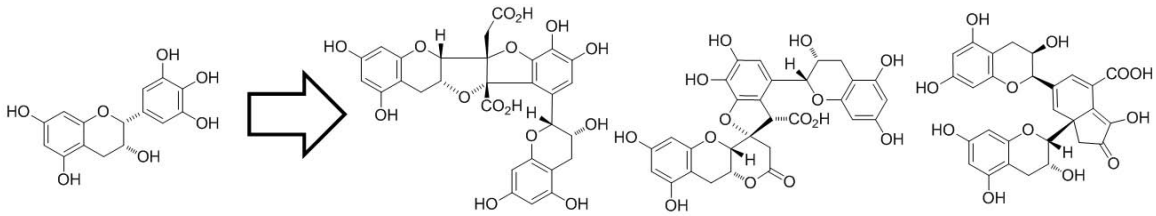
Graphical Abstract

Structures of enzymatic oxidation products of epigallocatechin

Yosuke Matsuo, Toshimi Hayashi, Yoshinori Saito, Isao Kouno, Takashi Tanaka*



Structures of enzymatic oxidation products of epigallocatechin

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ABSTRACT

To understand the structures of uncharacterized black tea polyphenols, the oxidation products of (−)-epigallocatechin were investigated. Enzymatic oxidation and subsequent heating of the reaction mixture afforded four new oxidation products (**6**, and **9**–**11**) along with theasinensins C and E (**5**), dehydrotheasinensin E (**12**), epitheaflagallin, hydroxytheaflavin, and desgalloyl oolongtheanin. The structures of the new compounds were determined chemically and spectroscopically. Isotheasinensin E (**6**) is a C-2 epimer of **5**, and compounds **9** and **10** are oxidation products of **12**. Another new compound, **11**, is a yellow pigment and presumed to be a degradation product of proepitheaflagallin. The results disclosed new oxidation mechanisms that occur during black tea production.

Keywords: Epigallocatechin; oxidation; theasinensins; thearubigins; Black tea

1. Introduction

Black tea, one of the most popular beverages worldwide, is produced by kneading and crushing fresh tea leaves (*Camellia sinensis*).[1] During the process, the catechins in the original fresh tea leaves are enzymatically oxidized to give complex mixtures of products.[2] The tea catechins are grouped into two types, based on their B-ring structures: pyrogallol-type [(–)-epigallocatechin (**1**) and its galloyl ester] and catechol-type [(–)-epicatechin and its galloyl ester]. Oxidative couplings between pyrogallol- and catechol-type catechins produce theaflavins,[3] which are well-known reddish-yellow pigments containing a characteristic benzotropolone moiety. However, oxidative couplings between two pyrogallol-type catechins are more important in production of black tea polyphenols, because **1** and its galloyl ester account for over 70% of total tea catechins.[4] Our previous studies indicated that the initial major oxidation products of **1** are quinone dimers, such as dehydrotheasinensin C (**2**) and proepitheaflagallin (**3**) (Fig. 1).[5–13] The quinone dimers are unstable and decompose in the final heating and drying process of black tea production to yield other characteristic black tea polyphenols such as theasinensins and epitheaflagallins.[2] However, these reactions are accompanied by production of numerous minor products, which are probably related to thearubigins,[14] which are heterogeneous mixtures of catechin oxidation products and have not yet been chemically characterized.[15,16] Thearubigins constitute up to 60% of the solids in black tea infusions, and are therefore very important with respect to color and taste. In the present study, we reexamined the large-scale in vitro oxidation of **1** and isolated minor degradation products of the unstable quinone dimers.

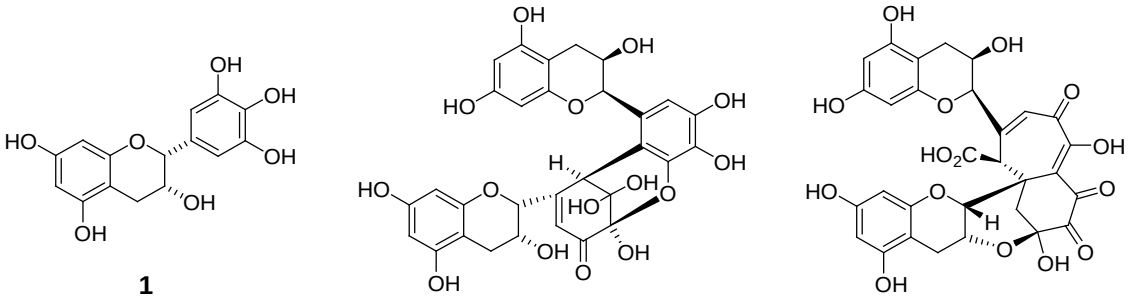


Fig. 1. Structures of **1** and its quinone dimers **2** and **3**.

2. Results and discussion

In this study, a Japanese pear fruit homogenate was used as the enzyme source, because it has strong catechin oxidation abilities and yields practically no oxidation products derived from its own constituents.[17] The homogenate was therefore directly applied to a large-scale model tea-fermentation experiment without purification of the enzymes. The reaction procedure was simple: an aqueous solution of **1** was added to the fruit homogenate and the mixture was vigorously stirred to mix it with air oxygen, which was the oxidant in this reaction. HPLC analysis of the initial reaction mixture showed broad peaks arising from **2** and **3**, together with **1**.[12] Subsequently, the mixture was heated to 80 °C. This procedure mimics the drying process in black tea production; it terminated the reaction by inactivating the enzymes and decomposed the unstable quinone dimers **2** and **3**.[5] The products were first fractionated using a Diaion HP20SS column chromatograph and then separated by combinations of column chromatography using Sephadex LH-20, Chromatorex ODS, Toyopearl HW40C, and MCI-gel CHP20P, to give theasinensins C (**4**) and E (**5**),[18] dehydrotheasinensin E (**12**),[17] desgalloyl oolongtheanin,[18] epitheaflagallin,[19] hydroxytheaflavin,[8] and four new compounds, **6** and

9–**11**. The major products were **4** and **5**, which are diastereomeric dimers of **1** with different atropisomerisms of the biphenyl bonds. These isomers are known to be produced by reduction–oxidation dismutation of **2**.[6]

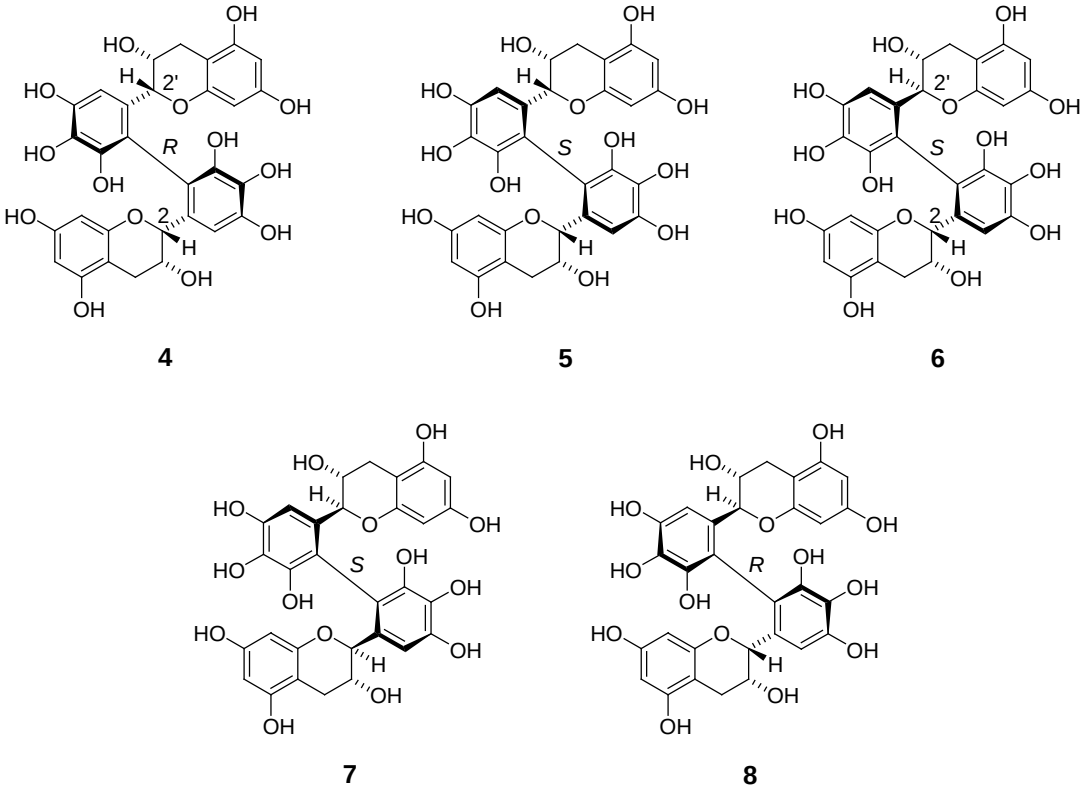


Fig. 2. Structures of theasinensins C (**4**) and E (**5**) and the stereoisomers **6**–**8**.

Isotheasinensin E (**6**) was shown to be an isomer of **4** and **5** by FABMS (*m/z*: 611 $[M + H]^+$) and comparisons of ^1H and ^{13}C NMR spectra. In contrast to the symmetrical structures of **4** and **5**, the NMR spectra had two sets of signals of flavan-3-ol units, and the large coupling constant of one of the two C-ring H-2 atoms (J = 9.3 Hz, H-2') indicated that **6** is a C-2 epimer of **4** or **5** ($J_{2,3}$ < 2 Hz). The epimerizations of **4** and **5** were therefore examined by autoclaving their respective aqueous solutions (120 °C for 30 min).[20] In both experiments, rotation of the biphenyl

bond (**4** → **5** and **5** → **4**) was not observed. Heating of **5** yielded **6** and a new isomer **7**, which has a symmetrical structure with two 2,3-*trans* C-rings ($J_{2,3}$ = 8.8 Hz), and was named neotheasinensin E. In contrast, heating of **4** under the same conditions yielded only one epimerization product, named isotheasinensin C (**8**), together with recovery of **4**. The ^{1}H NMR spectrum of **8** indicated that one of the two C-2 atoms of **4** was epimerized. Based on these results, the structure of **6** was confirmed, and it is shown in Fig. 2. The presence of isotheasinensin E (**6**) in commercial black tea was confirmed by chromatographic separation and spectroscopic identification (isolated yield: 0.008%). The results also revealed a difference in reactivities at the C-2 position, based on different configurations of the biphenyl bond.

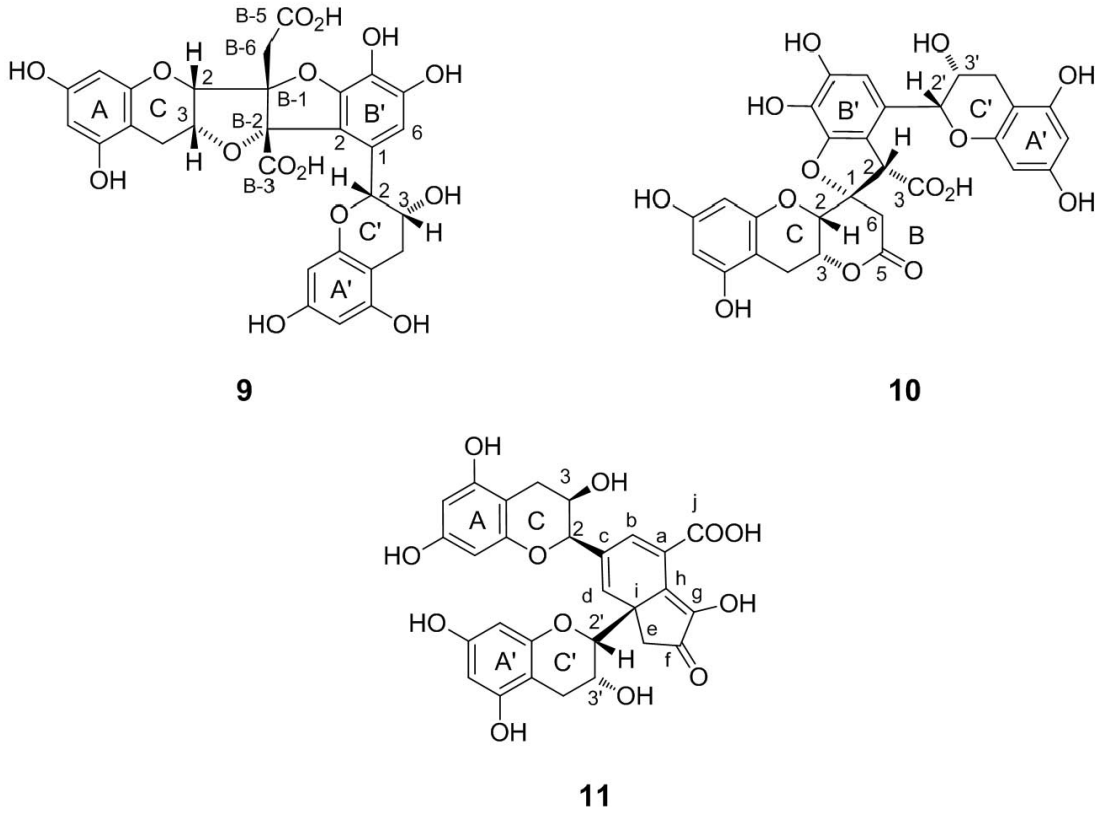


Fig. 3. Structures of new oxidation products **9**–**11**.

The new oxidation product **9** (Fig. 3) is a dimer of **1**, as shown by the $[M + H]^+$ peak at *m/z* 613 in the FABMS. The presence of two sets of flavan A- and 2,3-*cis* C-rings was apparent from the signals in the ^1H and ^{13}C NMR spectra (Table 1). An aromatic proton singlet at δ_H 6.91 (B′-ring H-6) and six aromatic carbon signals (B′-1–B'-6) indicated the presence of a pyrogallol ring, and its connection to C′-2 was shown by HMBC correlations of the C′-ring H-2 to B′-1, B′-2, and B′-6 (Fig. 4). The remaining part of the molecule consisted of two carboxyl groups (δ_C 170.4 and 170.8), two oxygenated quaternary carbons (δ_C 100.0 and 94.5), and a methylene carbon [δ_H 3.01 (2H, br s), δ_C 36.0]. In the HMBC spectrum, the ^4J correlations of the B′-ring H-6 and C′-ring H-2 with the quaternary carbon at δ_C 95.5 (B-2) indicated that the B-2 carbon was connected to B′-2. The B-2 carbon and the other oxygenated quaternary carbon at δ_C 100.0 (B-2) were correlated with the C-ring H-2 (δ_H 4.85) and methylene protons at δ_H 3.01 (B-6).

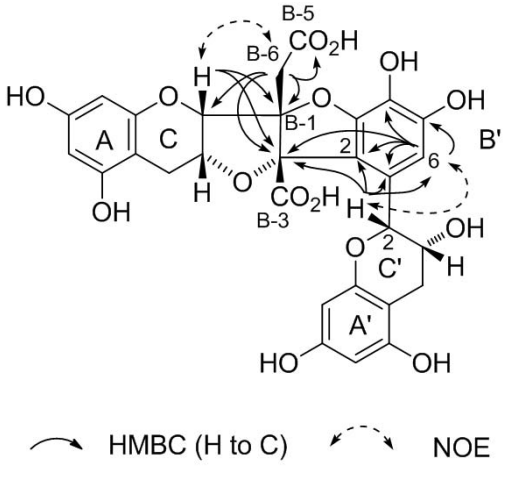


Fig. 4. Important HMBC and NOESY correlations of **9**.

Table 1. ^{1}H (500MHz), ^{13}C (125MHz) NMR data for **9** and **10** in acetone-d_6+D_2O (δ in ppm, *J* in Hz)

Position		**9**		**10**	
		^{1}H	^{13}C	^{1}H	^{13}C
C-ring	2	4.85 d (2.0)	77.9	4.19 br s	70.3
	3	4.35 br d (5.1)	73.5	5.32 dd (1.6, 2.6)	70.7
	4	2.87 dd (5.1, 18.0)	21.0	2.78-2.85 m	25.2
		2.96 br d (18.0)		3.01 dd (1.6, 17.6)	
A-ring	4a		98.7		98.0
	5		154.6 [a]		156.7 [a]
	6	6.04 d (2.4)	95.7 [b]	6.02 d (2.2)	95.6 [b]
	7		156.9 [a]		157.1 [a]
	8	5.88 d (2.4)	95.9 [b]	5.88 d (2.2)	96.2 [b]
	8a		157.2 [a]		157.1 [a]
C'-ring	2'	4.82 br s	76.3	4.85 br s	79.4
	3'	4.49 br s	65.8	4.15 br t (2.9)	67.0
	4'	2.77 2H, br s	29.0	2.78-2.85 m	29.4
A'-ring	4a'		99.6		99.5
	5'		157.3 [a]		157.2 [a]
	6'	5.99 d (2.4)	96.2 [b]	6.02 d (2.2)	96.2 [b]
	7'		157.4 [a]		157.3 [a]
	8'	5.89 d (2.4)	96.7 [b]	5.98 d (2.2)	97.0 [b]
	8a'		157.5 [a]		157.7 [a]
B	1		100.0		88.3
	2		94.5	4.95 br s	54.4
	3		170.4		171.8
	5		170.8		168.1
	6	3.01 2H, br s	36.0	2.85 br d (18.1)	34.4
				3.20 br d (18.1)	
B'-ring	1		128.8		128.7
	2		114.9		116.6
	3		148.3		148.0
	4		129.0		129.5
	5		149.9		147.2
	6	6.91 s	110.2	6.55 s	108.6

[a—c] Assignments may be interchanged in each column.

In addition, the methylene protons (B-6) were correlated with a carboxyl B-5 carbon resonating at δ_C 170.8. The other carboxyl carbon (B-3, δ_C 170.4) was concluded to be attached to the quaternary B-2 carbon, because it is apparent that this part (B-1–B-6) originated from the pyrogallol-type B-ring of **1**. The unusual low-field shifts of the two quaternary carbons, B-1 and B-2 (δ_C 100.0 and 94.5, respectively), suggested formation of ether linkages at these positions, and the low-field shift of the C-ring C-3 (δ_C 73.5, $\Delta\delta$ about 9) compared with those for **4** and **5** (δ_C 64.9 and 64.7, respectively) also indicated that the oxygen atom at C-3 participated in ether ring formation.

The unsaturation index (18) calculated from the molecular formula, which was deduced from the results of FABMS and elemental analysis, indicated the presence of two ether linkages. These spectroscopic findings and examination using Dreiding models led us to construct the structure shown in Fig. 3, in which two ether linkages were formed between the C-ring C-3 and B-2, and between B´-3 and B-1. The structure was supported by strong NOESY correlation between the C-ring H-2 and B-6 methylene protons. The NOE correlation also determined the configuration at the B-1 to be *S*. Furthermore, examination of molecular models indicated that the conjunction between the two five-membered rings should be *cis*, so the B-2 configuration was also *S*. Based on these results, the structure of **9** was concluded to be as shown in Fig. 3.

The ^{13}C NMR spectrum of **10** was related to that of **9**, exhibiting signals arising from two sets of flavan A- and C-rings, a pyrogallol-type B-ring, two carboxyl carbons (δ_C 171.8, B-3 and 168.1, B-5), an oxygenated quaternary carbon (δ_C 88.3, B-1), a methine carbon (δ_C 55.4, B-2), and a methylene carbon (δ_C 34.4, B-6) (Table 1). The HMBC correlations of the methine proton (δ_H 4.95, br s, B-2) with the pyrogallol ring carbons (B´-1, B´-2, and B´-3) indicated that this methine carbon was attached to the

pyrogallol B′-2 position (Fig. 5). The B-2 methine proton also correlated with carboxyl (B-3) and oxygenated quaternary (B-1) carbons, and the methine B-2 carbon showed cross peaks with the methylene protons (δ_H 2.85 and 3.20, br d, J = 18.1 Hz, B-6) and C-ring H-2 (δ_H 4.85, d, J = 2.0 Hz). Furthermore, the B-6 methylene protons showed correlations with carboxyl (B-5), quaternary (B-1), methine (B-2), and C-ring C-2 carbons. These HMBC correlations led us to construct the planar structure shown in Fig. 5. The molecular formula of **10** was deduced to be $C_{29}H_{24}O_{14}$ from the $[M + H]^+$ peak at *m/z* 597 in the FABMS and NMR spectroscopic analyses.

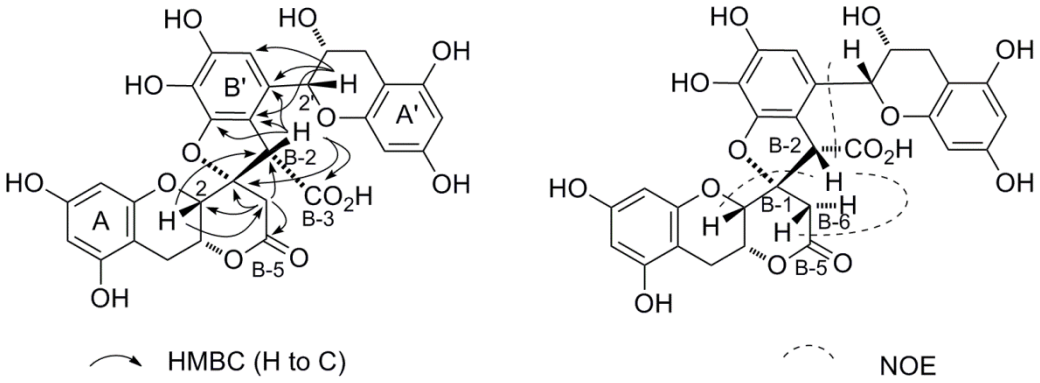
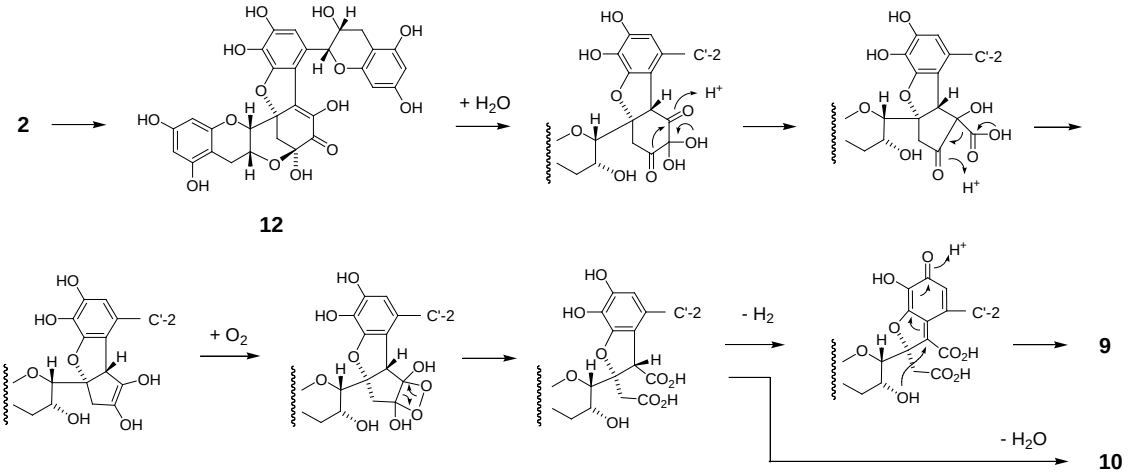


Fig. 5. Important HMBC and NOESY correlations of **10**.

The unsaturation index (18) and relatively large chemical shift value of the B-1 quaternary carbon (δ_C 88.3) suggested an ether linkage between B-1 and the phenolic hydroxyl group at B′-3. In addition, the large low-field shift of the C-ring H-3 (δ_H 5.32) compared with the C′-ring H-3 (δ_H 4.15) indicated esterification at the C-3 hydroxyl group. Although there are two carboxyl groups (B-5 and B-3), NOESY correlations of the methine proton B-2 with the C-ring H-2 and one of the B-6 methylene protons (δ_H 3.20) indicated that the C-3 hydroxyl group forms a lactone ring with the B-5 carboxyl

group (Fig. 5). The NOE correlations also confirmed the configurations at B-1 and B-2. Thus, the structure of product **10** was determined to be as shown in Fig. 3.

Compounds **9** and **10** were presumed to be oxidation products of dehydrotheasinensin E (**12**), which is an isomer of **2**, obtained as an oxidation product of **1** in this experiment (Scheme 1).[17]



Scheme 1. Plausible production mechanism of **9** and **10**.

Compound **11** was obtained as a yellow amorphous powder, with a UV absorption maximum at 384 nm. The molecular formula, $C_{28}H_{24}O_{12}$, was determined based on the results of elemental analysis and FABMS (*m/z* 553 $[M + H]^+$ and 575 $[M + Na]^+$). The ^1H and ^{13}C NMR spectra (Table 2) exhibited signals attributable to two sets of flavan A- and C-rings, the chemical shifts of which are similar to those of **1**. The remaining part of the molecule consisted of six olefinic carbons, a carbonyl carbon (δ_C 204.5, C-f), a carboxyl carbon (δ_C 172.3, C-j), an aliphatic quaternary carbon (δ_C 47.9, C-i), and a methylene carbon (δ_C 41.5, C-e). The allylic coupling of the C-ring H-2 (δ_H 4.53) with two olefinic methine protons at C-b (δ_H 7.01) and C-d (δ_H 6.42) observed in

the ^{1}H–^{1}H COSY spectrum, as well as the HMBC correlations of these protons, shown in Fig. 6, indicated the connectivity of these carbons.

Table 2. ^{1}H (500MHz), ^{13}C (125MHz) NMR data for **11** in acetone-d_6+D_2O (δ, ppm; J, Hz)

Position		^{1}H		^{13}C
C-ring	2	4.53	s	78.6
	3	4.23	br s	65.3
	4	2.79	dd (16.8, 4.5)	28.7
		2.71	dd (16.8, 2.6)	
A-ring	4a			99.4
	5			157.3 [b]
	6	5.99	d (2.3)	96.2
	7			157.2 [b]
	8	5.90	d (2.3)	95.4 [c]
	8a			156.3 [d]
C'-ring	2'	3.85	s	77.7
	3'	4.41	br s	63.2
	4'	2.73	br d (16.6)	29.3
		2.57	dd (16.6, 4.1)	
A'-ring	4a'			99.2
	5'			157.1 [b]
	6'	5.90	d (2.3)	95.9
	7'			156.9 [b]
	8'	5.69	d (2.3)	95.3 [c]
	8a'			156.2 [d]
B-rings	a			131.5
	b	7.01	d (1.4)	131.1
	c			136.5
	d	6.42	d (1.4)	134.4
	e	3.34	d (17.9)	41.5
		2.29	d (17.9)	
	f			204.5
	g			150.2
	h			136.8
	i			47.9
	j			172.3

[a—d] Assignmentsmay be interchanged in each column.

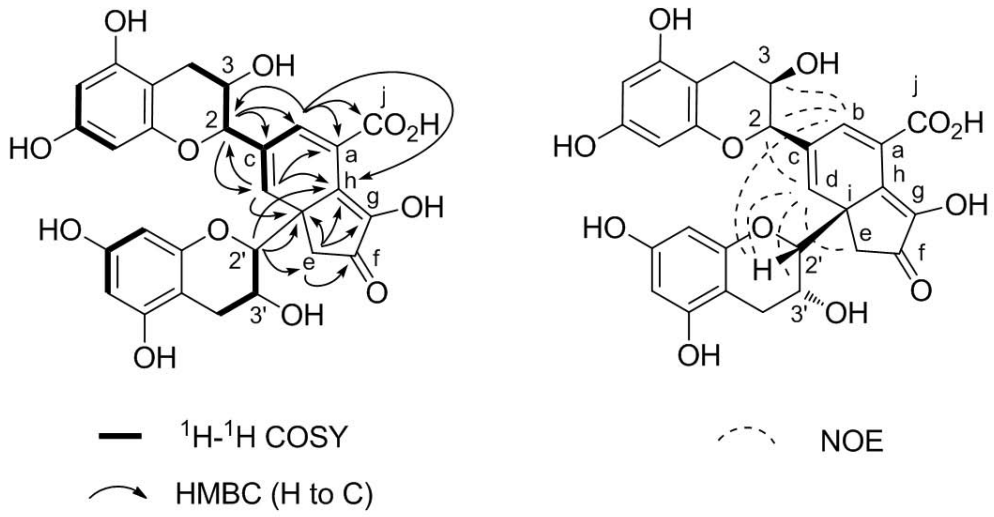
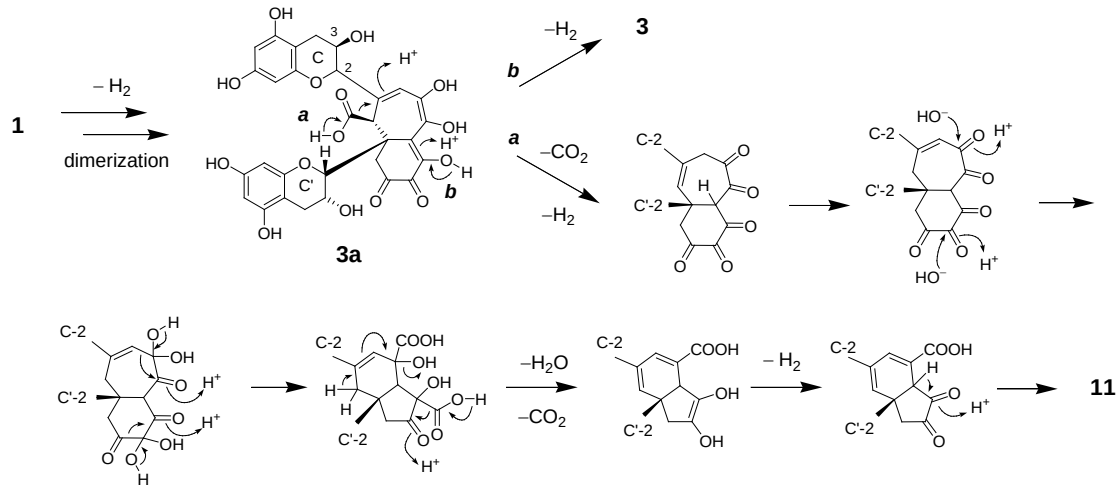


Fig. 6. Important HMBC and NOESY correlations of **11**.

In the HMBC spectrum, correlations of H-b with a carboxyl carbon (C-j) and two olefinic carbons (C-a and C-h) confirmed the location of the carboxyl group as C-a. Long-range couplings of the′ C -ring H-2 (δ_H 3.85) with C-e, C-h, and C-i, and correlations of the methylene H-e with C-f, C-g, C-h, and C-i revealed the presence of a cyclopentenone ring attached to C′-2. In addition, correlations of the olefinic proton H-d with C-h and C-i suggested the planar structure of **11**. The NOESY correlations shown in Fig. 6 are consistent with this structure. However, the configuration at C-i could not be determined spectroscopically. The configuration was presumed to be *S*, based on the following biogenetic considerations. Previously, we proposed the biogenesis of **3** by oxidation of **3a**, which is a dimer of **1**.[8,11,12] The pigment **11** was probably produced from the same intermediate **3a**, as shown in Scheme 2, in which the configuration at C-i was retained throughout the process. Accordingly, the proposed structure of **11** is as shown in Fig. 3.



Scheme 2. Plausible production mechanism of **3** and **11**.

Black tea polyphenols are a complex mixture of the oxidation products of four tea catechins; however, only a few per cent of the oxidation products have been chemically characterized. In this study, we attempted to characterize the minor oxidation products of **1** in a large-scale model tea-fermentation experiment. Since **1** and its 3-*O*-gallate account for over 70% of tea catechins, the oxidation products of **1** are important for identifying uncharacterized black tea polyphenols. Theasinensins C (**4**) and E (**5**) (16% in total) were major products, and a new compound **6** was obtained in 2.5% yield. Presence of **6** in commercial black tea was confirmed in this study. Other known products, namely desgalloyl oolongtheanin, epitheaflagallin, and hydroxytheaflavin, were obtained in 0.3–1.3% yields. The yields of the new products **9**, **10**, and **11** were only 0.08%, 0.17%, and 0.1%, respectively, and we could not identfy these products in commercial black tea due to presence of so many related compounds. In the initial reaction mixture, many other minor products were detected, which could not be purified in this study. These minor oxidation products, including those characterized in this experiment, probably contribute to the formation of thearubigins, a

heterogeneous mixture of uncharacterized black tea polyphenols.

3. Experimental section

3.1. Materials

(−)-Epigallocatechin was separated from a commercial tea catechin mixture according to the methods described by Nonaka et al. and purified by recrystallization from H_2O.[21]

3.2. Analytical procedures

UV spectra were obtained using a Jasco V-560 UV/vis spectrophotometer and optical rotations were measured using a Jasco DIP-370 digital polarimeter (Jasco Co., Tokyo, Japan). ^{1}H NMR, ^{13}C NMR, ^{1}H–^{1}H COSY, HSQC, and HMBC spectra were recorded on a Varian UNITY plus 500 (500 MHz for ^{1}H and 125 MHz for ^{13}C NMR) spectrometer (Varian, Palo Alto, CA, USA), and a JEOL JNM-AL400 (400 MHz for ^{1}H and 100 MHz for ^{13}C NMR) spectrometer (JEOL Ltd., Tokyo, Japan). Coupling constants are expressed in hertz and chemical shifts are given on the δ (ppm) scale. FABMS were recorded on a JEOL JMS-700N spectrometer (JEOL Ltd., Tokyo, Japan), and *m*-nitrobenzyl alcohol was used as the matrix. Elemental analysis was conducted using a PerkinElmer 2400 II analyzer (PerkinElmer Inc., Waltham, MA, USA). Column chromatography was performed using Diaion HP20SS (Mitsubishi Chemical Co., Tokyo, Japan), MCI-gel CHP20P (Mitsubishi Chemical Co.), Sephadex LH-20 (25–100 μm, GE Healthcare Bio-Science AB, Uppsala, Sweden), Chromatorex ODS (100–200 mesh; Fuji Silysia Chemical Ltd., Tokyo, Japan), and Toyopearl HW-40C (75 μm; Tosoh Bioscience Japan, Tokyo, Japan) columns. TLC was performed using pre-coated Kieselgel 60 F_{254} plates (0.2 mm thick; Merck, Darmstadt, Germany) with toluene–ethyl

formate–formic acid (1:7:1, v/v) or $CHCl_3$–MeOH–H_2O (14:6:1, v/v) as the solvent, and spots were detected by UV illumination (254 nm) and spraying with 2% ethanolic $FeCl_3$ and 10% sulfuric acid reagent, followed by heating. Analytical 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% to 75% in 15 min at a flow rate of 0.8 mL/min and detection with a Jasco MD-910 photodiode array detector.

3.3. Oxidation of epigallocatechin and heating of reaction mixture

This experiment was originally performed to confirm the production of epitheaflagallin and hydroxytheaflavin from **1**.[8] Japanese pear fruits (5.5 kg) were homogenized with 2.2 L of H_2O and filtered through four layers of gauze at 0 °C. The filtrate (6.7 L) was mixed with an aqueous solution of **1** (40 g/0.8 L) and vigorously stirred at room temperature for 3 h. The resulting mixture was heated at 80 °C for 30 min. After cooling, the mixture was poured into acetone (15 L) and stirred gently for 30 min; then the precipitates were removed by filtration. The filtrate was concentrated by rotary evaporation until the acetone had been removed and then subjected to Diaion HP20SS (5.5 cm i.d. × 55 cm) chromatography. Sugars and inorganic materials were eluted out with H_2O, and the products were eluted out with H_2O–MeOH (10% stepwise elution, each 1.0 L) to yield six fractions: fr 1 (13.2 g), fr 2 (5.7g), fr 3 (9.5 g), fr 4 (4.5 g), fr 5 (2.0 g), and fr 6 (1.4 g). Fr 1 was separated using Sephadex LH-20 (0–100% MeOH) to give four sub-fractions, and fr 1-2 was further separated by successive column chromatography using Chromatorex ODS (0–45% MeOH), MCI-gel CHP20P (0–40% MeOH), and Toyopearl HW40C (0–100% MeOH) to yield **11** (39.2 mg) and

dehydrotheasinensin E (**12**) (132.5 mg). Fr 2-3 was subjected to Diaion HP20SS (0–45% MeOH) to give isotheasinensin E (**6**) (393 mg) and a mixture of theasinensins C (**4**) and E (**5**) (6.3 g). Fr 2 was separated using Sephadex LH-20 (0–100% MeOH) to give 10 sub-fractions. Column chromatography of fr 2-7 using Chromatorex ODS (0–45% MeOH) yielded **1** (1.64 g), **4** (38.6 mg), and **5** (57.4 mg). Fr 2-9 was also subjected to Chromatorex ODS column chromatography (0–40% MeOH) to give **6** (594.9 mg). Fr 3 was applied to a column of Sephadex LH-20 (0–100% MeOH) to give two sub-fractions, and fr 3-1 (1.55 g) was separated by Chromatorex ODS (0–50% MeOH) and Sephadex LH-20 (0–80% MeOH containing 0.1% trifluoroacetic acid) to give **10** (68.1 mg). Fr 3-2 (7.5 g) was almost pure **1** and was not examined further. Fr 4 was subjected to Sephadex LH-20 column chromatography (60–100% MeOH) to give six sub-fractions. Fr 4-1 was successively separated using Chromatorex ODS (0–50% MeOH), Toyopearl HW-40F (0–80% MeOH), and Sephadex LH-20 (0–100% MeOH containing 0.1% trifluoroacetic acid) to yield **9** (30.0 mg). Column chromatography of Fr 4-4 using MCI-gel CHP20P (0–70% MeOH) and Chromatorex ODS (0–40% MeOH) afforded desgalloyl oolongtheanin (116.9 mg). Fr 6 was separated using Sephadex LH-20 (40–100% MeOH) and Chromatorex ODS (20–80% MeOH) to give epitheaflagallin (530.8 mg) and hydroxytheaflavin (185.2 mg).

*3.3.1. Isotheasinensin E (**6**).* White amorphous powder; $[\alpha]_D^{28}$ −37.4° (*c* = 0.3, MeOH); FABMS *m/z*: 611 $[M + H]^+$, 633 $[M + Na]^+$; HRFABMS *m/z*: 611.1408 $[M + H]^+$ (Calcd for $C_{30}H_{27}O_{14}$, 611.1401); UV (EtOH) λ_{max} (log ε): 271 nm (3.50); IR ν_{max}: 3378, 2929, 1622, 1604, 1514, 1464 cm^{-1}; ^1H NMR (acetone-d_6 + D_2O, 400 MHz) δ: 2.29 (1H, dd, *J* = 15.9, 10.2 Hz, H-4′), 2.38 (1H, dd, *J* = 16.6, 4.4 Hz, H-4), 2.67 (1H, br d, *J* = 16.6

Hz, H-4), 3.02 (1H, dd, *J* = 15.9, 6.3 Hz, H-4′), 4.04 (1H, ddd, *J* = 10.2, 9.3, 6.3 Hz, H-3′), 4.10 (1H, m, H-3), 4.30 (1H, d, *J* = 9.3 Hz, H-2′), 4.46 (1H, s, H-2), 5.81, 5.84, 5.93, 5.95 (each 1H, d, *J* = 2.4 Hz, H-6, H-8, H-6′, H-8′), 6.65, 6.92 (each 1H, s, B-H-6, B-H-6′); ^{13}C NMR (acetone-d_6 + D_2O, 75 MHz) δ: 29.6, 30.5 (C-4, C-4′), 64.6 (C-3), 68.7 (C-3′), 77.3 (C-2), 78.9 (C-2′), 95.3, 95.5, 95.8, 96.2 (C-6, C-8, C-6′, C-8′), 99.5, 100.4 (C-4a, C-4a′), 106.2, 109.0 (B-C-6, B-C-6′), 114.0, 116.0 (B-C-2, B-C-2′), 130.5, 130.7 (B-C-1, B-C-1′), 133.7, 134.4 (B-C-4, B-C-4′), 143.2, 143.5, 145.5, 146.6 (B-C-3, B-C-5, B-C-3′, B-C-5′), 156.9, 157.0, 157.2, 157.5 (2C), 157.62 (C-5, C-7, C-8a, C-5′, C-7′, C-8a′).

*3.3.2. Epimerizations of **4** and **5**.* An aqueous solution of theasinensin E (189.3 mg/189.3 mL) was autoclaved for 30 min at 120 °C. After cooling, the solvent was removed under reduced pressure. The residue was subjected to successive column chromatography over Sephadex LH-20 (2.5 cm i.d. × 20 cm, 0–50% MeOH) and Chromatorex ODS (2 cm i.d. × 22 cm, 0–30% MeOH) to yield isotheasinensin E (**6**) (79.4 mg, 41.9%) and neotheasinensin E (**7**) (36.1 mg, 19.1%). Autoclaving of an aqueous solution of theasinensin C (204.3 mg/204.3 mL) in a similar manner and separation by Chromatorex ODS column chromatography afforded isotheasinensin C (**8**) (32.5 mg, 15.9%) along with recovery of theasinensin C (78.0 mg, 38.2%).

*3.3.3. Neotheasinensin E (**7**).* White amorphous powder; $[\alpha]_D^{20}$ −79.1° (*c* = 0.1, MeOH); FABMS *m/z*: 611 $[M + H]^+$, 633 $[M + Na]^+$; HRFABMS *m/z*: 611.1401 $[M + H]^+$ (Calcd for $C_{30}H_{27}O_{14}$, 611.1401); UV (EtOH) λ_{max} (log ε): 271 (4.09) nm; IR ν_{max}: 3391, 2932, 1622, 1604, 1515, 1457 cm^{-1}; ^{1}H NMR (acetone-d_6 + D_2O, 400 MHz) δ: 2.29 (2H, dd, *J*

= 15.6, 9.3 Hz, H-4a), 2.92 (2H, dd, *J* = 15.6, 5.4 Hz, H-4b), 3.99 (2H, ddd, *J* = 9.3, 8.8, 5.4 Hz, H-3), 4.35 (2H, d, *J* = 8.8 Hz, H-2), 5.76, 5.95 (each 2H, d, *J* = 2.4 Hz, H-6, H-8), 6.63 (2H, s, B-H-6); ^{13}C NMR (acetone-d_6 + D_2O, 100 MHz) δ: 29.5 (C-4), 68.2 (C-3), 79.3 (C-2), 95.6, 96.0 (C-6, C-8), 100.4 (C-4a), 107.2 (B-C-6), 116.9 (B-C-2), 130.0 (B-C-1), 134.9 (B-C-4), 143.7, 146.4 (B-C-3, B-C-5), 156.92, 157.18, 157.28 (C-5, C-7, and C-8a).

*3.3.4. Isotheasinensin C (**8**).* White amorphous powder; $[\alpha]_D^{28}$ −8.1 (*c* = 0.3, MeOH); FABMS *m/z*: 611 $[M + H]^+$; HRFABMS *m/z*: 611.1405 $[M + H]^+$ (Calcd for $C_{30}H_{27}O_{14}$, 611.1401). UV (EtOH) λ_{max} (log ε): 270 (4.27) nm; IR ν_{max}: 3396, 2939, 1628, 1605, 1516, 1468 cm^{-1}; ^{1}H NMR (acetone-d_6 + D_2O, 400 MHz) δ: 2.32 (1H, dd, *J* = 15.6, 7.8 Hz, H-4′), 2.41 (1H, dd, *J* = 16.6, 4.9 Hz, H-4), 2.65 (1H, br d, *J* = 16.6 Hz, H-4), 2.66 (1H, dd, *J* = 15.6, 5.4 Hz, H-4′), 3.96 (1H, ddd, *J* = 7.8, 7.3, 5.4 Hz, H-3′), 4.15 (1H, br s, H-3), 4.44 (1H, d, *J* = 7.3 Hz, H-2′), 4.79 (1H, s, H-2), 5.79, 5.91 (each 1H, d, *J* = 2.0 Hz, H-6/H-8 or H-6′′/8′′), 5.90, 5.92 (each 1H, d, *J* = 2.4 Hz, H-6′/8′ or H-6/8), 6.53, 6.86 (each 1H, s, B-H-6 and B-H-6'); ^{13}C NMR (acetone-d_6 + D_2O, 100 MHz) δ: 28.3, 28.8 (C-4, C-4′), 64.9, 66.0 (C-3, C-3′′), 77.1 (C-2), 81.2 (C-2′), 95.3, 95.6, 95.8, 95.9 (C-6, C-8, C-6′, C-8′), 99.3, 100.1 (C-4a, C-4a′), 108.3 (2C, C-6′, C-6′), 113.0, 114.3 (B-C-2, B-C-2′), 129.4, 131.6 (B-C-1, B-C-1′), 132.8, 133.4 (B-C-4, B-C-4′), 144.1, 145.0, 145.5, 145.8 (C-3′, C-5′, C-3′′′ and C-5′′′), 156.7, 156.8, 157.0, 157.2, 157.3, 157.3 (C-5, C-7, C-8a, C-5′, C-7′, C-8a′).

*3.3.5. Isolation of isotheasinensin E (**6**) from black tea.* Commercial black tea (600 g), a blended tea produced in India and Sri Lanka, was extracted with boiling H_2O (5 L × 3),

and the extract was concentrated. The extract was successively partitioned with $CHCl_3$, EtOAc, and 1-BuOH. The 1-BuOH fraction (63.4 g) was separated into 10 fractions by Sephadex LH-20 column chromatography (5.0 cm i.d. × 35 cm) with 0–100% MeOH (10% stepwise) and then 60% acetone in H_2O. Fr 6 was further separated using a combination of column chromatography with MCI-gel CHP20P (0–50% MeOH) and Chromatorex ODS (0–40% MeOH) to give **6** (39.8 mg).

*3.3.6. Compound **9***. Tan amorphous powder; $[\alpha]_D^{28}$ +29.2° (*c* = 0.1, MeOH); *Anal*. Calcd for $C_{29}H_{24}O_{15}\cdot 7/2H_2O$: C, 52.10; H, 4.82. Found: C, 51.49; H, 5.06; FABMS *m/z*: 613 $[M + H]^+$, 635 $[M + Na]^+$. UV (EtOH) λ_{max} (log ε): 272 nm (3.42); IR ν_{max}: 3364, 1717, 1633, 1521 cm^{-1}; ^1H and ^{13}C NMR (acetone-d_6): see Table 1.

*3.3.7. Compound **10***. Brown amorphous powder; $[\alpha]_D^{28}$ +22.8° (*c* = 0.1, MeOH); *Anal*. Calcd for $C_{29}H_{24}O_{14}\cdot 4H_2O$: C, 52.10; H, 4.82. Found: C, 52.48; H, 4.90; FABMS *m/z*: 597 $[M + H]^+$; UV (EtOH) λ_{max} (log ε): 271 (3.69), 295 (sh) (3.51) nm; IR ν_{max}: 3377, 1716, 1632, 1519 cm^{-1}; ^1H and ^{13}C NMR (acetone-d_6): see Table 1.

*3.3.8. Compound **11***. Yellow amorphous powder; $[\alpha]_D^{24}$ −895.1° (*c* = 0.1, MeOH); *Anal*. Calcd for $C_{28}H_{24}O_{12}\cdot 7/2H_2O$: C, 54.64; H, 5.08. Found: C, 54.29; H, 4.89; FABMS *m/z*: 553 $[M + H]^+$, 575 $[M + Na]^+$; UV (EtOH) λ_{max} (log ε): 264 (4.12), 384 (3.85) nm; IR ν_{max}: 3364, 2938, 1683, 1629, 1606, 1518, 1469 cm^{-1}; ^1H and ^{13}C NMR (acetone-d_6): see Table 2.

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