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Structures of two new oxidation products of green tea polyphenols generated by model tea fermentation

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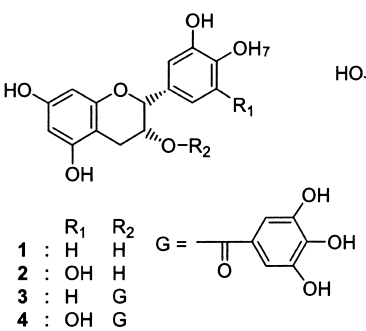
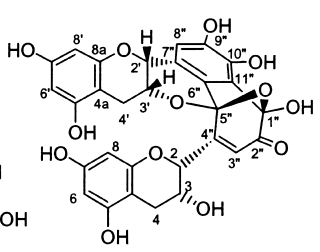
Abstract—To clarify the oxidation mechanism of green tea catechins during tea fermentation, pure catechins were oxidized with a catechin-free homogenate of tea leaf. Oxidation of a mixture of (−)-epicatechin and (−)-epigallocatechin yielded a new metabolite, named dehydrotheaflavin, produced by the oxidation of a benzotropolone moiety of the black tea pigment theaflavin. Similar oxidation of a mixture of (−)-epicatechin and (−)-epigallocatechin 3-*O*-gallate afforded a new dimer of (−)-epigallocatechin 3-*O*-gallate, which was generated by the oxidation and cycloaddition of two pyrogallol rings. Structures were determined by spectroscopic method, and the oxidation mechanisms for the formation of the products were proposed.

Teas are usually classified by the manufacturing process into three categories of fermented (black), unfermented (green) and semifermented (oolong). Among them, black tea accounts for almost 80% of the world tea production. However, the chemical constituents, especially polyphenolic compounds, of black tea remain to be clarified because of their complexity compared to those of green tea. The pigments in black tea infusions comprise two major polyphenolic substances, theaflavins and thearubigins. Theaflavins are well-characterized catechin dimers with a characteristic benzotropolone moiety.[1] On the other hand, thearubigins are a heterogeneous mixture of oxidation products of tea catechins, and little is presently known about their chemical structures.[2] Isolation of thearubigin components from black tea is difficult because they are produced from complex coupling reactions mainly between four major catechins, (−)-epicatechin (**1**), (−)-epigallocatechin (**2**) and their gallates (**3** and **4**, respectively). Therefore, model fermentation experiments using pure catechins are very useful for chemical studies on catechin oxidation during tea fermentation.[3] Coexistence of catechol- and pyrogallol-type of catechins in tea leaves makes the polyphenol oxidation highly characteristic, as represented by the formation of benzotropolone pigments. In a previous paper, we demonstrated that the enzymes preferentially oxidize the catechol-type catechins, and the resulting *o*-quinones in turn oxidize the pyrogallol-type catechins to their *o*-quinones.[4] Therefore, mixtures of the catechol-type (**1** or **3**) and pyrogallol-type catechins (**2** or **4**) were used as substrates for our model fermentation experiments to mimic the catechin oxidation in tea leaves. Our present study focused on the further metabolism of theaflavins and the *o*-quinones of pyrogallol-type catechins. This paper describes the determination of the structure of new oxidation products derived from theaflavin (**6**) and epigallocatechin 3-*O*-gallate (**4**) by the model fermentation experiment, and proposes oxidation mechanisms for their production.

1. Results and discussion

In this model fermentation experiment, a tea leaf homogenate was used as the enzymes, in which polyphenols were removed in advance by adsorption on polyvinylpolypyrrolidone (Polyclar AT®).[4,5] A mixture of **1** and **2** was treated with the tea homogenate and the mixture was successively extracted with AcOEt and 1-BuOH. The AcOEt extract was separated by a combination of column chromatography using Sephadex LH-20, MCI-gel CHP 20P and Chromatorex ODS to yield a new product **5** (0.007% yield from **1**),





Keywords: tea; polyphenol; oxidation; catechin; theaflavin.

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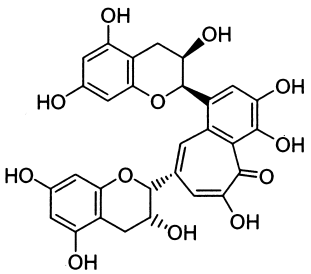
along with desgalloyloolongtheanin,[6] dehydrotheasinensin,[4] theaflavin (**6**),[1] theanaphthoquinone,[5] and theasinensins C and E.[6] Similar model fermentation using a mixture of **1** and **4** afforded a new dimer (**7**) (0.004% yield from **4**) together with theaflavin 3-*O*-gallate, theasinensins A and D,[6] galloyl oolongtheanin[6] and a known dimer **8**.[7] The known compounds were identified by comparisons of their spectral and physical data with those of authentic samples or the values described in the literature.



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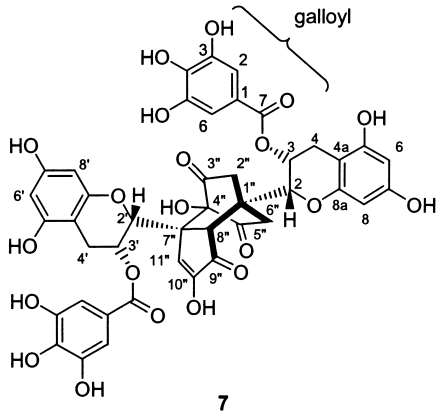

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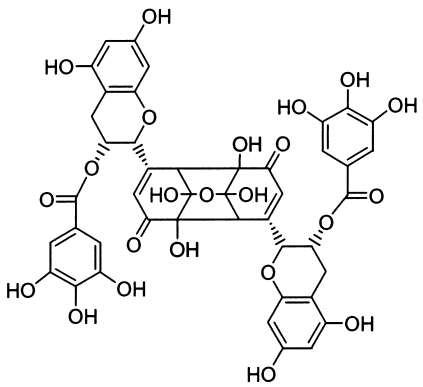

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Dehydrotheaflavin (**5**) was obtained as a reddish brown amorphous powder and showed UV absorption at 360, 296, and 279 nm. The IR absorption at 1676 cm^{-1} suggested presence of a conjugated carbonyl group. The FABMS (negative ion mode) exhibited the $(M-H)^-$ peak at *m/z* 577, indicating that the molecular weight of **5** was 14 mass units larger than that of theaflavin (**6**). In the ^1H and ^{13}C NMR spectra (Table 1), two sets of signals arising from catechin A- and C-rings, which were similar to those of **6**, were observed.[8] The remaining two methine proton signals in the ^1H NMR spectrum and 11 carbon signals in the ^{13}C NMR spectrum were attributable to a moiety originating from catechin B-rings. With the aid of HSQC and HMBC spectra, these signals were assigned to a conjugated carbonyl (C-2″), two acetals (C-1″ and C-5″), and eight sp^2 carbons including two methines (C-3″ and C-8″). The COSY spectrum revealed $^1H-^1H$ long-range coupling of the methine protons H-3″ and H-8″ with the H-2 and H-2′, respectively (Fig. 1). Furthermore, HMBC correlations (Fig. 1) from H-2′ and H-8″ to five aromatic carbons (C-6″–C-10″) suggested the presence of a catechol ring (C-6″–C-11″) similar to that of **6**. On the other hand, in the remaining part of the molecule consisting of C-1″–C-5″, the carbonyl C-2″ was deduced to be conjugated with the C-3″–C-4″ double bond from their chemical shifts (δ 130.3 and 157.0, respectively). In addition, HMBC correlations from the olefinic methine proton H-3″ to the C-2, C-4″ and two acetal carbons (C-1″ and C-5″), as well as correlations from the H-2 to C-3″, C-4″ and C-5″ were observed. These spectral observations and structural comparisons with **6** suggested that **5** was an oxidation product of **6**, in which two of three carbonyl groups of the seven-membered ring were hydrated to form acetals. Since the molecular formula of **5** was deduced to be $C_{29}H_{22}O_{13}$ from the FABMS, elemental analysis, and NMR data, the index of unsaturation was 19; indicating that the two acetal carbons took part in formation of two rings. The large low field shift of C-3′ (δ 69.2) accompanied by the large up field shift of C-2′ (δ 69.5), compared to the chemical shifts of C-3 (δ 63.9) and C-2 (δ 74.8), indicated that the hydroxyl group at C-3′ participated in an acetal ring formation with C-5″. This was supported by the absence of the C-3′ hydroxyl proton signal in the ^1H NMR spectrum, while the C-3 hydroxyl proton resonated at δ 3.50 and coupled with H-3 in the COSY spectrum. Further, it was deduced that another acetal ring was formed between C-1″ and C-5″, to account for the remaining unsaturation, because another acetal carbon C-1″ is located at the γ and δ positions from C-5″. Thus, the plane structure of **5** was presumed to be as shown in Fig. 1. Stereochemistry of **5** was determined by the NOESY experiment, which showed weak NOE correlations of H-8′ with the H-2 and one of the H_2-4 (Fig. 2). Since absolute configurations of the C-2 carbons of **1** and **2** were known to be *R* and **5** originated from **1** and **2**, these NOE correlations unequivocally confirmed the absolute configuration at C-1″ and C-5″ to be *R*. Consequently, the structure of dehydrotheaflavin was concluded to be as shown in the formula **5**.

The other product **7** showed two singlet signals due to two galloyl groups at δ 7.04 and 7.09 along with two sets of the signals arising from catechin A- and C-rings in the ^1H NMR spectrum (Table 1). A low field shift of the C-ring H-3 and H-3′ (δ 5.59 and 5.53, respectively) confirmed that the galloyl groups were located at the C-3 and C-3′ positions of the catechin C-rings. Since **7** was obtained as the oxidation product of a mixture of **1** and **4**, the spectral observation indicated that **7** is a dimer of **4**. This was supported by the appearance of the $(M-H)^-$ ion peak at *m/z* 855 in the FABMS. The remaining part of the molecule, which originated from two pyrogallol B-rings, consisted of 11 carbons including three carbonyls (C-3″, 5″, and 9″), one double bond (C-10″ and 11″), three quaternary carbons (C-1″, C-4″, and C-7″), a methine (C-8″), and two methylenes (C-2″ and C-6″). In the HMBC spectrum

Table 1. ^{13}C (125 MHz) and ^{1}H (500 MHz) NMR data for compounds **5** and **7** (in d_6-acetone)

Atom no.	**5**		**7**		
	^{13}C	^{1}H[a]	^{13}C	^{1}H[a]	HMBC (H to C)
2	74.8	4.50 (br s)	74.1	4.91 (br s)	3, 4, 1″, 2″, 8″
3	63.9	3.97 (br s)	65.8	5.59 (br d, 2.6)	4, 4a
4	27.8	2.40 (br d, 16.9)	27.8	2.85 (dd, 4.3, 17.4)	2, 3, 4a, 5, 8a
		2.19 (dd, 4.3, 16.9)		2.92 (br d, 17.4)	4a, 5, 8a
4a	99.3		98.9		
5	157.4[b]		157.2[b]		
6	96.2	5.93 (d, 2.3)	96.5	6.02 (d, 2.3)	4a, 5, 7, 8
7	157.1[b]		157.3[b]		
8	95.2	5.72 (d, 2.3)	96.1	6.18 (d, 2.3)	4a, 6, 7, 8a
8a	156.1		156.8[b]		
2′	69.5	4.72 (s)	75.1	4.57 (br s)	3′, 4′, 4″, 7″, 8″
3′	69.2	4.46 (dd, 1.4, 3.0)	66.5	5.53 (br d, 3.0)	4′, 4a′, galloyl 7
4′	25.7	3.03 (dd, 1.4, 17.4)	28.1	2.72 (br d, 17.6)	2′, 3′, 4a′, 5′, 8a′
		2.95 (dd, 3.0, 17.4)		2.88 (dd, 4.3, 17.6)	4a′, 5′, 8a′
4a′	98.2		98.7		
5′	157.7[b]		157.4[b]		
6′	96.8	6.12 (d, 2.3)	96.9	6.07 (d, 2.3)	4a′, 5′, 7′, 8′
7′	157.6[b]		157.5[b]		
8′	95.3	5.82 (d, 2.3)	95.2	5.92 (d, 2.3)	4a′, 6′, 7′, 8a′
8a′	155.7		156.2		
1″	106.4		39.4		
2″	198.3		44.0	3.48 (dd, 2.1, 19.2)	1″, 3″
				3.00 (d, 19.2)	1″, 3″, 8″, 9″
3″	130.3	6.34 (d, 1.6)	203.6		
4″	157.0		91.7		
5″	105.9		202.4		
6″	129.5		41.4	2.39 (dd, 2.1, 18.5)	1″, 5″
				2.71 (dd, 1.5, 18.5)	
7″	123.6		52.3		
8″	120.6	7.11 (s)	49.1	4.15 (d, 1.5)	2, 2′, 1″, 2″, 4″, 6″, 7″, 9″, 10″, 11″
9″	148.0		201.4		
10″	152.9		156.6		
11″	111.7		126.5	6.39 (s)	2, 4″, 7″, 8″, 9″
C-3 OH		3.59 (br s)			
Galloyl 1			121.3 (2C)		
Galloyl 2(6)			109.9[c], 110.6[d]	7.09[c], 7.04[d] (each 2H, s)	3′ (from δ 7.04), galloyl 1, 4–7
Galloyl 3(5)			146.0[c], 145.5[d]		
Galloyl 4			139.2[c], 139.7[d]		
Galloyl 7			166.0[c], 165.7[d]		

[a] Multiplicity and coupling constants (Hz) were shown in parentheses.
[b] Assignments may be interchanged.
[c] Galloyl ester at C-3.
[d] Galloyl ester at C-3′.

(Table 1), the H-2 of one of the catechin units was correlated with one of the quaternary carbons at δ 39.4 (C-1″), the methylene carbon at δ 44.0 (C-2″) and the methine carbon at δ 49.1 (C-8″). C-1″ was also correlated with the two methylene (H_2-2″ and one of the H_2-6″) and the methine (H-8″) protons. In addition, correlation of the two methylenes (H_2-2″ and H_2-6″) to C-8″ and two carbonyl

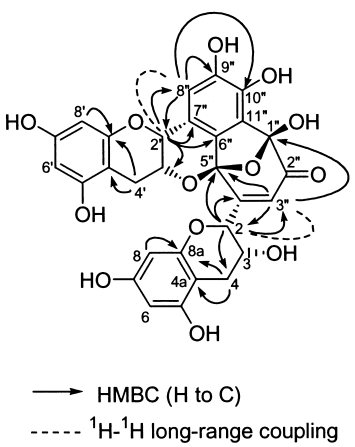


Figure 1. Selected HMBC correlations for **5**.

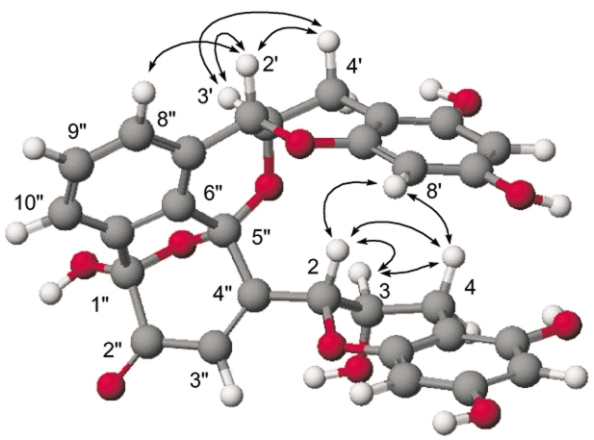


Figure 2. Important NOESY correlations for **5**. The model was drawn with an aid of CAChe® MOPAC.

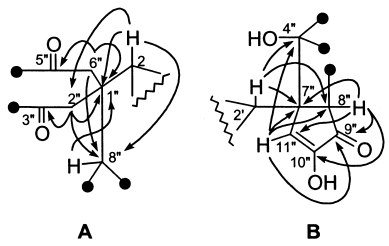


Figure 3. Partial structures of **7** and selected HMBC correlations.

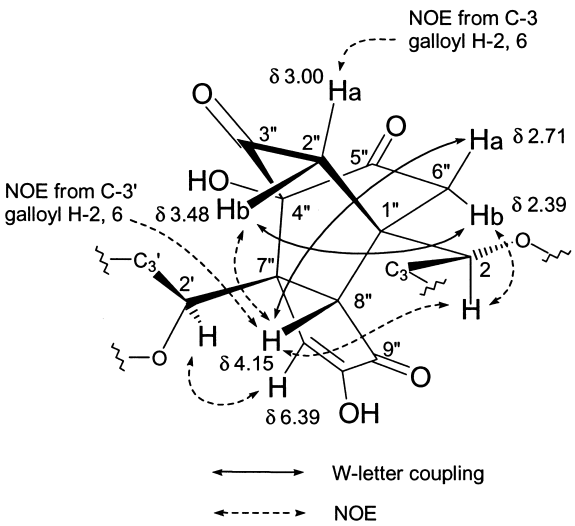
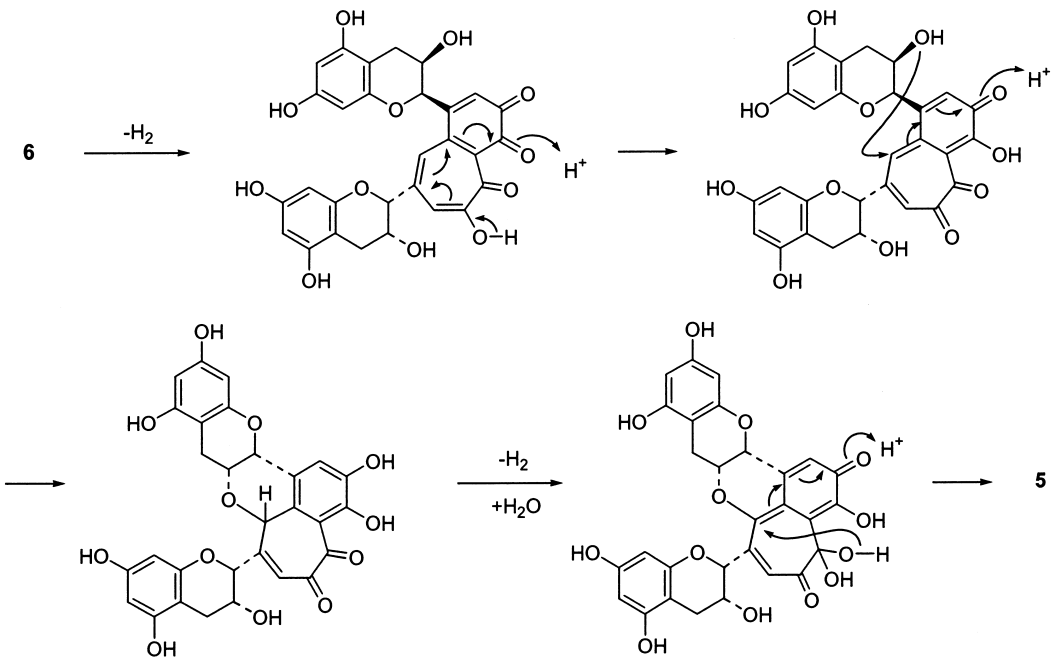


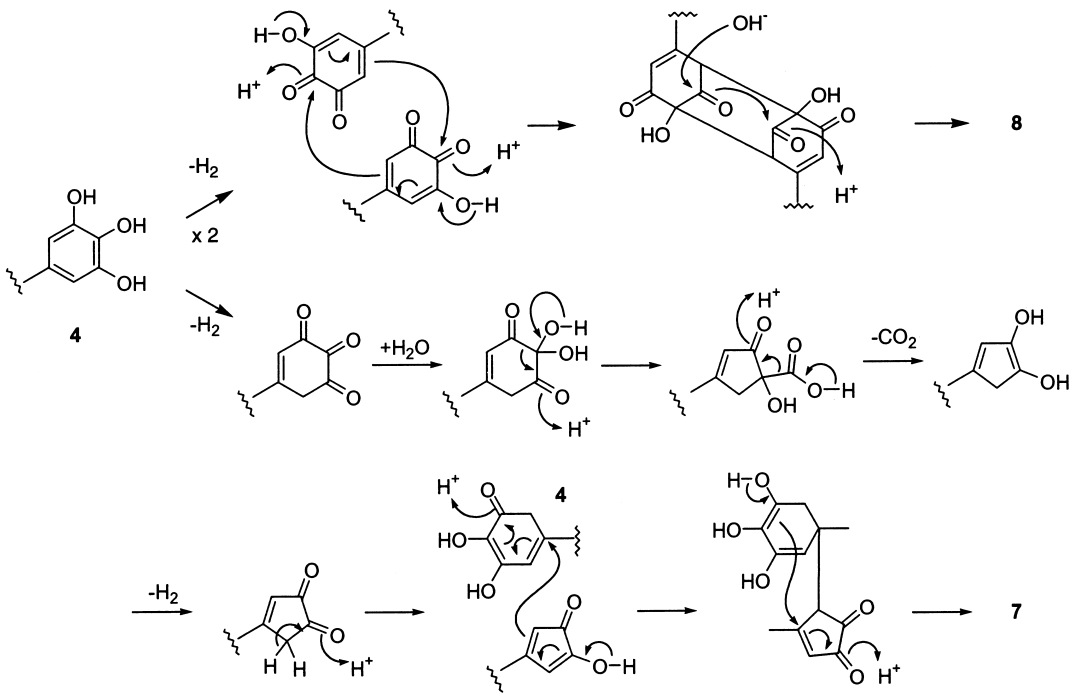
Figure 4. W-letter long-range couplings and NOESY correlations observed for **7**.

carbons at δ 203.6 and 202.4 (C-3″ and C-5″, respectively) suggested the presence of a partial structure A (Fig. 3). On the other hand, the H-2′ of the other catechin unit showed cross-peaks to the methine carbon C-8″ and the two quaternary carbons at δ 91.7 (C-4″) and 52.3 (C-7″). H-8″ was correlated in turn with the C-7″, a carbonyl carbon at δ 201.4 (C-9″), an oxygen-bearing olefinic carbon at δ 156.6 (C-10″), and an olefinic methine at δ 126.5 (C-11″). Further, the olefinic methine proton H-11″ (δ 6.39) was correlated with C-4″, C-7″, C-8″ and C-9″. These HMBC correlations permitted the construction of a partial structure B (Fig. 3). The occurrence of the cyclopentenone ring (C-7″ to C-11″) was also supported by the IR absorption at 1753 cm^{-1}, which appeared separately from the large ester absorption at 1707 cm^{-1}. The connectivity of the partial structures A and B was apparent from the HMBC correlations from the pivotal methine proton H-8″. Consequently, the presence of a tricyclo[5.2.2.$0^{2,6}$]undec-4-en-3,8,11-trione structure in the molecule of **7** was revealed (Fig. 4).

The two methylenes (C-2″ and C-6″) in the symmetrical cyclohexa-1,3-dione ring of the partial structure A were distinguishable from each other by four-bond W-letter coupling between H-8″ and H-6a″ (δ 2.71, $J_{6'',8''}$=1.5 Hz) and between H-6b″ (δ 2.39) and H-2b″ (δ 3.48, $J_{2'',6''}$=2.1 Hz) (Fig. 4). These long-range $^1H-^1H$ couplings indicated that the atoms of H-8″–C-8″–C-1″–C-6″–H-6a″ lie on the same plane, as do the atoms H-2b″–C-2″–C-1″–C-6″–H-6b″. Moreover, this observation implied that H_2-2″ and H-8″ were located in the same side, and therefore, H_2-6″ was on the opposite side of H-8″ and placed over the cyclopentenone ring. The NOESY correlation between H-8″ and H-2b″ was also consistent with the relative configuration (Fig. 4). Interestingly, a distinct four-bond H–C coupling from H-2a″ to C-9″ was observed in the HMBC spectrum (Table 1). This may be because the atoms of H-2a″–C-2″–C-1″–C-8″–C-9″ lie in a W-letter configuration. The absolute configuration of the tricycloundecentrione moiety was presumed based on the following NOESY observation. Taking into account the *R*-configuration at C-2 and C-2′ of the catechin C-rings, NOEs between H-2 and H-8″ and between the axial H-2a″ and the C-3 galloyl protons was suggestive of an absolute structure as shown in Fig. 4, in which C-8″ is of *S* configuration. Moreover,



Scheme 1. Proposed mechanism for formation of **4** from theaflavin (**6**).



Scheme 2. Proposed mechanisms for formation of **7** and **8** from **4**.

appearance of the NOE correlations from H-2′ to H-11″ and from the H-8″ to the C-3′ galloyl protons, and the absence of a correlation between H-2′ and H-8″, was also consistent with the configuration. Accordingly, the structure of the new oxidation product of **4** was concluded to be as shown in the formula **7**.

The results of our model fermentation experiment indicated that oxidation of the B-rings of catechins are important in the enzymatic oxidation of tea polyphenols. Production of **5** from theaflavin (**6**) was presumed as illustrated in Scheme 1. Although we previously reported on three other oxidation products of **6**,[5,9] the production of **5** seemed to be independent of any other oxidation products. On the other hand, product **7** was probably produced from **4** as shown in Scheme 2. The consecutive two-step Michael additions between two conjugated ketones were similar to that proposed for formation of **6** from *o*-quinones of **1** and **2**.[1] Interestingly, the known dimeric product **8** was first isolated as an oxidation product of **4** generated by treatment with 2,2′-azobis(2,4-dimethylvaleronitrile) in acetonitrile, and a mechanism involving radical coupling of phenols was proposed. However, in this experiment, it was presumed that **8** was formed by the coupling of two *o*-quinones of **4** as shown in Scheme 2, because the *o*-quinones of **4** were produced during enzymatic oxidation of a mixture of **1** and **2**, as reported in our previous paper.[4] An investigation is now in progress to clarify whether the new metabolites obtained by this model fermentation experiment occur in black tea or not.

2. Experimental

IR and UV spectra were obtained with JASCO FT/IR-410 and JASCO V-560 spectrophotometers. Optical rotations were measured with a JASCO DIP-370 digital polarimeter. CD spectra were measured with a JASCO J-720w apparatus. ^{1}H and ^{13}C NMR, ^{1}H–^{1}H COSY, NOESY, HSQC and HMBC spectra were recorded with Unity plus 500 spectrometer (Varian Inc., USA) operating at 500 MHz for ^{1}H, and 125 MHz for ^{13}C, respectively. FABMS were recorded on a JMS DX-303 spectrometer (JEOL Ltd, Japan), and *m*-nitrobenzyl alcohol used as a matrix. Elemental analysis was obtained with an MT-3 analyzer (Yanaco Analytical Instruments Co., Japan). Column chromatography was done on MCI-gel CHP 20P (Mitsubishi Chemical Co.), Chromatorex ODS (Fuji Silysia Chemical Ltd, Japan), Bondapak C_{18}/Porasil B (Waters), TSK gel Toyopearl HW-40F (TOSOH Co.) and Sephadex LH-20 (Pharmacia Fine Chemical Co.). 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 $CHCl_3$–MeOH–H_2O (14:6:1, v/v) and spots were detected by UV illumination, 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., Japan) with gradient elution from 10 to 30% (30 min) and 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 with an MD-910 photodiode array detector (JASCO Co., Japan). Epicatechin (**1**), epigallocatechin (**2**) and epigallocatechin 3-*O*-gallate (**4**) were isolated from commercial green tea according to Nonaka et al. and recrystallized from H_2O.[10]

2.1. Oxidation of 1 and 2 with a tea leaf homogenate

Fresh tea leaves (*Camelia sinensis* var. *assamica*, collected in May in Nagasaki Agricultural and Forestry Experimental Station) (720 g) were homogenized with H_2O (1800 mL) and Polyclar AT®, polyvinylpolypyrrolidone (GAF Co., 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 the resulting precipitates were filtered off. After concentration, the filtrate was extracted five times with ethyl acetate to yield ethyl acetate extract (3.2 g), which was subjected to Sephadex LH-20 column chromatography (25×2.5 cm) with EtOH containing increasing proportions of H_2O (0–20%) and finally with 50% acetone to give four fractions. The first fraction contained sugar. The second fraction was successively chromatographed over MCI-gel CHP20P and Chromatorex ODS with H_2O containing increasing proportions of MeOH (0–80%) to yield unchanged **1** (829 mg) and **5** (29.6 mg). The third fraction was similarly separated by MCI-gel CHP20P (H_2O–MeOH), Chromatorex ODS (H_2O–MeOH) and Sephadex LH-20 (60% MeOH) column chromatography to yield desgalloyloolongtheanin (234.8 mg), dehydrotheasinensin (246.6 mg), and theasinensins C (5.3 mg) and E (3.5 mg). The last fraction was separated by Chromatorex ODS column chromatography to give **6** (314.3 mg) and theanaphthoquinone (6.5 mg).

2.2. Oxidation of 1 and 4

An aqueous solution (100 mL) containing **1** (2.0 g) and **4** (9.5 g) was stirred with the tea leaf homogenate (1000 mL) prepared in the manner described above for 13 h at room temperature. The mixture was poured into acetone (1.5 L) and filtered. The filtrate was concentrated and successively extracted with ethyl acetate and 1-BuOH. The ethyl acetate extract (10.2 g) was subjected to Sephadex LH-20 column chromatography with EtOH containing increasing amounts of water (0–20%) to give three fractions containing phenolic substances. The first fraction was separated by MCI-gel CHP20P column chromatography to give gallic acid (96.7 mg) and a recovery of **1** (1.17 g). The second fraction was separated by a combination of column chromatography over MCI-gel CHP20P (H_2O–MeOH), Bondapak C_{18} (H_2O–MeOH) and Sephadex LH-20 (EtOH or 60% MeOH) to yield theasinensin D (30.1 mg), theaflavin 3-*O*-gallate (64.2 mg), **8** (30.8 mg), and **4** (1.43 g). The last fraction was successively separated by MCI-gel CHP20P (H_2O–MeOH), Bondapak C_{18} (H_2O–MeOH) and Sephadex LH-20 (60% MeOH) to give theasinensin A (165.6 mg), and galloyl oolongtheanin (18.8 mg). The 1-BuOH extract (11.8 g) was fractionated by Sephadex LH-20 column chromatography with EtOH containing increasing proportions of water and finally with 50% aqueous acetone. The fraction containing phenolic substances was subjected to a combination of column chromatographies on MCI-gel CHP20P (H_2O–MeOH), Sephadex LH-20 (H_2O–MeOH), Chromatorex ODS (H_2O–MeOH), and TSK gel Toyopearl HW-40F (60% MeOH) to give **7** (68.2 mg), **4** (21 mg), theasinensin A (19.0 mg).

2.2.1. Dehydrotheaflavin (5). Reddish brown amorphous powder, $[\alpha]_D=-474.8°$ (*c* 0.1, MeOH); IR (KBr) ν_{max} 3364, 1676, 1624, 1517, 1467 cm^{-1}; UV (EtOH) λ_{max} 360 (ε 2700), 296 (3600), 279 (3670) nm; CD (EtOH, *c* 2.8×10^{-5}) $[\theta]_{232}=+28{,}980$, $[\theta]_{279}=-2166$, $[\theta]_{293}=+451$, $[\theta]_{353}=-9287$; ^1H and ^{13}C NMR data, see Table 1; FABMS (negative ion mode) *m/z* 577 $[M-H]^-$. Anal. calcd for $C_{29}H_{22}O_{13}\cdot 4H_2O$: C, 53.44; H, 4.65. Found: C, 53.78; H, 4.68.

2.2.2. Epigallocatechin gallate dimer (7). Brown amorphous powder, $[\alpha]_D=-6.6°$ (*c* 0.4, MeOH); IR (KBr) ν_{max} 3300, 1753, 1707, 1628, 1615 cm^{-1}; UV (EtOH) λ_{max} 275 (ε 24,300) nm; CD (EtOH, *c* 2.1×10^{-5}) $[\theta]_{231}=-11{,}817$, $[\theta]_{280}=+20{,}922$; ^1H and ^{13}C NMR data, see Table 1; FABMS (negative ion mode) *m/z* 885 $[M-H]^-$. Anal. calcd for $C_{43}H_{34}O_{21}\cdot 6H_2O$: C, 51.92; H, 4.66. Found: C, 51.66; H, 4.66.

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