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Two Types of Oxidative Dimerization of the Black Tea Polyphenol Theaflavin

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Theaflavin and its galloyl esters are polyphenolic pigments of black tea. In the course of studies on the oxidation mechanism of tea polyphenols, two theaflavin oxidation products named bistheaflavins A and B were isolated, and their structures were elucidated on the basis of MS and NMR spectroscopic analyses. Treatment of a mixture of (−)-epicatechin and (−)-epigallocatechin with banana fruit homogenate yielded bistheaflavin A together with theaflavin and theanaphthoquinone. The symmetrical structure of bistheaflavin A suggested that this compound was formed by oxidative C−C coupling of two theaflavin molecules. In contrast, theaflavin in phosphate buffer (pH 7.3) was gradually oxidized to give bistheaflavin B and theanaphthoquinone. Bistheaflavin B possesses a bicyclooctane skeleton probably formed by intermolecular cyclization between dehydrotheaflavin and dihydrotheanaphthoquinone.

Keywords: *Theaflavin; oxidation; intermolecular cyclizatiion; black tea; polyphenol; catechin*

INTRODUCTION

Characteristic pigments of black tea consist of two major groups, theaflavins and thearubigins, which are formed by enzymatic oxidation of four major green tea flavan-3-ols [(−)-epicatechin (**1**), (−)-epigallocatechin (**2**), and their 3-*O*-gallates] (Figure 1) during tea fermentation (*1*). Theaflavins are flavan-3-ol dimers having a characteristic benzotropolone unit, and their structures and biogenesis are well studied. On the other hand, thearubigins are heterogeneous polymers (*2*), and the structures still remain largely unknown (*3*). Although possible participation of theaflavins in the formation of thearubigins has been suggested (*1*), the metabolism of theaflavin in tea fermentation remains to be clarified. In the course of our chemical studies on the oxidation of tea polyphenols, we have recently examined formation of theaflavin (**3**) from **1** and **2** by treatment with homogenate of fresh tea leaves or banana fruit and isolated a new dark-yellow pigment named theanaphthoquinone (**4**), which was generated by oxidation of **3** (*4*). Further investigation of the products obtained by oxidation with banana fruit led us to the isolation of an additional metabolite named bistheaflavin A (**5**). In addition, spontaneous oxidation of **3** under neutral conditions yielded **4** and another dimeric product named bistheaflavin B (**6**) (Figure 1). This paper deals with the structural characterization of these theaflavin dimers.

MATERIALS AND METHODS

General Procedures. 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 and 125 MHz for ^{13}C. Coupling constants are expressed in Hz, 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 a matrix for FAB-MS measurement.

Column chromatography was performed with MCI-gel CHP 20P (75−150 μm, Mitsubishi Chemical Co.) and with Sephadex LH-20 (25−100 μm, Pharmacia Fine Chemical Co. 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 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). Epicatechin and epigallocatechin were isolated from commercial green tea according to Nonaka et al. (*5*) and recrystallized from water.

Isolation of Bistheaflavin A (5). Commercial banana fruit flesh (*Musa acuminata* Colla cv. Giant Cavendish, 200 g) was homogenized in water (400 mL) and filtered through four layers of muslin. The filtrate (300 mL) was mixed with an aqueous solution (50 mL) of **1** (1.0 g, 3.5 mmol) and **2** (1.0 g, 3.3 mmol) and stirred vigorously for 5 h at 20 °C. Then the mixture was poured into acetone (700 mL) and insoluble precipitates were removed by filtering. The filtrate obtained was concentrated and extracted with ethyl acetate 4 × 300 mL). The organic layer was applied to a Sephadex LH-20 column (25 × 3.0 cm), and elution of the column with ethanol afforded **4** (280 mg, 0.524 mmol) and **3** (727 mg, 1.29 mmol). The fraction obtained by further elution of the column with aqueous acetone (1:1, v/v) was subjected to MCI gel CHP20P column chromatography with water containing increasing proportions of methanol to afford bistheaflavin A (**5**) (46 mg, 0.04 mmol).

Bistheaflavin A (5). Reddish-brown powder, $[\alpha]^{15}_{D}$ −48.3° (*c* 0.12, methanol). FABMS (negative-ion mode) *m*/*z* 1143 $[M-H]^-$. UV λ_{max} nm (log ϵ): 255 (4.36), 274sh (4.26), 320 (3.81). Anal. Calcd. for $C_{58}H_{48}O_{25}\cdot 6H_2O$: C, 55.59; H, 4.83. Found: C, 55.65; H, 4.90. ^{1}H and ^{13}C NMR data are presented in Table 1.

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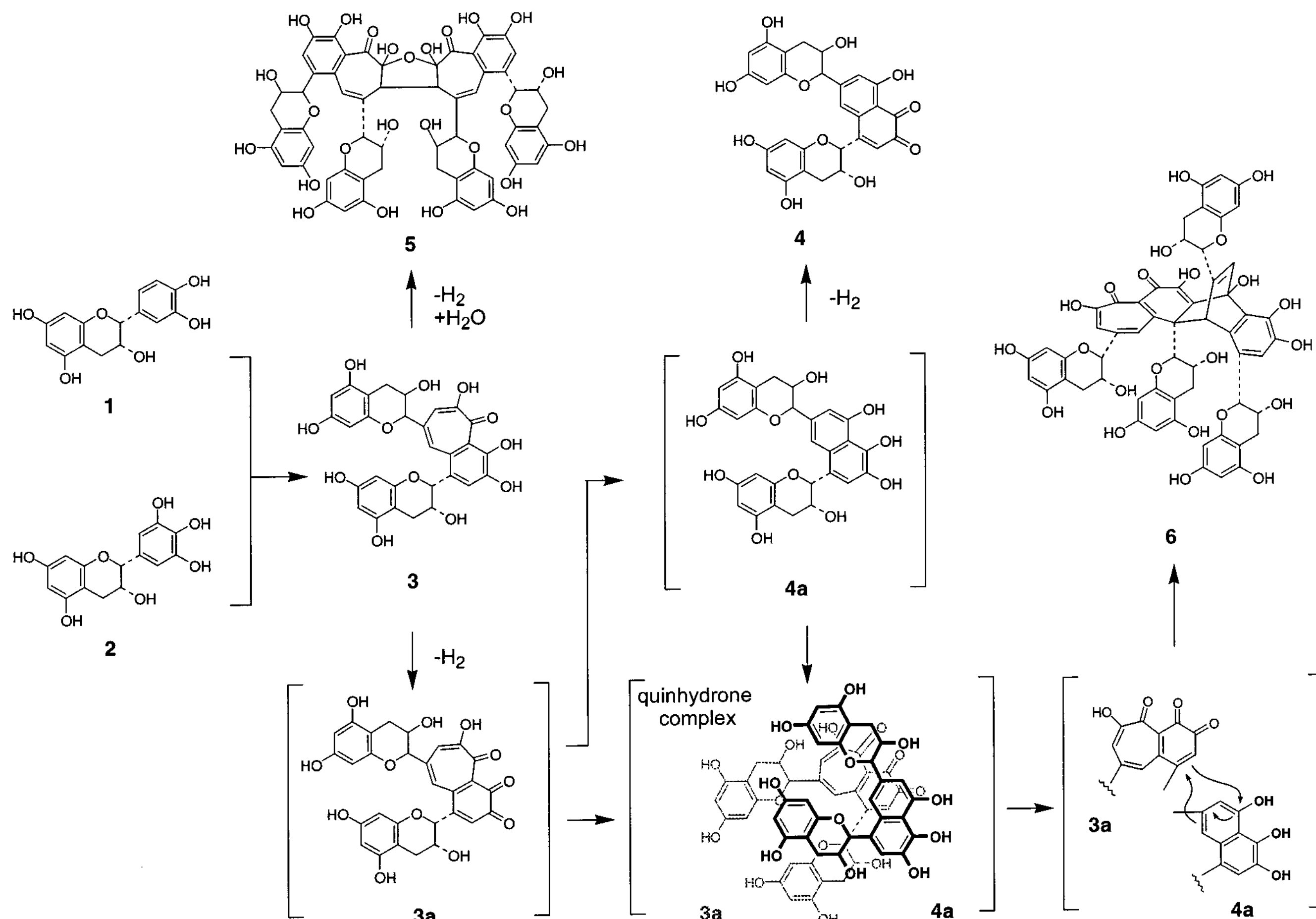

Figure 1. Structures of compounds **1**−**6** and proposed mechanisms for production of **5** and **6**.

Isolation of Bistheaflavin B (6). A solution of **3** (500 mg, 0.89 mmol) in 100 mL of phosphate buffer (0.1 M, pH 7.3) was stirred at 25 °C for 12 h. After acidification by addition of 0.1 M HCl, the mixture was directly applied to a MCI gel CHP 20P column (16 × 3.0 cm) eluted with water, and the phenolic compounds were adsorbed at the top of the gel. Subsequent elution of the column with water containing increasing proportions of methanol yielded bistheaflavin B (**6**) (70.8 mg, 0.06 mmol), **4** (26.6 mg, 0.05 mmol), and unchanged **3** (126.6 mg, 0.22 mmol).

*Bistheaflavin B (**6**).* Reddish-brown powder, $[\alpha]^{15}_{D}$ −72.8° (*c* 0.06, methanol). FABMS (negative-ion mode) *m/z* 1097 $[M-H]^-$, (positive mode) *m/z* 1099 $[M+H]^+$, 1121 $[M+Na]^+$. UV λ_{max} nm (log ϵ): 274 (4.10), 378 (3.70). Anal. Calcd. for $C_{57}H_{46}O_{23}\cdot 6H_2O$: C, 56.72; H, 4.84. Found: C, 56.98; H, 4.91. ^{1}H and ^{13}C NMR data: Table 2.

RESULTS AND DISCUSSION

Previously, we have studied the oxidation of (−)-epigallocatechin (**2**) with banana fruit homogenate. Although the reaction gave a complex mixture, the occurrence of the quinone metabolites and subsequent dimerization of the starting material were demonstrated (*6*). On the other hand, the oxidation of **2** with banana homogenate in the presence of (−)-epicatechin (**1**) was completely different and apparently simple: treatment of an equimolar mixture of **1** and **2** with banana fruit homogenate in contact with air yielded the black tea pigment **3** and a new metabolite **4** as the major products (*4*). The stable naphthoquinone structure of **4** was established by spectroscopic analyses of **4** and formation of its phenazine derivative. Further separation of the reaction mixture resulted in the isolation of a minor

Table 1. ^{1}H and ^{13}C NMR Data for Bistheaflavin A (5) (500 MHz for ^{1}H NMR, 125 MHz for ^{13}C NMR, in Acetone-d_6+D_2O)

position	δ_C	δ_H	HMBC (H to C)
2, 2*	81.78	4.256 (2H, br s)	c, d, e
3, 3*	63.67	4.256 (2H, br s)	
4, 4*	29.35	2.809 (4H, m)	4a, 5
4a, 4a*	99.50		
5, 5*	157.11[a]		
6, 6*	96.59	5.999 (2H, d, 1.6)	4a, 5, 7
7, 7*	157.35[a]		
8, 8*	95.75	5.789 (2H, d, 1.6)	4a, 8a, 7
8a, 8a*	156.21		
2′, 2′*	75.78	5.142 (2H, s)	g, f, k
3′, 3′*	65.37	4.256 (2H, br s)	4a′
4′, 4′*	29.60	2.789 (2H, dd, 16.8, 4.1)	
		2.617 (2H, br d, 16.8)	2′, 3′,4a, 5
4a′, 4a′*	99.67		
5′, 5′*	157.44[a]		
6′, 6′*	96.16	5.970 (2H, d, 1.6)	4a′, 5′, 7′
7′, 7′*	157.44[a]		
8′, 8′*	95.50	5.910 (2H, d, 1.6)	4a′, 8a′, 7′
8a′, 8a′*	156.99[a]		
a, a*	197.05		
b, b*	113.43		
c, c*	53.46	3.628 (2H, s)	2, a, b, c*, e
d, d*	136.67		
e, e*	128.85	7.187 (2H, s)	2, c, d, f, j, k, b(^4J)
f, f*	130.01		
g, g*	118.40	7.524 (2H, s)	2′, f, h, i, k, j(^4J)
h, h*	144.58		
i, i*	144.53		
j, j*	125.04		
k, k*	123.47		

[a] Assignments may be interchanged.

Table 2. ^{1}H and ^{13}C NMR Data for Bistheaflavin B (6) (500 MHz for ^{1}H NMR, 125 MHz for ^{13}C NMR, in Acetone-d_6+D_2O)

theaflavin part				theanaphthoquinone part			
position	δ_C	δ_H	HMBC (H to C)	position	δ_C	δ_H	HMBC (H to C)
t-2	78.72	4.986(d, 1.6)	t−4, t−8a, t−c, t−d, t−e	n-2	75.31	5.888(s)	n−3, n−4, n−c, n−d, n−j
t-3	66.43	4.348(ddd, 1.6, 4.4, 4.8)	t−4a	n-3	65.91	4.334(br s)	n−4a
t-4	27.71	2.784(dd, 4.8, 16.3)	t−2, t−3, t−4a, t−5, t−8a	n-4	29.14	2.852(dd, 4.3, 16.7)	n−4a, n−5, n−8
		2.488(dd, 4.4, 16.3)	t−2, t−3, t−4a, t−8a			2.653(br d, 16.7)	
t-4a	99.52			n-4a	99.87		
t-5	157.55[a]			n-5	157.49[a]		
t-6	97.04[b]	5.945[d] (d, 2.3)		n-6	96.29[b]	6.034[d] (d, 2.3)	
t-7	157.55[a]			n-7	157.45[a]		
t-8	96.96[b]	5.947[d] (d, 2.3)		n-8	96.17[b]	6.079[d] (d, 2.3)	
t-8a	156.02			n-8a	157.41[a]		
t-2′	81.19	3.930(s)	t−8a′, t−3′, t−f, t−g, t−k, n−e	n-2′	80.72	4.244(s)	n−4′, n−e, n−f, n−g
t-3′	62.69	4.034(br s)	t−4a′	n-3′	62.43	3.727(br s)	n−4′, n−4a′
t-4′	31.83	2.467(br d, 16.6)	t−2′, t−3′, t−4a′, t−8a′	n-4′	29.56	2.693(dd, 4.6, 17.2)	n−2′, n−3′
		2.378(dd, 4.0, 16.6)	t−4a′, t−8a′			2.621(dd, 2.5, 17.2)	
t-4a′	99.08			n-4a′	100.09		
t-5′	157.52[a]			n-5′	157.24[a]		
t-6′	96.96[b]	5.983[d] (d, 2.3)		n-6′	95.64[b]	6.126[d] (d, 2.3)	
t-7′	157.52[a]			n-7′	157.05[a]		
t-8′	96.60[b]	5.990[d] (d, 2.3)		n-8′	95.53[b]	6.161[d] (d, 2.3)	
t-8a′	156.79			n-8a′	156.47[a]		
t-a	185.50			n-a	144.60		
t-b	162.93			n-b	145.41		
t-c	115.21	7.060(s)	t−2, t−a, t−b, t−d, t−e	n-c	116.29	7.177(s)	n−2, n−a, n−b, n−d, n−j
t-d	148.39			n-d	129.72		
t-e	119.64	7.989(d, 1.0)	t−2, t−c, t−f, t−j	n-e	45.57	5.878(s)	t−f, t−g, t−k, t−h(^4J), n−2′, n−a(^4J), n−d, n−f, n−g, n−i, n−j
t-f	50.75						
t-g	127.01[c]			n-f	159.45		
t-h	148.90			n-g	126.95[c]	6.224(s)	n−2′, n−e, n−f, n−h, t−f(^4J), t−g
t-i	189.27			n-h	86.26		
t-j	116.07			n-i	113.50		
t-k	143.77			n-j	127.33		
t-b-OH		11.962(s)	t−b(^4J), t−c, t−d, t−j				

[a−d] Assignments may be interchanged.

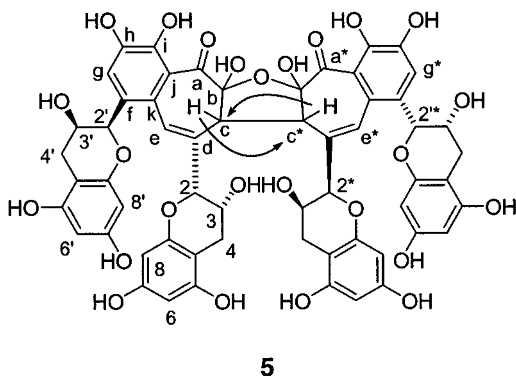

Figure 2. Important HMBC correlations (arrows) observed for **5**.

product named bistheaflavin A (**5**). Comparison of R_f values on TLC suggested that the polarity of **5** was much greater than the polarities of **3** and **4**. The ^{13}C NMR spectrum (Table 1) showed 29 carbon signals, with 18 of the signals being attributable to A and C rings of flavan-3-ols. However, the FABMS (negative-ion mode) exhibited a prominent $[M-H]^-$ peak at *m/z* 1143, indicating that this compound consists of four flavan-3-ol units. Considering the number of the carbon signals, this observation suggested that **5** has a symmetrical structure. In the ^{1}H NMR spectrum (Table 1), two olefinic singlets (δ 7.187 and 7.524) and an aliphatic methine singlet (δ 3.628) were observed together with two sets of signals arising from the flavan A- and C-rings. The long-range H−C correlations of the olefinic signal at δ 7.524 (H-g) observed in the HMBC spectrum (Table 1) suggested the presence of a catechol ring (C-f−C-k) attached to C-2′ of one of the flavan C-rings. In addition, the correlations of the remaining olefinic (δ 7.187, H-e) and aliphatic (δ 3.628, H-c) methine protons indicated occurrence of a seven-membered ring (C-a−C-j) fused to the catechol ring, which was structurally related to the benzotropolone unit of **3**. This partial structure was also supported by an ^{1}H-^{1}H COSY experiment which demonstrated the long-range coupling of H-2′ with H-g and H-e; and H-e with H-c, H-g, and H-2. In the HSQC spectrum, the methine proton signal at δ 3.628 correlated (^1J) with the carbon signal at δ 53.46. However, the HMBC spectrum also exhibited a strong cross-peak between these two signals, apparently indicating occurrence of long-range coupling (^2J or ^3J) between these two atoms. Because the aforementioned result of the FABMS indicated the symmetry of the molecule, this apparent contradiction could be explained by mutual two-bond (^2J) long-range coupling between the methine (c) and the corresponding position (c*) in the other part of the symmetrical molecule (Figure 2). Furthermore, the molecular mass (1144) and the chemical shift of C-b (δ 113.43) indicated formation of a hemiketal ring between C-b and C-b* (*7*). On the basis of the above spectroscopic observation, the whole structure of **5** was determined to be the dimer of theaflavin as shown in Figure 1. The NOESY correlations of H-e with H-2 and H-2′, H-g with H-2′, and H-c with H-2 were consistent with the structure. In addition, weak NOE correlation of one of the A-ring signals (probably H-8) with H-e, H-2, and H-3′ signals suggested the *trans*-configuration between H-c and H-c*. The absolute configuration of these methine carbons has not yet been determined. Bistheaflavin A was probably formed by oxidative C−C coupling between two theaflavin molecules (Figure 1). Although participation of peroxidase in thearubigin formation during tea fermentation has long been a subject for argument and degradation of **3** by peroxidase was reported recently (*8*), an attempt to derive **5** from **3** by treatment with horseradish peroxidase−H_2O_2 or fresh tea leaf homogenate was unsuccessful.

It is known that catechins and related polyphenols are spontaneously oxidized by atmospheric oxygen in aqueous solution. On this basis, we examined the autooxidation of **3** in 0.1 M phosphate buffer (pH 7.3) at room temperature and in contact with air. The HPLC analysis of the solution showed that two major products were generated as **3** decreased. One of the products was identified as **4** by direct comparison of the spectroscopic data, and the other product was found to be a new compound named bistheaflavin B (**6**). The product **6** was isolated as a reddish-brown amorphous powder and showed UV absorption at 274 and 378 nm. The FABMS (negative- and positive-ion modes) revealed the molecular weight to be 1098, which was comparable to that of **5** and coincident with the sum of the molecular weights of **3** (MW 564) and **4** (MW 534). The dimeric nature of this product was also indicated by the ^{1}H NMR and ^{1}H-^{1}H COSY spectra (Table 2), which exhibited four sets of signals due to protons at the 2-, 3-, 4-, 6-, and 8-positions of the flavan nucleus, similar to those of **1** and **2**. This observation also showed that the A and C rings of **3** did not undergo any change during this autoxidation. The remaining part of the molecule exhibited four olefinic proton signals [δ 7.060 (s, Ht-c), 7.989 (d, 1.0 Hz, Ht-e), 7.177 (s, Hn-c), and 6.224 (s, Hn-g)], an aliphatic proton signal [δ 5.878 (s, Hn-e)], and a hydroxyl proton signal [δ 11.962 (s, Ct-*b*-OH)]. The large downfield shift of the hydroxyl proton signal and slow exchange of this proton in a D_2O exchange experiment suggested the occurrence of intramolecular hydrogen bonding. In the ^{13}C NMR spectrum, besides the A and C ring-carbon signals, 21 carbon signals including two carbonyl [δ 185.50 (Ct-a) and 189.27 (Ct-i)], three aliphatic [δ 50.75 (Ct-f), 45.57 (Cn-e), and 86.26 (Cn-h)] and 16 olefinic carbons were observed (Table 2). In the HMBC spectrum (Table 2), the four flavan H-2 signals (t-2, t-2′, n-2, and n-2′) were correlated with 10 olefinic carbons (Ht-2 with Ct-c, Ct-d, Ct-e; Ht-2′ with Ct-f, Ct-g, Ct-k, Cn-e; Hn-2 with Cn-c, Cn-d, Cn-j; Hn-2′ with Cn-e, Cn-f, and Cn-g). In addition, the correlations of the four olefinic protons (Ht-c, Ht-e, Hn-c, Hn-g) and the aliphatic methine proton (Hn-e) with the four flavan C-2 signals established the relative positions of these carbons. The ^{1}H-^{1}H long-range couplings (Figure 3, dashed line) observed in the ^{1}H-^{1}H-COSY spectrum also supported the connectivities of these carbons. Occurrence of a catechol ring attached to Cn-2 was apparent from the correlations of Hn-c with Cn-2, Cn-a, Cn-b, Cn-d, and Cn-j. In addition, the HMBC correlations of the methine proton (Hn-e) with the aliphatic quaternary carbon (Ct-f) and nine olefinic carbons (Ct-g, Ct-k, Ct-h, Cn-a, Cn-d, Cn-f, Cn-g, Cn-i, and Cn-j), and the correlations of Hn-g with the methine Cn-e, the oxygen-bearing quaternary carbon (Cn-h) and three olefinic carbons (Cn-f, Ct-f, and Ct-g) revealed the presence of a benzobicyclooctene structure, as shown in Figure 3. Furthermore, the correlations between Ht-c, Ht-e, and the hydroxyl proton signal at δ 11.962 indicated the presence of a tropolone ring similar to that of **3**. Although no proton was correlated with the conjugated carbonyl carbon at δ 189.27 (Ct-i), the

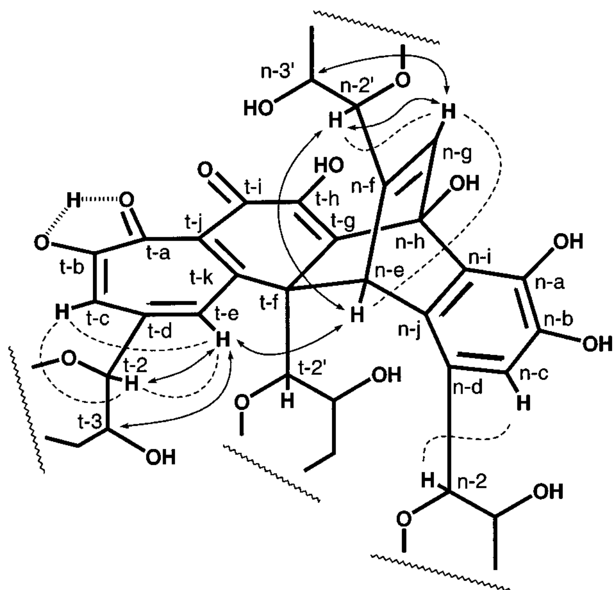

Figure 3. Important NOESY correlations (arrows) and $^{1}H-^{1}H$ long-range couplings (broken lines) observed for **6**.

location of this carbon was deduced from the structural comparison with the starting material **3**. These HMBC analyses permitted us to elucidate the partial structure shown in Figure 3. The NOESY spectrum of **6** showed the NOEs of Ht-e with Ht-2, Ht-3 and Hn-e; Hn-2′ with Hn-e and Hn-g; and Hn-g with Hn-3′ (Figure 3), which were also consistent with the above structure. On the basis of the spectroscopic evidence, the structure of bistheaflavin B was determined to be as shown by structure **6** (Figure 1). The absolute configuration of the bicyclooctene part (Cn-e and Cn-h) has not yet been determined. On the basis of the structure, it is postulated that **6** was generated by intermolecular cyclization between dehydrotheaflavin (**3a**) and dihydrotheanaphthoquinone (**4a**) (Figure 1). Haslam pointed out that the formation of quinhydrone type intermolecular $\pi-\pi$ complex between oxidized epigallocatehin (*o*-quinone) and un-oxidized epicatechin (catechol) is important in the biosynthesis of **3** (*9*). In the auto-oxidation process of **3**, oxidized catechol (**3a**) and un-oxidized catechol (**4a**) probably formed a similar intermolecular $\pi-\pi$ complex prior to the intermolecular cyclization.

This work has demonstrated two types of oxidative dimerization of theaflavin (Figure 1), and these reactions might participate in the formation of thearubigins in tea fermentation. Because self-association of theaflavin forms an antiparallel dimer at the benzotropolone rings (*10*), bistheaflavin A (**5**) might be formed by oxidative coupling between the stacked benzotropolone rings. On the other hand, the generation of **6** by autooxidation of **3** in aqueous solution indicated the importance of the quinhydrone type complex in the oxidation of polyphenols.

ABBREVIATIONS USED

COSY, correlation spectroscopy; FABMS, fast atom bombardment mass spectroscopy; HMBC, heteronuclear multiple bond connectivity; HPLC, high-performance liquid chromatography; HSQC, heteronuclear single quantum coherence; NOESY, ^{1}H NMR nuclear Overhauser and exchange spectroscopy.

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