JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2005,** *53,* 7571−7578 **7571**

A Novel Black Tea Pigment and Two New Oxidation Products of Epigallocatechin-3-*O*-gallate

TAKASHI TANAKA,* YOSUKE MATSUO, AND ISAO KOUNO

Graduate School of Biomedical Sciences, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki 852-8521, Japan

During tea fermentation, oxidation−reduction dismutation of a number of quinone metabolites of tea catechins yields numerous minor products, which make it difficult to separate and purify black tea polyphenols. In this study, epigallocatechin-3-*O*-gallate was enzymatically oxidized and then the unstable quinone metabolites in the oxidation mixture were hydrogenated with 2-mercaptoethanol to reduce production of inseparable minor dismutation products. As a result, three new oxidation products including a new black tea pigment were isolated, and their structures were determined based on chemical and spectroscopic data. Dehydrotheasinensin AQ is a new reddish-orange pigment with a 1,2-diketone structure, and its presence in commercial black tea was confirmed. In addition, a new quinone dimer with a complex caged structure and a trimer of epigallocatechin-3-*O*-gallate were also isolated and their production mechanisms are proposed. The presence of this trimer suggested participation of galloyl quinones in production of minor polyphenols in black tea.

KEYWORDS: Black tea; dehydrotheasinensin AQ; quinone dimer; polyphenol; oxidation

INTRODUCTION

Manufactured and consumed worldwide, black tea is an important polyphenol-rich beverage with a higher polyphenol content than coffee, cocoa, and green tea (*1*−*3*). During its fermentation, four major tea catechins originally contained in fresh leaves are enzymatically oxidized and converted to various oxidation products comprising black tea polyphenols. Of these oxidation products, characteristic pigments have been particularly studied since the end of the 1950s (*4*). Black tea pigments are usually classified into two major groups, theaflavins and thearubigins. Theaflavin and its galloyl esters (**3**) (**Figure 1**) are well-characterized catechin dimers with a characteristic benzotropolone unit produced by oxidative coupling between pyrogallol type catechins [(−)-epigallocatechin and (−)-epigallocatechin-3-*O*-gallate (**1**)] and catechol type catechins [(−)-epicatechin and (−)-epicatechin-3-*O*-gallate (**2**)] (**Figure 1**) (*5*). The theaflavin content of black tea leaves is usually 0.8−2.8% depending on the conditions of fermentation (*6*−*9*). On the other hand, thearubigins constitute up to 60% of the solids in black tea infusions (*6*) and are therefore much more important with respect to coloration and taste. However, the definition of thearubigins remains ambiguous and little is known about their chemical structures despite substantial efforts by many research groups (*10*, *11*). Recently, structures with benzotropolone units derived by oxidative coupling between galloyl groups and catechol type catechin B-rings have been proposed as nonpolar thearubigin components (*12*).

In addition to theaflavins and thearubigins, many unidentified colorless oxidation products are produced during tea fermentation (*13*, *14*). Recently, we demonstrated a new oxidation mechanism of (−)-epigallocatechin-3-*O*-gallate (**1**), the most abundant tea catechin in fresh tea leaves, during tea fermentation (*15*−*17*). In this mechanism, dehydrotheasinensin A (**4**) and EGCg quinone dimer A (**5**) (*18*) are produced as major products by stereoselective dimerization of the *o*-quinone (**1a**) (**Figure 2**). Compound **4** is unstable on heating to yield theasinensins A (**6**) and D (**7**), a pair of atropisomers of B- and B′-linked dimers of **1**. In addition to these reduction products, a number of oxidation products, including oolongtheanin (**10**) and **11**, are generated simultaneously; therefore, the decomposition of **4** involves oxidation−reduction dismutation (*15*). Because theasinensins are major black tea polyphenols comparable to theaflavins, this mechanism is important in the formation of black tea polyphenols (*14*). However, the yield of **4** and **5** from **1** in a previous in vitro enzymatic oxidation experiment did not exceed 40%, suggesting the presence of different oxidation mechanisms. Our lack of understanding of the oxidation of **1** is mainly due to the presence of numerous minor inseparable products, some of which probably originate from the dismutation of **4** or related unknown quinone metabolites (*15*). We previously demonstrated that the dismutation of **4** occurs in the final stage of tea fermentation, when the fermented leaves are heated and dried (*16*). In addition, we also showed that the unstable quinone **4** is easily hydrogenated by treatment with a moderate reducing agent such as ascorbic acid or thiol compounds, yielding theasinensin A (**6**) stereoselectively (*15*).

In the present study, on the basis of the above findings, we chemically reduced **4** and related unstable quinones produced

* To whom correspondence should be addressed. Tel: 81(0)95 819 2433. Fax: 81(0)95 819 2477. E-mail: t-tanaka@net.nagasaki-u.ac.jp.

10.1021/jf0512656 CCC: $30.25
Published on Web 08/27/2005

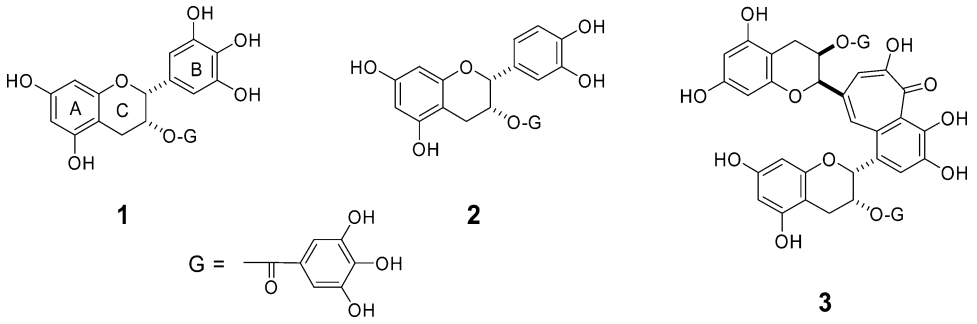

Figure 1. Structures of compounds **1**–**3**.

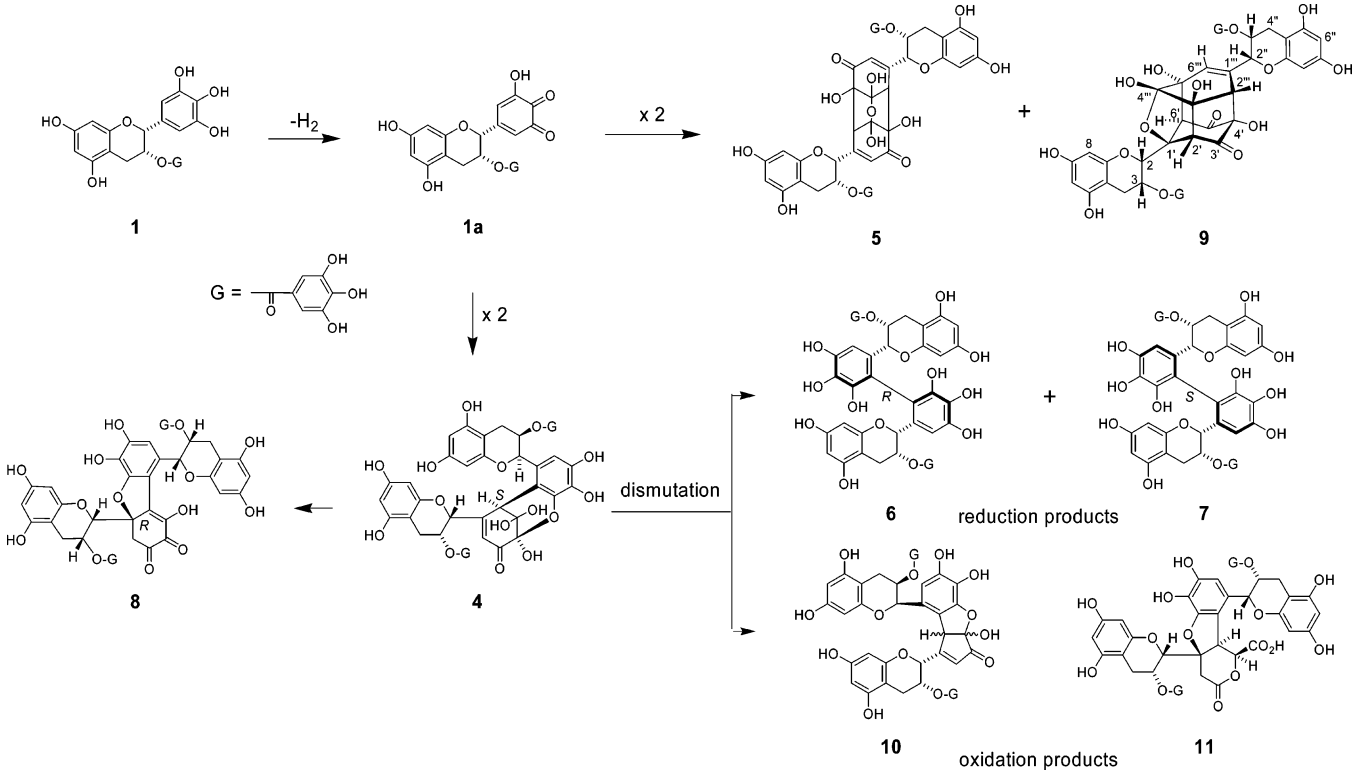

Figure 2. Oxidation of epigallocatechin-3-*O*-gallate.

in the initial enzymatic oxidation reaction mixture in order to prevent the dismutation reaction. This procedure was expected to allow us to isolate unknown and unstable quinone metabolites related to **4** as reduction products. In addition, the composition of the reaction mixture was expected to become simplified owing to the absence of dismutation products; therefore, chromatographic separations were also expected to become easier. On the basis of the structure elucidation and biogenesis of the catechin oxidation products, a new oxidation mechanism for production of one component of black tea polyphenols is proposed.

MATERIALS AND METHODS

General Procedures. Ultraviolet (UV) spectra were obtained with a Jasco V-560 UV/vis spectrophotometer, and optical rotations were measured with a Jasco DIP-370 digital polarimeter. ^{1}H, ^{13}C NMR, ^{1}H−^{1}H correlation spectroscopy (COSY), ^{1}H NMR nuclear Overhauser and exchange spectroscopy (NOESY), heteronuclear single quantum coherence (HSQC), and heteronuclear multiple bond connectivity (HMBC) spectra were recorded in a mixture of acetone-d_6 and D_2O (19:1, v/v) at 27 °C with a Varian Unity plus 500 spectrometer operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C. Coupling constants are expressed in Hz, and chemical shifts are given on a δ (ppm) scale with tetramethylsilane as an internal standard. HMQC (J_{CH} = 140 Hz), HMBC ($^nJ_{CH}$ optimized for 8 Hz), and NOESY (mixing time 0.50 s) experiments were performed using standard Varian pulse sequences. Mass spectra (MS) were recorded on a JEOL JMS-700N spectrometer, and glycerol or *m*-nitrobenzyl alcohol was used as the matrix for fast atom bombardment mass spectroscopy (FAB-MS) measurements.

Column chromatography was performed with Diaion HP20SS, MCI-gel CHP 20P (75−150 μm) (Mitsubishi Chemical Co., Japan), Sephadex LH-20 (25−100 μm) (Pharmacia Fine Chemical Co., Ltd.), and Chromatorex ODS (Fuji Silysia Chemical Ltd., Japan). Thin-layer chromatography (TLC) was performed on 0.2 mm precoated Kieselgel 60 F_{254} plates (Merck) with benzene−ethyl formate−formic acid (1:7:1, v/v) or chloroform−methanol−water (14:6:1, v/v). Spots were detected by UV illumination and by spraying with 2% ethanolic $FeCl_3$ or 10% sulfuric acid reagent followed by heating. Analytical high-performance liquid chromatography (HPLC) was performed on a 250 mm × 4.6 mm i.d. Cosmosil 5C_{18}-AR II column (Nacalai Tesque Inc.) with gradient elutions of CH_3CN in 50 mM H_3PO_4 from 10 to 30% in 30 min and 30−75% in 15 min at a flow rate of 0.8 mL/min and detection with a Jasco MD-910 photodiode array detector. Epigallocatechin-3-*O*-gallate (**1**) was isolated from commercial green tea and recrystallized from water.

Enzymatic Oxidation and Treatment with 2-Mercaptoethanol. Japanese pear fruits (562 g) were homogenized with 500 mL of H_2O and filtered through four layers of gauze (*19*). The filtrate was then mixed with an aqueous solution of **1** (10 g/300 mL) (**Figure 3A**) and

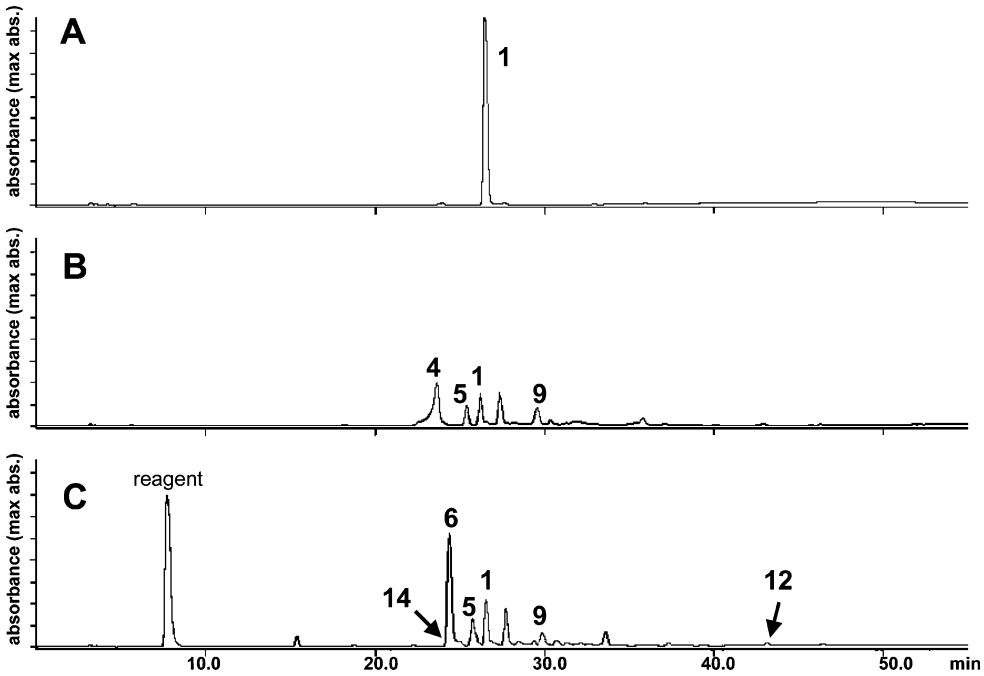

Figure 3. HPLC profiles of the reaction mixture. (**A**) Before addition of pear homogenate. (**B**) After stirring for 3 h with pear homogenate. (**C**) After addition of 2-mercaptoethanol (18 h).

vigorously stirred for 3 h. At this stage, HPLC analysis of the reaction mixture indicated a reduction of **1** and accumulation of **4** and **5** together with several unknown products (**Figure 3B**). The mixture was poured into 8% 2-mercaptoethanol in EtOH (1.3 L) at −20 °C and gently stirred at room temperature for 18 h. HPLC analysis showed that **4** was completely converted to **6** (**Figure 3C**). Insoluble materials in the mixture were consequently filtered off, and the filtrate was concentrated until EtOH was removed in vacuo. The resulting aqueous solution was applied to a 35 cm × 4 cm i.d. MCI-gel CHP20P column with H_2O containing increasing proportions of MeOH (10% stepwise elution from 0 to 80%, each 500 mL) to give two $FeCl_3$ positive fractions, A (eluted out with 30−60% MeOH) and B (eluted out with 70−80% MeOH). Fraction A was further fractionated by chromatography over a 27 cm × 4 cm i.d. Sephadex LH-20 column with H_2O containing 0−100% MeOH (20% stepwise elution, each 500 mL) yielding seven fractions. The first three fractions eluted out with 0−60% MeOH and contained 2-mercaptoethanol and gallic acid. Fraction A-4 (4.83 g) was further separated by a 33 cm × 4 cm i.d. Sephadex LH-20 column with EtOH (1 L) and then with 50% aqueous acetone (1 L) to give two fractions, A-4-1 and A-4-2. The fraction A-4-1 was applied to a 26 cm × 4 cm i.d. MCI-gel CHP20P column with 0−50% MeOH (5% stepwise elution, each 100 mL) to give **1** (519 mg). The fraction A-4-2 was subjected to chromatography on a 26 cm × 4 cm i.d. MCI-gel CHP20P column with 0−50% MeOH (5% stepwise elution, each 100 mL) to give **5** (554 mg), **6** (486 mg), and a crude crop of **9**, which was purified by chromatography on a 26 cm × 4 cm i.d. Chromatorex ODS column with 0−40% MeOH (5% stepwise elution, each 100 mL) to yield **9** (305 mg). Fraction A-5 (4.42 g) was chromatographed over a 25 cm × 3 cm i.d. Sephadex LH-20 column with 0−20% H_2O in EtOH (10% stepwise elution, each 300 mL) to give **6** (2.75 g) and a fraction containing mainly **6**, **14**, and isomers of **14**. This fraction was applied to a 27 cm × 3 cm i.d. Chromatorex ODS column with 0−40% MeOH (5% stepwise elution, each 100 mL) and then to a 21 cm × 2.5 cm i.d. Sephadex LH-20 column with 80−100% MeOH (10% stepwise elution, each 200 mL) and then MeOH−H_2O−acetone (90:5:5, v/v/v, 200 mL) to give a crude crop of **14**. Purification of the compound was achieved by chromatography on a 21 cm × 2.5 cm i.d. MCI-gel CHP20P column 0−40% MeOH (5% stepwise elution, each 100 mL) to yield **14** (43 mg). Fraction A-6 was subjected to a 21 cm × 2.5 cm i.d. MCI-gel CHP20P column with 20−60% MeOH (5% stepwise elution, each 100 mL) to give **8** (51.3 mg). Fraction B was separated by chromatography on a 19 cm × 2 cm i.d. Sephadex LH-20 column with 80−100% MeOH (10% stepwise elution, each 100 mL) to give epitheaflagallin 3-*O*-gallate (**12**) (60.4 mg).

Dehydrotheasinensin AQ (8). Red amorphous powder; $[\alpha]_D^{25}$ −603.8° (*c* 0.12, MeOH). FAB-MS *m*/*z* 913 $[M + H]^+$, *m*/*z* 935 $[M + Na]^+$. UV λ_{max} nm (log ϵ): 447 (4.06), 277 (4.53). IR ν_{max} cm^{-1}: 3355, 1698, 1686, 1604, 1521. Anal. calcd for $C_{44}H_{32}O_{22}\cdot 3/2H_2O$: C, 56.23; H, 3.75. Found: C, 56.45; H, 4.02. ^1H and ^{13}C NMR data: See **Table 1**.

Preparation of a Quinoxaline Derivative (8a). *o*-Phenylenediamine (5 mg) in acetic acid (0.1 mL) was added to a solution of **8** (15 mg) in 0.5 mL of EtOH, and the mixture was stirred at 25 °C for 2 h. The mixture was directly applied to a 15 cm × 10 cm i.d. Sephadex LH-20 column eluted with MeOH and then MeOH−H_2O−acetone (95:5:5, v/v/v) to give the quinoxaline derivative **8a** (7.7 mg).

Quinoxaline Derivative (8a). Reddish brown powder; $[\alpha]_D^{25}$ −300.7° (*c* 0.06, methanol). FAB-MS *m*/*z* 985 $[M + H]^+$. UV λ_{max} nm (log ϵ): 379 (3.80), 272 (4.70). ^1H NMR (400 MHz, in acetone-d_6): δ 8.23, 8.01 (each 1H, dd, J = 1.7, 7.5 Hz, Q-5 and Q-8), 7.73 (2H, m, Q-6 and Q-7), 7.35 (2H, s, galloyl-2, 6), 7.08 (2H, s, galloyl-2, 6), 7.02 (1H, s, H-6‴), 6.12 (1H, s, H-2″), 6.06, 6.01 (each 1H, d, J = 2.3 Hz, H-6″, 8″), 5.89, 5.85 (each 1H, br d, J = 3.2 Hz, H-3, 3″), 5.81 (1H, d, J = 2.3 Hz, H-6), 4.98 (1H, d, J = 2.3 Hz, H-8), 4.39, 3.66 (each 1H, d, J = 15.9 Hz, H-6′), 4.19 (1H, br s, H-2), 3.28 (1H, dd, J = 4.4, 17.3 Hz, H-4″), 2.94 (overlapped with HOD signal, H-4, 4″), 2.77 (1H, J = 4.4, 17.6 Hz, H-4).

HPLC Analysis of Black Tea. Commercial black tea (a blended tea produced in India and Sri Lanka, purchased from Mitsui Norin Co., Ltd.) (10 g) was extracted with 70% acetone (2×), and then, the organic solvent was removed by evaporation. The resulting aqueous solution was successively partitioned with ether, $CHCl_3$, and EtOAc, and then, the EtOAc layer was concentrated (0.9 g). The EtOAc extract was applied to a 10 cm × 2 cm i.d. Sephadex LH-20 column and eluted with 80−100% MeOH and then with 50% aqueous acetone to give a fractions mainly containing theaflavins. HPLC analysis of the theaflavin fraction showed a peak at 34.0 min corresponding to **8**, the UV spectrum of which was also identical with that of **8**.

EGCg Quinone Dimer B (9). Tan amorphous powder; $[\alpha]_D^{25}$ −16.4° (*c* 0.18, acetone). FAB-MS *m*/*z* 931 $[M + H]^+$, 949 $[M + H_2O + H]^+$. MALDI TOF-MS *m*/*z*: 953 $[M + Na]^+$, 971 $[M + H_2O + Na]^+$. UV λ_{max}^{EtOH} nm (log ϵ): 278 (4.33). IR ν_{max} cm^{-1}: 3405,

Table 1. ^{1}H and ^{13}C NMR Data for Dehydrotheasinensin AQ (**8**) and EGCg Quinone Dimer B (**9**) (500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR)[a]

		8			**9**			
position		δ_C	δ_H	HMBC (H to C)	δ_C	δ_H	HMBC (H to C)	NOESY
2		76.5	4.25 (br s)	3, 4, 1′, 2′	77.2	4.33 (s)	3, 4, 1′, 2′, 6′	3, 4, 2′, 6′
3		65.9	5.91 (br s)	2, 4, 4a, gal-7 (δ 166.1)	64.6	5.83 (br s)	4a, gal-7 (δ 167.6)	2, 4, 2′, 6′
4		26.7	3.04 (br d, 17.4)	2, 3, 4a, 5, 8a	27.0	2.95 (br d, 17.5)	2, 3, 4a, 5, 8a	2, 3, 4
			2.86 (dd, 4.1, 17.4)	4a, 5, 8a		2.87 (dd, 4.2, 17.5)	4a, 5, 8a	3, 4
4a		98.8				98.8		
5		157.6[b]				157.8[b]		
6		97.3	5.98 (d, 2.3)	4a, 5, 7, 8	96.9	6.06 (d, 2.3)	4a, 5, 7, 8	
7		157.6[b]			157.6[b]			
8		95.4	5.63 (d, 2.3)	4a, 6, 7, 8a	95.7[c]	5.83 (d, 2.3)	4a, 6, 7, 8a	
8a		154.8			155.7			
1′		88.6			78.3			
2′		136.1			63.6	3.39 (br d, 0.9)	2, 1′, 3′, 6′, 2‴, 3‴, 5‴	2, 3, 2‴, 3‴-OH
3′		142.5			200.7			
4′		178.4			81.9			
5′		187.1			199.6			
6′		47.7	4.02 (d, 15.2)	2, 1′, 2′, 4′, 5′	69.8	3.37 (d, 1.8)	2, 1′, 2′, 4′, 5′, 4‴, 5‴	2, 3, 6‴
			3.34 (d, 15.2)	2, 1′, 2′, 5′				
2″		76.4	6.05 (br s)	3″, 4″, 1‴, 2‴, 6‴	76.9	4.60 (br s)	3, 1‴, 2‴, 6‴	3″, 4″, 2‴, 6‴
3″		69.8	5.78 (br d, 4.6)	4″, 4a″, gal-7 (δ 165.9)	65.1	5.94 (br d, 4.6)		2″, 4″, 2‴
4″		26.3	3.18 (dd, 4.6, 17.4)	4a″, 5″, 8a″	26.5	3.06 (dd, 4.6, 17.5)	4a″, 5″, 8a″	2″, 3″
			2.91 (br d, 17.4)	2″, 3″, 4a″, 5″, 8a″		2.93 (br d, 17.5)	2″, 3″, 4a″, 5″, 8a″	3″
4a″		99.30			98.9			
5″		157.5[b]			157.7[b]			
6″		96.5	6.06 (d, 2.3)	4a″, 5″, 7″, 8″	96.8	6.13 (d, 2.3)	4a″, 5″, 7″, 8″	
7″		157.4[b]			157.4[b]			
8″		95.8	6.00 (d, 2.3)	4a″, 7″, 6″	95.6[c]	6.04 (d, 2.3)	4a″, 7″, 6″, 8a″	
8a″		157.3[b]			156.2			
1‴		129.6			136.8			
2‴		112.8			54.0	3.17 (br d, 1.1)	2, 5′, 1‴, 3‴, 4‴, 5‴, 6‴	2′, 2″, 3″, 3‴-OH
3‴		152.8			79.7			
4‴		129.9			110.1			
5‴		151.2			97.4			
6‴		112.3	7.10 (s)	2′, 2″, 1‴, 2‴, 3‴, 4‴, 5‴	127.7	6.03 (br d, 1.8)	2″, 1‴, 4‴	6′, 2″, 3″
3′-OH			8.77 (s)	2′, 3′, 4′				
3‴-OH						4.95 (s)	2′, 3‴	2′, 2‴
galloyl	1	121.9, 121.2			121.3, 121.0			
	2, 6	110.3, 109.9	7.20, 7.07 (each 2H, s)	gal-1, 2, 3, 4, 5, 6, 7	110.6, 110.5	7.19, 7.02 (each 2H, s)	gal-1, 2, 3, 4, 5, 6, 7	
	4	139.4, 138.7			139.3, 139.2			
	3, 5	146.1, 145.9			145.8, 145.6			
	7	166.1, 165.9			167.6, 166.8			

[a] Compounds **8** and **9** were measured in acetone-d_6 and in acetone-d_6 + benzene-d_6 (9:1), respectively. [b,c] Assignments may be interchanged in each column.

1770, 1683, 1608, 1519, 1465. Anal. calcd for $C_{44}H_{34}O_{23}\cdot 7/2H_2O$: C, 53.18; H, 4.16. Found: C, 53.21; H, 4.00. ^{1}H and ^{13}C NMR data: See **Table 1**.

EGCg Trimer (14). White amorphous powder; $[\alpha]_D^{25}$ −205.9° (*c* 0.18, acetone). MALDI TOF-MS *m/z*: 1393 $[M + Na]^+$. FAB-MS (positive mode) *m/z*: 1371 $[M + H]^+$, (negative mode) *m/z*: 1369 $[M - H]^-$. HR FAB-MS *m/z*: 1371.2312 (calcd for $C_{66}H_{51}O_{33}$, 1371.2310). UV λ_{max}^{EtOH} nm (log ϵ): 275 (4.48). ^{1}H NMR (500 MHz, in acetone-d_6): δ 7.14 (1H, s, G-6′), 7.09, 6.97 (each 2H, s, G-2, 6, 2″, 6″), 6.95 (1H, d, J = 0.5 Hz, B-6′), 6.88 (1H, d, J = 0.5 Hz, B-6″), 6.79 (1H, d, J = 0.5 Hz, B-6), 6.03, 6.00 (each 1H, d, J = 2.2 Hz), 5.95 (3H, br d, J = 2.2 Hz), 5.92 (1H, d, J = 2.2 Hz), 5.35 (1H, br d, J = 4.6 Hz, H-3), 5.14 (1H, br d, J = 4.8 Hz, H-3′), 5.03 (1H, br d, J = 3.9 Hz, H-3″), 4.78 (1H, br s, H-2), 4.68 (1H, br s, H-2′), 4.60 (1H, br s, H-2″), 2.93 (1H, brd, J = 17.9 Hz, H-4′), 2.85 (1H, brd, J = 17.2 Hz, H-4), 2.81 (1H, brd, J = 17.2 Hz, H-4″), 2.56 (1H, dd, J = 4.8, 17.9 Hz, H-4′), 2.43 (1H, dd, J = 4.6, 17.2 Hz, H-4), 2.31 (1H, dd, J = 3.9, 17.2 Hz, H-4″). ^{13}C NMR (125 MHz, in acetone-d_6): δ 166.53(2C) (G-7, 7″), 166.20 (G-7′), 158.95, 158.71, 158.41(2C), 157.47(2C), 157.28(3C) (C-5, 7, 8a, 5′, 7′, 8a′, 5″, 7″, 8a″), 146.36, 146.12, 145.87-(2C), 145.81(3C), 145.53, 144.96 (B-3, 5, 3′, 5′, 3″, 5″, G-3, 5, 3′, 5′), 144.55 (G-5′), 144.29 (G-3′), 138.82(2C) (G-4, G-4″), 138.20 (G-4′), 139.95 (B-5′), 133.75 (B-4), 133.45 (B-4″), 129.39 (B-1), 129.15 (B-1′), 128.81 (B-1″), 122.85 (G-1′), 122.01, 121.67 (G-1, 1″), 117.09 (G-2′), 114.62 (B-2″), 112.40 (B-2′), 111.95 (B-2), 111.68 (G-6′), 110.14(2C), 109.97(2C) (G-2, 6, 2″, 6″), 108.42 (B-6′), 108.05 (B-6), 107.72 (B-6″), 99.12 (C-4a″), 98.88 (C-4a′), 98.57 (C-4a), 96.51, 96.35(3C), 96.02, 95.75 (C-6, 8, 6′, 8′, 6″, 8″), 76.46 (C-2″), 76.12

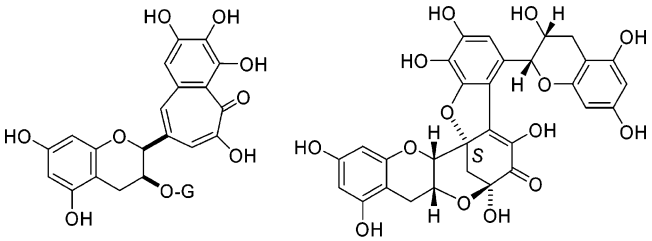

Figure 4. Structures of compounds **12** and **13**.

(C-2′), 76.02 (C-2), 68.70 (C-3″), 68.59 (C-3), 68.37 (C-3′), 27.36 (C-4″), 26.90(2C) (C-4, 4′). Important HMBC correlations (H→C): G-6′→G-1′, 2′, 5′, 4′ 7′; H-3′→G-7′, C-4a′; B-6″→C-2″, B-1″, 2″, 3″, 4″, 5″; H-2″→B-1″, 2″, 6″, C-3″; H-3→G-7, C-4a; H-2→B-1, 2, 6; H-2′→B-1′, 2′, 6′.

RESULTS AND DISCUSSION

(−)-Epigallocatechin 3-*O*-gallate (**1**) was enzymatically oxidized with Japanese pear fruit homogenate, which was previously shown to have a strong ability to convert epigallocatechin and epicatechin into theaflavin (*19*). HPLC analysis of the reaction mixture showed peaks attributable to **1**, dehydrotheasinensin A (**4**), quinone dimer A (**5**), and a new product **9** (**Figure 3B**). 2-Mercaptoethanol was added to the mixture to hydrogenate the unstable quinone products into stable phenols, and HPLC analysis of the resulting solution showed complete conversion of **4** into theasinensin A (**6**) (**Figure 3C**). Preliminary experiments using several reducing agents, such as ascorbic acid, sodium cyanoborohydride, and sodium bisulfite, showed 2-mercaptoethanol to be the most effective for this purpose. Separation of the products was performed by chromatography over MCI-gel CHP20P, Sephadex LH-20, and Chromatorex ODS, resulting in isolation of six products, **5**, **6**, **8**, **9**, **14**, and epitheaflagallin 3-*O*-gallate (**12**) (*20*) (total: 48%), along with recovery of **1** (0.5%). It should be noted that the black tea pigment **12**, which possesses a benzotropolone ring related to that of theaflavin (**Figure 4**), was produced by oxidation of **1** without participation of catechol type catechins.

Dehydrotheasinensin AQ (**8**) was obtained as a yellow amorphous powder and showed UV absorptions at 277 and 447 nm. On the basis of numbers of carbon signals observed in the ^{13}C NMR spectrum, FAB-MS [*m*/*z* 913 $(M + H)^+$, 935 $(M + Na)^+$], and elemental analysis, the molecular formula was shown to be $C_{44}H_{32}O_{22}$. The ^{1}H and ^{13}C NMR spectra (**Table 1**) indicated the presence of two galloyl esters in the molecule. This was consistent with dark blue coloration with the $FeCl_3$ reagent. The spectra also showed two sets of flavan-3-ol A and C rings in the molecule, and the coupling patterns of the signals were similar to those of **1**. Large downfield shifts of C rings H-3 (δ?5.91) and H-3″ (δ 5.78) confirmed the location of the two galloyl esters at these positions. In the HMBC spectrum (**Table 1**), the H-2″ signal was correlated with three aromatic carbons (C-1‴, C-2‴, and C-6‴), and the aromatic methine proton H-6‴ showed cross-peaks with five aromatic carbons (C-1‴−C-5‴) in addition to C-2″. The chemical shifts of these carbons indicated that a pyrogallol ring was attached to C-2‴. H-6‴ showed a long-range (^4J) correlation with an sp^2 carbon at δ 136.1 (C-2′), which correlated with H-2. Because H-2 also showed cross-peaks with an oxygenated quaternary carbon (δ 88.6, C-1′) and a methylene carbon (δ 47.7, C-6′), another B ring is not aromatic. In turn, the H-6′ methylene protons showed correlations with two carbonyl carbons (C-4′ and C-5′). Furthermore, an enolic hydroxyl proton at δ 8.77 was correlated with C-2′, C-4′, and a remaining sp^2 carbon at δ 142.5 (C-3′). These HMBC correlations permitted construction of a 3-hydroxy-3-cyclohexene-1,2-dione structure for this B ring. The unsaturation index (*21*) calculated from the molecular formula and chemical shifts of C-1′ (δ 88.6) and C-3‴ (δ 152.8) suggested occurrence of an ether linkage between C-1′ and C-3‴. The planar structure of **8** was consequently deduced from these spectroscopic data.

The presence of a 1,2-diketone structure was confirmed by reaction with *o*-phenylenediamine, which yielded the quinoxaline derivative **8a**. Interestingly, in the ^{1}H NMR spectrum of **8a**, the A ring H-8 resonated at a significantly higher field (δ 4.99, Δδ about 1.0 ppm) as compared to those of usual flavan-3-ols (around δ 6.0). This unusual upfield shift could only be explained by the shielding effect caused by the quinoxaline residue. As shown in **Figure 5**, there are two possible configurations at C-1′ (A and B). The bulky galloyl group was oriented to the opposite side of the other flavan-3-ol unit owing to steric hindrance; therefore, in structure B (C-1′ *S*), H-8 could not be shielded by the quinoxaline moiety. On the other hand, in structure A (C-1′ *R*), where the galloyl group is oriented outside the molecule, H-8 is located directly underneath the quinoxaline plane and was strongly shielded. The configuration of C-1′ was thus concluded to be *R*.

HPLC analysis of the crude theaflavin fraction obtained from commercial black tea demonstrated the presence of **8** (**Figure 6**). The structure of this new pigment is closely related to that of dehydrotheasinensin A (**4**) and was probably produced by intramolecular addition of a phenolic hydroxyl group at C-3‴ to the conjugated double bond of **4**. The configuration of C-1′ of **8** was consistent with that of **4** and the atropisomerism of its reduction product **6**.

Product **9** was obtained as a white amorphous powder, and its ^{1}H and ^{13}C NMR spectra showed signals arising from two sets of 3-*O*-galloyl-flavan-3-ol A and C rings (**Table 1**). The remaining 12 signals were attributable to two carbonyl (C-3′ and C-5′), two olefinic (C-1‴ and C-6‴), three aliphatic methine (C-2′, C-6′, and C-2‴), one acetal (C-4‴), and four oxygenated quaternary (C-1′, C-4′, C-3‴, and C-5‴) carbons. The total number of carbon signals observed in the spectrum indicated that this compound was a dimer of **1**. Results of FAB-MS (*m*/*z* 931 $[M + H]^+$), MALDI TOF-MS (*m*/*z*: 953 $[M + Na]^+$) and elemental analysis suggested the molecular formula $C_{44}H_{34}O_{23}$. Mutual HMBC correlations (**Table 1**) between CH-2, C-1′, CH-2′, and CH-6′ indicated that the C ring C-2 was attached to C-1′, and this was supported by NOESY correlations (**Table 1**) and long-range ^{1}H−^{1}H couplings between H-2, H-2′, and H-6′. On the other hand, the HMBC and NOESY correlations between CH-2″, C-1‴, CH-2‴, and CH-6‴ suggested the relative locations of these carbons shown in formula **9** (**Figure 2**). This was supported by observation of ^{1}H−^{1}H long-range couplings between H-2″ and H-2‴, H-2″ and H-6‴, and H-2‴ and H-6‴. In the HMBC spectrum, H-2′ and H-6′ were correlated with carbonyl carbons C-3′ and C-5′, respectively, and H-6′ was also correlated with the oxygenated carbon C-4′. The remaining two oxygenated quaternary carbons C-3‴ and C-5‴ and the acetal carbon C-4‴ showed correlation peaks with H-2‴, while the acetal carbon C-4‴ was coupled with H-6‴. In addition, C-5‴ was correlated through ^4J coupling with the H-2‴ located at a homoallylic position. Because C-1″−C-6″ and C-1‴−C-6‴ originated from pyrogallol B rings of **1**, these HMBC correlations suggested the disposition of these carbons as shown in formula **9**. The ^{1}H NMR spectrum exhibited a D_2O exchangeable singlet due to a hydroxyl group correlated with

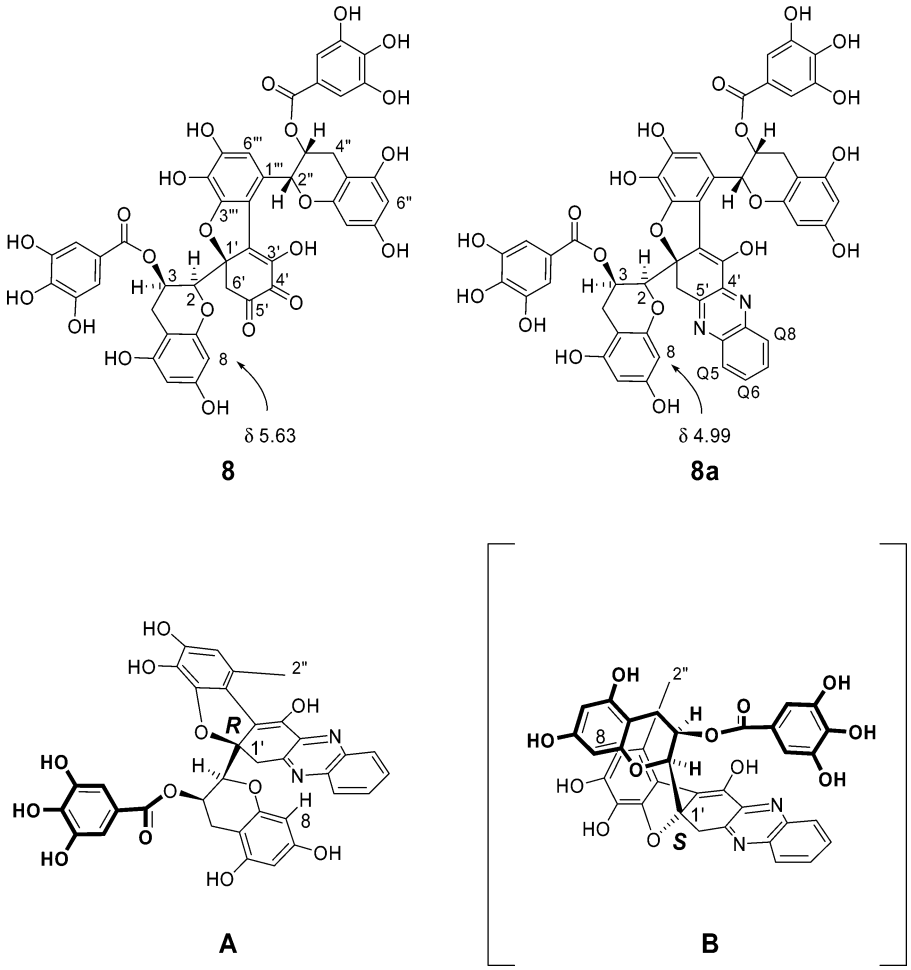

Figure 5. Structures of **8** and **8b**. Structures A and B represent **8a** and its 1′-*S* isomer, respectively.

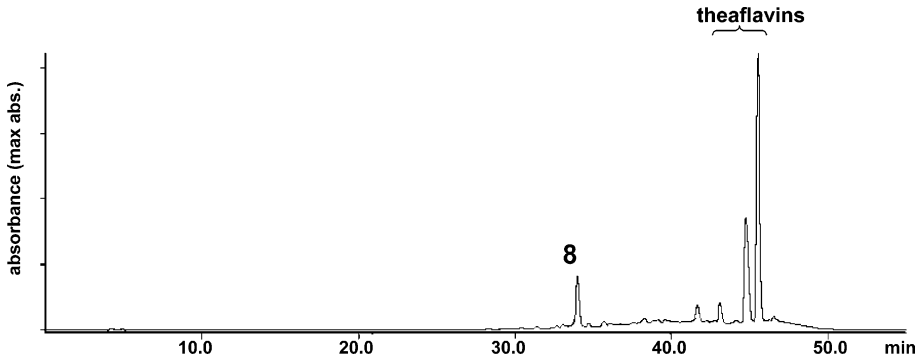

Figure 6. HPLC profile (at 450 nm) of a crude theaflavin fraction of commercial black tea.

C-3‴ and C-2′ in the HMBC spectrum, suggesting a C−C linkage between C-2′ and C-3‴. This was supported by the appearance of HMBC correlations of H-2′ with C-3‴ and C-2‴, and observation of NOESY correlations of the hydroxyl proton with H-2′ and H-2‴. Another C−C linkage between C-3′ and C-5‴ was deduced from HMBC correlations of H-5′ with C-4‴ and C-5‴. Furthermore, HMBC correlations of H-2‴ with the C-5′ carbonyl carbon suggested additional C−C bonding between C-4′ and C-2‴. The presence of these C−C linkages was supported by NOESY correlations between H-6′ and H-6‴ and H-2′ and H-2‴, respectively. Taking the unsaturation index[28] calculated from the molecular formula and chemical shift of the acetal carbon C-4‴ (δ 110.1) into account, formation of an acetal ring between C-1′ and C-4‴ was suggested. On the basis of the above spectroscopic observations, the structure of **9** could be represented by formula **9** and tentatively named EGCg quinone dimer B. The structure of this compound was related to that of EGCg quinone dimer A (**5**), and a plausible mechanism for formation of **9** from **1** is illustrated in **Figure 7**, in which successive addition of electron-rich carbons to carbonyl groups forms the caged structure. However, we could not determine the absolute configuration of this compound.

Compound **14** was isolated as a white amorphous powder, and MALDI TOF-MS (*m*/*z*: 1393 $[M + Na]^+$) and FAB-MS (positive mode: *m*/*z* 1371 $[M + H]^+$; negative mode: *m*/*z* 1369 $[M - H]^-$) indicated that it is a trimer of **1**. The high resolution FAB-MS showed a $[M + H]^+$ peak at *m*/*z* 1371.2312, indicating the molecular formula $C_{66}H_{50}O_{33}$. The ^1H and ^{13}C NMR spectra were related to those of **6** and **7**; however, they exhibited signals arising from three sets of flavan-3-ol A and C rings and two galloyl groups. In the ^1H NMR spectrum, four one-proton aromatic singlets were observed as well as two two-proton

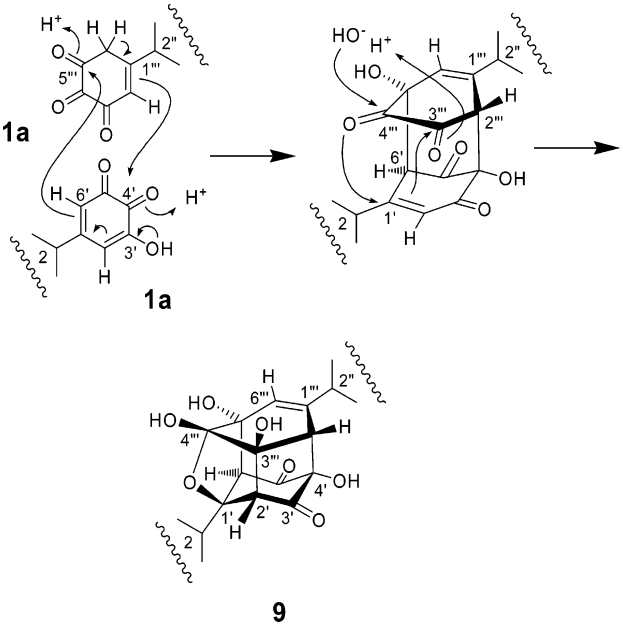

Figure 7. Proposed mechanism for production of quinone dimer **9**.

singlets due to galloyl groups. These results suggested that the B ring C-2 of an additional epigallocatechin-3-*O*-gallate was attached to the C-2 of a galloyl group of theasinensin through a C−C bond. This was supported by HMBC spectroscopic analysis (**Figure 8**), which showed a correlation of H-3′ with the carboxyl group (C−G7′) of the galloyl group attached to the additional epigallocatechin-3-*O*-gallate moiety. The aromatic methine proton of the galloyl group (H−G6′, δ 7.14) showed HMBC correlations with C−G1′ (δ 122.85), C−G2′ (δ 117.09), C−G5′ (δ 144.55), C−G4′ (δ 138.20), and C−G7′ (δ 166.2), and chemical shifts of these carbons were slightly different from those of usual galloyl groups. Consequently, the structure of this trimer was determined to be as shown by formula **14**. The absolute configuration of the B−B′ ring biphenyl bond of the theasinensin moiety in **14** was suggested to be *R*, because theasinensin A (**6**), which has a *R* biphenyl bond, was stereoselectively formed by reduction of **4** with 2-mercaptoethanol, and theasinensin D (**7**) was not detected in this experiment (*15*). In addition, by analogy with formation of theasinensins (**6** and **7** in **Figure 2**), product **14** was presumed to be produced by reduction of product **14a**, which is formed by oxidative coupling of quinones **4a** and **1a**, as shown in **Figure 8**. During the purification procedure, the presence of an isomer of **14** showing similar chromatographic behavior was suggested. Although purification failed, this isomer might be an atropisomer differing in configuration of the galloyl−B″ ring biphenyl bond. In the course of our studies on catechin oxidation, this product represents the first example showing participation of galloyl groups in intermolecular oxidative coupling. As for the benzotropolone moiety, some interesting pigments produced by condensation between galloyl groups and catechol rings have previously been reported (*12*, *21*).

In this experiment, reduction of unstable quinone metabolites, including **4**, with 2-mercaptoethanol in the initial oxidation mixture of **1** effectively reduced production of inseparable minor dismutation products, which interfere with chromatographic purification of metabolites. As a result, we isolated three new oxidation products including a new black tea pigment. The new pigment **8** was structurally related to dehydrotheasinensin E (**13**) (**Figure 4**), which was produced by oxidative coupling of (−)-epigallocatechin (*19*). In the structure of **13**, one carbonyl carbon of 1,2-diketone formed an intramolecular hemiacetal ring with the C ring hydroxyl group. In addition, the absolute configuration of **13** at the benzylic methine was shown to be *S*, which is opposite to that of **8**.

Production of the quinone dimers **5** and **9** indicated the high reactivity of quinone **1a**. These quinone dimers were unstable and decomposed when their aqueous solutions were heated; therefore, it is possible that they do not exist in commercial black tea. Trimer **14** was probably generated by reduction of **14a**, which has a structure analogous to **4**. If **14a** is produced at the fermentation stage of black tea production, it will undergo oxidation−reduction dismutation in a manner similar to that of **4**, resulting in generation of a complex mixture of reduction and oxidation products. Therefore, production of **14** suggested

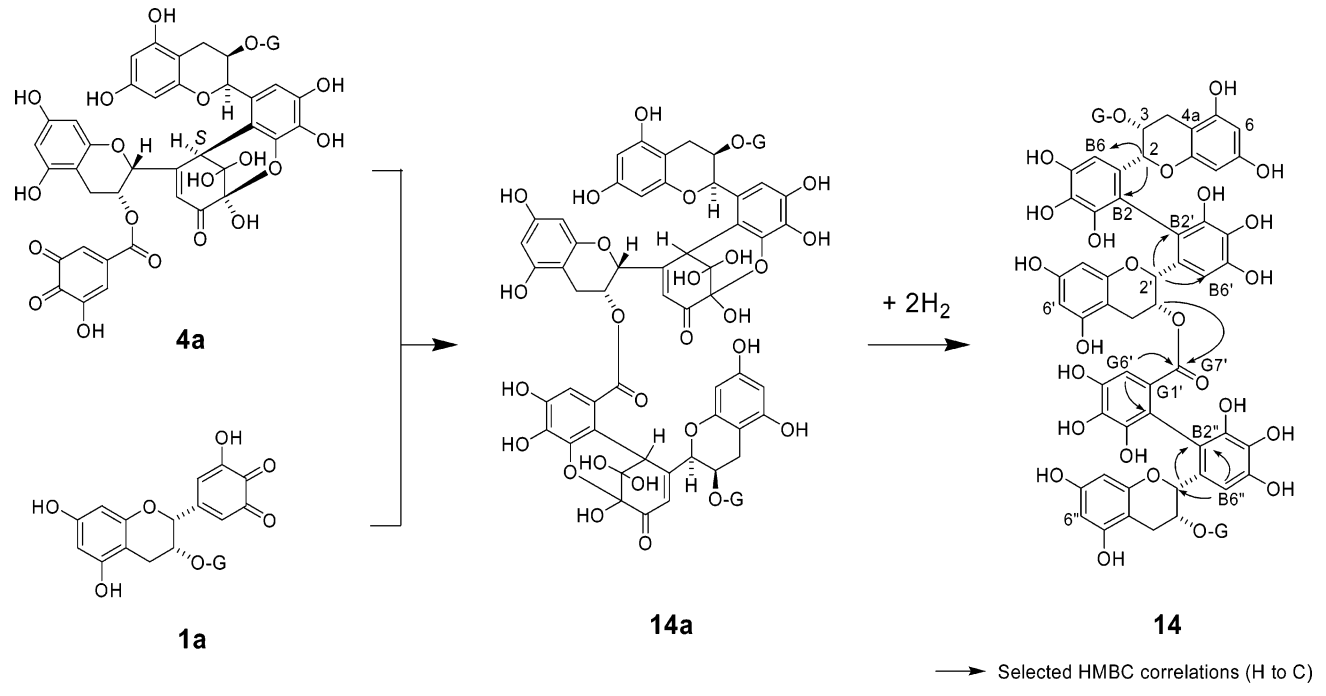

Figure 8. Proposed mechanism for production of trimer **14** and selected HMBC correlations.

that generation and subsequent intermolecular coupling of galloyl quinones contribute to formation of minor polyphenols in black tea. Studies to show the evidence of **14a** being generated during tea fermentation are now in progress.

ABBREVIATIONS USED

COSY, correlation spectroscopy; FABMS, fast atom bombardment mass spectroscopy; HMBC, heteronuclear multiple bond connectivity; HPLC, high-performance liquid chromatography; HSQC, heteronuclear single quantum coherence; NOESY, ^{1}H NMR nuclear Overhauser and exchange spectroscopy.

ACKNOWLEDGMENT

We are grateful to K. Inada and N. Yamaguchi (Nagasaki University) for NMR and MS measurements.

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Received for review May 31, 2005. Revised manuscript received July 26, 2005. Accepted July 26, 2005. This work was supported by a Grant-in-Aid for Scientific Research 12680594 from the Japan Society for the Promotion of Science.

JF0512656