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Oxidation mechanism of black tea pigment theaflavin by peroxidase

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ABSTRACT

A large number of black tea polyphenols remain uncharacterized because of the complexity of catechin oxidation reactions that occur during tea fermentation. In the course of our studies on black tea polyphenols, we examined the enzymatic degradation of theaflavins, which are black tea pigments having a benzotropolone chromophore. Oxidation of theaflavin with peroxidase afforded a new product named theacoumarin A together with known pigment theanaphthoquinone. The structure of the new compound was determined by spectroscopic examination and a production mechanism via theanaphthoquinone is proposed.

Plant polyphenols have been demonstrated to show a wide range of biological activities,[1] and black tea, one of the most popular beverages worldwide, is an important source of polyphenols for humans. Black tea is produced by crushing and kneading the fresh leaves of *Camellia sinensis*, which contains epicatechin (**1**), epigallocatechin (**2**), and their galloyl esters as major polyphenols. During processing, the tea catechins are oxidized by reaction with oxygen by catalysis with endogenous enzymes, polyphenol oxidase and peroxidase,[2] to afford various oxidation products.[3] The most important products are theaflavins, mainly including theaflavin (**3**), theaflavin-3-*O*-gallate (**4**), theaflavin-3′-*O*-gallate (**5**), and theaflavin-3,3′-di-*O*-gallate (**6**), which are reddish-yellow pigments with the benzotropolone chromophore (Fig. 1). The pigments are produced by oxidative coupling between pyrogallol-type and catechol-type catechins.[4] Theaflavins contribute largely to the quality, taste, and color of black tea, and are shown to have various biological activities, such as radical scavenging,[5] α-glucosidase inhibition,[6] lipase inhibition,[7] anti-inflammatory activity,[8] and prevention of mouse type IV allergy.[9] However, theaflavins are degraded enzymatically in the process of black tea production, and their degradation is considered to be related to production of uncharacterized black tea polyphenols.[2a,10] Previously, we revealed that theaflavin (**3**) is oxidized by polyphenol oxidase in the presence of epicatechin (**1**) to give theanaphthoquinone (**7**) as a major product,[11] along with several minor products.[12] Degradation of **3** is also mediated by peroxidase to afford **7**;[13] however, its degradation reaction has not been examined in detail.[2a,14] In this study, we examined the oxidation reaction of **3** with peroxidase.

First, we examined the time course of oxidation of a mixture of epicatechin (**1**) and epigallocatechin (**2**) in the presence of horseradish peroxidase (Fig. 2A).[15–17] After 10 min, theaflavin (**3**) was observed as the major product. Then, theanaphthoquinone (**7**) appeared, along with the disappearance of **3** (t = 30 min). Subsequently, a new product (**8**) gradually increased, which was accompanied by a decrease of **7** (t = 60, 120 min). Therefore, compound **8** was presumed to be an oxidation product of **7**. We also investigated the time course of oxidation of **3** (Fig. 2B); the results supported the production of **8** from **3** via **7**. To elucidate the structure of **8**, we performed the oxidation reaction on a large scale.[18] Catechins **1** (1.0 g) and **2** (1.0 g) were dissolved in phosphate buffer at pH 5.0 and stirred with horseradish peroxidase and H_2O_2 for 3 h. Separation of the reaction mixture by Sephadex LH-20 and MCI-gel CHP20P column chromatography afforded **8** (25.3 mg).

Compound **8**[19] showed an $[M+H]^+$ peak of m/z 523 by FABMS. ^{13}C NMR and elemental analysis revealed the molecular formula of **8** to be $C_{27}H_{22}O_{11}$. Two sets of signals arising from the A-ring and C-ring of the flavan-3-ol skeleton were observed in the ^1H and ^{13}C NMR spectra, and their signals were assigned by ^1H–^1H COSY, HSQC, and HMBC spectra (Table 1). The remaining 11 carbon signals in the ^{13}C NMR were attributed to the moiety derived from catechin B-rings. In the HMBC spectrum (Fig. 3), correlations from C′-ring H-2′ (δ_H 5.08) to C-5″ (δ_C 113.2), C-6″ (δ_C 136.8), C-7″ (δ_C 118.07 or δ_C 118.10), and from H-3′ (δ_H 4.28) to C-6″ were observed. These correlations indicated the connectivity of C-5″–C-6″–C-7″, and the connection between C-2′ and C-6″. In

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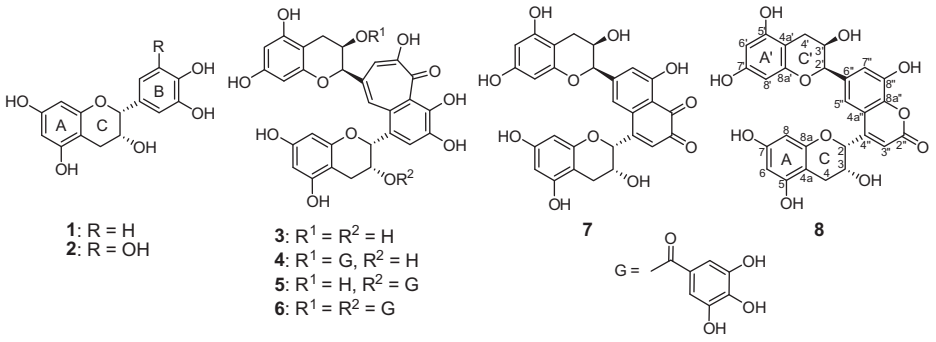


Figure 1. Structures of **1**–**8**.

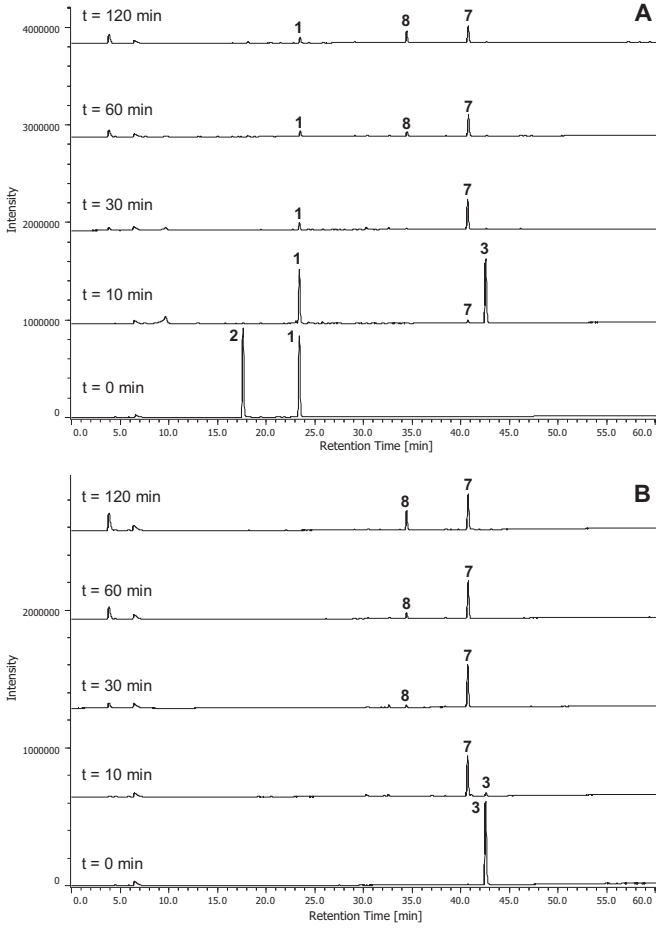


Figure 2. (A) HPLC-DAD chromatogram (max absorbance) of the reaction mixture of epicatechin (**1**) and epigallocatechin (**2**) by peroxidase. (**3**: theaflavin; **7**: theanaphthoquinone; **8**: theacoumarin A) (B) HPLC chromatogram of the reaction mixture of theaflavin (**3**) by peroxidase.

addition, HMBC correlations from H-5″ to C-4a″ (δ_C 118.10 or δ_C 118.07), C-6″, C-7″, and C-8a″ (δ_C 142.4), and correlations from H-7″ to C-5″, C-6″, C-8″ (δ_C 145.3), and C-8a″ revealed that the six carbons (C-4a″, 5″, 6″, 7″, 8″, 8a″) formed a benzene ring, and C-8″ and C-8a″ were oxygenated based on their ^{13}C NMR chemical shifts. Another C-ring H-2 (δ_H 5.35) was correlated with C-3″ (δ_C 114.3), C-4″ (δ_C 153.5), and C-4a″ in the HMBC spectrum. Furthermore, the correlations from H-3″ (δ_H 6.68) to C-2″ (δ_C 161.0), C-4″, and C-4a″ revealed the connectivity of C-2″–C-3″–C-4″–C-4a″. This indicated that the α,β-conjugated carbonyl group

Table 1
^1H (500 MHz) and ^{13}C (125 MHz) NMR data for **8** (in acetone-d_6 + D_2O, δ in ppm, J in Hz)

Position	δ_H	δ_C	HMBC (H to C)
2	5.35 (br s)	75.0	4, 3″, 4″, 4a″
3	4.35 (m)	64.1	2, 4a
4	2.79 (br d, 16.9)	28.7	2, 3, 4a, 5, 8 (^4J), 8a
	2.93 (dd, 4.4, 16.9)		
4a		99.40[a]	
5		157.51[b]	
6	6.05 (d, 2.3)	96.3	4a, 5, 7, 8
7		157.47[b]	
8	5.99 (d, 2.3)	95.4	4a, 6, 7, 8a
8a		155.8	
2′	5.08 (br s)	78.5	4′, 8a′, 5″, 6″, 7″
3′	4.28 (m)	66.5	4a′, 6″
4′	2.84 (dd, 4.8, 16.5)	28.2	2′, 3′, 4a′, 5′, 8a′
	2.59 (dd, 4.5, 16.5)		
4a′		99.36[a]	
5′		157.42[b]	
6′	6.04 (d, 2.3)	96.6	4a′, 5′, 7′, 8′
7′		157.39[b]	
8′	5.94 (d, 2.3)	95.2	4a′, 6′, 7′, 8a′
8a′		156.2	
2″		161.0	
3″	6.68 (br s)	114.3	2, 2″, 4″, 4a″
4″		153.5	
4a″		118.10[c]	
5″	7.33 (d, 1.5)	113.2	2′, 4″, 4a″, 6″, 7″, 8″ (^4J), 8a″
6″		136.8	
7″	7.40 (d, 1.5)	118.07[c]	2′, 4a″ (^4J), 5″, 6″, 8″, 8a″
8″		145.3	
8a″		142.4	

[a–c] Assignments may be interchanged.

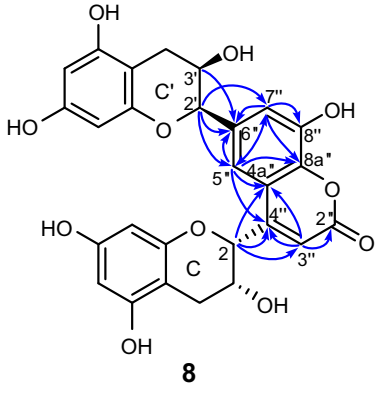
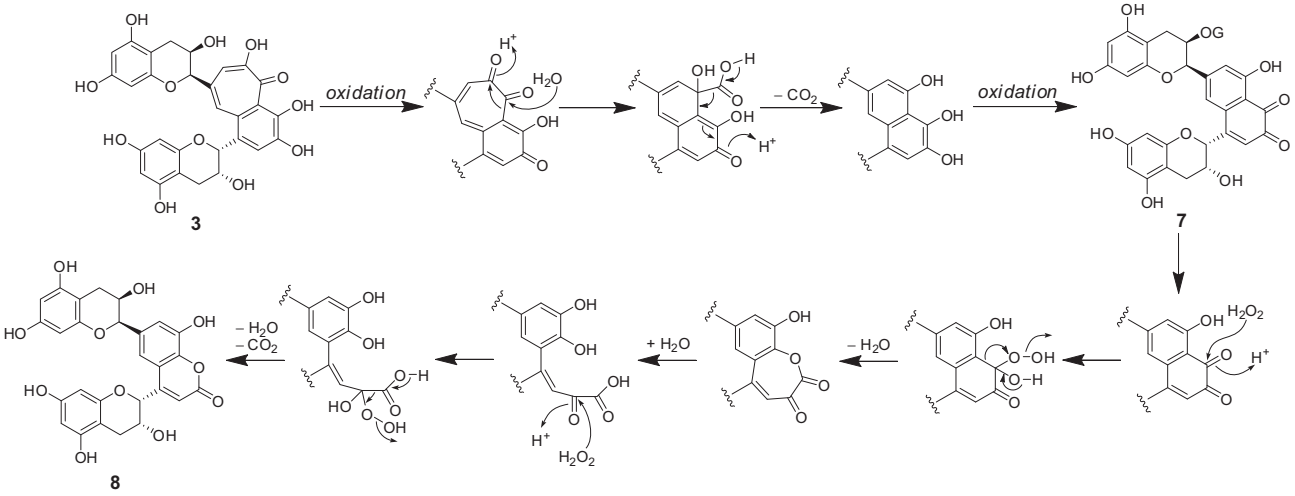


Figure 3. Important HMBC correlations of **8**.



Scheme 1. Plausible production mechanism of **8** from **3**.

C-2″–C-4″ is connected to C-2. The IR spectrum also supported the presence of a conjugated carbonyl group (1695 cm^{-1}). Taking the molecular formula into account, connection between C-2″ and C-8a″ through an ester bond was deduced; thus, 11 carbons derived from two B-rings form a coumarin skeleton. Based on these results, the structure of **8** was determined as shown in Figure 1, and **8** was named as theacoumarin A.

A plausible mechanism for the production of **8** is shown in Scheme 1. After oxidation of the benzotropolone ring of **3**, a benzylic acid-type rearrangement, decarboxylation, and oxidation afford **7**.[11] Subsequent Baeyer–Villiger oxidation, which includes the addition of H_2O_2 to the dicarbonyl moiety and dehydration via rearrangement, affords a lactone intermediate. Finally, ring opening of the lactone by hydration, addition of H_2O_2 with decarboxylation, followed by formation of the lactone ring yield **8**. During black tea production, H_2O_2 is produced by reduction of O_2 in the course of enzymatic oxidation of catechins.[20]

In summary, theacoumarin A (**8**) was identified as a major product of the oxidation of theaflavin (**3**) by peroxidase, and its structure was determined on the basis of spectroscopic data. Our previous study showed that theanaphthoquinone (**7**) is produced from **3** by polyphenol oxidase in the presence of epicatechin; however, the oxidation reaction of **7** has not been observed.[12,21] This study revealed that **7** is oxidized by peroxidase to afford **8**. The production of **8** from **3** via **7** by peroxidase is assumed to occur during the process of black tea production. The oxidation of theaflavin (**3**) and its galloyl esters (**4**–**6**) by peroxidase, including the production of **8** and related compounds, is expected to contribute to the generation of uncharacterized black tea polyphenols. In addition, degradation of theaflavins is considered to affect the quality of black tea.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tetlet.2015.07.037.

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15. Compounds **1** (2.0 mg) and **2** (2.0 mg) were dissolved in 0.2 M phosphate buffer at pH 5.0 (2.0 mL), then 100 μL of a buffer solution of horseradish peroxidase (1.0 mg/mL) (Type II, 150–250 units/mg; Sigma–Aldrich)[17] and 10 μL of 5% H_2O_2 were added and stirred. For the first 60 min, 20 μL of 5% H_2O_2 was added with every 10 min. EtOH containing 1% trifluoroacetic acid (100 μL) was poured into the reaction mixture (100 μL) and the resulting mixture was filtered through a membrane filter (0.45 μm). The filtered solution (5 μL) was analyzed by analytical HPLC.[16]
16. Analytical HPLC was performed on a Cosmosil $5C_{18}$-ARII column (250 × 4.6 mm i.d.; Nacalai Tesque, Kyoto, Japan) with gradient elution from 4% to 30% (39 min) and from 30% to 75% (15 min) of CH_3CN in 50 mM H_3PO_4 (column temperature: 35 °C; flow rate: 0.8 mL/min).
17. In this study, horseradish peroxidase was used instead of tea peroxidase because the former is commercially available (Sigma–Aldrich). In addition, a previous study showed that horseradish peroxidase can catalyze the production of theaflavins from tea catechins,[14] which indicated that the catechin oxidizing capacity of horseradish peroxidase is similar to that of tea peroxidase.

18. Compounds **1** (1.0 g) and **2** (1.0 g) were dissolved in 0.2 M phosphate buffer at pH 5.0 (75 mL), then horseradish peroxidase (5.0 mg) and 30% H_2O_2 (2 mL) were added and stirred. For the first 40 min, 2 mL of 30% H_2O_2 was added with every 10 min. After 3 h, reaction solution was directly applied to Sephadex LH-20 (3 × 28 cm, H_2O–MeOH–50% aq acetone) to afford 10 fractions. HPLC analysis of each fraction indicated that **8** was contained in fraction 8.[16] Purification of fraction 8 using MCI-gel CHP20P (2 × 23 cm, 30–100% aq MeOH) and Sephadex LH-20 (1.5 × 10 cm, EtOH) afforded **8** (25.3 mg).
19. Theacoumarin A (**8**): A brown amorphous powder; $[\alpha]_D^{27}$ −211.4 (*c* 0.1, MeOH); FABMS *m*/*z*: 523 $[M+H]^+$; Anal. Calcd for $C_{27}H_{22}O_{11}\cdot 1.5H_2O$: C, 59.02, H, 4.59. Found: C, 58.95, H, 4.61; IR ν_{max} (dry film) cm^{-1}: 3382, 2932, 1695, 1630, 1612, 1591, 1518, 1470; UV λ_{max} (MeOH) nm (log ε): 293 (4.07), 256 (4.20).
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