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Production mechanism of proepitheaflagallin, a precursor of benzotropolone-type black tea pigment, derived from epigallocatechin via a bicyclo[3.2.1]octane-type intermediate

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ABSTRACT

An unstable intermediate proepitheaflagallin B (**2**), a precursor of proepitheaflagallin (**3**), was isolated as an enzymatic oxidation product of (−)-epigallocatechin (**1**), and the structure of **2** was determined based on spectroscopic data. The structure and its decomposition revealed that the detailed production mechanism of proepitheaflagallin (**3**) via a bicyclo[3.2.1]octane-type intermediate was related to that of major black tea pigments, theaflavins.

Black tea is a very popular polyphenol-rich beverage and has various health benefits.[1] It is produced by fermentation of fresh tea leaves (*Camellia sinensis*). In the fermentation process, polyphenols in fresh tea leaves, (−)-epicatechin, (−)-epicatechin 3-*O*-gallate, (−)-epigallocatechin (**1**), and (−)-epigallocatechin 3-*O*-gallate, are oxidized with polyphenol oxidase, an endogeneous enzyme of tea leaves, to give complex oxidation products.

Theaflavins[2] and theasinensins[3] are well-known black tea polyphenols produced by oxidative dimerization of tea catechins and have beneficial biological activities, such as antioxidant and antimicrobial activities.[4] However, the major part of black tea polyphenols is mainly composed of structurally unknown catechin oxidation products.[5] Separation of the unknown polyphenols from black tea is difficult because they are a complex mixture of numerous oxidation products having closely related structures and various molecular weights. In addition, spectroscopic methods directly applied to a mixture of the polyphenols gave only limited information on their chemical structures.[6] Therefore, in order to chemically understand the black tea polyphenols, we are studying the enzymatic oxidation mechanism of tea catechins by model oxidation experiments using plant homogenates with high polyphenol oxidase activities.[7] Our study disclosed that some important black tea polyphenols, such as theasinensins and epitheaflagallin,[8] are produced via unstable intermediates.[7b–d,f] The reaction of the intermediate also produced many unidentified byproducts, which may be related to the uncharacterized black tea polyphenols. Thus, understanding of the reaction mechanism of the unstable intermediates is important in black tea chemistry. Proepitheaflagallin (**3**)[7f] is the unstable intermediate produced from (−)-epigallocatechin (**1**), one of the major tea catechins of *C. sinensis* var. *sinensis*. In the present study, we disclosed that **3** was produced via a new precursor named proepitheaflagallin B (**2**). The structure is related to a hypothetical precursor of theaflavins.[2a] This Letter details the isolation and structural elucidation of **2** and its production and degradation mechanisms.

An aqueous solution of (−)-epigallocatechin (**1**, 1.5 g) was stirred vigorously with Japanese pear homogenate having high polyphenol oxidase activity[7a] for 60 min. The reaction mixture was acidified with trifluoroacetic acid (TFA) to inactivate the enzyme activity and to prevent degradation of unstable reaction products. An analytical HPLC[9] revealed the production of dehydrotheasinensin C[7f] (t_R = 9.9 min), quinone dimer[7f,10] (t_R = 13.2 min), proepitheaflagallin (**3**)[7f] (t_R = 24.9 min) and an unknown product **2** (t_R = 23.3 min). After filtration, the filtrate was applied to MCI-gel CHP20P (75–150 μm, Mitsubishi Chemical Co.; 0–100% aq MeOH containing 0.1% TFA) and the fractions containing **2** and **3** were collected. Subsequent separation of the fraction using YMC-GEL

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ODS-AQ (12 nm, S-50 μm, YMC Co., Ltd; 4–50% aq MeOH containing 0.1% TFA), evaporation under reduced pressure below 40 °C, and lyophilization afforded **2**[11] (28.2 mg, yield 1.9%) along with **3** (62.8 mg). In the process of separating **2** and **3**, analytical HPLC showed a gradual decrease in compound **2** (t_R = 23.3 min) accompanied by an increase in compound **3** (t_R = 24.9 min). TLC analysis also supported the fact that **2** was gradually converted into **3**. These observations strongly suggested that **2** is an unstable precursor of **3**.

The molecular formula of **2** was determined to be $C_{30}H_{24}O_{14}$ by HRFABMS. The ^{1}H and ^{13}C NMR spectra (Table 1) suggested the presence of two pairs of A- and C-rings in flavan-3-ol, and these signals were easily assigned by ^{1}H–^{1}H COSY, HSQC, and HMBC spectra. The remaining 12 carbon signals in the ^{13}C NMR spectrum were attributable to a B-ring part originated from two pyrogallol B-rings of **1**. The HMBC correlations (Table 1) of H-2′ (δ_H 3.96) with C-e (δ_C 48.0), C-f (δ_C 119.9), and C-k (δ_C 53.7), and the correlations of H-e (δ_H 3.67) and H-f (δ_H 6.80) with C-j (δ_C 129.9) indicated that C-e, C-f, C-j, and C-2′ were connected to a quaternary carbon, C-k. Furthermore, H-f was correlated with C-g (δ_C 150.2) and C-h (δ_C 178.8), and a ^4J correlation between H-e and C-i (δ_C 147.3) was also observed. In addition, the chemical shift of the carbonyl carbon signal at C-h (δ_C 178.8) indicated that this carbonyl group is located between two double bonds. These findings and the chemical shifts of C-g and C-i revealed the presence of a 2,6-dihydroxy-2,5-cyclohexadien-1-one ring moiety (C-f–C-k). The connectivity of C-d, C-2, C-c, and C-e was determined from the HMBC correlations of H-c (δ_H 6.27) and H-e to C-2 (δ_C 78.9), H-2 (δ_H 4.77) to C-d (δ_C 159.1). Although the conjugated ketone carbon C-b (δ_C 191.8) showed no correlation peak, the HMBC correlations of H- e to C-a (δ_C 86.3), C-l (δ_C 106.0) and H-c to C-a, and the chemical shift of the olefinic carbon at C-c (δ_C 125.2) and C-d (δ_C 159.1) suggested that C-b was located between C-a and C-c, and C-a–C-e and C-l formed a six-membered ring. The linkage between C-a and C-j was determined from the appearance of weak HMBC correlations of H-f to C-a (^4J). These HMBC correlations revealed the presence of a bicyclo[3.2.1]octane system in **2**. The chemical shift of C-l (δ_C 106.0) indicated that this carbon is an acetal, and that this carbon was connected to the C-3′ of C-ring through an ether bond. This was based on observation of a remarkable up-field shift of C-4′ (δ 24.5; usually observed at δ ca. 28)[7a,f] and the molecular formula deduced from HRFABMS. The spectroscopic observations described above confirmed the planar structure of **2**. The absolute configuration of compound **2** is deduced to be as shown in Figure 1 because compound **2** was produced from **1** and the precursor of **3**.

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

Position	^{1}H	^{13}C	HMBC (H to C)
2	4.77 (br s)	78.9	3, c, d
3	4.52 (m)	63.5	4a
4	2.83 (2H, m)	27.4	2, 3, 4a, 5, 8a
4a		99.5	
5		157.4[c]	
6	6.01 (d, 2.3)[a]	96.5[d]	4a, 5, 7, 8
7		157.7	
8	5.89 (d, 2.3)[a]	95.5[d]	4a, 6, 7, 8a
8a		155.7[c]	
2′	3.96 (d, 2.1)	81.9	3′, e, f, k
3′	4.89 (m)	69.9	2′, 4a′
4′	2.89 (dd, 17.3, 1.7)	24.5	2′, 3′, 4a′, 5′, 8a′
	2.76 (dd, 17.3, 4.8)		
4a′		98.4	
5′		157.1[e]	
6′	6.05 (d, 2.3)[b]	96.6[f]	4a′, 5′, 7′, 8′
7′		157.7	
8′	6.00 (d, 2.3)[b]	95.5[f]	4a′, 6′, 7′, 8a′
8a′		155.3[e]	
a		86.3	
b		191.8	
c	6.27 (br s)	125.2	2, a, e
d		159.1	
e	3.67 (br s)	48.0	2, 2′, a, c, d, i (^4J), j, k, l
f	6.80 (s)	119.9	2′, a (^4J), e, g, h, j, k
g		150.2	
h		178.8	
i		147.3	
j		129.9	
k		53.7	
l		106.0	

[a–f] May be interchanged in the same column.

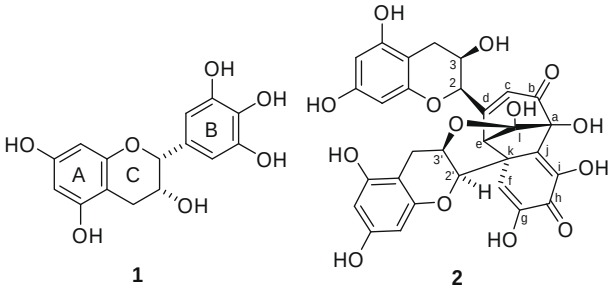
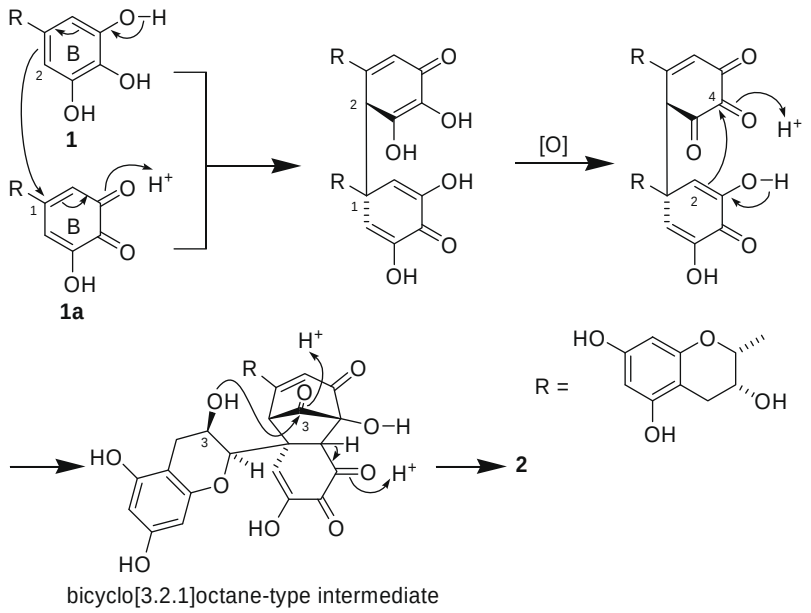


Figure 1. Structures of **1** and **2**.

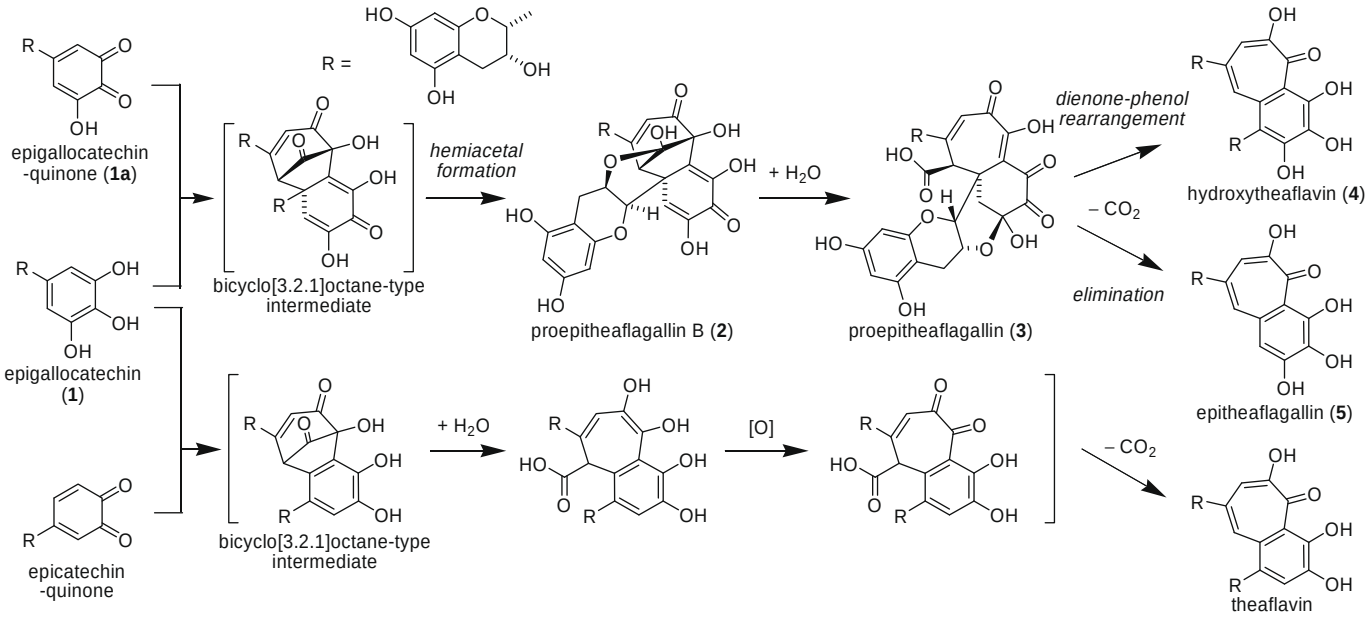
The production mechanism of the bicyclo[3.2.1]octane structure of **2** was proposed as shown in Scheme 1. First, an intermolecular C–C bond was formed between **1** and epigallocatechin-quinone (**1a**) by a 1,4-addition of the electron-rich B-ring C-2 of **1** to the α,β-unsaturated carbonyl group of **1a**. Successive oxidation of B-ring of the **1** unit and the intramolecular 1,2-addition of C-2 of the **1a** unit to the carbonyl carbon at C-4 of the **1** unit formed the bicyclo[3.2.1]octane-type intermediate. Subsequent hemiacetal formation between the hydroxyl group at C-3 of the C-ring and the carbonyl group at B-ring C-3 of the **1** unit affords **2**.

Proepitheaflagallin (**3**) is produced by enzymatic dimerization of **1** and is spontaneously decomposed to give hydroxytheaflavin (**4**) and epitheaflagallin (**5**), which have 1′,2′,3′-trihydroxybenzotropolone rings.[7f] The reactions include a dienone-phenol rearrangement in the 1,4-cyclohexadiene moiety[12] or elimination of the flavan-3-ol A, C-ring of **3** as shown in Scheme 2. The structures of these compounds derived from **1** were related to those of theaflavins, which are produced by an oxidative cross-coupling between catechol and the pyrogallol B-rings of tea catechins. Some production mechanisms of theaflavins have been proposed so far.[2a,5,13] Recently, Yanase et al. have proposed that the benzotropolone skeleton is produced via a bicyclo[3.2.1]octane-type intermediate produced by condensation between a pyrogallol B-ring and *o*-quinone derived from a catechol B-ring.[14] They succeeded to isolate the bicyclooctane intermediate; however, the intermediate was produced from simple model compounds by a non-enzymatic oxidation reaction in an aprotic solvent.[14] In the present study, we demonstrated the formation of the bicyclo[3.2.1]octane-type intermediate **2** by enzymatic oxidation of tea catechin under aqueous conditions. It is interesting to note that the production mechanism of theaflavin is very similar to our mechanism proposed for the production of **3**, **4** and **5** (Scheme 2) as all go through a bicyclo[3.2.1]octane-type intermediate.

The bicyclo[3.2.1]octane-type intermediates of theaflavin and its model compounds are unstable and are easily degraded to a benzotropolone ring by addition of a water molecule to the carbonyl group and cleavage of the adjacent C–C bond, and by subsequent oxidation.[14] In contrast, the bicyclo[3.2.1]octane structure of



Scheme 1. Proposed production mechanism of proepitheaflagallin B (**2**).



Scheme 2. Proposed production mechanism of **2**, **3**, and theaflavin via a bicyclo[3.2.1]octane-type intermediate.

2 has a hemiacetal structure with the hydroxyl group at C-ring C-3, which stabilizes the skeleton. At the final stage of the theaflavin production, decarboxylation and subsequent aromatization easily occur to give a benzotropolone ring. In the proepitheaflagallin (**3**) pathway, however, **3** is relatively stable at room temperature due to the presence of a substituent group at C-k and the hemiacetal formation between the C-ring C-3 hydroxyl group and C-g. Degradation of **3** to **4** or **5** by dienone–phenol rearrangement and elimination of the A- and C-ring, respectively, proceed slowly at room temperature.

Theaflavins are the major products of oxidative coupling between catechol-type catechins and pyrogallol-type catechins. On the other hand, the major products of oxidative coupling between two molecules of pyrogallol-type catechins are dehydrotheasinensins, which afford theasinensins and oolongtheanins through a spontaneous degradation reaction.[7d,f] Although compared with these major products, compounds **2** and **3** are unstable and are only detected as minor products of the enzymatic oxidation of tea catechins,[7f] understanding these unstable oxidation products is important to clarify the structurally unknown catechin oxidation products which account for the major part of the black tea polyphenols.

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