Structures of Epicatechin Gallate Trimer and Tetramer Produced by Enzymatic Oxidation

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During black tea production, catechins and their galloyl esters are enzymatically oxidized to generate a complex mixture of black tea polyphenols. The role of galloyl ester groups in this process has yet to be determined. Enzymatic oxidation of epicatechin 3-*O*-gallate (1) yielded two new oxidation products, theaflavate C and bistheaflavate A, along with theaflavate A (2), a known dimer of 1 generated by coupling of the B-ring with the galloyl group. Theaflavate C is a trimer of 1 and possesses two benzotropolone moieties generated by the oxidative coupling of the galloyl groups with the catechol B-rings. Bistheaflavate A was found to be a tetramer produced by intermolecular coupling of two benzotropolone moieties of 2. From the structures of the products, it was deduced that oxidative coupling of galloyl groups resulted in extension of the molecular size of the products in catechin oxidation.

Key words epicatechin 3-*O*-gallate; benzotropolone; oxidation; black tea; polyphenol

Black tea accounts for almost 80% of the world tea production, and therefore represents one of the most important beverages in the world. Additionally, black tea is rich in polyphenols compared to other beverages,[1] and the various health benefits associated with its consumption, including antioxidative,[2] anticancer[3,4] and anti-inflammatory[5,6] activity, have recently been investigated. Black tea is produced by crushing the fresh leaves of *Camellia sinensis*, and the constituents of the leaves are enzymatically converted to numerous secondary products that contribute to the characteristic color and flavor of black tea.[7,8] In this process tea catechins, which are the major constituents of fresh tea leaves and mainly comprise (−)-epicatechin, (−)-epigallocatechin and their galloyl esters, are oxidized to yield a complex mixture of secondary polyphenols including theaflavins,[9] theasinensins[10,11] and oolongtheanins.[11] However, the major components of the secondary polyphenols have not been chemically characterized due to their complexity and the difficulties involved in their separation and purification.[12] In our previous studies concerning the enzymatic oxidation of tea catechins, oxidative coupling reactions of catechin B-rings were demonstrated.[13—22] On the other hand, 60—80% of the total tea catechins possess galloyl esters located at the C-3 hydroxy group and although oxidation of galloyl groups must be important in the formation of black tea polyphenols,[23] only limited examples of oxidative coupling of galloyl groups has been reported.[22,24—26] Our previous *in vitro* experiments showed that enzymes preferentially oxidize the catechol B-rings of epicatechin and the resulting quinone, which is a potential oxidizing reagent, subsequently oxidizes pyrogallol rings, the redox potential of which is lower than that of the catechol ring.[27] The catechol quinone also undergoes coupling reactions with other aromatic rings.[9,14,15] Taking the reaction mechanism into account, (−)-epicatechin 3-*O*-gallate (**1**), which possesses both the catechol ring and galloyl groups, was used in the present study to examine the oxidation of galloyl groups. In this paper, we describe the structure and mechanism of production of catechin trimer (**3**) and tetramer (**4**) produced by the oxidation of galloyl groups.

The enzyme source used in our study should be noted here. Given that catechin oxidation in fresh tea leaf is a complex chemical process that acts on individual tea catechins, *in vitro* model fermentation experiments using pure catechins were employed in this study. However, the tea leaf homogenate or crude enzymes prepared from tea leaf did not display sufficient activity required for performing large scale experiments necessary to supply sufficient amounts of oxidation products needed for isolation and structure determination.[16] Previously, we showed that homogenates of Japanese pear or banana could synthesize typical black tea polyphenols from tea catechins.[15,17,19] The reactions proceeded in a manner similar to the oxidation catalyzed by tea leaf enzymes, and the products were consistent with catechin oxidation in fresh tea leaf. Consequently, Japanese pear homogenate was employed as the enzyme source in the present study.

Results

An aqueous solution of **1** was mixed with a homogenate of Japanese pear and stirred vigorously at room temperature. After the color of the reaction mixture gradually changed to reddish-yellow due to the benzotropolone chromophores, the reaction was terminated by the addition of acetone. Products were separated by repeated column chromatography using Sephadex LH-20 and Chromatorex ODS to give two new compounds named theaflavate C (**3**) and bistheaflavate A (**4**), together with a known pigment **2**. Pigment **2** was the major product of the reaction and identified to be theaflavate A,[24] which was generated by intermolecular oxidative coupling of the galloyl group and catechol B-ring of **1**. The presence of **2** in commercial black tea was previously confirmed by HPLC analysis.

Theaflavate C (**3**) was obtained as a red amorphous powder and showed UV absorptions at 279 and 405 nm, which were similar to those of **2**, suggesting the presence of benzotropolone moieties in the molecule. The FAB-MS data exhibited a $[M+H]^+$ ion peak at *m*/*z* 1263, and HR-FAB-MS (*m*/*z* 1263.2267 for $C_{64}H_{47}O_{28}$) confirmed the molecular formula $C_{64}H_{46}O_{28}$, indicating that **3** is a trimer of **1**. The ^{1}H-NMR spectrum (Table 1) showed signals arising from three

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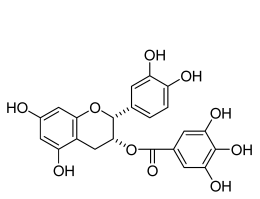

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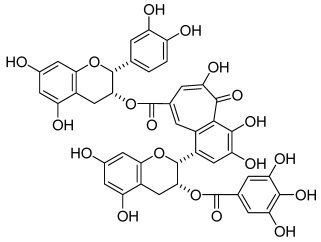

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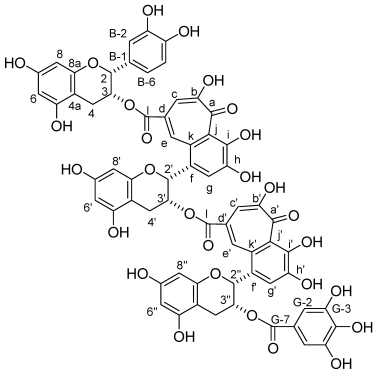

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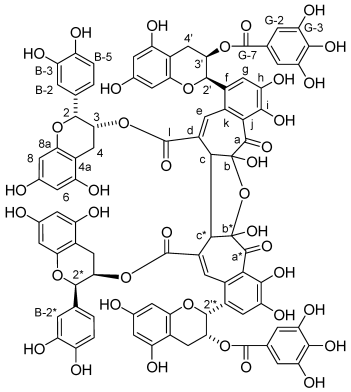

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Table 1. ^{1}H- and ^{13}C-NMR Data for Theaflavate C (**3**) (500 MHz for ^{1}H-NMR, 125 MHz for ^{13}C-NMR, in acetone-d_6+D_2O)

Position	δ_C	δ_H	HMBC (H to C)	Position	δ_C	δ_H	HMBC (H to C)
2	77.3	5.22 (br s)	3, B-1, B-2, B-6	B-1	130.8		
3	71.6	5.73 (br s)	4a	B-2	113.8	6.99 (d, 2.0)	2, B-3, B-4, B-6
4	26.3	3.16 (dd, 5.0, 18.0)	3, 4a, 8a	B-3	145.9		
		3.08 (br d, 18.0)		B-4	145.5		
4a	98.8			B-5	115.9	6.63 (d, 8.0)	B-1, B-3, B-4
5	157.3[a)]			B-6	118.5	6.97 (dd, 2.0, 8.0)	2, B-2, B-4
6	96.9	6.07 (d, 2.0)	4a, 5, 7, 8				
7	157.9[a)]			a	185.9		
8	96.3	6.24 (d, 2.0)	4a, 6, 7, 8a	b	154.7		
8a	156.7[a)]			c	115.2	7.62 (s)	b, d, e, l
2′	75.1	5.76 (br s)	3′, f, g	d	124.2		
3′	68.2	5.48 (br s)	4a′	e	131.7	8.41 (s)	c, d, f, j, l
4′	26.9	2.95[b)] (m)	4a′	f	133.2		
4a′	99.0			g	122.7	7.95 (s)	2′, f, h, i, k
5′	157.5[a)]			h	149.0		
6′	97.6	6.19 (d, 2.0)	4a′, 5′, 7′, 8′	i	151.6		
7′	157.8[a)]			j	122.2		
8′	96.3	6.22 (d, 2.0)	4a′, 6′, 7′, 8a′	k	126.2		
8a′	156.6[a)]			l	166.2		
2″	75.0	5.87 (br s)	3″, f′, g′				
3″	70.6	5.71 (br d, 3.0)	4a″	a′	185.9		
4″	26.9	3.54 (dd, 5.0, 18.0)	2″, 3″, 4a″, 8a″	b′	154.7		
		3.19 (br d, 18.0)		c′	115.7	7.73 (s)	b′, d′, e′, l′
4a″	99.1			d′	124.9		
5″	157.9[a)]		4a″, 5″, 7″, 8″	e′	131.5	8.55 (s)	c′, d′, f′, j′, l′
6″	96.1	6.10 (2H, s)		f′	132.5		
7″	158.0[a)]		4a″, 6″, 7″, 8a″	g′	122.0	7.92 (s)	2″, f′, h′, i′, k′
8″	97.2	6.10 (2H, s)		h′	149.0		
8a″	156.6[a)]			i′	151.9		
Galloyl-1	121.0			j′	122.2		
Galloyl-2, 6	109.8	6.88 (2H, s)	Galloyl-1, 3, 4, 6, 7	k′	126.8		
Galloyl-3, 5	145.9			l′	166.7		
Galloyl-4	139.0						
Galloyl-7	165.7						

a) Assignments may be interchanged. *b*) Signals were overlapped with the solvent signal.

sets of flavan A- and C-rings. Acylation at all of the C-3 hydroxyl groups was apparent from the chemical shifts of H-3, H-3′ and H-3″. However, only one galloyl signal (δ 6.88) was observed in the spectrum. In the ^{1}H–^{1}H COSY spectrum, the H-2 signal at δ 5.22 showed allylic long-range correlations with aromatic proton signals at δ 6.99 (d, J=2.0 Hz) and δ 6.97 (dd, J=2.0, 8.0 Hz),[14,16,19] attributable to the H-2 and H-6 of a catechol-type B-ring, respectively. The remaining H-2′ and H-2″ were correlated with aromatic proton signals resonating at lower field [H-2′→δ 8.41 (H-e) and δ 7.95 (H-g); H-2″→δ 8.55 (H-e′) and δ 7.92 (H-g′)]. These aromatic signals and the signals at δ 7.62 and 7.73, H-c and H-c′, respectively, were related to those of the benzotropolone moiety of **2** and suggested the occurrence of two benzotropolone moieties. This was supported by the HMBC correlations of these aromatic protons listed in Table 1. In addition to the aforementioned ^{1}H–^{1}H long-range couplings of H-2′ and H-2″, HMBC correlations of H-g and H-g′ to C-2′ and C-2″, respectively, confirmed that the C-f and C-f′ of the benzotropolone catechol ring were attached to C-2′ and C-2″, respectively. The correlations of the ester carbonyl carbon C-l with H-c and H-e, and C-l′ with H-c′ and H-e′, showed that the carboxyl carbons (C-l and C-l′) were attached to C-d and C-d′ of the benzotropolone seven-membered ring. It is known that benzotropolone moieties can be generated by the condensation of catechol and pyrogallol rings with accompanied decarboxylation,[9] therefore it was deduced that the benzotoropolone moieties in **3** formed by intermolecular condensation of catechol-B-rings and the galloyl groups of **1**. Thus, the structure of theaflavate C was assigned as shown in formula **3**.

Bistheaflavate A (**4**) was obtained as a brown amorphous powder and showed UV absorptions at 276 and 354 nm. The ^{1}H- and ^{13}C-NMR spectra (Table 2) were related to those of **2** and showed signals arising from two sets of flavan A- and C-rings, one catechol-type B-ring and one galloyl group. Additionally, twelve carbon signals including a conjugated ketone (δ 197.1) and an ester carbonyl carbon (δ 165.3) in the ^{13}C-NMR spectrum were observed along with three singlet signals at δ 4.02, 7.53 and 7.87 in the ^{1}H-NMR spectrum. Although the NMR spectral data suggested that **4** was a dimeric compound, HR-FAB-MS revealed the molecular formula to be $C_{86}H_{65}O_{39}$ (*m/z*: 1721.3114 for $C_{86}H_{65}O_{39}$ $[M+H]^+$), and thus indicated that this product is a tetramer of **1** with a symmetrical structure. The ^{1}H–^{1}H COSY spectrum showed long-range correlations of the C-ring H-2 signal at δ 4.78 with catechol H-2 (δ 6.62) and H-6 (δ 6.59), indicating that the catechol ring is located at the C-2 position. In the HMBC spectrum (Table 2), the adjacent H-3 showed a correlation peak with the ester carbonyl carbon at δ 165.3 (C-l), which was also correlated with H-c (δ 4.02) and H-e (δ 7.87). Furthermore, the HMBC correlations of H-c, H-e and H-g (δ 7.53) suggested the presence of a benzotropolone-related structure in **4**. However, chemical shifts of the carbon signals differed significantly from those observed in **3**; in particular, C-c (δ 52.7) and C-b (δ 111.9) were shifted markedly to upper field [C-c, δ 115.2; C-b, δ 154.7 for **3**] and C-a (δ 197.1) was shifted to lower field (δ 185.9 for **3**). These observations indicated saturation of the C-b–C-c double bond and the acetal carbon at the C-b position. In the HSQC spectrum, the H-c methine proton signal at δ 4.02 correlated (^1J) with the carbon signal at δ 52.7. However, the HMBC spectrum also exhibited a strong cross peak between these two signals, suggesting the occurrence of long-range coupling (^2J or ^3J) between these two atoms. Since the aforementioned FAB-MS result indicated the symmetry of the molecule, this apparent contradiction could be explained by mutual two-bond (^2J) long-range coupling of the methine (C-c) with the corresponding position (C-c*) in the other part of the symmetrical molecule. Furthermore, the index of hydrogen deficiency (55) calculated from the molecular formula and the chemical shift of C-b (δ 111.9) suggested formation of a

Table 2. ^{1}H- and ^{13}C-NMR Data for Bistheaflavate A (**4**) (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)	Position	δ_C	δ_H	HMBC (H to C)
2, 2*	78.0	4.78 (br s)	3, B-1, B-2, B-6	B-1, B-1*	130.0		
3, 3*	69.8	5.14 (br s)	4a, l	B-2, B-2*	115.7	6.62 (d, 2.0)	B-3, B-4, B-5, B-6
4, 4*	27.1	2.73 (d, 18.0)	2, 3, 4a, 5, 8a	B-3, B-3*	144.8		
		2.88 (dd, 5.0, 18.0)		B-4, B-4*	145.4		
4a, 4a*	98.5			B-5, B-5*	115.7	6.52 (d, 8.0)	B-1, B-3, B-4, B-6
5, 5*	156.4[a]			B-6, B-6*	119.3	6.59 (dd, 2.0, 8.0)	B-2, B-4, B-5
6, 6*	95.3[b]	5.94[c] (d, 2.0)	4a, 5, 7, 8	a, a*	197.1		
7, 7*	157.6[a]			b, b*	111.9		
8, 8*	96.4[b]	6.05[c] (d, 2.0)	4a, 6, 7, 8a	c, c*	52.7	4.02 (s)	a, b, c*, d, e, l
8a, 8a*	157.1[a]			d, d*	129.6		
				e, e*	137.7	7.87 (s)	b(^4J), c, d, f, j, k, l
2′, 2′*	74.6	5.48 (br s)	3′, f, g, k	f, f*	130.0		
3′, 3′*	67.7	5.48 (br s)	4a′, galloyl-7	g, g*	117.9	7.53 (s)	2′, f, h, i,, k
4′, 4′*	26.2	2.83 (dd, 5.0, 18.0)		h, h*	147.4		
		2.92 (br d, 18.0)	2′, 3′, 4a′, 5′, 8a′	i, i*	144.6		
4a′, 4a′*	98.5			j, j*	125.4		
5′, 5′*	157.2[a]			k, k*	120.1		
6′, 6′*	95.6[b]	6.02[c] (d, 2.0)	4a, 5, 7, 8	l, l*	165.3		
7′, 7′*	157.2[a]			Galloyl-1	120.9		
8′, 8′*	96.6[b]	6.12 (d, 2.0)	4a, 6, 7, 8a	Galloyl-2, 6	109.8	6.97 (s)	Gallloyl-1, 3, 4, 5, 6, 7
8a′, 8a′*	157.2[a]			Galloyl-3, 5	145.7		
				Galloyl-4	138.9		
				Galloyl-7	166.3		

a, *b*, *c*) Assignments may be interchanged in each column.

hemiketal ring between C-b and C-b*. The structural feature was similar to that of bistheaflavin A, which was a dimer of theaflavin.[14] Location of the galloyl group was apparent from the HMBC correlation of H-3′ with the ester carbonyl carbon (δ 166.3) of the galloyl group, and connection of C-2′ to C-f was confirmed by correlation of H-2′ with C-f, C-g and C-k. On the basis of these spectral observations, the structure of bistheaflavate A was assigned as shown in formula **4**. The geometry of the bisacetal furan ring including C-c and C-c* carbons was presumed to be in the *trans* configuration. Molecular modeling suggested that positioning of the two large flavan-3-ol moieties attached at C-1 and C-1* on the same side of the molecule in the *cis* configuration would generate unfavorable steric hindrance.

Discussion

The oxidative coupling of two catechins is dependent on structural and redox potential factors. With the enzymatic oxidation of (+)-catechin[28] or (−)-epicatechin,[15] the B-ring *o*-quinone, an electron deficient initial intermediate, attacks the electron rich A-ring C-8 of another molecule to generate dimeric products. Similarly, the oxidation of (−)-epigallocatechin 3-*O*-gallate yields dimers produced by the oxidative condensation of two pyrogallol-B-rings as the major product.[19] When a mixture of (−)-epicatechin and (−)-epigallocatechin is oxidized, theaflavin is produced by the condensation of catechol- and pyrogallol-B-rings.[15] Along with theaflavin production, the oxidative condensation of two pyrogallol B-rings of epigallocatechin also occurred.[17] In these *in vitro* model fermentation experiments, only a limited amount of galloyl groups participated in the oxidation reactions[22] since the redox potential of the galloyl group is higher than that of other aromatic rings and its reactivity with *o*-quinones is comparatively low.[27] However, over half of the total tea catechins possess galloyl esters at the C-3 position, and the oxidation of galloyl esters may be involved in the production of minor black tea constituents and which, although chemically ambiguous, comprise the major portion of total polyphenols.[12] In the present *in vitro* experiment, condensation of the B-ring *o*-quinone with the galloyl group occurred preferentially. The mechanism postulated for the production of compounds **2**—**4** is shown in Chart 1. The results indicated that production of the benzotropolone moiety from the galloyl group extended the molecular size. Additionally, the results also revealed the occurrence of oxidative coupling of the resulting two benzotropolone moieties, which is similar to the dimerization reaction observed for theaflavins.[14] Furthermore, these reactions may participate in the formation of unknown black tea polyphenols with larger molecular size referred to as thearubigins.[29]

Experimental

General Infrared (IR) and ultraviolet (UV) spectra were obtained using JASCO FT/IR-410 and JASCO V-560 spectrophotometers, and optical rotations were measured using 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 using 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. HMQC, HMBC and NOESY experiments were performed using standard Varian pulse sequences. Fast atom bombardment (FAB) and high resolution FAB (HR-FAB) mass spectra (MS) were recorded on a JEOL JMS-700N spectrometer with glycerol or *m*-nitrobenzyl alcohol used as the matrix. Column chromatography was performed using Sephadex LH-20 (Pharmacia Fine Chemical Co.), MCI-gel CHP 20P (Mitsubishi Chemical Co.) and Chromatorex ODS (Fuji Silysia Chemical Ltd., Japan) columns. Thin-layer chromatography (TLC) was performed on 0.2-mm-thick precoated Kieselgel 60 F_{254} plates (Merck) using toluene–ethyl formate–formic acid (1 : 7 : 1, v/v) or cellulose F_{254} (Merck) uisng 2% AcOH. Spots were detected by UV illumination, sprayed with 2% methanolic $FeCl_3$ or 10% sulfuric acid reagent, and then heated. Analytical reverse-phase HPLC was performed on a Cosmosil 5C_{18}-AR II column (Nacalai Tesque Inc.; 4.6 mm i.d.×250 mm) using an elution gradient of 4—30% (39 min) and 30—75% (15 min) CH_3CN in 50 mM H_3PO_4 (flow rate 0.8 ml/min; detection uisng a JASCO photodiode array detector MD-910). Preparative HPLC was performed on a Cosmosil 5C_{18}-AR-II column (Nacalai Tesque Inc.; 10 mm i.d.×250 mm) using a linear elution gradient of 20—70% CH_3CN in 0.5% TFA. Japanese pear was purchased at a local market and epicatechin 3-*O*-gallate (**1**) was extracted and separated from commercial green tea and purified by crystallization from H_2O.[10]

Enzymatic Oxidation of 1 Japanese pear (500 g) was homogenized in H_2O (500 ml) and filtered through four layers of gauze. The homogenate (1000 ml) was mixed with an aqueous solution of **1** (5.0 g in 200 ml) and vigorously stirred for 6 h at room temperature. The reaction was monitored by reversed-phase HPLC. The mixture was poured into acetone (3 l), gently stirred for 30 min, and then insoluble material was removed by filtration. The filtrate was concentrated by evaporation until the acetone was completely removed. The resulting aqueous solution was subjected to Sephadex LH-20

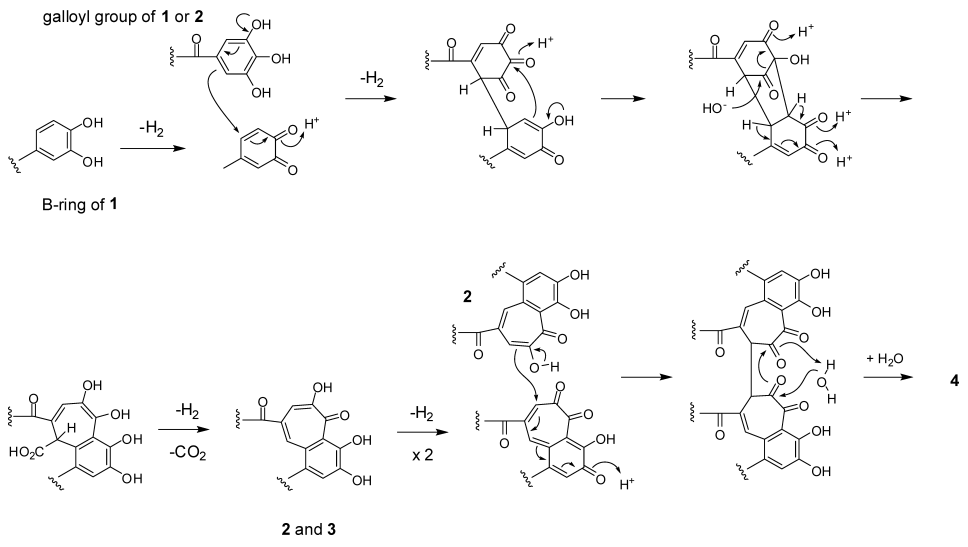

Chart 1. A Plausible Production Mechanism of **2**, **3** and **4**

column chromatography (4.5 cm i.d.×26 cm) using H_2O containing an increasing proportion of MeOH (10% stepwise elution from 0 to 100%, each 300 ml) to yield six fractions. The first fraction mainly contained sugars and was not examined further. Fraction 2 was applied to a MCI-gel CHP 20P column (3.0 cm i.d.×20 cm) and eluted with H_2O–MeOH (10% stepwise elution from 0 to 60%, each 100 ml) to give **1** (2.1 g). Fraction 3 (571 mg) was successively subjected to chromatography over Chromatorex ODS (1.5 cm i.d.×18 cm, 5% stepwise elution from 0 to 60% MeOH, each 100 ml), and Sephadex LH-20 (2.0 cm i.d.×32 cm, 10% stepwise elution from 30 to 100% MeOH, each 100 ml) to yield a crude sample of **4**. Purification of **4** was achieved by gel filtration chromatography over Sephadex LH-20 (1.0 cm i.d.×13 cm) using 8 M urea in 60% acetone[30] followed by removal of the urea using a MCI-gel CHP-20P column (1.0 cm i.d.×13 cm, 0 to 100% MeOH) to give **4** (22.9 mg). Fraction 4 was separated by Chromatorex ODS column chromatography (1.5 cm i.d.×18 cm, 5% stepwise elution from 0 to 60% MeOH, each 100 ml) to afford **2** (712 mg). Fraction 6 (310 mg) was subjected to Sephadex LH-20 column chromatography (2.0 cm i.d.×32 cm, 10% stepwise elution from 50 to 100% MeOH, each 100 ml) to yield **3** (99.3 mg). Fraction 5 (218.9 mg) contained **2** and **3**, and was not separated further.

Theaflavate C Red amorphous powder. $[\alpha]_D$ −358.8° (c=0.05, MeOH). IR ν_{max} cm^{-1}: 3381, 1704, 1607, 1520, 1471. UV λ_{max} (MeOH) nm (log ε): 279 (4.7) and 405 (4.2). HR-FAB-MS m/z: 1263.2267 $[M+H]^+$ (Calcd for $C_{64}H_{47}O_{28}$: 1263.2252). ^{1}H- and ^{13}C-NMR data: see Table 1. Assignments of the signals were achieved with the aid of ^{1}H–^{1}H COSY, HSQC, and HMBC spectra.

Bistheaflavate A Brown amorphous powder. $[\alpha]_D$ −419.2° (c=0.12, MeOH). IR ν_{max} cm^{-1}: 3406, 1699, 1631, 1610, 1519, 1470. UV λ_{max} (MeOH) nm (log ε): 276 (4.6) and 354 (4.1). HR-FAB-MS m/z: 1721.3114 $[M+H]^+$ (Calcd for $C_{86}H_{65}O_{39}$: 1721.3101). ^{1}H- and ^{13}C-NMR data: see Table 2. Assignments of the signals were achieved with the aid of ^{1}H–^{1}H COSY, HSQC, and HMBC spectra.

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