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Theadibenzotropolone A, a new type pigment from enzymatic oxidation of (−)-epicatechin and (−)-epigallocatechin gallate and characterized from black tea using LC/MS/MS

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Abstract—Theaflavins and thearubigins are major pigments of black tea. In the course of studies on the oxidation mechanism of tea polyphenols, a new type of tea pigment, theadibenzotropolone A, together with theaflavin 3-gallate were formed by the reaction of (−)-epicatechin (EC) and (−)-epigallocatechin gallate (EGCG) with horseradish peroxidase in the presence of H_2O_2. The structure of theadibenzotropolone A was elucidated on the basis of MS and 2D NMR spectroscopic analyses. The observation that galloyl ester groups of theaflavins can be oxidized to form dibenzotropolone skeletons strongly implied that this type of oxidation as an important pathway to extend the molecular size of thearubigins. The existence of this compound in black tea was characterized by LC/ESI-MS/MS. Theadibenzotropolone A is the first theaflavin type trimer of catechins in black tea.

Tea is one of the most popular beverages in the world. Three types of tea are produced from the leaves of *Camellia sinensis*—green tea, oolong tea, and black tea. Green tea (non-fermented) and oolong tea (semi-fermented) are more popular in China, Japan, Korea and some African countries, whereas black tea (fermented) is preferred in India and the Western countries. Theaflavins and thearubigins are major pigments in black tea, and it is generally accepted that these are produced by oxidation of flavan-3-ols (catechins) during tea fermentation.[1] Theaflavins, which are orange or orange–red in color, possess a benzotropolone skeleton that is formed from co-oxidation of appropriate pairs of catechins, one having a *vic*-trihydroxy structure, and the other having an *ortho*-dihydroxy group.[2,3] It is known that theaflavins make important contributions to the properties of black tea, such as color,[4] 'mouthfeel'[5] and extent of tea cream formation.[6] Their structures are well studied.[7–10] On the other hand, thearubigins, which are red–brown or dark brown, are heterogeneous polymers.[11] So far information about their formation, structures and contribution to black tea quality is very limited. A partial structure of thearubigins from black tea was elucidated by using chemical degradation, which indicated them to be heterogeneous polymers of flavan-3-ols and flavan-3-ol gallates having bonds at C-4, C-6, C-8, C-2′, C-5′, and C-6′ in the flavan-3-ol units.[12] In addition, the possible participation of theaflavins in the formation of thearubigins has been suggested.[1] It was shown in a recent study that horseradish peroxidase would oxidize theaflavins into thearubigins in the presence of H_2O_2.[13] While the contribution of peroxidase to the metabolism of theaflavin and thearubigin in tea fermentation remains to be clarified. In the course of our studies on the functions of peroxidase in tea fermentation, we examined the oxidation of two tea catechins, EC (**1**) and EGCG (**2**) by treatment with horseradish peroxidase in the presence of H_2O_2. In addition to theaflavin 3-gallate, a new type pigment, theadibenzotropolone **A** (**4**), was isolated. This paper reports the isolation, structural elucidation and characterization from black tea by LC/ESI-MS/MS. of this new pigment.

Keywords: theadibenzotropolone A; black tea; peroxidase; H_2O_2; enzyme oxidation.

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EC (**1**) (1.0 g) and EGCG (**2**) (1.0 g) were dissolved in a mixture of acetone–pH 5 buffer (1:10, v/v, 50 mL), which contained 5 mg of horseradish peroxidase. While being stirred, 2 mL of 3.13% H_2O_2 was added four times over 45 min. One major reaction product, compound **3**, was formed. After the addition of 0.5 mL of H_2O_2, stirring was continued for an additional 30 min, the amount of compound **3** decreased, while another reaction product, compound **4** was formed. Compound **3** (120 mg) and **4** (18 mg), the two major reaction products, and the recovered EC (180 mg) and EGCG (70 mg) were isolated using a combination of Sephadex LH-20 (40% acetone/water) column and RP C-18 (50% MeOH/water) column chromatography.

Compound **3** was identified as theaflavin 3-gallate by comparison with the standard sample isolated from black tea. They showed the same APCI MS, ^{1}H, ^{13}C NMR spectra data.

The molecular formula of **4** was determined to be $C_{50}H_{38}O_{21}$ by negative-ion ESI-MS ($[M-H]^-$ at m/z 973) as well as from its ^{13}C NMR data, which indicate that this compound consists of three flavan-3-ol units. This was confirmed by the ^{1}H and ^{13}C NMR data. The ^{13}C NMR spectrum of **4** displayed 50 carbon signals, 27 of which were assigned to the A and C rings of flavan-3-ols. In addition, the ^{1}H NMR spectrum exhibited three sets of signals due to protons at the 2-, 3-, 4-, 6-, and 8- positions of the flavan-3-ol nucleus. These observations also indicate that the A and C rings of **4** did not undergo any change during oxidation. In comparison with the ^{1}H NMR spectrum of **3**, compound **4** is distinguished by the absence of galloyl ester signals, a large downfield shift of H-3′, an additional set of A and C ring signals from flavan-3-ol, and three more olefinic proton signals (δ 7.54 brs H-c; 7.79 s H-g; 8.30 brs H-e). In the ^{13}C NMR spectrum of **4**, besides the A and C ring signals, there were observed 23 carbon signals including two carbonyls (δ 186.5 s C-a; 185.5 s C-a′), one ester carbonyl (δ 167.7 s C-l) and 20 olefinic carbons (Table 1). All of these spectral features support the presence of two benzotropolone groups in compound **4**. Thus, the galloyl ester group on **3** can react with the B-ring of EC to form another benzotropolone. This assertion is supported by the 2D HMBC NMR spectrum. HMBC spectral analysis (Fig. 2) yielded correlation peaks between H-c (δ 7.54) and C-a (δ 186.5), C-b (δ 155.1), C-d (δ 123.9), C-e (δ 132.4), C-l (δ 167.7); H-e (δ 8.30) and C-c (δ 114.9), C-d (δ 123.9), C-f (δ 134.8), C-l (δ 167.7), C-j (δ 122.7); H-g (δ 7.79) and C-2 (δ 76.8), C-h (δ 149.7), C-i (δ 152.0), C-k (δ 126.3). Thus, the correlation peaks of H-c and H-e with the ester carbonyl carbon C-l, and H-g with C-2 of the flavan-3-ol unit all indicate the presence of a benzotropolone group formed by the galloyl ester group on **3** with the B-ring of EC. Another three olefinic protons (δ 7.28 brs H-c′; 7.56 brs H-e′; 7.71 s H-g′) show similar correlation patterns to those of H-c, H-e, and H-g. H-c′ has cross peaks with C-a′ (δ 185.5), C-b′ (δ 155.6), C-d′ (δ 133.4), C-e′ (δ 125.9), and C-2′ (δ 79.9); H-e′ shows correlation peaks with C-c′ (δ 117.7), C-d′ (δ 133.4), C-f′ (δ 131.2), C-j′ (δ 121.8) and C-2′ (δ 79.9); H-g′ has correlations with C-f′ (δ 131.2), C-h′ (δ 146.9), C-i′ (δ

Table 1. δ_H (600 MHz) and δ_C (150 MHz) NMR spectra data of compound **4** (CD_3OD) (δ in ppm, J in Hz)

	δ_H	δ_C		δ_H	δ_C
2	5.11 brs	76.8 d	8″	5.52 brs	96.1 d
3	3.94 brs	66.3 d	9″		157.8 s
4	2.62 d 17.4	29.6 t	10″		99.9 s
	2.55 dd 17.4, 1.8		a		186.5 s
5		156.5 s	b		155.1 s
6	6.05 brs	95.9 d	c	7.53 brs	114.9 d
7		158.5 s	d		123.9 s
8	6.02 brs	97.3 d	e	8.30 brs	132.4 d
9		156.8 s	f		134.8 s
10		99.2 s	g	7.79 s	123.5 d
2′	5.21 brs	79.9 d	h		149.7 s
3′	5.86 brs	70.9 d	i		152.0 s
4′	3.13 dd 17.4, 4.2	26.0 t	j		122.7 s
	3.03 dd 17.4, 3.6		k		126.3 s
5′		157.5 s	l		167.7 s
6′	5.91 brs	97.1 d	a′		185.5 s
7′		158.1 s	b′		155.6 s
8′	5.82 d 1.2	96.3 d	c′	7.28 brs	117.7 d
9′		157.0 s	d′		133.4 s
10′		99.8 s	e′	7.56 brs	125.9 d
2″	5.38 brs	76.9 d	f′		131.2 s
3″	4.12 brs	66.0 d	g′	7.71 s	124.1 d
4″	2.84 17.4, 3.6	29.5 t	h′		146.9 s
	2.72 d 17.4		i′		151.6 s
5″		157.5 s	j′		121.8 s
6″	5.89 d 1.2	96.9 d	k′		128.2 s
7″		157.8 s			

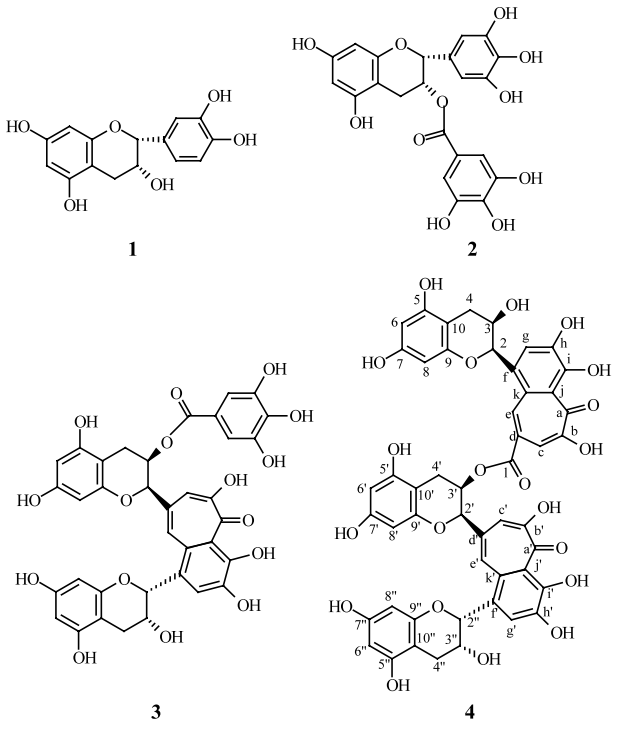

Figure 1. Structures of compounds **1**–**4**.

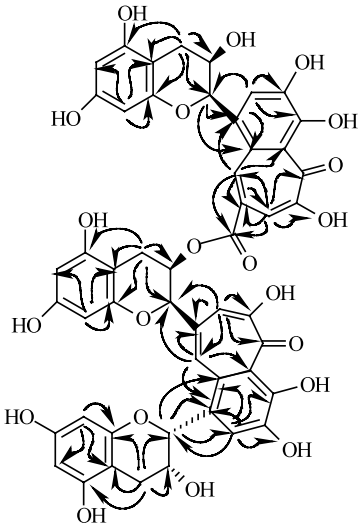

Figure 2. Significant HMBC (H→C) correlations of compound **4**.

151.6), C-k′ (δ 128.2) and C-2″ (δ 76.9) (Fig. 2). These through-bond connectivities proved that H-c′, H-e′ and H-g′ belong to the benzotropolone of the theaflavin part of **4**. Thus, the structure of **4** was deduced as shown (Fig. 1) and named theadibenzotropolone **A**. The complete interpretation of the NMR data was based on the results of COSY, HMQC and HMBC experiments (Table 1).

Efforts were made to establish the presence of theadibenzotropolone **A** in black tea using selected-ion monitoring (SIM) chromatograms of the standard (compound **4**) and the theaflavin fraction of black tea isolated from the 80% acetone aqueous extraction of black tea by Sephadex LH-20 column chromatography (40% acetone/water). Fig. 3 shows the respective LC/MS/MS chromatograms and fragment ion mass spectra of compound **4** and the theaflavin fraction of black tea. As shown in this figure, compound **4** and the peak in the theaflavin fraction of black tea exhibited almost not only the same chromatographic retention time and molecular masses, but also had the same fragment ion mass spectra. This proved the presence of theadibenzotropolone **A** in black tea.

It is well known that the major oxidizing enzyme in tea is polyphenol oxidase. It is the action of this enzyme that mainly produces the theaflavins. So, the formation in black tea of theadibenzotropolone **A** may come from the reaction between theaflavin 3-gallate and the free catechin EC by the action of peroxidase in the presence of H_2O_2. This was confirmed by the reaction of theaflavin 3-gallate and EC using the same conditions as that of EC and EGCG. All of the theaflavin 3-gallate can change to theadibenzotropolone **A** if there is enough free EC. Thus, the synthesis of theadibenzotropolone **A** via enzymatic oxidation of EC (**1**) and EGCG (**2**) and EC and theaflavin 3-gallate (**3**) is highly significant. It is generally accepted that theaflavins possess a benzotropolone skeleton that is formed from co-oxidation of appropriate pairs of catechins, one having a *vic*-trihydroxyl structure and the other having an *ortho*-dihydroxy group. The formation of theaflavin type compounds involves the oxidation of the B rings to the quinones followed by a Michael addition of the gallocatechin quinone to the catechin quinone, prior to carbonyl addition across the ring and subsequent decarboxylation.[7,14] Since theaflavin 3-gallate has a *vic*-trihydroxy structure, and EC has an *ortho*-dihydroxy group, they can form a benzotropolone skeleton. The formation of theadibenzotropolone **A** confirmed this hypothesis. Theadibenzotropolone **A** provides the first evidence to suggest that the galloyl ester group of theaflavin 3-gallate is as reactive as the B-ring (*vic*-trihydroxy) of EGCG or EGC (epigallocatechin) and the galloyl ester group of ECG (epicatechin gallate) in the same mechanism as the formation of theaflavins and theaflavate **A** and **B**[15,16] (Fig. 4). The observation that the galloyl ester group of theaflavins can be oxidized to form dibenzotropolone skeletons strongly implied that this type of oxidation is an important pathway to extend the molecular size of thearubigins. Theadibenzotropolone **A** is the first theaflavin type trimer of catechins in black tea. In addition, it may be a good method to study the minor constituents of black tea by combining the enzymatic oxidation reaction and LC/MS/MS analysis.

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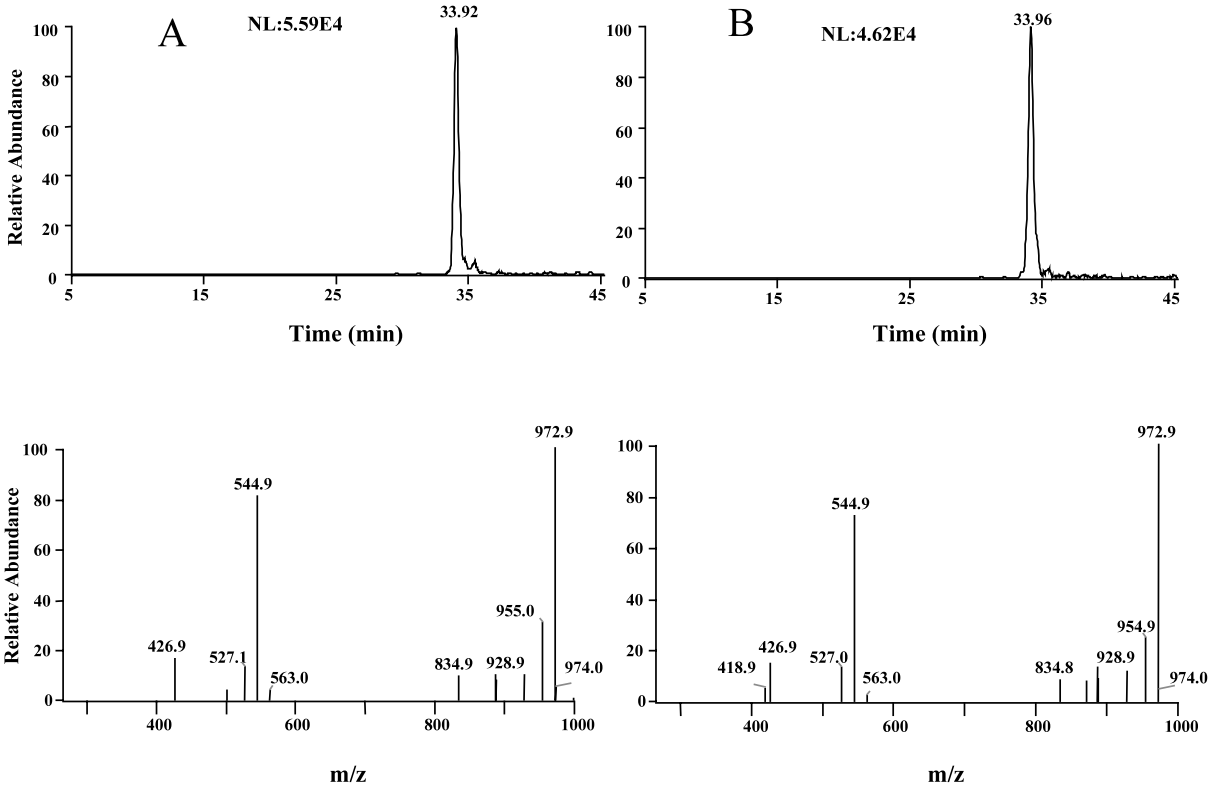

Figure 3. Negative ion LC/ESI-MS/MS chromatograms and fragment ion mass spectra of compound **4** (A) and the peak in the theaflavin fraction of black tea (B).

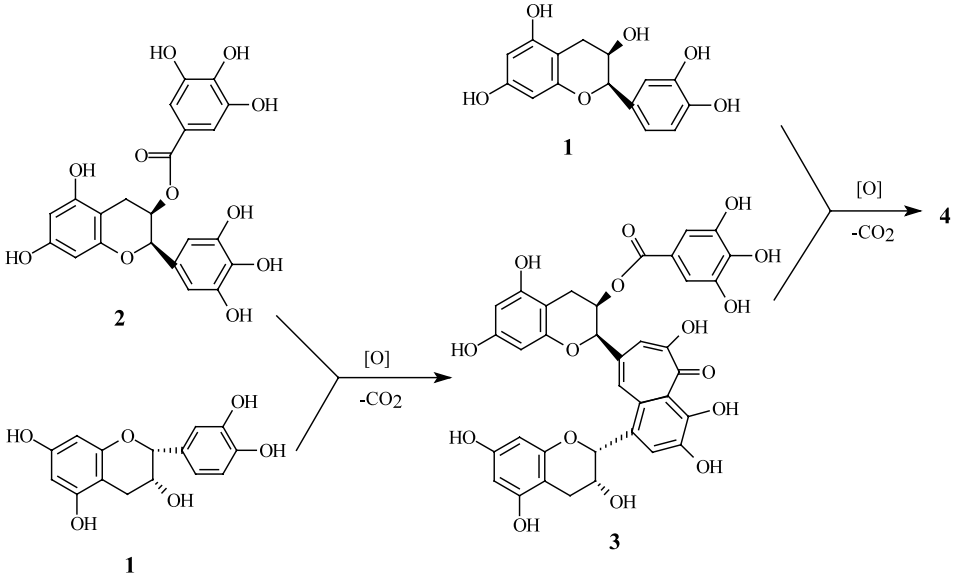

Figure 4. Proposed mechanism for the formation of compound **4**.

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