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Bioorganic & Medicinal Chemistry 12 (2004) 3009–3017
Bioorganic & Medicinal Chemistry

New dibenzotropolone derivatives characterized from black tea using LC/MS/MS

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Received 9 December 2003; revised 2 March 2004; accepted 14 March 2004
Available online 20 April 2004

Abstract—Theaflavins and thearubigins are major pigments in black tea, and it is generally accepted that they are produced by oxidation of flavan-3-ols (catechins) during tea fermentation. In the course of studies on the oxidation mechanism of tea polyphenols, especially the formation of thearubigins, a method combining the enzymatic synthesis and LC/ESI-MS/MS analysis was developed to search for new higher molecular weight polymers from black tea. Three new dibenzotropolones, theadibenzotropolone **A**, **B**, and **C**, together with one new tribenzotropolone, theatribenzotropolone **A**, were formed by the reaction of theaflavins and tea catechins with horseradish peroxidase in the presence of H_2O_2. The structures of these new benzotropolone derivatives were elucidated on the basis of MS and 2D NMR spectroscopic analyses. The existence of these compounds in black tea was characterized by LC/ESI-MS/MS. Theadibenzotropolone **A** and **B** were the first benzotropolone-type trimers of catechins found in the black tea extract. The observation that galloyl ester groups of theaflavins can be oxidized to form di- or tri-benzotropolone skeletons strongly implied that this type of oxidation is an important pathway to extend the molecular size of thearubigins.

1. Introduction

The role of tea in health promotion and disease prevention has been supported by results from large number of studies in cell culture and tumor bioassays in animal models carried out over more than a decade.[1,2] 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), which accounts for almost 80% of the world tea production, is preferred in India and the Western countries.

Theaflavins and thearubigins are major pigments in black tea, and it is generally accepted that they are produced by oxidation of flavan-3-ols (catechins) during tea fermentation.[3] 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.[4,5] It is known that theaflavins contribute importantly to the properties of black tea, such as color,[6] 'mouthfeel'[7] and extent of tea cream formation.[8] Their structures are well studied.[9–12] On the other hand, thearubigins, which are red-brown or dark brown, are heterogeneous polymers.[13] 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.[14] In addition, the possible participation of theaflavins in the formation of thearubigins has been suggested.[3] It was shown in a recent study that horseradish peroxidase could oxidize theaflavins into thearubigins in the presence of H_2O_2 by a model fermentation.[15]

However, it is very difficult to isolate thearubigins from black tea extract since they are the complex polymers

Keywords: Theadibenzotropolone **A**, **B** and **C** theatribenzotropolone **A**; Peroxidase; LC/MS/MS; Thearubigin; Theaflavin; Black tea.
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doi:10.1016/j.bmc.2004.03.027

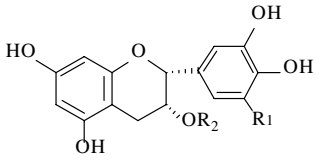
produced from coupling reactions mainly between five major tea catechins ((−)-epigallocatechin gallate (EGCG), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECG), (−)-epicatechin (EC), and catechin (C)). Thus, model fermentation experiments using pure catechins are very useful to understand the formation of black tea components, especially thearubigins. As an important analytical tool, LC/MS has been widely used to identify unknown peaks from biological samples. In the course of our studies on the structures of thearubigins, a method combining the enzymatic synthesis and LC/ESI-MS/MS analysis was developed to search for new higher molecular weight polymers from black tea. Enzymatic synthesis can provide the structure information of some new pigments formed by the oxidation of tea catechins; whereas LC/MS/MS can establish the existence of these new pigments in black tea extract.

Our preliminary communication[16] has reported the enzymatic synthesis, structural elucidation and characterization of a new type of pigment, theadibenzotropolone **A** (**9**), from black tea by LC/ESI-MS/MS. In addition, two additional peaks corresponding to the same molecular weight as that of theadibenzotropolone **A** were found in black tea extract. To identify these unknown peaks and find more new peaks, two new dibenzotropones, theadibenzotropolone **B** and **C**, together with one new tribenzotropolone, theatribenzotropolone **A** were synthesized by the reaction of theaflavins and tea catechins (Fig. 1) with horseradish peroxidase in the presence of H_2O_2. This paper reports the enzymatic synthesis and structural elucidation by 2D NMR of theadibenzotropolone **B** (**10**) and **C** (**11**) and theatribenzotropolone **A** (**13**), and the attempt of the characterization from black tea by LC/ESI-MS/MS of these new tea catechin trimers and tetramer.

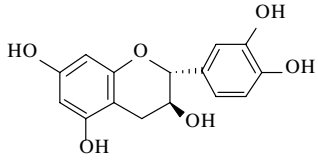
2. Results and discussion

Our preliminary communication[16] has reported 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), and can further react with EC to form the new theaflavin-type tea catechin trimers, theadibenzotropolone **A** (**9**), which was characterized from subfraction 12 of the ethyl acetate fraction of black tea extract by LC/ESI-MS/MS. In addition, two additional peaks corresponding to the same molecular weight as that of theadibenzotropolone **A** were found in subfraction 13. To identify these unknown peaks, two new dibenzotropolones, theadibenzotropolone **B** and **C**, together with one new tribenzotropolone, theatribenzotropolone **A**, were synthesized by the reaction of theaflavins and tea catechins with horseradish peroxidase in the presence of H_2O_2.

Theaflavin 3-gallate (**5**) could not only react with EC (**1**) to form theadibenzotropolone **A**, but also react with catechin (**4**) to form the isomer of theadibenzotropolone **A**, theadibenzotropolone **B** (**10**). The molecular formula



1. R_1=R_2=H
2. R_1=H, R_2=galloyl
3. R_1=OH, R_2=galloyl



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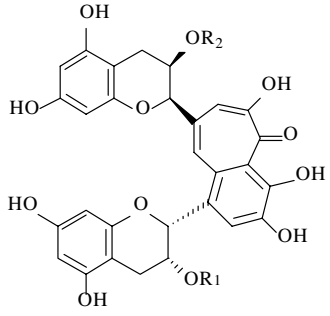

5. R_1=H, R_2=galloyl
6. R_1=galloyl, R_2=H
8. R_1=R_2=galloyl

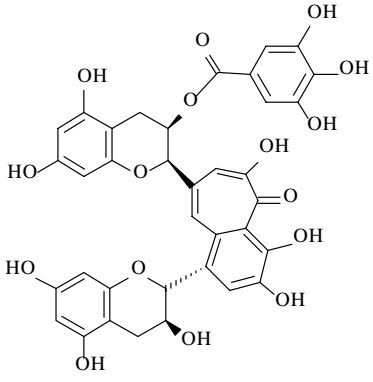

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Figure 1. Structures of compounds **1**–**8**.

of **10** was determined to be $C_{50}H_{38}O_{21}$ by negative-ion APCI-MS ($[M-H]^-$ at m/z 973) as well as from its ^{13}C NMR data, which indicated that this compound consisted of three flavan-3-ol units. Its ^1H and ^{13}C NMR spectra showed very similar patterns as those of compound **9**. The ^1H 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 **9** did not undergo any change during oxidation. In comparison with the ^1H NMR spectrum of **5**, compound **10** 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 7.51 br s H-c; 7.57 s H-g; 8.76 br s H-e). The ^{13}C NMR spectrum of **10** displayed 50 carbon signals, 27 of which were assigned to the A and C rings of flavan-3-ols. Besides the A and C ring signals, 23 carbon signals were observed including two carbonyls (δ 187.5 s C-a; 186.2 s C-a′), one ester carbonyl (δ 168.0 s C-l) and 20 olefinic carbons (Table 1). All of these spectral features supported the presence of two benzotropolone groups in compound **10**. Thus, the galloyl ester group on **5** could react with the B-ring of catechin to form another benzotropolone. This assertion was supported by the HMBC NMR spectrum. The HMBC spectral analysis (Fig. 3) yielded correlation peaks between H-c (δ 7.51) and C-a (δ 187.5), C-b (δ 155.5), C-d (δ 123.9), C-e (δ 135.5), C-l (δ 168.0); H-e (δ 8.76) and C-c (δ 115.2), C-d (δ 123.9), C-f (δ 135.8), C-l (δ 168.0), C-j (δ 123.1); H-g (δ 7.57) and C-2 (δ 79.6), C-h (δ 150.2), C-i (δ 153.0), C-k (δ 129.3). 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 indicated the presence of a benzotropolone group formed by the galloyl ester group on **5** with the B-ring of catechin. Another three olefinic protons (δ 7.29 br s H-c′; 7.64 br s H-e′; 7.81 s H-g′) showed similar correlation patterns to those of H-c, H-e, and H-g. H-c′ had cross peaks with C-a′ (δ 186.2), C-b′ (156.2), C-d′ (δ 134.0), C-e′ (δ 126.1), and C-2′ (δ 79.6); H-e′ showed correlation peaks with C-c′ (δ 117.6), C-d′ (δ 134.0), C-f′ (δ 132.0), C-j′ (δ 122.6), and C-2′ (δ 79.6); H-g′ had correlations with C-f′ (δ 132.0), C-h′ (δ 147.2), C-i′ (δ 152.0), C-k′ (δ 129.1), and C-2″ (δ 76.9) (Fig. 3). These through-bond connectivities proved that H-c′, H-e′, and H-g′ belonged to the benzotropolone of the theaflavin part of **10**. Thus, the structure of **10** was deduced as shown (Fig. 2) and named theadibenzotropolone **B**. The complete interpretation of the NMR data was based on the results of HMQC and HMBC experiments (Tables 1 and 2).

Table 1. δ_C (150 MHz) NMR spectra data of compounds **10** and **11** (CD_3OD) (δ in ppm)

	10	11		10	11
2	79.6 d	75.9 d	9″	156.9 s	156.5 s
3	69.5 d	65.7 d	10″	99.9 s	100.8 s
4	29.0 t	28.4 t	a	187.5 s	186.5 s
5	157.7 s	157.0 s	b	155.5 s	155.0 s
6	96.7 d	96.2 d	c	115.2 d	115.4 d
7	157.1 s	157.4 s	d	123.9 s	124.1 s
8	95.9 d	95.5 d	e	135.5 d	132.2 d
9	158.0 s	157.3 s	f	135.8 s	135.0 s
10	100.7 s	99.4 s	g	122.7 d	123.6 d
2′	79.6 d	79.1 d	h	150.2 s	150.0 s
3′	70.2 d	70.3 d	i	153.0 s	152.2 s
4′	25.4 t	24.2 t	j	123.1 s	122.8 s
5′	156.5 s	154.9 s	k	129.3 s	126.4 s
6′	97.1 d	96.4 d	l	168.0 s	168.0 s
7′	156.4 s	157.8 s	a′	186.2 s	185.8 s
8′	95.7 d	95.2 d	b′	156.2 s	155.3 s
9′	158.9 s	158.0 s	c′	117.6 d	118.8 d
10′	99.1 s	99.1 s	d′	134.0 s	132.9 s
2″	76.9 d	78.4 d	e′	126.1 d	128.3 d
3″	64.4 d	70.1 d	f′	132.0 s	132.6 s
4″	29.2 t	28.6 t	g′	124.5 d	122.7 d
5″	156.6 s	155.0 s	h′	147.2 s	147.5 s
6″	96.8 d	95.6 d	i′	152.0 s	152.4 s
7″	157.9 s	156.2 s	j′	122.6 s	121.8 s
8″	95.8 d	95.0 d	k′	129.1 s	130.4 s

Whereas the isomer of theaflavin 3-gallate, neotheaflavin 3-gallate (**7**), could react with EC to form theadibenzotroplone **C** (**11**), it could not react with catechin to generate compound **12**. The negative-ion APCI-MS of **11** displayed a molecular ion peak at m/z $[M-H]^-$ 973, supporting a molecular formula of $C_{50}H_{38}O_{21}$. The NMR spectra of **11** displayed signal patterns similar to those of **10**. The ^1H NMR spectrum of **11** also showed three sets of signals, due to protons, at the 2-, 3-, 4-, 6-, and 8- positions of the flavan-3-ol nucleus, and two sets characteristic signals for the benzotropolone group (δ 7.61 br s H-c; 7.80 s H-g; 8.37 br s H-e; 7.30 br s H-c′; 7.49 s H-g′; 7.97 br s H-e′). The ^{13}C NMR spectrum of **11** also displayed 50 carbon signals. Besides the A and C ring signals, 23 carbon signals were observed including two carbonyls (186.5 s C-a; 185.8 s C-a′), one ester carbonyl (δ 168.0 s C-l), and 20 olefinic carbons (Table 1). All of these spectral features support the presence of two benzotropolone groups in compound **11**. Therefore, just as we expected that the galloyl ester group on **7** could react with the B-ring of EC to form another benzotropolone. This was demonstrated by the HMBC data. The cross peaks involved H-c (δ 7.61) and C-a (δ 186.5), C-b (δ 155.0), C-d (δ 124.1), C-e (δ 132.2), C-l (δ 168.0); H-e (δ 8.37) and C-c (δ 115.4), C-d (δ 124.1), C-f (δ 135.0), C-l (δ 168.0), C-j (δ 122.8); H-g (δ 7.80), and C-2 (δ 75.9), C-h (δ 150.0), C-i (δ 152.2), C-k (δ 126.4). 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 indicated the presence of a benzotropolone group formed by the galloyl ester group on **7** with the B-ring of EC. Another three olefinic protons (δ 7.30 br s H-c′; 7.97 br s H-e′; 7.49 s H-g′) showed similar correlation patterns to those of H-c, H-e, and H-g. H-c′ had cross peaks with C-a′ (δ 185.8), C-b′ (δ 155.3), C-d′ (132.9), C-e′ (δ 128.3), and C-2′ (δ 79.1); H-e′ showed correlation peaks with C-c′ (δ 118.8), C-d′ (δ 132.9), C-f (δ 132.6), C-j′ (δ 121.8), and C-2′ (δ 79.1); H-g′ had correlations with C-f′ (δ 132.6), C-h′ (δ 147.5), C-i′ (δ 152.4), C-k′ (δ 130.4), and C-2″ (δ 78.4) (Fig. 3). These data indicated that H-c′, H-e′, and H-g′ belonged to the benzotropolone of the theaflavin part of **11**.Therefore, the structure of **11** was deduced as shown (Fig. 2) and named theadibenzotropolone **C**. The complete interpretation of the NMR data was based on the results of HMQC and HMBC experiments (Tables 1 and 2).

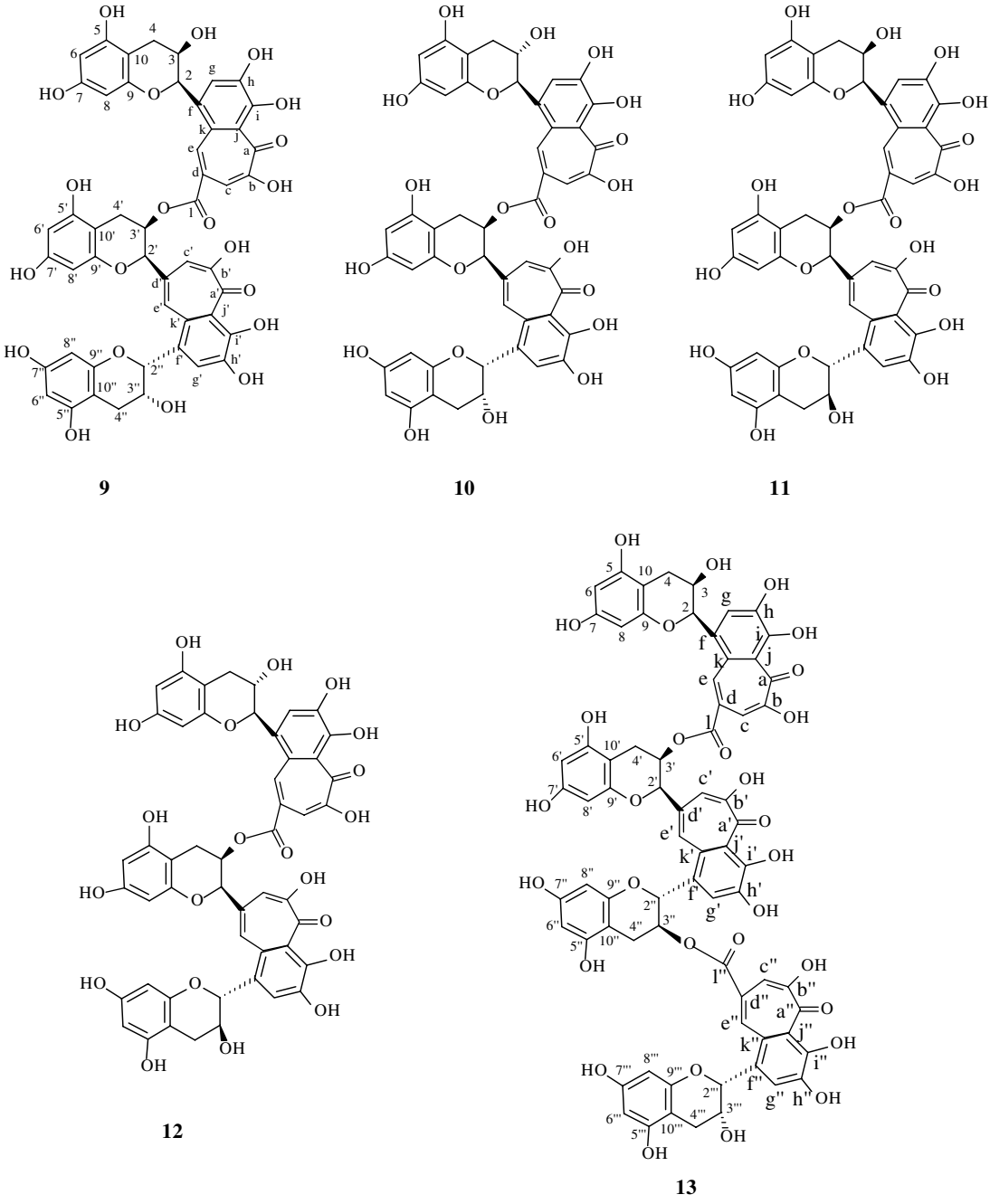
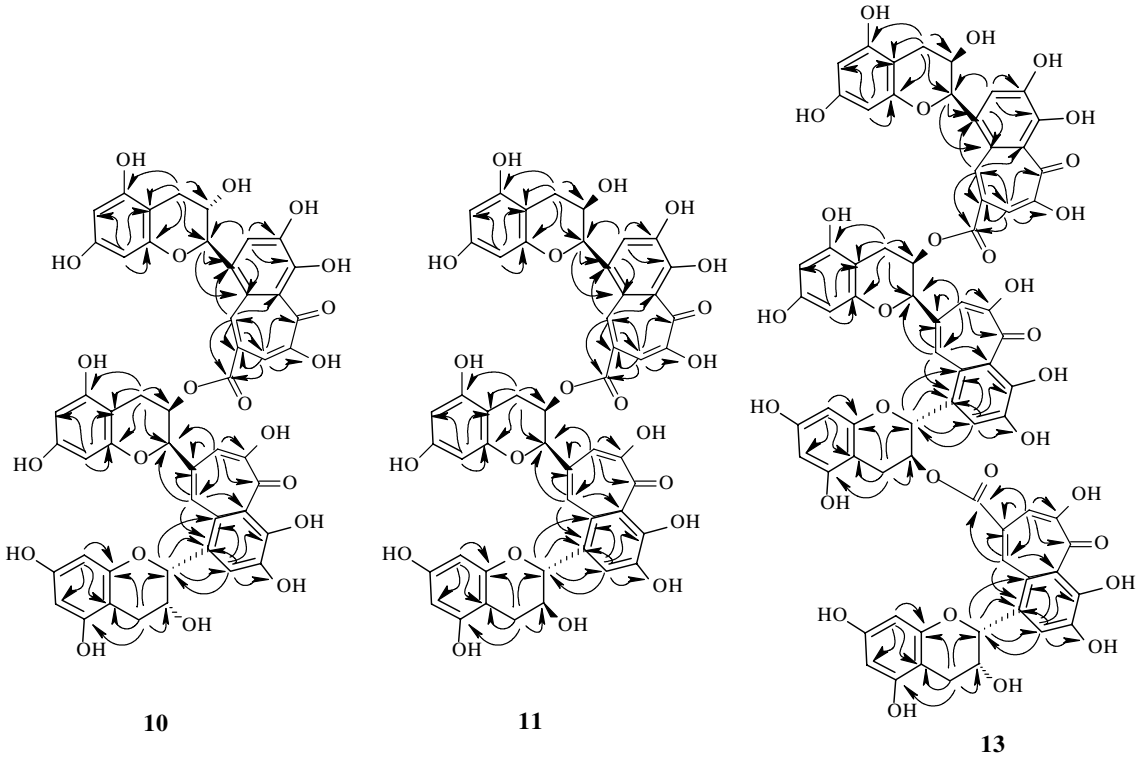


Figure 2. Structures of compounds **9**–**13**.

Interestingly, theatribenzotropolone **A** (**13**), instead of the two isomers of theadibenzotropolone that we expected, was obtained by the reaction between theaflavin 3,3′-digallate (**8**) and EC. Compound **13** was assigned the molecular formula $C_{71}H_{52}O_{30}$ based on negative-ion APCI-MS ($[M-H]^-$ at m/z 1383) as well as from its ^{13}C NMR data, which indicated that this compound consisted of four flavan-3-ol units. The ^1H NMR spectrum exhibited four sets of signals, due to protons, at the 2-, 3-, 4-, 6-, and 8- positions of the flavan-3-ol nucleus and three sets of characteristic signals for the benzotropolone group (δ 7.46 br s H-c; 7.82 s H-g; 8.32 br s H-e; 7.25 br s H-c′; 7.44 s H-g′; 7.73 br s H-e′; 7.15 br s H-c″; 7.71 s H-g″; 7.78 br s H-e″). The ^{13}C NMR spectrum of **13** displayed 71 carbon signals, 36 of which were assigned to the A and C rings of flavan-3-ols. Besides the A and C ring signals, 35 carbon signals were observed including three carbonyls (δ 186.5 s C-a; 185.5 s C-a′; 186.4 s C-a″), two ester carbonyls (δ 167.4 s C-l and 166.2 s C-l″) and 30 olefinic carbons (Table 3). All of these spectral features supported the presence of three benzotropolone groups in compound **13**. Thus, the two galloyl ester groups on **8** could react with the B-ring of EC to form two additional benzotropolones. This hypothesis was supported by the HMBC NMR spectrum. The HMBC spectrum (Fig. 3) indicated correlation peaks between H-c (δ 7.46) and C-a (δ 186.2), C-b (δ 154.7), C-d (δ 124.0), C-e (δ 133.2), C-l (δ 167.4); H-e (δ 8.32) and C-c (δ 115.2), C-d (δ 124.0), C-f (δ 134.2), C-l (δ 167.4), C-j (δ 123.2); H-g (δ 7.82), and C-2 (δ



10 **11** **13**

Figure 3. Significant HMBC (H → C) correlations of compounds **10**, **11**, and **13**.

Table 2. δ_H (600 MHz) NMR spectra data of compounds **10** and **11** (CD_3OD) (δ in ppm, J in Hz)

	10	**11**		**10**	**11**
2	5.14 d 8.4	5.14 br s	2″	5.48 br s	5.11 d 8.4
3	3.86 m	4.05 m	3″	4.21 m	3.80 m
4	2.74 dd 16.2 4.8	2.58 d 16.8	4″	2.89 dd 16.8, 3.6	2.82 dd 16.2, 6.0
	2.35 dd 16.2, 9.0	2.52 dd 16.8, 3.6		2.78 d 16.8	2.44 dd 16.2, 9.6
6	5.82 d 1.8	5.85 d 1.8	6″	5.77 d 1.8	5.83 d 1.8
8	5.93 d 1.8	5.76 d 1.8	8″	5.92 d 1.8	5.25 d 1.8
2′	5.18 br s	5.22 br s	c	7.51 br s	7.61 br s
3′	5.82 m	5.78 m	e	8.76 br s	8.37 br s
4′	3.08 dd 17.4, 4.2	3.07 m	g	7.57 s	7.80 s
	2.96 dd 17.4, 3.6		c′	7.29 br s	7.30 br s
6′	5.99 br s	6.01 d 1.8	e′	7.64 br s	7.97 br s
8′	5.99 br s	5.96 d 1.8	g′	7.81 s	7.49 s

76.6), C-h (δ 149.4), C-i (δ 151.6), C-k (δ 126.1); H-c″ (δ 7.15) and C-a″ (δ 186.4), C-b″ (δ 154.6), C-d″ (δ 123.3), C-e″ (132.1), C-l″ (δ 166.2); H-e″ (δ 7.78) and C-c″ (δ 115.1), C-d″ (δ 123.3), C-f″ (δ 134.8), C-l″ (δ 166.2), C-j′ (δ 123.2); H-g″ (δ 7.71) and C-2′ (δ 76.6), C-h″ (δ 149.2), C-i″ (δ 151.5), C-k″ (δ 127.0). Thus, the correlation peaks of H-c and H-e with the ester carbonyl carbon C-l and H-c″ and H-e″ with the ester carbonyl carbon C-l″, and H-g with C-2 of the flavan-3-ol unit and H-g″ with C-2′ indicated the presence of two benzotropolone groups formed by the galloyl ester group on **8** with the B-ring of EC. Another three olefinic protons (δ 7.25 br s H-c′; 7.73 br s H-e′; 7.44 s H-g′) showed similar correlation patterns to those of the above two sets of benzotropolone protons (H-c, H-e, and H-g, and H-c″, H-e″, and H-g″). H-c′ had cross peaks with C-a′ (δ 185.4), C-b′ (δ 155.7), C-d′ (132.3), C-e′ (δ 124.9), and C-2′ (δ 79.4); H-e′ showed correlation peaks with C-c′ (δ 117.4), C-d′ (δ 132.3), C-f (δ 130.1), C-j′ (δ 122.5) and C-2′ (δ 79.4); H-g′ had correlations with C-f′ (δ 130.1), C-h′ (δ 146.4), C-i′ (δ 151.2), C-k′ (δ 128.9), and C-2″ (δ 75.6) (Fig. 3). These through-bond connectivities proved that H-c′, H-e′, and H-g′ belonged to the benzotropolone of the theaflavin part of **13**. Thus, the structure of **13** was deduced as shown (Fig. 2) and named theatribenzotropolone **A**. The complete interpretation of the NMR data was based on the results of HMQC and HMBC experiments (Table 3).

Our previous communication[16] reported characterization of theadibenzotropolone **A** from subfraction 12 of the ethyl acetate fraction of black tea extraction by using selected-ion monitoring (SIM) chromatograms. In addition, subfraction 13 showed three peaks corresponding to the same molecular ion as that of theadibenzotropolone **A**. In order to identify these peaks, we

Table 3. δ_H (600 MHz) and δ_C (150 MHz) NMR spectra data of compound 13 (CD_3 OD) (δ in ppm, J in Hz)

	δ_H	δ_C		δ_H	δ_C
2	5.12 br s	76.6 d	9′		156.7 s
3	4.08 br s	65.7 d	10′		100.1 s
4	2.65 d 17.4	29.1 t	a		186.2 s
	2.39 dd 17.4, 3.6		b		154.7 s
5		157.1 s	c	7.46 br s	115.2 d
6	5.91 br s	96.2 d	d		124.0 s
7		156.8 s	e	8.32 br s	133.2 d
8	5.94 br s	95.7 d	f		134.2 s
9		156.9 s	g	7.82 s	122.4 d
10		99.9 s	h		149.4 s
2′	5.17 br s	79.4 d	i		151.6 s
3′	5.98 br s	70.2 d	j		123.2 s
4′	3.16 dd 17.4, 4.2	26.7 t	k		126.1 s
	3.05 d 17.4		l		167.4 s
5′		156.8 s	a′		185.4 s
6′	5.93 d 1.8	97.4 d	b′		155.7 s
7′		156.3 s	c′	7.25 br s	117.4 d
8′	6.05 d 1.8	95.4 d	d″		132.3 s
9′		157.8 s	e′	7.73 br s	124.9 d
10′		99.1 s	f′		130.1 s
2″	5.66 br s	75.6 d	g′	7.44 s	121.9 d
3″	5.64 m	69.8 d	h′		146.4 s
4″	3.09 18.0, 3.6	26.9 t	i′		151.2 s
	2.94 d 18.0		j′		122.5 s
5″		157.4 s	k′		128.9 s
6″	5.89 d 1.2	97.0 d	a″		186.4 s
7″		157.2 s	b″		154.6 s
8″	5.52 br s	95.3 d	c″	7.15 br s	115.1 d
9″		158.3 s	d″		123.3 s
10″		99.3 s	e″	7.78 br s	132.1 d
2′	4.96 br s	76.6 d	f″		134.8 s
3′	3.83 br s	66.3 d	g″	7.71 s	122.5 d
4′	2.70 d 16.2	29.6 t	h″		149.2 s
	2.60 d 16.2		i″		151.5 s
5′		157.0 s	j″		123.2 s
6′	5.89 d 1.2	96.9 d	k″		127.0 s
7′		156.6 s	l″		166.2 s
8′	5.52 br s	95.8 d			

analyzed the LC/MS/MS spectra of these three peaks and our three dibenzotropolone standards. Figures 4 and 5 showed the respective LC/MS/MS chromatograms and fragment ion mass spectra of compounds **9**–**11** and the peaks found in subfraction 13. As shown in these two figures, compound **9** and the peak at retention time 26.86 in subfraction 13 exhibited not only the same chromatographic retention time and molecular masses, but also the same fragment ion mass spectra. Whereas compound **10** showed the same chromatographic retention time, molecular mass, and fragment ion mass spectra as those of the peak at retention time 27.29. These findings proved the presence of theadibenzotropolone **A** and **B** in black tea. The amount of theadibenzotropolone **A** in black tea (220 ug/kg dry tea leaves) is about three fold higher than that of theadibenzotroplone **B** (70 ug/kg). The third peak (retention time 30.24) in subfraction 13 could not be identified as our standard theadibenotropolone **C** since they had different retention times. However, the MS/MS spectrum of this peak showed similar fragment ions as those of our three standards, which indicated this peak represented the isomer of these theadibenzotropolone-type structures. We have tried to synthesize the isomers of these dibenzotropolone-type structures (**9**–**11**) by reacting theaflavin 3′-gallate (**6**) with EC and catechin in the peroxidase/H_2O_2 system, but were not successful. Since no dibenzotropolone-type compounds were obtained from the reaction between theaflavin 3,3′-digallate and EC, and compound **12** was not obtained by reacting neotheaflavin 3-gallate with catechin, we could not identify the structure of the third peak in subfraction 13. The reason that most oxidation reactions worked with EC, but catechin did not work for some, maybe due to the redox potential value of catechin (79 mV at pH 13.5) is higher than that of EC (48 mV at pH 13.5).[29] Effort was also made to identify theatribenzotropolone **A** from black tea using selected-ion monitoring (SIM) chromatograms. However, no peak showed up in all of the fractions of the black tea extract.

There are two major enzymes involve in the fermentation process of making black tea.[17] One is polyphenol oxidase (PPO), which plays a key role in the oxidation of flavanols to black tea components, such as theaflavins (TF) and thearubigins (TR). Many studies have been carried out on the PPO-catalyzed formation of black tea oxidation products.[18–23] The other is peroxidase (POD), which also can catalyze the oxidation of *o*-diphenols to their quinones by using a peroxide, such as hydrogen peroxide, formed during the action of PPO on certain flavanols. However, the contribution of peroxidase to the formation of black tea pigment, especially thearubigins, in tea fermentation is still not clear. Our results here clearly showed that some theaflavins can further react with tea catechins to form di- or tri-benzotropolone-type compounds in peroxidase/H_2O_2 system.

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[9,24] (Fig. 6). Since theaflavin mono- or di-gallate, such as theaflavin 3-gallate, neotheaflavin 3-gallate, and theaflavin 3,3′-digallate, have *vic*-trihydroxy structure, and EC or catechin has an *ortho*-dihydroxy group, they can form a benzotropolone skeleton. The formation of theadibenzotropolone **A**, **B**, and **C**, and theatribenzotropolone **A** confirmed this hypothesis. These compounds provided the first evidence to suggest that the galloyl ester group of theaflavin was as reactive as the B-ring (*vic*-trihydroxy) of EGCG or EGC and the galloyl ester group of ECG in the same mechanism as the formation of theaflavins and theaflavate **A** and **B**.[25,26] The observation that the galloyl ester group of theaflavins could be oxidized to form di- or tribenzotropolone skeletons strongly implied that this type of oxidation is an important pathway to extend the molecular size of thearubigins. Theadibenzotropolone **A**

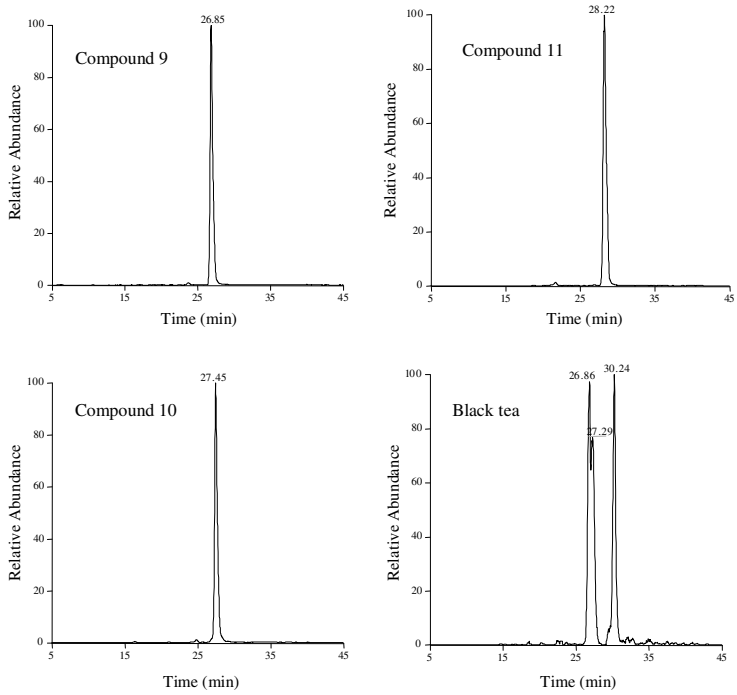


Figure 4. LC/MS chromatograms of compounds **9**–**11** and the peaks found in subfraction 13 of the ethyl acetate fractions of black tea extract.

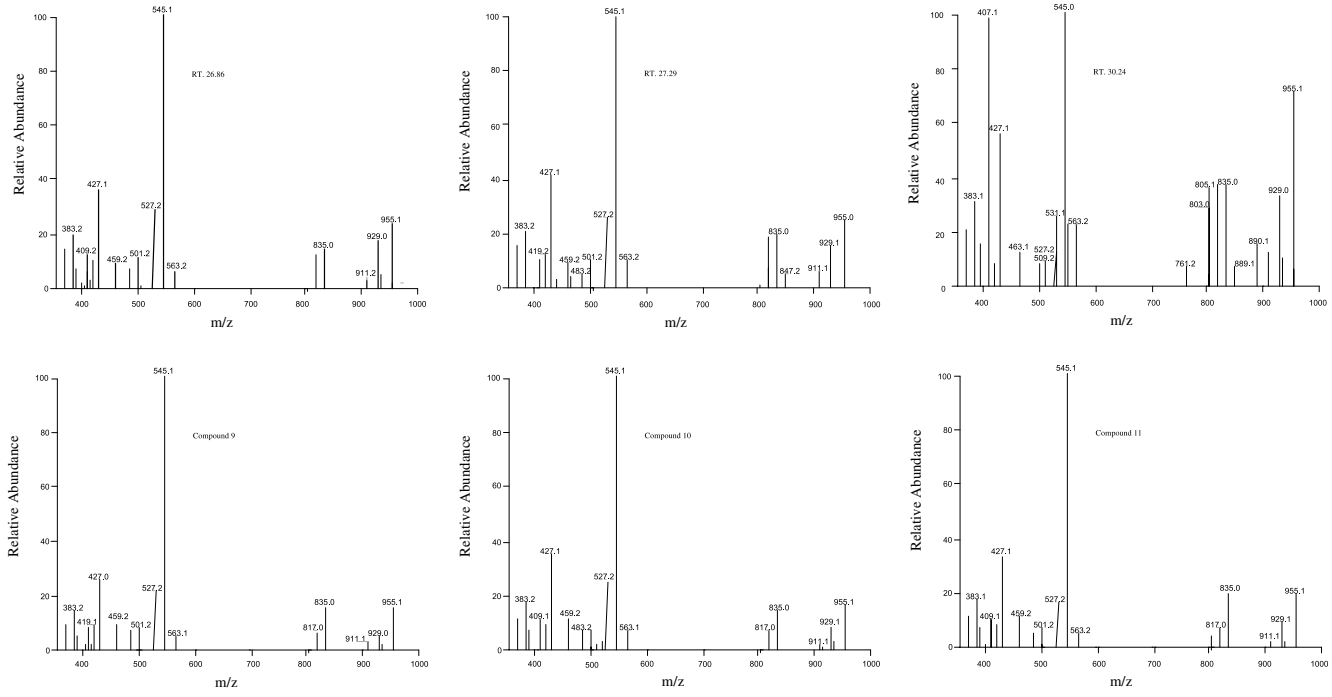


Figure 5. Negative ion LC/ESI-MS/MS spectra of compounds **9**–**11** and the three peaks found in subfraction 13 of the ethyl acetate fractions of black tea extract.

and **B** are the first theaflavin-type trimer of catechins in black tea.

Besides the four major tea catechins (EC, EGC, EGCG, and ECG), their related epimers (C, GC, GCG, and CG) also exist in tea leaves. Therefore, more stereo isomers can be formed during fermentation process of making black tea. It is challenging to isolate and identify these compounds, especially the minor constituents. Structural information of these minor constituents can help us to fully understand the fermentation process of black tea and to approach the formation of the so-called polymer thearubigins, which count as 20% by weight of black tea extract. In other words, this can help us further

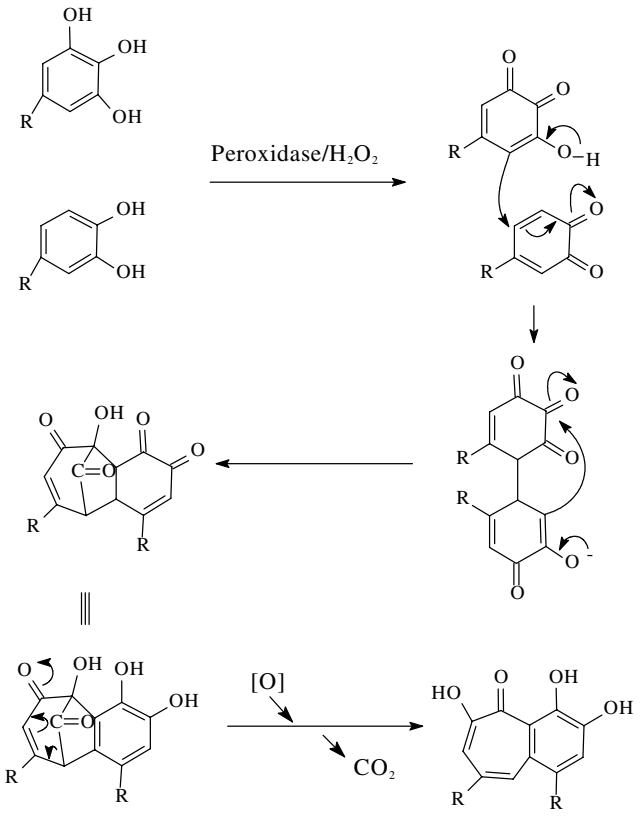


Figure 6. Proposed mechanism for the formation of benzotropolone skeleton.

understand the health benefits of drinking black tea. Thus, the method that we develop here is important. It can be applied to study the structures of other polymers whose formation involve in a similar enzymatic process.

3. Experimental

3.1. General procedures

^{1}H (600 MHz), ^{13}C (150 MHz), and all 2D NMR spectra were acquired on a Varian ^{Unity}INOVA 600 NMR spectrometer (Palo Alto, CA) equipped with a *z*-gradient inverse-detection triple resonance probe. ^{1}H–^{13}C HMQC and HMBC experiments were performed as described previously.[27] The general mass spectrometry was performed on a VG Platform II single quadrupole mass spectrometer (Micromass Co., MA) equipped with an atmospheric pressure ion source and atmospheric pressure chemical ionization (ApCI) interface. The effluent from the LC column was delivered to the ion source (150 °C) through a heated nebulizer probe (450 °C) using nitrogen as drying gas (300 L/h) and sheath gas (150 L/h). Negative ions were acquired in full scan. RP C-18 silica gel and Sephadex LH-20 gel were purchased from Sigma Chemical Co. (St. Louis, MO). Thin-layer chromatography was performed on Sigma-Aldrich TLC plates (250 μm thickness, 2–25 μm particle size), with compounds visualized by spraying with 5% (v/v) H_2SO_4 in ethanol solution. Horseradish peroxidase, epicatechin, catechin, H_2O_2, and CD_3OD were purchased from Aldrich Chemical Co. (Milwaukee, WI). Theaflavin 3-gallate, theaflavin 3′-gallate, theaflavin 3,3′-digallate, neotheaflavin 3-gallate were synthesized previously in our lab.[28]

3.2. Synthesis of theadibenzotropolone A, B, C, and theatribenzotropolone A

3.2.1. Theadibenzotropolone A (9). EC (**1**) (1.0 g) and EGCG (**3**) (1.0 g) were dissolved in a mixture of acetone-pH 5 buffer (1:10, v/v, 50 mL), which contained 5 mg horseradish peroxidase. While being stirred, 2 mL of 3.13% H_2O_2 was added four times during 45 min. One major reaction product, compound **5**, was formed. After addition of 0.5 mL of H_2O_2, stirring was continued for an additional 30 min; the amount of compound **5** decreased, while another reaction product, compound **9** was formed. Compound **5** (120 mg) and **9** (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. In addition, following the same procedure, 300 mg EC (**1**) can react with 100 mg theaflavin 3-gallate (**5**) to generate 10 mg theadibenzotropolone **A**. ^{1}H and ^{13}C NMR: see literature 16.

3.2.2. Theadibenzotropolone B (10). Following the procedure for the synthesis of **9**, 500 mg catechin (**4**) and 200 mg theaflavin 3-gallate (**5**) were used to synthesize 30 mg theadibenzotropolone **B**. ^{1}H and ^{13}C NMR: see Table 1. Negative APCI-MS m/z 973 $[M-H]^-$.

3.2.3. Theadibenzotropolone C (11). Following the procedure for the synthesis of **9**, 500 mg EC (**1**) and 200 mg neotheaflavin 3-gallate (**7**) were used to synthesize 35 mg theadibenzotropolone **C**. ^{1}H and ^{13}C NMR: see Table 1. Negative APCI-MS m/z 973 $[M-H]^-$.

3.2.4. Theatribenzotropolone A (13). Following the procedure for the synthesis of **9**, 500 mg EC (**1**) and 200 mg theaflavin 3,3′-digallate (**8**) were used to synthesize 25 mg theatribenzotropolone **A**. ^{1}H and ^{13}C NMR: see Table 2. Negative APCI-MS m/z 1383 $[M-H]^-$.

3.3. Preparation of black tea fractions

Yunnan black tea (908 g), which was purchased from the local Chinese supermarket, was extracted with 80% acetone (five times) at room temperature for two weeks. The extract was concentrated to dryness under reduced pressure, and the residue was dissolved in water and partitioned with chloroform, ethyl acetate, and *n*-butanol, respectively. The ethyl acetate fraction was subjected to Sephadex LH-20 eluted by acetone/water solvent system (30–60%) to give 14 subfractions.

3.4. HPLC/ESI-MS Detection of theadibenzotropolone A, B, C, and theatribenzotropolone A in black tea fractions

LC/MS analysis was carried out with a Finnigan Spectra System, which consisted of a Finnigan model P4000 pump, a model AS3000 refrigerated autosampler, and a Finnigan LCQ Deca mass detector (ThermoFinigan, San Jose, CA) incorporated with electrospray ionization (ESI) interface. A Supelco Discovery HS C_{18} column (75×2.1 mm i.d.; particle size, 3 μm) was used for separation with a flow rate of 0.2 mL/min. The column elution started with 100% solvent A (10% aqueous methanol). The linear gradient was changed to 20% B (70% aqueous methanol) at 8 min, and 100% B at 34 min to clean the column and then reequilibrate to 100% A from 37 to 52 min for the next run. The LC elute was introduced into the ESI interface. The negative ion polarity mode was set for ESI ion source with the voltage on the ESI interface maintained at approximately −4 kV. Nitrogen gas was used as the sheath gas at a flow rate of 80 arb and the auxiliary gas at 10 arb, respectively. The heated capillary temperature and voltage were maintained at 260 °C and −24 V, respectively. The tube lens offset voltage was set at −55 V. The structural information of the standard theadibenzotroplones and theatribenzotropole were obtained by tandem mass spectrometry (MS/MS) through collision-induced dissociation (CID) with a relative collision energy setting of 25%.

Acknowledgements

This work was supported by NIH Grant PO1 CA88961. Funds to purchase the 600 MHz NMR spectrometer at CUNY College of Staten Island were provided by the New York State Dormitory Authority and the New York State Higher Education Applied Technology Program.

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