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A new mechanism for oxidation of epigallocatechin and production of benzotropolone pigments

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Abstract—Enzymatic oxidation of (−)-epigallocatechin gave two new quinone dimers, dehydrotheasinensin C and proepitheaflagallin. Dehydrotheasinensin C has a hydrated cyclohexenetrione structure and its oxidation–reduction dismutation reaction yielded black tea polyphenols, theasinensins C and E, and desgalloyl oolongtheanin. The structure of proepitheaflagallin was determined based on spectroscopic data of its quinoxaline derivatives prepared by condensation with *o*-phenylenediamine. Proepitheaflagallin was decomposed on heating to give epitheaflagallin and hydroxytheaflavin. The former is a known black tea pigment and the latter is a new pigment with 1′,2′,3′-trihydroxy-3,4-benzotropolone moiety. The results revealed a new mechanism for the production of these pigments from epigallocatechin.

1. Introduction

Tea plant (*Camellia sinensis*) was originally used as a medicine and beverage in East Asia thousands of years ago and is currently of agricultural and commercial importance worldwide.[1] In addition to caffeine, the presence of four catechin monomers (epigallocatechin (**1**), epicatechin, and their 3-*O*-galloyl esters) in a high concentration (10–25% dry weight) is what makes tea plant distinctive from other polyphenol-rich plants.[1,2] The catechin composition of green tea, which is commonly consumed in China and Japan, is similar to that of fresh tea leaves, because the polyphenoloxidase is inactivated by steaming or roasting immediately after harvesting of fresh leaves. On the other hand, black tea contains a complex mixture of oxidation products produced by enzymatic oxidation of the original catechins, because the fresh leaves are rolled and crushed during black tea manufacturing, and thus, the catechins are mixed with active enzymes and oxygen molecules. The oxidation reaction yields numerous products, only some of which have so far been chemically clarified.[3,4]

Theaflavins are well known reddish-yellow pigments characteristic of black tea and with a unique 1′,2′-dihydroxy-3,4-benzotropolone moiety.[5] The pigments are formed by oxidative coupling between catechol-type catechins (epicatechin and its gallate) and pyrogallol-type catechins (**1** and its gallate). The presence of minor pigments with related benzotropolone structures has also been reported in black tea,[6–14] and these pigments were shown to be produced by a mechanism similar to that of theaflavin synthesis. Epitheaflagallin (**14**) and its 3-*O*-gallate, on the other hand, have 1′,2′,3′-trihydroxy-3,4-benzotropolone moiety, the hydroxylation pattern of which is different from those of theaflavins. These compounds were originally synthesized by in vitro experiments,[15] and their presence in commercial black tea was later disclosed by Nonaka et al.[16] It was presumed that **14** is produced by oxidative coupling between the B-ring of **1** and 1,2,3-trihydroxybenzene (pyrogallol) or gallic acid based on the results of in vitro enzymatic and chemical synthesis.[2]

Since the contents of **1** and its 3-*O*-galloyl ester account for over 70% of total tea catechins in tea leaves, oxidation of these pyrogallol-type catechins is most important in production of black tea polyphenols. Previously, we disclosed that dimers of the B-ring quinones of **1** and its gallate accumulate at the initial stage of tea fermentation.[17] Furthermore, a subsequent study showed that dehydrotheasinensin A (**2a**), one of the quinone dimers produced from (−)-epigallocatechin-3-*O*-gallate, is easily converted to stable products such as theasinensins[18] and oolongtheanins[19] by oxidation–reduction dismutation.[20] Since theasinensins are major black tea constituents,[2] this oxidation pathway is important in the production of black tea polyphenols. The results also indicated that pyrogallol-type catechin B-rings are much more easily oxidized compared to the galloyl groups. However, participation of the galloyl groups in catechin oxidation has also been reported recently,[7,11–14,21] and it was suggested that oxidation of galloyl groups generates minor products, resulting in a complex reaction mixture. Therefore, to further understand the oxidation of tea catechins with pyrogallol type B-rings, **1** should be used as the substrate rather than its

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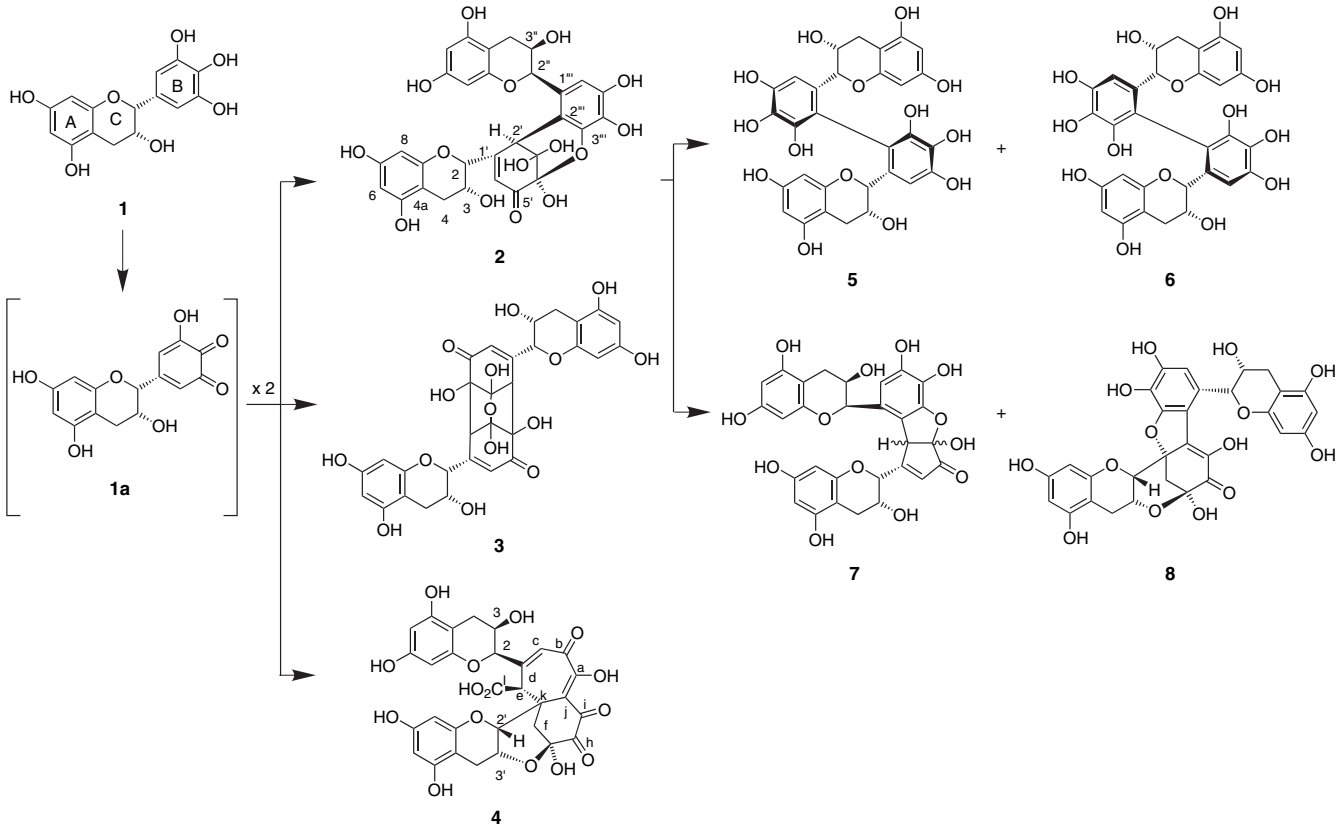
3-*O*-gallate. In the present study, we examined enzymatic oxidation of **1** and found a novel oxidation pathway producing **14** and a related new pigment.

2. Results and discussion

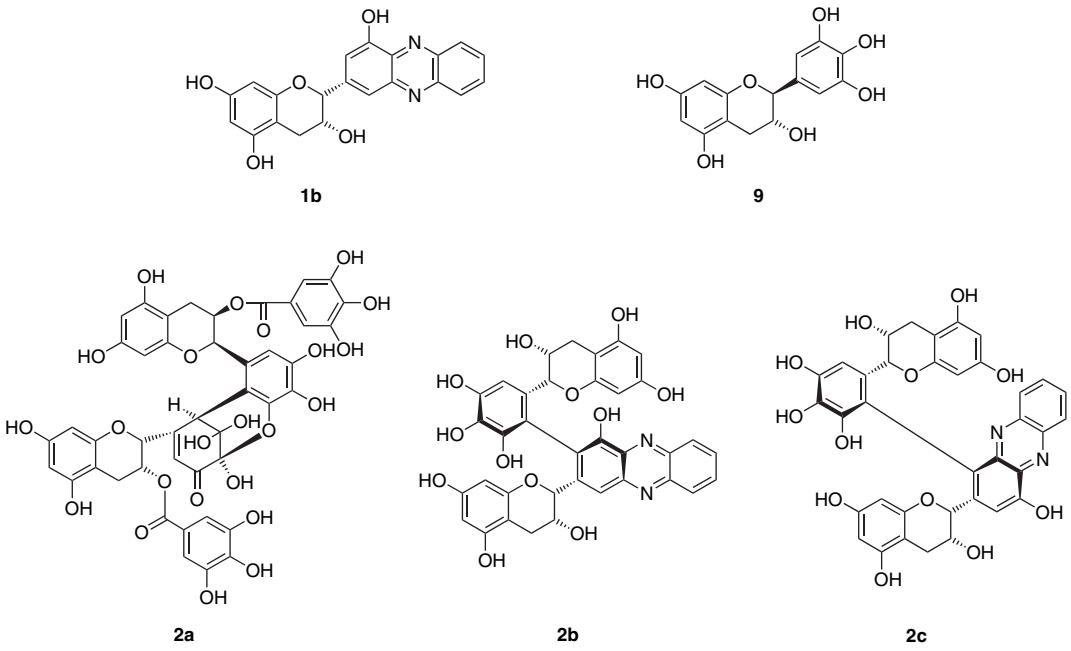
2.1. Enzymatic oxidation of epigallocatechin and isolation of quinone metabolites

Substrate **1**, which was prepared from commercial green tea,[18] was oxidized by mixing with a Japanese pear homogenate until the substrate disappeared. Japanese pear was used because it has strong catechin oxidation activity and does not result in interfering side products derived from compounds originally contained in the pear fruits.[22] The reaction mixture was cooled and acidified prior to separation, because quinone dimers are relatively stable in weakly acidic conditions.[20] After filtration, the filtrate was separated by MCI-gel CHP20P column chromatography to give compound **2**, and further chromatography of the remaining fractions yielded compounds **3** and **4** (Scheme 1). Among these products, compound **3** was identified as a symmetrical quinone dimer formerly synthesized as the radical oxidation product of **1** in an aprotic solvent.[23]

Major product **2** was obtained as a white amorphous powder and its NMR data were closely related to those of dehydrotheasinensin A (**2a**). In the ^{13}C NMR spectrum, signals of a conjugated ketone (δ 191.2), a trisubstituted double bond (δ 161.5 and 122.5), a benzyl methine (δ 45.3), and two hemiacetal carbons (δ 91.6 and 95.5) indicated the presence of a hydrated form of cyclohexenetrione moiety. In addition, the appearance of a $[M+H]^+$ ion peak at *m*/*z* 627 in the FABMS and the lack of signals of galloyl groups in the NMR spectra suggested that **2** is a desgalloyl analog of **2a**. On hydrogenation with dithiothreitol, **2** was converted to theasinensin C (**5**),[18] which has an *R*-biphenyl bond, showing that configuration of the benzyl methine carbon (C-2′) was *S*.[20] In addition, treatment of **2** with *o*-phenylenediamine yielded a phenazine derivative **2b**, which was previously obtained by similar treatment of crushed fresh tea leaves.[17] In neutral phosphate buffer, **2** was decomposed to give **5**, theasinensin E (**6**), which is an atropisomer of **5**,[19] desgalloyl oolongtheanin (**7**),[19] and dehydrotheasinensin E (**8**)[22] (Scheme 1). The former three products were produced by oxidation–reduction dismutation, which also occurs in the case of **2a**.[20] Product **8** was formed by isomerization at the C-2′ position of **2** and subsequent intramolecular acetal formation. These results allowed us to conclude the structure of **2**, which we named dehydrotheasinensin C. Products **2** and **3** were considered to be dimerization products of the B-ring *o*-quinone **1a**, which was initially generated by enzymatic hydrogenation. When *o*-phenylenediamine was directly added to the initial enzymatic oxidation mixture, phenazine derivatives **2b** and **2c**[17] were produced as the major products, and the phenazine derivative (**1b**)[24] derived from the monomer quinone (**1a**) was only obtained as one of the minor products. This result indicated that stereoselective dimerization of the quinone **1a** predominantly occurred and this reaction is the most important oxidation route of **1**.



Scheme 1. Production of **2**–**4** from **1** and decomposition of **2**.

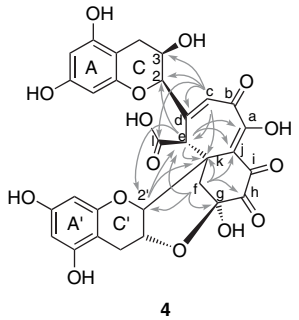


1b 9

2a 2b 2c

Product **4** was obtained as brown amorphous powder and showed a $[M+H]^+$ peak at *m*/*z* 625 in FABMS, indicating that this compound is another oxidation product with a dimeric structure. This was supported by the observation of two sets of signals for the catechin A- and C-rings in the ^1H and ^{13}C NMR spectra. In the ^{13}C NMR spectrum, the remaining signals were attributed to an acetal (*δ* 94.0, C-g), a methylene (*δ* 37.4, C-f), a methine (*δ* 49.2, C-e), a quaternary carbon (*δ* 44.6, C-k), four olefinic carbons (*δ* 152.3, C-a; 128.2, C-c; 156.2, C-d; 120.5, C-j), two conjugated carbonyl carbons (*δ* 185.6, C-b; 191.6, C-h), and a carboxyl carbon (*δ* 166.0, C-l). The presence of a carboxyl group was supported by the appearance of a $[M-CO_2+H]^+$ peak at *m*/*z* 581 in FABMS. Since the molecular weight was calculated to be 624 from the results of FABMS, the molecular formula was suggested as $C_{30}H_{24}O_{15}$, and this was supported by elemental analysis. However, the ^{13}C NMR spectrum showed only 29 carbon signals, suggesting that one carbonyl carbon signal (C-i) was not detected in the spectrum, probably due to severe broadening caused by keto–enol tautomerization or hydration.

In the HMBC spectrum (Fig. 1), proton signals of the olefinic methine H-c and aliphatic methine H-e were correlated to the C-ring of C-2, and H-e and the aliphatic methylene H-f were correlated with C-2′. H-c was also coupled with C-d and the conjugated carboxyl carbon C-l. In addition, correlations of H-e with C-k, and C-j, H-f with C-g, C-h, C-k, and C-j, and other correlations illustrated in Figure 1 suggested connection between C-c–C-d (–C-2)–C-e (–C-l)–C-k (–C-2′, –C-j)–C-f–C-g–C-h. Observation of allyl ^1H–^1H coupling of H-c with H-2 and H-e in the ^1H–^1H COSY spectrum supported this structure. Formation of a hemiacetal ring between the acetal carbon C-g and C-3′ hydroxyl group was deduced from resonance of the C-ring of C-4′ at a higher field (*δ* 25. 5) compared to usual catechin C-4 carbons (*δ* 28), which was similarly observed in the case of **8** (*δ* 25.0).[22] C-b showed no correlation peaks with any protons; however, its chemical shift (*δ* 185.6) indicated that this carbonyl carbon is located between two double bonds. Furthermore, appearance of a ^4J correlation between H-e and C-a suggested that C-a is located between C-j and C-b. At this stage, a novel structure represented by formula **4** or its tautomer is proposed for this unstable oxidation product. However, we could not obtain further spectral evidence to conclude the complete structure of **4** because of tautomerization and gradual decomposition of the compound during NMR measurements.

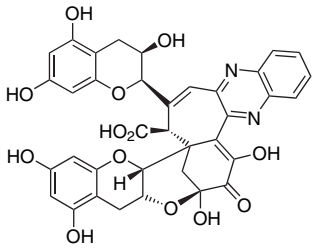


4

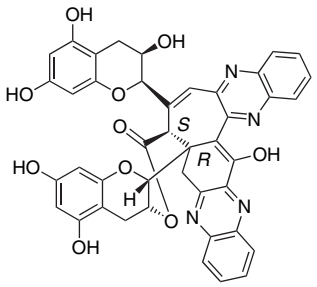
Figure 1. Selected HMBC correlations for **4**.

2.2. Phenazine derivatives of the quinone metabolites

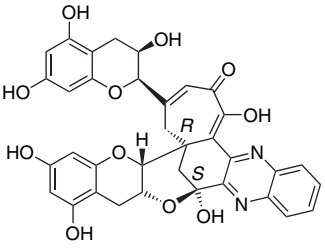
To obtain further evidence on the structure of the new metabolite **4**, we next performed an experiment to trap the unstable oxidation products as quinoxaline derivatives by condensation with *o*-phenylenediamine, because compound **4** apparently has an α-diketone or equivalent structure.[17,20] After **1** was oxidized enzymatically, *o*-phenylenediamine was directly added to the reaction mixture, resulting in isolation of four new condensation products, **10** (yield 0.3%), **11** (0.6%), **12** (0.9%), and **13** (0.08%), along with **2b** (yield 33%), **2c** (6%), **1b** (3%), **3** (0.8%), **5** (0.5%), and **9** (0.3%), and recovery of **1** (7.7%). The results confirmed the predominant formation of **2** from **1** in the reaction mixture.



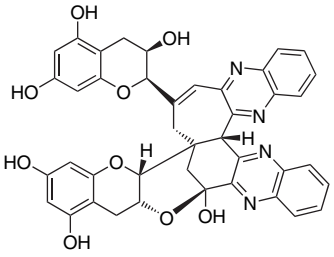
10



11



12



13

Product **10** was obtained as a brown amorphous powder and showed UV absorption at 354 nm. FABMS exhibited a $[M+H]^+$ peak at *m/z* 697 and $[M-CO_2+H]^+$ peak at 653. Since ^1H and ^{13}C NMR spectra (Table 1) indicated the presence of one quinoxaline ring,[17] the molecular weight of 696 coincided with that of the expected condensation product between **4** and *o*-phenylenediamine. The ^1H and ^{13}C NMR spectral data and HMBC correlations (Fig. 2) were also related to those of **4**, indicating a partially identical structure. The location of the quinoxaline ring at C-a (δ 152.7) and C-b (δ 152.4) was determined based on the HMBC correlation between these carbons and H-c. A chemical shift in C-h (δ 193.2) indicated that this carbonyl group is conjugated with a double bond. In addition, correlations of H-f with C-e, C-k, C-j, C-g, and C-h, and ^4J correlation of H-e to an oxygen-bearing olefinic carbon (C-i) allowed construction of a cyclohexenone ring. Furthermore, the upfield shift of C-4′ suggested formation of a hemiacetal ring between C-3′ and C-g. Thus, the plane structure of this quinoxaline derivative was concluded to be as shown in formula **10**. The NOESY correlations of H-e with H-f, H-2, H-3, and H-2′, and H-c with H-2 and H-3 supported this structure; however, the absolute configuration at C-e and C-k could not be determined at this stage, but was presumed to be the same as that of the quinoxaline derivatives **12** and **13** described below.

Product **11** was shown to have two quinoxaline units using FABMS (*m/z* 751 $[M+H]^+$) and according to the ^1H and ^{13}C NMR spectra (Table 1). Other NMR signals were related to those of **10**, except for the appearance of an sp^2 carbon signal at δ 152.6 (C-g) instead of an acetal carbon and a large low field shift of the C-ring of H-3′ (δ 5.60) compared to that of **10** (δ 4.83). The HMBC correlations (Fig. 2) revealed the presence of similar carbon strings from C-c to C-h through C-k. Correlations of H-c with C-a and C-b, and H-f with C-g and C-h confirmed the location of the quinoxaline units. The carboxyl group (δ 172.4, C-l), which was correlated with H-e, showed a small correlation peak with H-4′. Taking the large low field shift of H-3′ into account, the C-3′ hydroxyl group was believed to be esterified by this carboxyl group. Thus, the plane structure of **11** was deduced from these spectral data. As for the relative stereochemistry at C-e and C-k, H-e showed strong NOESY correlations with both methylene protons of C-f, indicating that H-e and H-f were located on the same side of the molecule.

Product **12** exhibited a $[M+H]^+$ peak at *m/z* 653 in FABMS, indicating a molecular weight 44 mass units smaller than that of **10**. In addition, the absence of the carboxyl carbon signal in the ^{13}C NMR spectrum suggested that **12** is a quinoxaline derivative of the decarboxylated form of **4**. The ^1H and ^{13}C NMR spectra revealed the presence of two methylenes (C-e and C-f), and HMBC correlations of these methylenes showed that the structure of **12** was partially similar to that of **10**, except for the absence of the carboxyl group at C-e (Fig. 2). The chemical shift of a carbonyl carbon (δ 186.1) indicated its location between two double bonds, and the HMBC correlations of this carbon with H-2 and H-e suggested that it is attributable to C-b. Correlations of H-c with C-a (δ 163.6), and H-e with C-j (δ 113.4) supported construction of a seven-membered ring structure. Although C-i (δ 151.7) showed no correlation peaks, correlation of C-h with H-f showed the location of the quinoxaline ring at C-h and C-i. The UV absorption of this product at 405 nm reflected the long conjugation of double bonds including the quinoxaline ring. The formation of an ether linkage between the C-3′ hydroxyl group and acetal carbon C-g was indicated by observation of an upfield shift of C-4′ and a ^4J HMBC correlation between H-4 and C-g. As for stereochemistry, strong NOEs between H-e and H-f in the NOESY spectrum indicated that these protons were located on the same side of the molecule. In addition, an NOE cross peak between H-2 and H-8′ was also observed (Fig. 3). Since the absolute configuration at C-2 and C-2′ was *R*, this observation indicated

Table 1. ^{1}H (500 MHz), ^{13}C (125 MHz) NMR data for phenazine derivatives **10–13** (δ in ppm, J in Hz)

Position	**10**		**11**		**12**		**13**	
	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
2	4.65 (s)	81.4	4.94 (s)	80.7	4.88 (s)	80.5	4.79 (s)	80.1
3	4.40 (m)	67.3	4.57 (br s)	62.4	4.61 (br s)	62.8	4.63 (br s)	63.0
4	2.80–2.76 (2H, m)	28.8	2.94 (dd, 16.9, 2.1) 2.88 (dd, 16.9, 4.6)	27.2	2.97 (dd, 16.8, 4.1) 2.90 (dd, 16.8, 2.4)	29.7	2.93 (br d, 16.7) 2.85 (dd, 16.7, 4.6)	29.4
4a		99.7		99.4		99.5		99.3
5		157.2[c]		155.9[c]		157.7[b]		157.5[b]
6	5.99 (d, 2.5)[a]	96.2[d]	6.07 (d, 1.7)[a]	96.4[d]	6.05 (d, 2.2)[a]	96.6[c]	6.01 (br s)	96.5[c]
7		156.9[c]		157.5[c]		157.6[b]		157.5[b]
8	5.95 (d, 2.5)[a]	96.0[d]	6.05 (d, 1.7)[a]	95.3[d]	5.94 (d, 2.2)[a]	95.6[c]	6.01 (br s)	96.4[c]
8a		156.7[c]		157.4[c]		157.5[b]		157.0[b]
2′	5.04 (s)	70.9	4.33 (s)	73.1	4.13 (s)	72.4	4.24 (s)	73.1
3′	4.83 (m)	66.3	5.60 (br s)	72.4	4.03 (m)	66.0	3.66 (m)	66.2
4′	2.80–2.76 (2H, m)	25.6	2.80 (2H, m)	25.5	2.70 (dd, 17.4, 2.4) 2.64 (dd, 17.4, 4.6)	25.7	2.60 (dd, 17.6, 5.3) 2.50 (dd, 17.6, 1.6)	25.1
4a′		98.7		97.4		99.0		99.1
5′		156.6[c]		154.8[e]		157.2[b]		156.3[b]
6′	5.86 (d, 2.5)[b]	95.4[d]	5.97 (d, 2.1)[b]	95.4[f]	5.96 (d, 2.4)	96.5[c]	6.04 (d, 2.3)[a]	95.5[c]
7′		156.1[c]		157.8		155.8[b]		155.4[b]
8′	5.89 (d, 2.5)[b]	95.3[d]	5.98 (d, 2.1)[b]	96.9[f]	5.90 (d, 2.4)	95.2[c]	5.96 (d, 2.3)[a]	95.0[c]
8a′		56.0[c]		157.2[e]		155.6[b]		155.4[b]
a		152.7[e]		150.5		163.6		158.4
b		152.4[e]		152.6		186.1		155.0
c	6.91 (s)	129.7	7.46 (s)	130.4	6.77 (s)	128.3	7.42 (d, 1.6)	127.1
d		151.0		143.9		152.4		145.4
e	4.20 (s)	51.4	4.13 (s)	44.9	3.18 (d, 16.2) 2.96 (d, 16.2)	39.9[d]	3.29 (br d, 14.9) 1.91 (br d, 14.9)	34.3
f	2.63 (d, 13.5) 2.45 (d, 13.5)	40.7	4.04 (d, 16.0) 3.77 (d, 16.0)	38.0	2.52 (d, 13.2) 2.15 (d, 13.2)	39.9[d]	2.42 (d, 13.0) 2.36 (d, 13.0)	33.9
g		93.9		152.6		93.1		94.5
h		193.2		144.2		153.4		151.4
i		150.2		152.0		151.7		154.0
j		130.6[f]		115.2		113.4	4.91 (s)	53.4
k		55.1		54.3		41.2		53.3
l		174.9		172.4				
a-OH					16.60 (s)			
g-OH					6.30 (s)			
PHE	8.02–8.00 (1H, m) 7.95–7.93 (1H, m) 7.73–7.70 (2H, m)	141.2 140.4 130.6[f] 130.3 129.0 128.8	8.18–8.16 (1H, m) 8.09–8.04 (3H, m) 7.91–7.87 (2H, m) 7.83–7.77 (2H, m)	143.1, 142.2 141.8, 140.8 131.7, 131.4 131.3, 130.7 129.9, 129.7 129.7, 129.3	8.13 (1H, m) 8.10 (1H, m) 7.92 (1H, m) 7.85 (1H, m)	140.1 137.8 132.6 130.9 129.7 126.8	8.18 (1H, m) 8.00 (1H, m) 7.86–7.81 (2H, m) 7.78–7.74 (1H, m) 7.69 (1H, m) 7.54 (1H, m) 7.39 (1H, m)	143.5, 142.0 141.1, 140.2 131.2, 130.9 130.5, 130.1 129.8, 129.6 129.4, 129.3

[a–f] Assignments may be interchanged in each column.

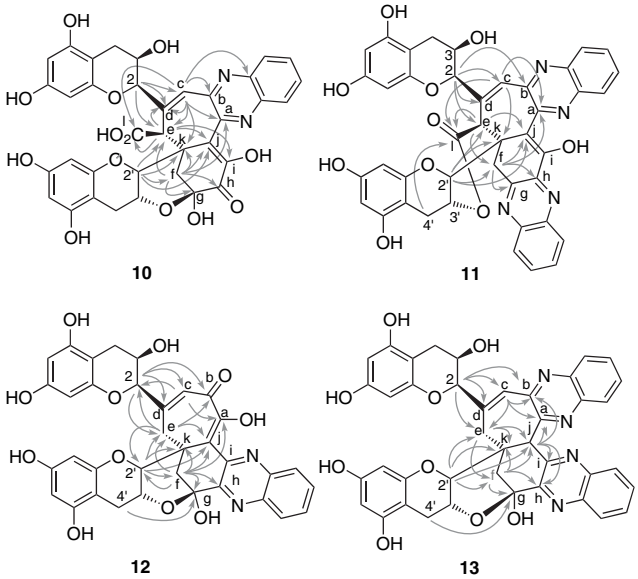


Figure 2. Selected HMBC correlations for **10–13**.

that the configurations at C-g and C-k were *S* and *R*, respectively.

The ^{1}H NMR spectra of product **13** were related to those of **12** (Table 1), showing signals of two sets of catechin A- and C-rings, two methylenes (H-e and H-f), and an olefinic methine (H-c). However, two sets of signals arising from quinoxaline moieties and a singlet aliphatic methine signal at δ 4.91 also appeared. In the ^{13}C NMR spectrum, a methine carbon (C-j) and two sp^{2} carbons bearing nitrogen atoms (C-a and C-b) were observed instead of the enone unit at C-b, C-a, and C-j in **12**. Remaining carbon signals and their HMBC correlations (Fig. 2) were similar to those of **12**, supporting close structural relationships between **12** and **13**. The C-a and C-b of **13** were correlated with both the H-c and newly appeared methine proton (H-j) in the HMBC spectrum, indicating that **13** was generated by condensation of additional *o*-phenylenediamine to the C-a and C-b positions of **12**. This was supported by the observation of a [M+Na]$^{+}$ peak at *m*/*z* 747 in the MALDI-TOF MS. In the NOESY

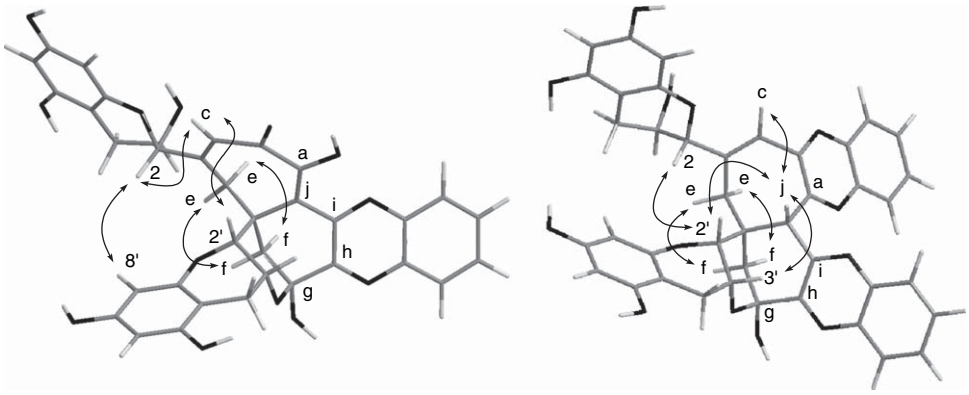


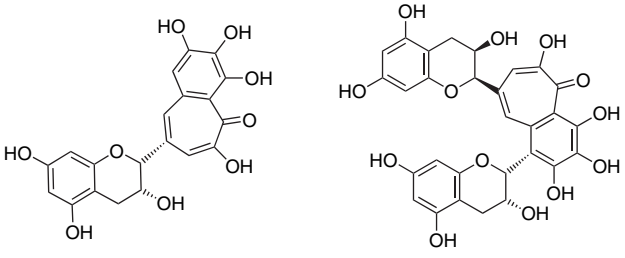
Figure 3. Selected NOESY correlations for **12** and **13**.

spectrum (Fig. 3), H-j showed NOE correlations with H-2′, H-3′, and H-c. In addition, NOEs between H-e and H-f, and between H-2 and H-2′ indicated that the configurations at C-g, C-j, and C-k were *S*, *R*, and *R*, respectively.

Production of the derivatives **10**–**13** not only supported the proposed structure of **4**, but also indicated the occurrence of decarboxylation at the C-e in the molecule of **4** in the following metabolism.

2.3. Degradation products of the quinone metabolites

The unstable product **4** was expected to be a precursor of some black tea polyphenols, as we have shown that **2** was a precursor of important black tea polyphenols **5**, **6**, and **7**. So, we examined decomposition of **4** on heating, which is expected to occur at the final stage of black tea manufacturing. An aqueous solution of **4** was heated at 80 °C for 10 min and then analyzed by HPLC. Photodiode array detection revealed production of two pigments, **14** and **15**; however, isolation of these pigments directly from the reaction mixture failed owing to the shortage of **4**. Isolation of **14** and **15** was achieved by performing a large-scale experiment in which the initial enzymatic oxidation products of **1** were heated and the unstable products including **2** and **4** were converted to more stable products (**5**, **6**, **7**, **8**, **14**, and **15**). Here, it might be of interest to note that MALDI-TOF MS analysis of the total mixture of phenolic products suggested production of a trimer and tetramer of **1**, though the peak intensity was much smaller than those of the dimeric products.



Pigment **14** was identified as epitheaflagallin, having a characteristic 1′,2′,3′-trihydroxy-3,4-benzotropolone unit, by comparisons of ^{1}H and ^{13}C NMR, UV and MS spectra.[16] At this stage, the unstable intermediate **4** was named proepitheaflagallin because it was found to be a precursor of the black tea pigment **14**.

Pigment **15** showed characteristic UV absorptions at 286, 309, 378, and 428 nm, which closely resembled to those of **14**. The ^{1}H and ^{13}C NMR spectra were also related to those of **14**, suggesting the presence of a trihydroxybenzotropolone unit. However, two sets of signals arising from catechin A- and C-rings were also observed. In addition, the absence of an aromatic singlet attributable to H-f of **14** in the ^{1}H NMR spectrum and low field shift of the C-f signal (δ 115.4, **14**: δ 111.8) in the ^{13}C NMR spectrum indicated that **15** has additional catechin A- and C-rings at the C-f position of compound **14**. This structure was confirmed by the HMBC correlations shown in Figure 4. Interestingly, the chemical shift of the C-i hydroxyl proton (δ 15.21) indicated hydrogen bonding of this hydroxyl group with the adjacent carbonyl group at C-a. This pigment was found to be a new compound and named hydroxytheaflavin.

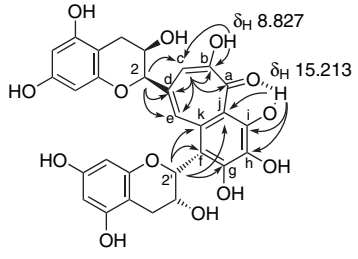
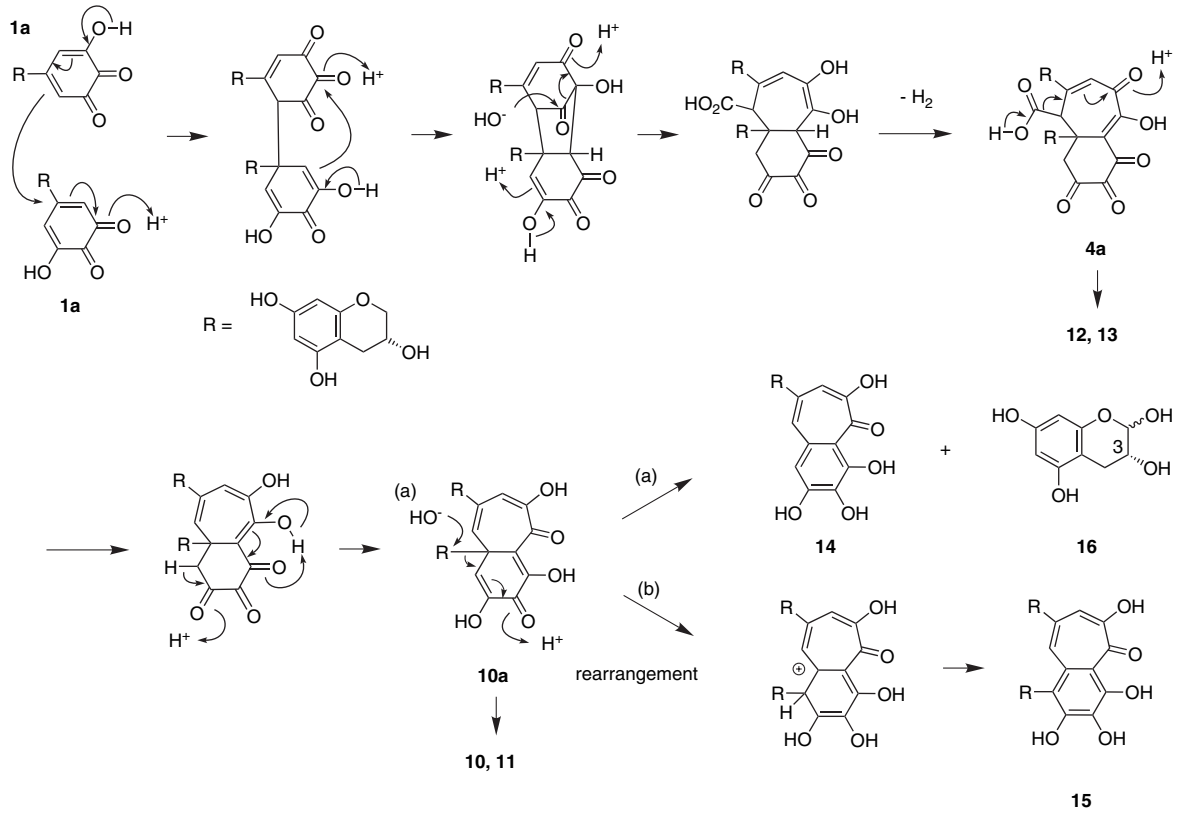


Figure 4. Selected HMBC correlations for **15**.

3. Conclusion

Many theaflavin related pigments, namely, theaflavins, isotheaflavins,[6,7] neotheaflavins,[7,8] theaflavic acids,[9,10] theaflavates,[7,11,12] theadibenzotropolones,[13,14] and theatribenzotropolone A,[14] have been isolated and synthesized. All were produced by oxidative condensation between a catechol ring and pyrogallol ring. Theaflagallins, including **14**, are exceptional because they were produced by condensation



Scheme 2. Proposed mechanisms for production of **14** and **15** from **1**.

between two pyrogallol rings.[15,16] Since pyrogallol itself does not occur in fresh tea leaf, **14** and its gallate found in black tea were considered to have formed by condensation with gallic acid via decarboxylation.[2,16] However, our results revealed that **14** was produced from **1** alone even in the absence of gallic acid, and we succeeded in confirming the key intermediate of this mechanism. Details of the proposed production of **14** and **15** are shown in Scheme 2. In the last step, migration or elimination of the flavan C-ring occurs; this was supported by the fact that compound **16**, which corresponds to the eliminated flavan A, C-rings were actually isolated from black tea.[20] Recently, we also demonstrated that the 3-*O*-galloyl ester of **14** is produced by enzymatic oxidation of the 3-*O*-gallate of **1**,[20] and the reaction probably proceeds in a similar manner.

4. Experimental

4.1. General

UV spectra were obtained with a JASCO V-560 UV/VIS spectrophotometer. Optical rotations were measured with a JASCO DIP-370 digital polarimeter. ^{1}H, ^{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) at 27 °C with a Varian Unity plus 500 spectrometer operating at 500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR. Coupling constants are expressed in Hertz, and chemical shifts are given on a δ (ppm) scale with tetramethylsilane as an internal standard. MS were recorded on a JEOL JMS DX-303 spectrometer, and glycerol or *m*-nitrobenzyl alcohol was used as the matrix for the FABMS measurements. MALDI-TOF MS was measured on a Voyager-DE PRO (Applied Biosystems), and 2,5-dihydroxy benzoic acid (10 mg/mL) was used as the matrix.

Column chromatography was performed with Diaion HP20SS, MCI-gel CHP20P (75–150 μm, Mitsubishi Chemical Co.), Sephadex LH-20 (25–100 μm, Pharmacia Fine Chemical Co. Ltd), TSK-gel Toyopearl HW-40 (TOSO Co. Ltd), and Chromatorex ODS (Fuji Silysia Chemical Ltd). TLC was performed on precoated Kieselgel 60 F_{254} plates (0.2 mm thick, Merck) with benzene–ethyl formate–formic acid (1:7:1, v/v) or chloroform–methanol–water (14:6:1, v/v) and spots were detected by ultraviolet (UV) illumination and by spraying with 2% ethanolic $FeCl_3$ or 10% sulfuric acid reagent followed by heating. Analytical HPLC was performed on a Cosmosil 5C_{18}-AR II (Nacalai Tesque Inc.) column (250×4.6 mm i.d.) 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 (flow rate, 0.8 mL/min; detection: JASCO photodiode array detector MD-910). Epigallocatechin was isolated from commercial green tea and recrystallized from water.

4.2. Enzymatic oxidation of epigallocatechin and isolation of quinone metabolites

Japanese pear fruits (200 g) were homogenized with 200 mL of H_2O and filtered through four layers of gauze at 0 °C. The filtrate was mixed with an aqueous solution of **1** (1.5 g/75 mL) and vigorously stirred for 90 min at room temperature. The mixture was acidified with 2 mL of trifluoroacetic

acid (TFA) and directly applied to a column of MCI-gel CHP20P (3 cm i.d.×30 cm). After washing with 0.1% TFA, the column was eluted with 0–50% MeOH in 0.1% TFA (5% stepwise gradient elution, each 200 mL), and fractions (each 15 mL) were analyzed by HPLC. Fractions containing **2** were collected and lyophilized to give a white powder (422 mg). Fractions containing **3** and **4** were separately collected and purified by Chromatorex ODS (H_2O–MeOH) to give **3** (47 mg) and **4** (50 mg). Compound **3** was identified with a known oxidation product of **1** by comparison of the spectral data.[23]

4.2.1. Dehydrotheasinensin C (2). White amorphous powder, $[\alpha]_D^{20}$ −74.5 (*c* 0.1, MeOH); IR ν_{max} 3365, 2920, 1696, 1627, 1606 cm^{-1}; UV (MeOH) λ_{max} 269(ε 3640) nm; ^1H NMR (500 MHz, d_6-acetone) δ 2.70 (1H, dd, *J*=16.4, 4.4 Hz, H-4″), 2.73 (2H, br s, H-4), 2.95 (1H, dd, *J*=16.4, 4.4 Hz, H-4″), 4.17 (1H, m, H-3), 4.34 (1H, m, H-3″), 4.42 (1H, s, H-2′), 4.73 (1H, br s, H-2), 5.62 (1H, br s, H-2″), 5.94, 5.97, 5.97, 6.00 (each 1H, d, *J*=2.2 Hz, H-6, 8, 6″, 8″), 6.43 (1H, s, H-6′), 6.80 (1H, s, H-6‴); important long-range ^1H–^1H couplings observed in ^1H–^1H COSY, H-2/H-2′, H-2/H-6′; ^{13}C NMR (125 MHz, d_6-acetone) δ 27.5, 29.0 (C-4, C-4″), 45.3 (C-2′), 64.4 (C-3), 65.6 (C-3″), 75.5 (C-2″), 78.2 (C-2), 91.6 (C-3′), 95.0, 95.2, 95.5, 95.9, 96.1 (C-6, C-8, C-4′, C-6″, C-8″), 99.0, 99.1 (C-4a, C-4a′), 108.2 (C-6‴), 112.0 (C-2‴), 122.5 (C-6′), 127.3 (C-1‴), 132.4 (C-4‴), 142.3 (C-3‴), 145.1 (C-5‴), 155.4, 156.4, 156.7, 156.9 (2C), 156.9 (C-5, C-7, C-8a, C-5″, C-7″, C-8a″), 161.5 (C-1′), 191.2 (C-″); important HMBC correlations (H to C), H-2/C-1′, C-2′, C-6′, H-2′/C-1′, C-3′, C-4′, C-6′, C-1‴, C-2‴, C-3‴, H-2″/C-1‴, C-2‴, C-6‴, H-6‴/C-1‴, C-2‴, C-4‴, C-5‴; FABMS *m*/*z* 627 $[M+H]^+$, 609 $[M-H_2O+H]^+$; Anal. Calcd for $C_{30}H_{26}O_{15}\cdot 7H_2O$: C, 47.88; H, 5.36. Found: C, 47.89; H, 5.21.

4.2.1.1. Treatment of 2 with *o*-phenylenediamine. Compound **2** (2 mg) was treated with a solution of *o*-phenylenediamine (2 mg) in 5% AcOH in EtOH (1 mL) at room temperature for 1 h. HPLC analysis of the reaction mixture showed a product peak at t_R 30.3 min corresponding to the phenazine derivative **2b**.

4.2.1.2. Degradation of 2 under neutral conditions. Compound **2** (100 mg) was dissolved in 0.1 mM Na–K phosphate buffer (pH 7.1) (100 mL) and stirred for 3 h at room temperature. The mixture was acidified to pH 2 by addition of a few drops of diluted HCl and directly applied to a Sephadex LH-20 column (2 cm i.d.×20 cm) with water containing increasing proportions of MeOH (0–80%) to give **5** (24.0 mg), **6** (3.3 mg), **7** (22.3 mg), and **8** (6.8 mg).

4.2.2. Proepitheaflagallin (4). Brown amorphous powder, $[\alpha]_D^{20}$ 92.5 (*c* 0.1, MeOH); IR ν_{max} 3403, 1706, 1631, 1519, 1468 cm^{-1}; UV (MeOH) λ_{max} 271(ε 9490) nm; ^1H NMR (500 MHz, d_6-acetone) δ 2.09 (1H, d, *J*=13.0 Hz, H-f'), 2.58 (1H, d, *J*=13.0 Hz, H-f), 2.82 (2H, m, H-4′), 2.84 (1H, dd, *J*=16.8, 2.7 Hz, H-4), 2.91 (1H, dd, *J*=16.8, 4.3 Hz, H-4), 3.88 (1H, br s, H-2′), 4.47 (1H, br s, H-3), 4.52 (1H, m, H-3″), 4.81 (1H, s, H-2), 4.81 (1H, s, H-e), 5.94, 5.97, 5.95, 5.97, 6.03, 6.07 (each 1H, d, *J*=2.3 Hz, H-6, 8, 6″, 8″), 6.67 (1H, s, H-c); important long-range ^1H–^1H coupling observed in ^1H–^1H COSY, H-2/H-c, H-e/H-c, H-2′/H-f (δ 2.08); ^{13}C NMR (125 MHz, d_6-acetone) δ 25.5 (C-4′), 29.0 (C-4), 37.4 (C-f), 44.6 (C-k), 49.2 (C-e), 65.2 (C-3), 66.2 (C-3′), 94.0 (C-g), 95.2, 95.4, 96.6, 96.7 (C-6, C-8, C-6″, C-8″), 98.3, 99.1 (C-4a, C-4a′), 120.5 (C-j), 128.2 (C-c), 152.3 (C-a), 155.2, 155.3, 157.0, 157.5, 157.6 (2C) (C-5, C-7, C-8a, C-5′, C-7′, C-8a′), 156.2 (C-d), 116.0 (C-l), 185.6 (C-b), 191.6 (C-h) C–I was not detected; important HMBC correlations (H to C), H-c/C-2, C-3, C-d, C-e, C-k, C-l, H-e/C-2, C-2′, C-a, C-c, C-d, C-f, C-k, C-j, C-l, H-2/C-d, C-c, C-e, H-2′/C-e, C-f, C-k, H-f/C-e, C-k, C-j, C-g, C-h, C-2′; FABMS *m*/*z* 625 $[M+H]^+$, 581 $[M-CO_2+H]^+$; Anal. Calcd for $C_{30}H_{24}O_{15}\cdot 3.5H_2O$: C, 52.41; H, 4.54. Found: C, 52.14; H, 4.58.

4.3. Enzymatic oxidation of epigallocatechin and treatment with *o*-phenylenediamine

Japanese pear fruits (4 kg) were homogenized with 1.6 L of H_2O and filtered through four layers of gauze at 0 °C. The filtrate (4.0 L) was mixed with an aqueous solution of **1** (30 g/1.0 L) and vigorously stirred for 2.5 h at room temperature. The mixture was poured into EtOH (15 L) containing *o*-phenylenediamine (11.5 g) and AcOH (750 mL), and stirred gently for 30 min. After removal of insoluble precipitates by filtration, the filtrate was concentrated and applied to Diaion HP20SS (5.5 cm i.d.×55 cm) column chromatography. After washing the column with H_2O to remove sugars and AcOH, the products were eluted out with H_2O containing 0–100% MeOH (10% stepwise elution, each 1.0 L) yielding nine fractions: fr. 1 (4.0 g), fr. 2 (0.89 g), fr. 3 (16.5 g), fr. 4 (2.59 g), fr. 5 (1.81 g), fr. 6 (3.18 g), fr. 7 (1.07 g), fr. 8 (1.77 g), and fr. 9 (0.56 g). Fr. 1 was successively subjected to Sephadex LH-20 (0–60% MeOH, gradient elution), Diaion HP20SS (0–50% MeOH), and Chromatorex ODS (0–50% MeOH) chromatography to give **1** (2.3 g), **3** (265 mg), **5** (145 mg), and **9** (93 mg). Crystallization of fr. 2 from water yielded **1** (0.5 g). The recovery of starting material **1** was 9.3%. Separation of fr. 3 by successive chromatography over MCI-gel CHP20P (10–100% MeOH) and Sephadex LH-20 (20–100% MeOH) yielded **2b** (10.24 g) and **2c** (1.84 g), which were identified as isomers of phenazine derivatives prepared in our previous work.[17] Fr. 4 was applied to Sephadex LH-20 column chromatography (30–90% MeOH) and then further separated by Chromatorex ODS (20–80% MeOH) and MCI-gel CHP20P (25–75% MeOH) chromatography to yield a phenazine derivative **10** (97.2 mg). Fr. 6 was separated into two fractions, fr. 6-1 and fr. 6-2, by Sephadex LH-20 chromatography (50–90% MeOH). The concentration of aqueous MeOH solution of fr. 6-1 yielded reddish-brown powder (806 mg), which was identified as a phenazine derivative of epigallocatechin (**1b**). Fr. 6-2 gave microcrystalline powder of **12** (272 mg) from aqueous MeOH. Fr. 7 was separated by Chromatorex ODS (40–90% MeOH) and Sephadex LH-20 (30–90% MeOH) chromatography to give **1b** (200 mg). Fr. 8 was separated into two fractions, fr. 8-1 and fr. 8-2, by Sephadex LH-20 chromatography (30–100% MeOH). Fr. 8-1 was successively chromatographed over MCI-gel CHP20P (30–80% MeOH), Sephadex LH-20 (60–80% MeOH), Toyopearl HW-40 (40–80% MeOH), and Sephadex LH-20 (EtOH) to give **13** (23.6 mg). Chromatography of fr.

8-2 over Sephadex LH-20 (50–90% MeOH) yielded **11** (190.2 mg).

4.3.1. Phenazine derivative 10. Brown amorphous powder, $[\alpha]_D^{26}$ 104.5 (*c* 0.1, MeOH); IR (dry film) ν_{max} 3383, 1696, 1627, 1609, 1519, 1469 cm^{-1}; UV (MeOH) λ_{max} 354 (ε 7520) nm; ^1H and ^{13}C NMR data, see Table 1; important NOESY correlations: H-c/H-2 and H-3, H-e/H-2, H-3, H-2′, H-f (δ 2.63), and H-f (δ 2.452); FABMS *m/z* 697 $[M+H]^+$, 653 $[M-CO_2+H]^+$; Anal. Calcd for $C_{36}H_{28}N_2O_{13}\cdot 4.5H_2O$: C, 55.60; H, 4.80; N, 3.60. Found: C, 55.45; H, 4.82; N, 3.57.

4.3.2. Phenazine derivative 11. Brown amorphous powder, $[\alpha]_D^{26}$ 196.8 (*c* 0.1, MeOH); IR (dry film) ν_{max} 3366, 1715, 1626, 1609, 1519, 1467 cm^{-1}; UV (MeOH) λ_{max} 249 (ε 30,900), 382 (16,300) nm; ^1H and ^{13}C NMR data, see Table 1; important NOESY correlations: H-c/H-2 and H-3, H-e/H-2, H-3, H-f (δ 4.039), and H-f (δ 3.768), H-f (δ 4.039)/H-2′, H-e, and H-f (δ 3.768), H-f (δ 3.768)/H-2, H-e, and H-f (δ 4.039); FABMS *m/z* 751 $[M+H]^+$; Anal. Calcd for $C_{42}H_{30}N_4O_{10}\cdot 5.5H_2O$: C, 59.36; H, 4.86; N, 6.59. Found: C, 59.49; H, 4.78; N, 6.25.

4.3.3. Phenazine derivative 12. Yellow powder (from H_2O–MeOH, 4:1), $[\alpha]_D^{26}$ −104.5 (*c* 0.1, acetone); IR (dry film) ν_{max} 3394, 1697, 1631, 1521, 1469 cm^{-1}; UV (MeOH) λ_{max} 252 (ε 18,000), 405 (12,700) nm; ^1H and ^{13}C NMR data, see Table 1; important NOESY correlations: H-2/H-8′, H-c/H-2 and H-2′, H-e (δ 3.181)/H-f (δ 2.153) and H-e (δ 2.960), H-e (δ 2.960)/H-f (δ 2.524) and H-e (δ 3.181); FABMS *m/z* 653 $[M+H]^+$; Anal. Calcd for $C_{35}H_{28}N_2O_{11}\cdot 4.5H_2O$: C, 57.30; H, 5.08; N, 3.82. Found: C, 57.01; H, 5.39; N, 3.76.

4.3.4. Phenazine derivative 13. Brown amorphous powder, $[\alpha]_D^{26}$ 234.1 (*c* 0.1, MeOH); IR (dry film) ν_{max} 3369, 1715, 1626, 1489, 1467 cm^{-1}; UV (MeOH) λ_{max} 235 (ε 62,400), 324 (18,100) nm; ^1H and ^{13}C NMR data, see Table 1; important NOESY correlations: H-2/H-2′, H-j/H-2′, H-3′, and H-c, H-e (δ 3.287)/H-2, H-3 and H-e (δ 1.914), H-e (δ 1.914)/H-f (δ 2.361); TOF MS *m/z* 747 $[M+Na]^+$, 763 $[M+K]^+$; Anal. Calcd for $C_{41}H_{32}N_4O_9\cdot 6.5H_2O$: C, 58.50; H, 5.39; N, 6.66. Found: C, 58.59; H, 4.90; N, 6.54.

4.4. Degradation of 4

Aqueous solution of **4** (0.1 mg/0.1 mL) was heated at 80 °C for 10 min and then 5 μL of the solution was analyzed by HPLC. HPLC showed peaks arising from two pigments at 38.3 and 42.1 min, respectively. The retention times and UV absorptions of these peaks coincided with those of **14** and **15**, respectively.

4.5. Oxidation of epigallocatechin and heating of the reaction mixture

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, and then the mixture was analyzed by HPLC at intervals of 30 min. After 3 h, the starting material had almost disappeared and a large peak of **2** and moderate peak of **4** appeared. The mixture was poured into a stainless beaker (10 L) and heated until the temperature was elevated to 80 °C in order to inactivate the enzymes and degrade the unstable quinone dimers. The stainless beaker was then placed in ice water to cool down the reaction mixture. To the mixture, acetone (15 L) was added and stirred gently for 30 min, and then the precipitates were removed by filtration. A sample (50 mL) of the filtrate was concentrated and applied to a Diaion HP20SS (2 cm i.d.×10 cm). After washing the column with water, the polyphenols were eluted out with 70% MeOH and concentrated to dryness. The total mixture of products thus obtained was subjected to MALDI-TOF MS analysis. The spectrum showed large peaks at *m/z* 603 (relative intensity 37%), 633 (100%), and 649 (52%) corresponding to the $[M+Na]^+$ of **7**, $[M+Na]^+$ and $[M+K]^+$ of **5** or **6**, respectively. The spectrum also showed peaks at 936 (14%), 952 (12%), 972 (7%), 1212 (18%), 1228 (12%), and 1240 (10%). The remainder of filtrate was concentrated until acetone was removed and subjected to Diaion HP20SS (5.5 cm i.d.×55 cm), then the column was washed with H_2O. The products were eluted out with H_2O–MeOH (10% stepwise elution, each 1.0 L), yielding six fractions: fr. 1 (13.2 g), fr. 2 (5.7 g), fr. 3 (9.5 g), fr. 4 (4.5 g), fr. 5 (2.0 g), and fr. 6 (1.4 g). HPLC and TLC analyses indicated the presence of **5** and **6** in fr. 1, **1** in fr. 2, **1** and **7** in fr. 3, and **7** and **8** in fr. 4 as the major constituents of each fraction. HPLC analysis showed that fr. 6 contained the pigments produced from **4**. This fraction was separated by Sephadex LH-20 (40–100% MeOH) and Chromatorex ODS (20–80% MeOH) to give **14** (530.8 mg) and **15** (185.2 mg). Pigment **14** was identified as epitheaflagallin by comparison of spectral data and co-HPLC with authentic sample.[16]

4.5.1. Hydroxytheaflavin. Red amorphous powder, $[\alpha]_D^{20}$ −230.6 (*c* 0.1, MeOH); IR (dry film) ν_{max} 3366, 1627, 1604, 1517, 1467, 1430 cm^{-1}; UV (EtOH) λ_{max} 286 (ε 21,500), 309 (25,300), 378 (4990), 428 (2910) nm; ^1H NMR (500 MHz, d_6-acetone) δ 2.80 (1H, dd, *J*=16.8, 2.0 Hz, H-4), 2.93 (1H, dd, *J*=16.8, 4.3 Hz, H-4), 2.95 (1H, br d, *J*=17.0 Hz, H-4′), 3.06 (1H, dd, *J*=17.0, 4.3 Hz, H-4′), 4.36 (1H, br s, H-3), 4.72 (1H, br s, H-3′), 4.95 (1H, s, H-2), 5.94 (1H, s, H-2′), 5.96, 6.04 (each 1H, d, *J*=2.3 Hz, H-8 and H-6), 5.97, 6.09 (each 1H, d, *J*=2.3 Hz, H-8′ and H-6′), 7.51 (1H, d, *J*=0.9 Hz, H-c), 8.10 (1H, br s, H-e), 8.83 (1H, br s, OH at C-b), 15.21 (1H, s, OH at C-i); ^{13}C NMR (125 MHz, d_6-acetone) δ 29.4 (C-4), 29.9 (C-4′), 66.4 (C-3), 66.8 (C-3′), 78.6 (C-2′), 81.7 (C-2), 95.6, 96.9 (C-8 and C-6), 95.7, 96.5 (C-8′ and C-6′), 99.5 (C-4a), 99.6 (C-4a′), 115.4 (C-f), 117.3 (C-c), 117.4 (C-j), 127.8 (C-e), 132.0 (C-k), 135.8 (C-d), 136.0 (C-h), 151.8 (C-i), 152.2 (C-g), 154.9 (C-b), 156.6, 156.7, 157.6 (2C), 157.7, 157.9 (C-5, C-5′, C-7, C-7′, C-8a, and C-8a′), 183.1 (C-a); HR-FABMS *m/z* 581.1294 $[M+H]^+$ (Calcd for $C_{29}H_{25}O_{13}$: 581.1295).

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