, while at the same time **4** appeared in the reaction mixture. This suggested that **3** was oxidized to **4** by epicatechin quinone (**1a**) (*13*). This was supported by the treatment of **3** with banana homogenate.

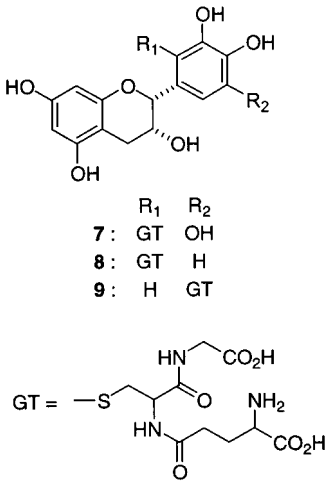

Figure 5. Structures of glutathione conjugates of **1** and **2**.

In the presence of **1**, 87% of **3** was decomposed within 30 min and a significant amount of **4** was generated (peak area of **4** was 25% of that of **3** at 0 min). Without **1**, 88% of **3** remained unchanged at 30 min and only a very small amount of **4** (<2% of that of **3** at 0 min) was produced.

It is noteworthy that the color of the reaction mixture dramatically changed from reddish orange to greenish black when **2** was exhausted and **3** began to be oxidized. A similar color change was also observed immediately after the addition of a banana homogenate to the mixture of **1** and **3**. This color change is probably caused by the formation of a quinhydrone type $\pi-\pi$ complex between **3** and **1a** (*14*). The black color steadily changed to light brown within 10 min, by which time **3** was almost consumed.

In this work, we have demonstrated that many plants are capable of synthesizing **3** from **1** and **2**. The yields of **3** on treatment with Japanese pear and loquat homogenates were much higher than that of fresh tea leaves. Addition of these plant homogenates into green tea infusion produced theaflavins, **3** and its 3-*O*-, 3′-*O*-, and 3, 3′-di-*O*-gallates, indicating that the black tea pigments can be produced from green tea without any chemical reagents (*15*). Theaflavins are brilliant reddish-orange pigments important for the quality of black tea and possess beneficial effects for human health, such as antioxidative and antimutagenic effects and inhibition of glucosyltransferase and α-amylase (*16*−*21*). Our results provide a new method for the production of theaflavins or theaflavin-containing food materials from green tea. The theaflavin content of the product can be varied according to species and amounts of plant homogenates and reaction time. Furthermore, our research disclosed the mechanism of the oxidation of **3** to **4** and the formation of **6** from **2**. In these reactions as well as theaflavin synthesis, epicatechin quinone (**1a**) plays an important role as an oxidizing agent of **2** and **3**. Because the content of theaflavins represents a high variance component of the selling price (*22*), generation of **4** and **6** is undesirable for black tea manufacturing. If these side reactions can be artificially diminished during tea fermentation, the concentration of theaflavins of the product will increase. Optimization of the theaflavin formation based on these results and further chemical study on the catechin oxidation products is now in progress.

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LITERATURE CITED

(1) Davies, A. P.; Goodsall, C.; Cai, Y.; Davis, A. L.; Lewis, J. R.; Wilkins, J.; Wan, X.; Clifford, M. N.; Powell, C.; Parry, A.; Thiru, A.; Safford, R.; Nursten, H. E. Black tea dimeric and oligomeric pigments—structures and formation. In *Plant Polyphenols 2: Chemistry, Biology, Pharmacology, Ecology*; Gross, G. G., Hemingway, R. W., Yoshida, T., Eds.; Kluwer Academic/Plenum Publishers: New York, 1999; pp 697−724.

(2) Tanaka, T.; Betsumiya, Y.; Mine, C.; Kouno, I. Theanaphthoquinone, a novel pigment oxidatively derived from theaflavin during tea-fermentation. *Chem. Commun.* **2000**, 1365−1366.

(3) Nonaka, G.; Kawahara, O.; Nishioka, I. Tannins and related compounds. XV. A new class of dimeric flavan-3-ol gallates, theasinensins A and B, and proanthocyanididin gallates from green tea leaf (1). *Chem. Pharm. Bull.* **1983**, *31*, 3906−3914.

(4) Tanaka, T.; Kondou, K.; Kouno, I. Oxidation and epimerization of epigallocatechin in banana fruits. *Phytochemistry* **2000**, *53*, 311−316.

(5) Weinges, K.; Mattauch, H.; Wilkins, C.; Frost, D. Spectroskopiche und chemische konstitutionsaufklarung des dehydrodicatechins A. *Liebigs Ann. Chem.* **1971**, *754*, 124−136.

(6) Van Soest, T. C. Aufklarung der moleckularstruktur der dehydrodicatechins A durch rontgenstrukturanalyse seines bromoheptamethylathers. *Liebigs Ann. Chem.* **1971**, *754*, 137−138.

(7) Guyot, S.; Vercauteren, J.; Cheynier, V. Structural determination of colourless and yellow dimers resulting from (+)-catechin coupling catalysed by grape polyphenoloxidase. *Phytochemistry* **1996**, *42*, 1279−1288.

(8) Tanaka, T.; Takahashi, R.; Kouno, I.; Nonaka, G. Chemical evidence for the de-astringency (insolubilization of tannins) of persimmon fruit. *J. Chem. Soc., Perkin Trans. 1* **1994**, 3013−3022.

(9) Hashimoto, F.; Nonaka, G.; Nishioka, I. Tannins and related compounds. LXIX. Isolation and structure elucidation of B,B′-linked bisflavanoids, theasinensins D−G and oolongtheanin from Oolong tea (2). *Chem. Pharm. Bull.* **1988**, *36*, 1676−1684.

(10) Robertson, A. Effects of physical and chemical conditions on the *in vitro* oxidation of tea leaf catechins. *Phytochemistry* **1983**, *22*, 889−896.

(11) Robertson, A.; Bendall, D. S. Production and HPLC analysis of black tea theaflavins and thearubigins during *in vitro* oxidation. *Phytochemistry* **1983**, *22*, 883−887.

(12) Monks, T. J.; Lau, S. S. Biological reactivity of polyphenolic-glutathione conjugates. *Chem. Res. Toxicol.* **1997**, *10*, 1296−1313.

(13) Bajaj, K. L.; Anan, T.; Tsushida, T.; Ikegawa, K. Effects of (−)-epicatechin on oxidation of theaflavins by polyphenol oxidase from tea leaves. *Agric. Biol. Chem.* **1987**, *51*, 1767−1772.

(14) Haslam, E. *Practical Polyphenolics, From Structure to Molecular Recognition and Physiological Action*; Cambridge University Press: Cambridge, U.K., 1998; pp 335−373.

(15) Collier, P. D.; Bryce, T.; Mallows, R.; Thomas, P. E. The theaflavins of black tea. *Tetrahedron* **1973**, *29*, 125−142.

(16) Hattori, M.; Kusumoto, I. T.; Namba, T.; Ishigami, T.; Hara, Y. Effect of tea polyphenols on glucan synthesis by glucosyltransferase from Streptococcus mutans. *Chem. Pharm. Bull.* **1990**, *38*, 717−720.

(17) Shiraki, M.; Hara, Y.; Osawa, T.; Kumon, H.; Nakayama, T.; Kawakishi, S. Antioxidative and antimutagenic effects of theaflavins from black tea. *Mutat. Res.* **1994**, *323*, 29−34.

(18) Matsumoto, N.; Kohri, T.; Okushio, K.; Hara, Y. Inhibitory effects of tea catechins, black tea extract and oolong tea extract on hepatocarcinogenesis in rat. *Jpn J. Cancer Res.* **1996**, *56*, 1034−1038.

(19) Matsumoto, N.; Okushio, K.; Hara, Y. Effect of black tea polyphenols on plasma lipids in cholesterol-fed rats. *J. Nutr. Sci. Vitaminol.* **1998**, *44*, 337−342.

(20) Hara, Y.; Honda, M. The inhibition of α-amylase by tea polyphenols. *Agric. Biol. Chem.* **1990**, *54*, 1939−1945.

(21) Honda, M.; Hara, Y. Inhibition of rat small intestinal sucrase and α-glucosidase activities by tea polyphenols. *Biosci., Biotechnol., Biochem.* **1993**, *57*, 123−124.

(22) Hilton, P. J. In vitro oxidation of flavanols from tea leaf. *Phytochemistry* **1972**, *11*, 1243−1248.

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