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Production of theasinensins A and D, epigallocatechin gallate dimers of black tea, by oxidation–reduction dismutation of dehydrotheasinensin A

Takashi Tanaka, Sayaka Watarumi, Yosuke Matsuo, Midori Kamei and Isao Kouno*

Department of Molecular Medicinal Sciences, Graduate School of Biomedical Sciences, Nagasaki University, Bunkyo Machi 1-14, Nagasaki 852-8521, Japan

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Abstract—Theasinensins A and D are B,B′-linked dimers of (−)-epigallocatechin 3-*O*-gallate connected through *R* and *S* biphenyl bonds, respectively, and are major constituents of black tea. Enzymatic oxidation of epigallocatechin 3-*O*-gallate produced dehydrotheasinensin A, and the structure was shown to be equivalent to an *o*-quinone of theasinensin A. When the aqueous solution of dehydrotheasinensin A was heated, theasinensin D was produced along with galloyl oolongtheanin. On the other hand, dehydrotheasinensin A was converted to theasinensins A and D along with oxidation products in phosphate buffer at pH 6.8 at room temperature. The results strongly suggested that theasinensins in black tea were produced by oxidation–reduction dismutation of dehydrotheasinensin.

1. Introduction

Tea is made from the young leaves of the tea plant, *Camellia sinensis*, which contains four major catechins, (−)-epigallocatechin, (−)-epicatechin and their 3-*O*-galloyl esters. In green tea manufacturing, enzymes catalyzing oxidation of polyphenols are inactivated by steaming or roasting immediately after crop; therefore, polyphenol composition of the products is similar to those of fresh leaves. In contrast, in black tea manufacturing, the catechins are oxidized by the enzymes during tea fermentation process prior to inactivation of the enzymes by drying with hot-air. Theaflavins[1,2] and theasinensins[3,4] are the major oxidation products found in black tea.[5] The former are reddish-orange pigments of black tea having a characteristic benzotropolone ring produced by oxidative condensation between (−)-epicatechins and (−)-epigallocatechins. The latter are colorless catechin dimers, in which two catechin B-rings are connected through C–C bond. Since (−)-epigallocatechin 3-*O*-gallate (**1**) is dominant among the tea catechins (45–65% of total tea catechins), theasinensins A (**5**) and D (**6**), isomeric dimers of **1** differing in configuration of their biphenyl bonds, are the most important black tea polyphenols.

Recently, we demonstrated that unstable theasinensin quinones (**3a**) or their equivalents are accumulated in tea leaves during tea fermentation and the quinones are converted to theasinensins when the leaves were heated.[6] In a continuation of that study, we have now succeeded in producing and isolating dehydrotheasinensin A (**3**), an entity of the theasinsisin precursor, by enzymatic oxidation of **1**. Dehydrotheasinensin A is equivalent to a hydrated *o*-quinone of the theasinensin A and is easily converted to **5** and **6** by oxidation–reduction dismutation. This paper describes the structure elucidation of **3** and mechanism of theasinensin production.

2. Results and discussion

Previously, we reported that various plant homogenates oxidize tea catechins to produce black tea polyphenols.[7] Among the plants tested, Japanese pear showed the highest activity and no interfering products originating from constituents of the fruit itself were observed. In the present experiment, (−)-epigallocatechin 3-*O*-gallate (**1**) was vigorously stirred with Japanese pear homogenate at room temperature for 2 h. HPLC analysis of the reaction mixture showed a large peak, the retention time of which coincided with that of a major theasinensin precursor produced in fermented tea leaves.[6] Since it was previously shown that theasinensin quinones are unstable and decompose when extracts are concentration by rotary evaporator,[6] the reaction mixture was directly applied to MCI gel CHP20P column chromatography without concentration. It was also

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* Corresponding author. Fax: +81-95-819-2432; e-mail: ikouno@net.nagasaki-u.ac.jp

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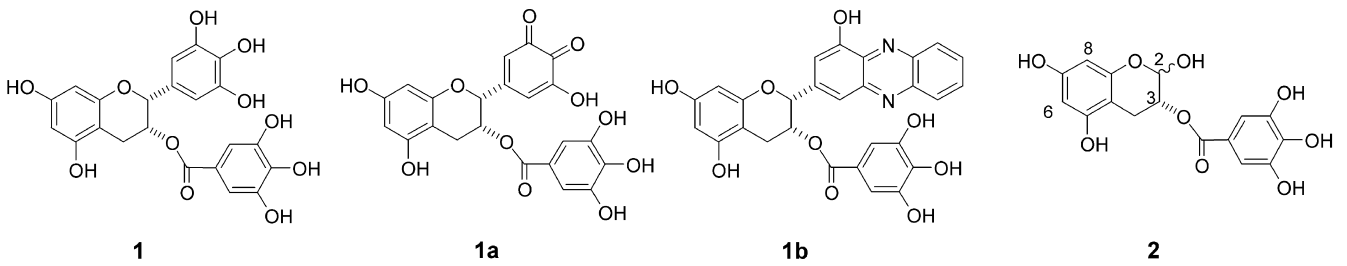
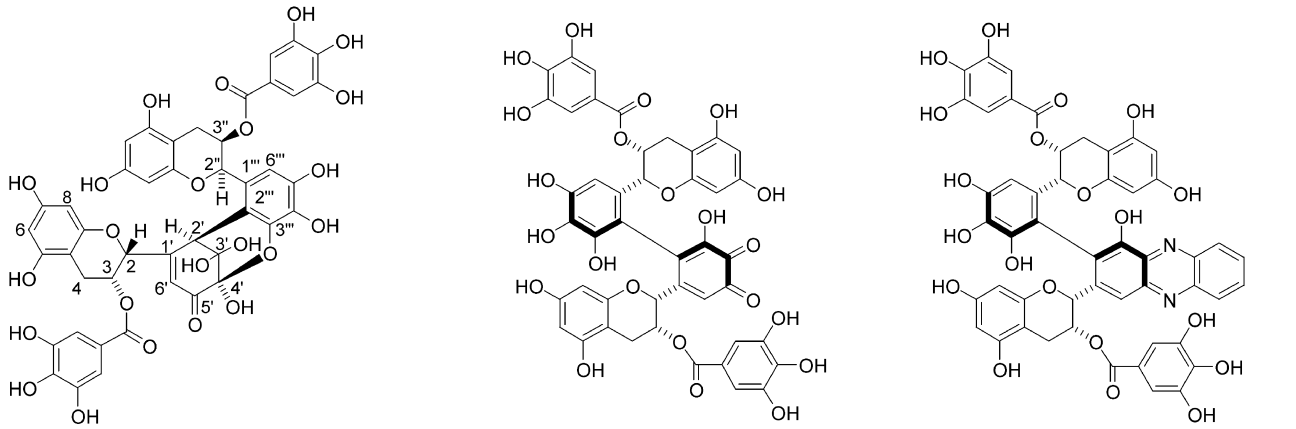


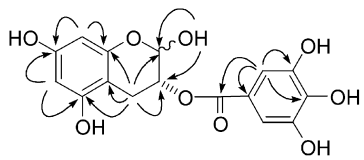
1 1a 1b 2



3 3a 3b

found that addition of trifluoroacetic acid (TFA) in an elution solvent of the column (0.1%) increased the stability of **3**. Elution with aqueous MeOH containing 0.05% TFA yielded two new oxidation products **2** (1.8%) and **3** (20.7%), along with a known dimer **4** (8.9%). The product **4** was identified based on the results of ^{1}H–^{1}H-COSY, HSQC, and HMBC experiments and ^{1}H and ^{13}C NMR spectroscopic comparison with previously described data;[8,9] however, its stereochemistry still remained to be clarified. This product was probably produced by double 1,2-addition between two molecules of **1a** as shown in Scheme 1 (route A).[9] Interestingly, treatment of **4** with *o*-phenylenediamine at 80°C afforded a phenazine derivative **1b** together with **1**. The production of **1** could be accounted for by oxidation–reduction dismutation of **1a**, which was regenerated from **4**.

The minor product **2** was obtained as a tan amorphous powder and showed a $[M+H]^{+}$ peak at *m/z* 351 in FABMS. This product was detected as a broadened peak on reversed-phase HPLC analysis and ^{1}H and ^{13}C NMR signals were observed in duplicate. These phenomena were caused by the equilibrium between epimers at an acetal carbon (δ 90.98, 91.12), which correlated with a hydroxyl proton (δ 6.316, 6.214, each d, OH) in the HMBC spectrum. The presence of a galloyl group (δ 7.030, 7.158, H-2,6) at C-3 position was obvious from the chemical shift of H-3 (δ 5.184 and 5.216).[3] The complete structure of **2** was determined by ^{1}H–^{1}H COSY, HSQC and HMBC spectral analyses (Fig. 1). Heating of an aqueous solution of **4** at 100°C yielded **2** along with **1**. Since **1a** was regenerated from **4** on heating as mentioned above, **2** could be a product of oxidation–reduction dismutation of **1a**. The product **2** was also isolated from commercial black tea, suggesting that similar reactions occur during black tea manufacturing.



2

Figure 1. HMBC correlations for **2**.

The product **3** was obtained as a pale yellow amorphous powder, $[\alpha]_{D}=-254.4°$ (in acetone), and showed dark blue coloration with ethanolic $FeCl_3$ reagent. The $[M+H]^{+}$ peak at *m/z* 931 in FABMS suggested that **3** is a dimer of **1**. The presence of the *ortho*-quinone structure or its equivalents was confirmed by condensation with *o*-phenylenediamine to yield a phenazine derivative **3b**, which was previously isolated from fermented tea leaves treated with *o*-phenylenediamine.[6] Compound **3** was observed as a broad peak on reversed-phase HPLC analysis probably because of the equilibrium between hydrated quinone structures. The ^{1}H and ^{13}C NMR spectra of **3** measured in d_6-acetone exhibited complex signals due to the equilibrium; however, well dissolved signals arising from a dominant form of the hydrated quinone (over 80%) were distinctively observed and used for spectral interpretation (Table 1). The ^{1}H and ^{13}C NMR signals assignable to catechin A- and C-rings and two galloyl ester groups were similar to those of **1**, indicating that these parts were not involved in the dimerization reaction. In the ^{13}C NMR spectrum, the remaining 12 signals were attributable to one ketone (C-5′), two acetal (C-3′,4′), one aliphatic methine (C-2′), two olefinic (C-1′,6′) and six aromatic (C-1‴-C-6‴) carbons, which constitute B-rings of the catechin units. In

Table 1. ^{13}C (125 MHz) and ^{1}H (500 MHz) NMR data for compounds **3** and **8** (in d_6-acetone)

Atom no.	^{13}C	^{1}H	HMBC (H to C)	^{13}C	^{1}H	HMBC (H to C)
2	76.59	5.034 (s)	3,1′,2′,6′	77.88	4.281 (br s)	3,4,3″,4″,5″
3	66.71	5.521 (br s)	Galloyl C-7 (to δ 165.51), 4a	65.48	5.806 (br s)	4a
4	26.78	2.892 (2H, m)	2,4a, 8a	26.61	3.004 (br d, 17.4)	2,3,4a,5,8a
					2.849 (dd, 4.4, 17.4)	4a,5,8a
4a	98.88			98.91		
5	157.72			157.43		
6	97.00	5.947 (d, 2.3)	4a,5,7,8	97.03	6.031 (d, 2.3)	4a,5,7,8
7	157.30			157.50		
8	95.49	6.030 (d, 2.3)	4a,6,7,8a	95.83	5.991 (d, 2.3)	4a,6,7,8a
8a	155.80			155.95		
1′	160.70			170.11		
2′	45.92	4.447 (s)	2,1′,3′,4′,5′(^4J),6′,1‴, 2‴, 3‴	76.81	5.515 (d, 6.2)	1″,3″,4″,6″,2‴
3′	91.91			49.29	4.698 (d, 6.2)	2,1″,2″,4″,1‴,2‴,3‴
4′	96.64			89.49		
5′	191.70			36.47	3.802 (d, 16.4)	2,3″,4″,6″
					3.237 (d, 16.4)	2,3″,4″,6″
6′	122.63	6.297 (s)	2,1′,2′,4′,2‴(^4J)	168.94		
2″	74.84	5.725 (s)		75.85	5.303 (br s)	3″,4″,1‴,2‴,6‴
3″	68.90	5.622 (br s)	Galloyl C-7 (to δ 166.13), 4a″	68.21	5.806 (br s)	4a″
4″	26.96	3.055 (br d, 17.3)	2″,3″,4a″,8a″	27.07	3.159 (dd, 4.4, 17.4)	4a″,5″,8a″
		3.196 (dd, 4.4, 17.3)	4a″		2.954 (br d, 17.4)	2″,3″,4a″,5″,8a″
4a″	98.78			99.26		
5″	157.60			157.55		
6″	96.44	6.003 (d, 2.3)	4a″,5″,7″,8″	96.57	6.043 (d, 2.3)	4a″,5″,7″,8″
7″	157.30			157.61		
8″	95.76	6.046 (d, 2.3)	4a″,7″,6″,8a″	95.65	5.950 (d, 2.3)	4a″,7″,6″,8a″
8a″	157.01			157.16		
1‴	126.86			128.42		
2‴	112.18			114.48		
3‴	142.52			147.93		
4‴	133.45			130.14		
5‴	146.08			148.13		
6‴	109.79	7.003 (s)	2″,1‴,2‴,3‴(^4J),4‴,5‴	109.81	6.828 (s)	3″(^4J),2″,1‴,2‴,3‴′(^4J),4‴,5‴
Galloyl 1	121.42, 121.72			121.47, 121.83		
Galloyl 2(6)	109.91, 110.00	6.965, 7.067 (each 2H, s)	Galloyl 1, 4, 5, 6, 7	109.94, 110.03	7.126, 7.063 (each 2H, s)	Galloyl 1, 4, 5, 6, 7
Galloyl 3(5)	145.87 (4C)			145.91, 146.13		
Galloyl 4	138.87, 138.79			138.79, 139.21		
Galloyl 7	165.51, 166.13			165.84, 166.09		

[a] Multiplicity and coupling constants (Hz) were shown in parentheses.
[b,c] Assignments may be interchanged.
[d] Galloyl ester attached to C-3.
[e] Galloyl ester attached to C-3.

the HMBC spectrum (Table 1), an aromatic methine singlet (H-6‴) was correlated with the C-ring C-2″ carbon and five aromatic carbons (C-1‴–C-5‴) including weak ^4J coupling with the carbons at *para*-position (C-3‴). Furthermore, the chemical shifts of these aromatic carbon signals were similar to those of **5**;[7] therefore, these signals indicated the presence of a pyrogallol ring connected to C-2″. This was supported by observation of ^{1}H–^{1}H long-range correlations between the H-6‴ and H-2″ in the ^{1}H–^{1}H COSY spectrum. The ^{1}H–^{1}H long-range couplings through sp^2 carbons were also observed between H-2″/H-2′, H-2/H-2′, and H-2/H-6′. In addition, HMBC correlations of H-2, H-2′ and H-6′ with remaining six B-ring carbons (C-1′–C-6′) and C-2 (Table 1) suggested that a cyclohexenone ring was attached to the C-2 position. Correlations of the H-2′ methine proton with the pyrogallol C-1‴, C-2‴, and C-3‴ carbons indicated a connection between C-2′ and pyrogallol C-2‴. The number of the ^{13}C NMR signals, FABMS and elemental analysis indicated that the molecular formula of **3** was $C_{44}H_{34}O_{23}$. The degree of unsaturation (28) calculated from the molecular composition suggested the presence of an additional ether ring between phenolic hydroxyl group and one of the acetal rings (C-3′ or C-4′). Similar hydrated quinone structures were found in a group of ellagitannins having dehydrohexahydroxydiphenoyl esters, which were isolated as major metabolites from some Geraniaceous and Euphorbiaceous plants (Fig. 2).[10,11] Comparison of ^{13}C NMR chemical shifts of the cyclohexenone and pyrogallol rings of **3** with those of two equilibrium forms of the dehydrohexahydroxydiphenoyl group strongly suggested that the position of the acetal ring in **3** was between the C-4′ acetal carbon and the hydroxyl group at C-3‴. Accordingly, the plane structure of **3** was determined as shown in the formula **3**. Assignments of the ^{1}H and ^{13}C NMR signals arising from the minor equilibrium form of **3** could not be completed because of overlapping of some signals and insufficient HMBC correlations; therefore, at present we do not have sufficient evidence for an equilibrium of **3** similar to the dehydrohexahydroxydiphenoyl groups. Absolute configuration of the C-2′ methine carbon was determined to be *S*, because reduction of **3** with ascorbic acid, cysteine methyl ester, dithiothreitol, and mercaptoacetic acid yielded theasinensin A (**5**), which has *R* biphenyl bond.[4] This was based on the fact that reduction of (*R*)-dehydrohexahydroxydiphenoyl esters yields *R*-hexahydroxydiphenoyl groups.[10,11] The results indicated that the production of **3** from **1** is highly stereoselective. A plausible mechanism of the production of **3** from **1a** is shown in Scheme 1 (route B): 1,4-addition of the relatively electron-rich C-2′ carbon of *ortho*-quinone **1a** to an unsaturated carbonyl group of another molecule of **1a** gave **3**. The direct formation of **3** from **5** was unlikely because enzymatic oxidation of **5** or **6** under similar condition did not afford **3**.

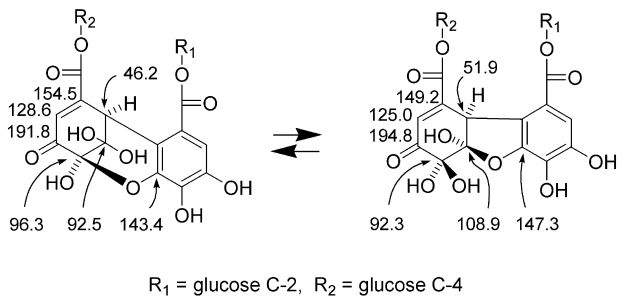
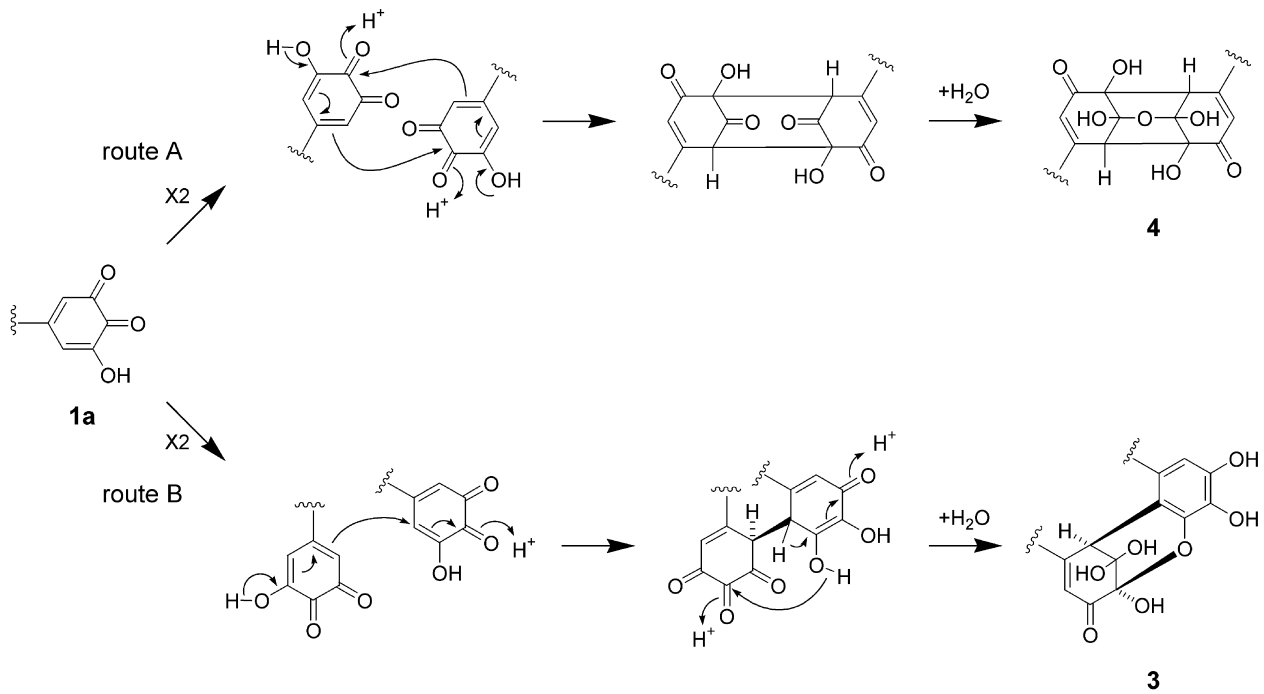
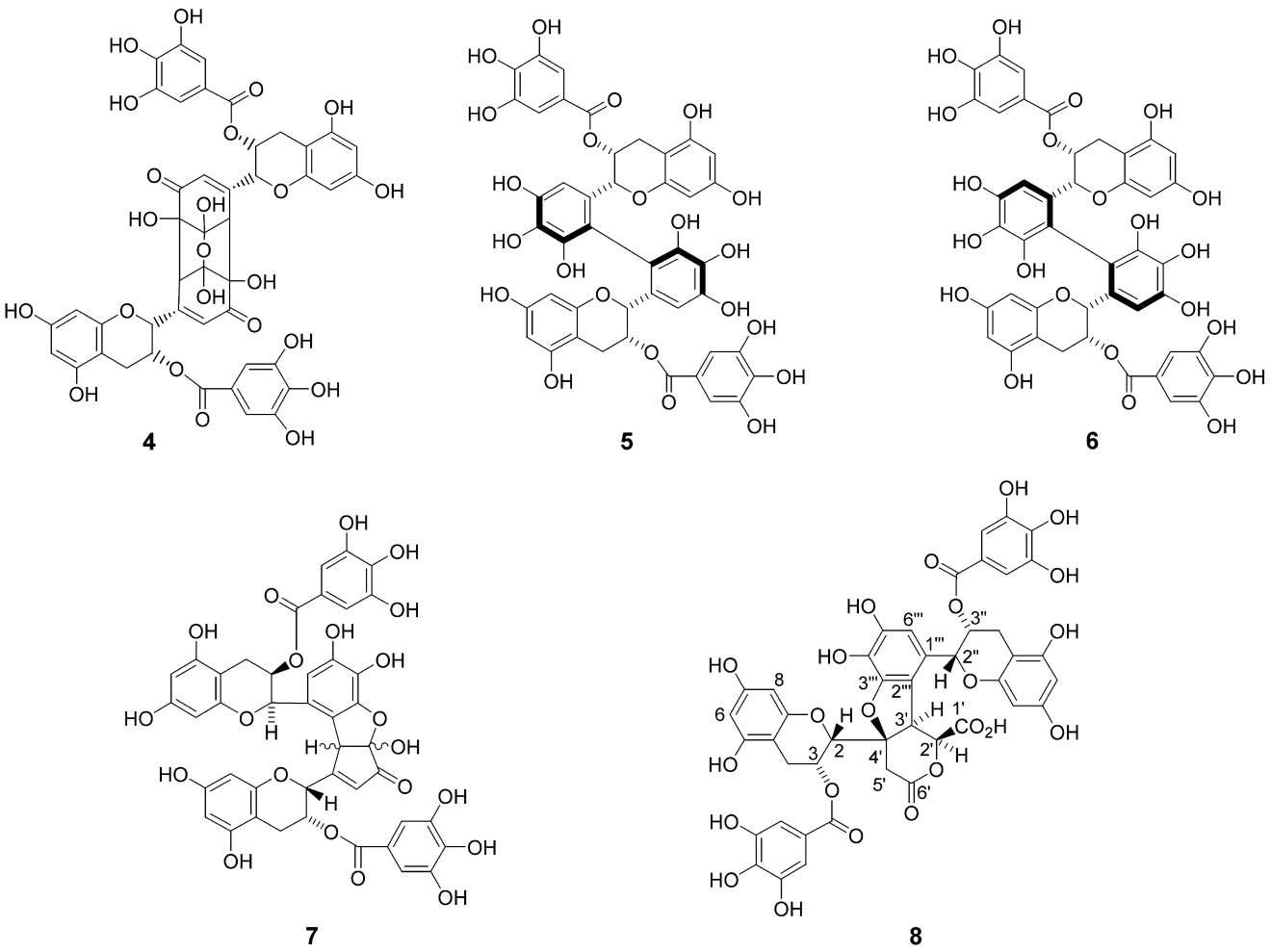


Figure 2. Structures and ^{13}C NMR chemical shifts of dehydrohexahydroxydiphenoyl esters.

As mentioned above, the reduction of **3** with ascorbic acid or thiol compounds yielded theasinensin A (**5**), which have a *R*-biphenyl bond. However, when the aqueous solution of **3** was heated at 80°C, oxidation–reduction dismutation occurred and theasinensin D (**6**) with *S*-biphenyl bond was produced along with galloyloolongtheanin (**7**).[3] In this

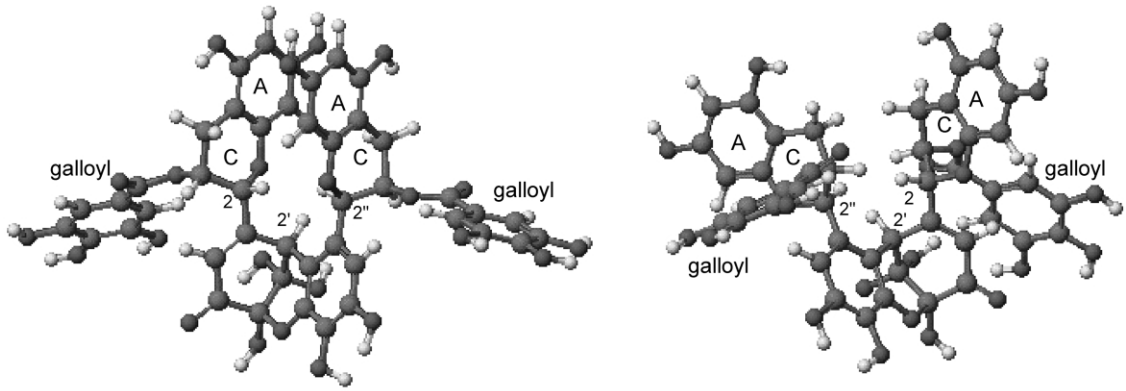


Scheme 1. Plausible mechanism for formation of **3** and **4** from **1a**.



reaction, **5** was not detected in the reaction mixture. Since racemization between **5** and **6** did not occur under similar conditions, the result suggested that the inversion occurred prior to the dismutation reaction. Calculation of the heat of formation of **3** (−785 Kcal/mol) and its C-2′ (*S*) isomer (**3c**) (−790 Kcal/mol) with the aid of CAChe® MOPAC suggested that the isomer **3c** is more stable than **3**, probably because of steric hindrance between two sets of flavan A, C-rings in **3** (Fig. 3). Therefore, it was deduced that, when an aqueous solution of **3** was heated, first **3** isomerized to **3c** and subsequently the resulting **3c** underwent oxidation–reduction dismutation. The stereostructure of **3** in Figure 3 suggested that the π–π stacking between less hindered sides of two flavan A rings and the hydrophobic interaction between two sets of the A and C rings contributed to the stereoselective coupling of two molecules of **1a** and the stability in aqueous solution at ambient temperature.

On the other hand, **3** was gradually decomposed in a phosphate buffer of pH 6.8 at 20°C to give a mixture of **5**, **6**, **7** along with an unknown product **8a**, which was detected on HPLC analysis. However, **8a** was unstable and converted to **8** during a separation procedure by column chromatography. The molecular weight of **8** was shown to be the same as that of **3** by FABMS [*m*/*z* 931 $(M+H)^+$]. The NMR signals arising from two sets of A- and C-rings and galloyl groups were also similar to those of **3**. In addition, the presence of a pyrogallol-type B-ring was apparent from the signals of C-1‴–C-6‴ (Table 1). Another B-ring was comprised of two carboxyl [δ 170.11 (C-1′), 168.94 (C-6′)], two methine [δ 76.81 (C-2′), 49.29 (C-3′)], one oxygenated quaternary [δ 89.49 (C-4′)] and one methylene [δ 36.47 (C-5′)] carbons. In the HMBC spectrum (Table 1), correlation of H-3′ with C-1‴, C-2‴, and C-3‴ of the pyrogallol ring indicated that C-3′ was attached to the pyrogallol C-2‴. Connection of C-4′ to the C-ring C-2 was evidenced by the HMBC correlation of H-2 with C-3′, C-4′ and C-5′. Location of the two carboxyl carbons C-1′ and C-6′ was apparent from their long-range coupling with H-2′ and H-5′, respectively. Furthermore, correlation between H-2′ and C-6′ indicated the formation of a δ-lactone ring between these positions. From the result of elemental analysis and the above spectral data, the molecular formula of **3** was deduced to be $C_{44}H_{34}O_{23}$, which implied the presence of another ring in the molecule. There are two possible ways to form an additional ring: one is an ether ring between C-3‴ and C-4′, and the other is a γ-lactone ring between C-1′ and C-4′. However, the latter was unlikely because the NOESY spectrum showed weak but distinctive NOE correlation between the H-2′ and one of the H-5′ (δ 3.802), together with strong NOEs between H-2′ and H-3′ (Fig. 4). These NOEs indicated that H-2′ is oriented to an axial position and the C-1′ carboxyl group is located at a equatorial position; therefore, formation of a γ-lactone ring with C-4′ is impossible and the structure was concluded to be as shown in the formula **8**. Absolute configuration of the



3 **3c**

Figure 3. Models of **3** and **3c** drawn with the aid of CAChe® MOPAC.

δ-lactone ring was tentatively assigned on the assumption that the configuration at C-3′ was retained during the reaction. Since **8** was produced via the unstable precursor **8a**, as mentioned above, characterization of the structure of **8a** will be important in understanding the mechanism of dismutation reaction of **3**. Recently, Yoshida et al. presented a structure of an oxidation product generated by autoxidation of **1** in a phosphate buffer solution at pH 7.[12] The plane structure of their oxidation product is the same as that of **8**, and these compounds may be identical.

We demonstrated that **3** was produced not only by enzymatic oxidation but also by autoxidation of **1** in a phosphate buffer (pH 7.4); therefore, mechanism of theasinensin production by autoxidation in neutral conditions might be the same as the mechanism for enzymatic oxidation. Because of the instability of **3** at pH 7 as described above, **3** was not accumulated in the reaction mixture and was only detected at an initial stage of the autoxidation. From these results, it was deduced that formation of **5** and **6** from **3** during black tea manufacturing was a non-enzymatic process, and that it occurred spontaneously when the leaves were heated and dried. Furthermore, production of **3** on oxidation of **1** with potassium ferricyanide was confirmed by HPLC analysis and subsequent treatment with *o*-phenylenediamine yielding **3b**.

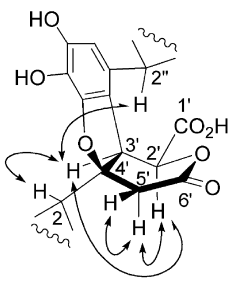
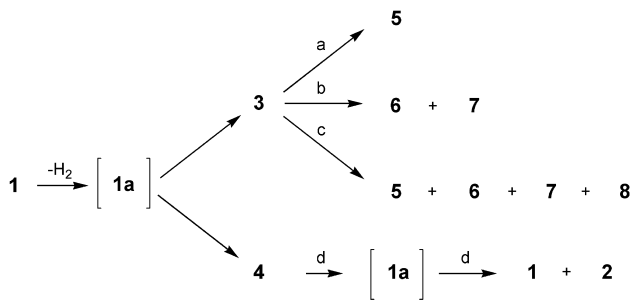


Figure 4. Important NOESY correlations for **8**.

3. Conclusion

Our results indicated that tea enzymes oxidized **1** to an *o*-quinone **1a**, which was subsequently dimerized by stereoselective 1,4- and 1,2-additions to give **3** and **4** (Scheme 2). Production of **4** from **1a** was a reversible reaction, and heating of the aqueous solution of **4** afforded **1a**, which subsequently underwent oxidation–reduction dismutation to give **1** along with oxidation products including **2**. Reduction of another product **3** with ascorbic acid or thiol compounds at ambient temperature yielded theasinensin A (**5**) having a *R* biphenyl bond. On the other hand, when **3** was heated, theasinensin D (**6**) having a *S* biphenyl bond was produced together with oxidation products including galloyl oolongtheanin (**7**). In a reaction at an elevated temperature, **3** probably isomerized to **3c**, which is thermodynamically more stable than **3**; and the resulting **3c** underwent dismutation reaction. Furthermore, dismutation of **3** occurred in an aqueous solution at pH 7 to give **5**, **6** and **7** together with an oxidation product **8**. In these dismutation reactions, the composition of the oxidation products seemed to be very complex compared to that of the reduction products and it was difficult to identify all of them. As shown in our earlier paper,[6] similar dismutation reactions occur in the process of heating and drying during black tea manufacturing, and most of the theasinensins are probably synthesized via **3** and its desgalloyl analogs. Other reductants, such as ascorbic acid, may also contribute to the theasinensin synthesis. Surprisingly, Roberts, who opened up the chemistry of black tea polyphenols, had already presumed at the end of the 1950s that a B,B′-ring linked dimer of epigallocatechin gallate, probably identical to **5** and/or **6**, might be formed by oxidation–reduction



Scheme 2. Oxidation of **1** and following reactions (a) reduction with ascorbic acid or thiol compounds; (b) dismutation in an aqueous solution at 80°C, 15 min; (c) dismutation in a phosphate buffer (pH 6.8) at room temperature, 15 h; (d) dismutation in an aqueous solution at 100°C, 15 min.

dismutation of the dimer quinone **3a**;[13,14] however, his greatly insightful proposal has long been ignored because no chemical evidence could be demonstrated. However, our result described in this report substantiates his assumption.

4. Experimental

IR and UV spectra were obtained with JASCO FT/IR-410 and JASCO V-560 spectrophotometers. Optical rotations were measured with a JASCO DIP-370 digital polarimeter. CD spectra were measured with a JASCO J-720w apparatus. ^{1}H and ^{13}C NMR, ^{1}H–^{1}H COSY, NOESY, HSQC and HMBC spectra were recorded with a Unity plus 500 spectrometer (Varian Inc, USA) operating at 500 MHz for ^{1}H, and 125 MHz for ^{13}C, respectively. FABMS were recorded on a JMS DX-303 spectrometer (JEOL Ltd., Japan), and *m*-nitrobenzyl alcohol or glycerol used as a matrix. Elemental analysis was obtained with a Perkin–Elmer 2400 II analyzer (Perkin–Elmer, Inc.). Column chromatography was done on MCI-gel CHP 20P (Mitsubishi Chemical Co.), Chromatorex ODS (Fuji Silysia Chemical Ltd., Japan), TSK gel Toyopearl HW-40F (TOSOH Co.) and Sephadex LH-20 (Pharmacia Fine Chemical Co.). Thin layer chromatography (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 $CHCl_3$–MeOH–H_2O (14:6:1, v/v) and spots were detected by UV illumination, sprayed with 2% ethanolic $FeCl_3$ or 10% sulfuric acid reagent, and followed by heating. Analytical high pressure liquid chromotography (HPLC) was performed on a Cosmosil 5C_{18}-AR II, 250×4.6 mm i.d. column (Nacalai Tesque Inc., Japan) with gradient elution from 10 to 30% (30 min) and 30–75% (15 min) of CH_3CN in 50 mM H_3PO_4 at a flow rate of 0.8 mL/min, and detected with a MD-910 photodiode array detector (JASCO Co., Japan)]. (−)-Epigallocatechin 3-*O*-gallate (**1**) were isolated from commercial green tea according to Nonaka et al. and recrystallized from H_2O.[3] Japanese pear fruits were purchased in a local market.

4.1. Oxidation of 1 with a Japanese pear homogenate

Japanese pear fruits (100 g) were homogenized with H_2O (100 mL) and filtered through four layers of gauze. The homogenate (120 mL) was added to a solution of **1** (0.5 g, 1.092 mmol) in H_2O (50 mL) and vigorously stirred at room temperature for 2 h. Vigorous stirring is necessary to supply sufficient oxygen molecules, which are indispensable to progress the reaction. The mixture was filtered through filter paper (No. 101) and the filtrate was directly applied to an MCI gel CHP20P column (2 cm×15 cm) without concentration. After washing the column with 0.1% TFA in H_2O, elution was undertaken with 0.1% TFA containing increasing proportions of MeOH (20–30%) to give **3** (103.5 mg, 0.11 mmol). The fractions containing **3** were concentrated by rotary evaporator below 35°C until MeOH was removed and then lyophilized. Elution of the column with 30–40% MeOH in 0.1% TFA afforded a fraction containing **4**, which was subjected to Sephadex LH-20 column chromatography with 80% MeOH to give **2** (7.0 mg, 0.02 mmol) and **4** (44.7 mg, 0.048 mmol).

4.1.1. Compound 2. A tan amorphous powder, $[\alpha]_D^{20}$=+0.5° (*c* 0.1, MeOH); IR (KBr) ν_{max} 3389, 1694, 1632 cm^{-1}; UV (EtOH) λ_{max} (ε 10600) 277 nm; ^{1}H NMR (500 MHz, d_6-acetone) δ 2.830 (1/3H, dd, *J*=10.4, 15.6 Hz, H-4), 2.838 (2/3H, dd, *J*=2.0, 17.2 Hz, H-4), 2.949 (2/3H, dd, *J*=4.8, 17.2 Hz, H-4), 3.006 (1/3H, dd, *J*=6.1, 15.6 Hz, H-4), 5.184 (1/3H, ddd, *J*=2.3, 6.1, 10.4 Hz, H-3), 5.216 (2/3H, ddd, *J*=2.0, 2.8, 4.8 Hz, H-3), 5.520 (2/3H, dd, *J*=2.8, 5.0 Hz, H-2), 5.585 (1/3H, dd, *J*=2.3, 5.3 Hz, H-2), 5.891 (1/3H, d, *J*=2.3 Hz, H-8), 5.899 (2/3H, *J*=2.3 Hz, H-8), 6.051 (1/3H, d, *J*=2.3 Hz, H-6), 6.056 (2/3H, d, *J*=2.3 Hz, H-6), 6.214 (1/3H, d, *J*=5.3 Hz, C-2–OH), 6.316 (2/3H, d, *J*=5.0 Hz, C-2–OH), 7.030 (4/3H, s, galloyl H-2,6), 7.158 (2/3H, s, galloyl H-2,6);^{13}C NMR (125 MHz, d_6-acetone) δ 21.12, 21.83 (C-4), 67.73, 69.45 (C-3), 90.98, 91.12 (C-2), 96.15, 96.25, 96.40, 96.60 (C-6,8), 99.06, 99.62 (C-4a), 109.84, 109.99 (galloyl C-2,6), 121.51 (galloyl C-1), 138.90 (galloyl C-4), 145.93, 145.98 (galloyl C-3,5), 153.72, 153.86 (C-8a), 156.74, 156.92, 157.66, 158.01 (C-5,7), 166.16, 166.27 (galloyl C-7); FABMS *m/z* 351 $[M+H]^+$. Anal. calcd for $C_{16}H_{14}O_9$ 5/2H_2O: C, 48.61; H, 4.84. Found: C, 48.54; H, 4.72.

4.1.2. Treatment of 4 with *o*-phenylenediamine. To a solution of **4** (10 mg) in EtOH (1.0 mL) was added *o*-phenylenediamine (5 mg) and AcOH (0.3 mL), and the mixture was heated at 80°C for 2.5 h. The mixture was applied to a Sephadex LH-20 column (1.0 cm×15 cm) with EtOH to give **1** (2.7 mg, 27.6%) and phenazine derivative **1b** (3.3 mg, 29.2%): a red amorphous powder, $[\alpha]_D^{20}$=−240.4° (*c* 0.2, MeOH); IR (KBr) ν_{max} 3325, 1692, 1608 cm^{-1}; UV (EtOH) λ_{max} 370 (ε 8920), 269 (61400) nm; ^{1}H NMR (500 MHz, d_6-acetone) δ 3.058 (1H, dd, *J*=1.7, 17.5 Hz, H-4), 3.218 (1H, dd, *J*=2.0, 17.5 Hz, H-4), 5.593 (1H, br s, H-2), 5.904 (1H, br t, *J*=1.3 Hz, H-3), 6.143, 6.206 (each 1H, d, *J*=2.3 Hz, H-6 and H-8), 7.008 (2H, s, galloyl-H-2,6), 7.527 (1H, d, *J*=1.6 Hz, H-2′), 7.913 (2H, m, H-10′, H-11′), 8.016 (1H, br s, H-6), 8.195 (2H, m, H-9′, H-12′); ^{13}C NMR (125 MHz, d_6-acetone) δ 26.63 (C-4), 68.88 (C-3), 78.04 (C-2), 95.84, 96.78 (C-6,8), 98.87 (C-4a), 109.26 (C-2′), 109.77 (galloyl C-2,6), 117.74 (C-6′), 121.29 (galloyl C-1), 130.01, 130.29, 131.29, 131.73 (C-9′,10′,11′,12′), 135.63 (C-4′), 138.87 (galloyl C-4), 142.15 (C-1′), 144.01, 144.39, 144.82 (C-5′,7′,8′), 145.88 (galloyl C-3,5), 153.36 (C-3′), 156.53, 157.54, 157.92 (C-5,7,8a), 165.90 (galloyl C-7); FABMS *m/z* 529 $[M+H]^+$. Anal. calcd for $C_{28}H_{20}O_9N_2$ 5/4H_2O: C, 61.04; H, 4.12; N, 5.08. Found: C, 61.07; H, 4.38; N, 4.69.

4.2. Decomposition of 4

A solution of **4** (11.5 mg) in H_2O (1.15 mL) was heated at 100°C for 15 min and the mixture was subjected to column chromatography over Sephadex LH-20 (1.0 cm×15 cm) with 80% MeOH to give **1** (5.3 mg) and **2** (0.6 mg).

4.3. Isolation of 2 from black tea

Commercial black tea (600 g), a blended tea produced in India and Sri Lanka, was extracted six times with boiling

water (4 L), and the extract concentrated to about 2 L. After decaffeination by partitioning with $CHCl_3$, the extract was successively partitioned with AcOEt and 1-BuOH to give AcOEt soluble (19.5 g) and 1-BuOH soluble (63.4 g) fractions. The 1-BuOH fraction was separated into 11 fractions by Sephadex LH-20 column chromatography (5.0 cm×35 cm) with H_2O containing increasing proportions of MeOH. The fraction 7, obtained by elution of 60–70%MeOH, was further separated by a combination of column chromatography of MCI-gel CHP20P (H_2O–MeOH), Chromatorex ODS (H_2O–MeOH), Sephadex LH-20 (EtOH) and TSK gel Toyopearl HW-40F (H_2O–MeOH) to yield **2** (75.2 mg).

4.4. Dehydrotheasinensin A (3)

Off-white amorphous powder, $[\alpha]_D^{20}=-254.4°$ (*c* 0.3, acetone); IR (KBr) ν_{max} 3365, 1696, 1610, 1518, 1456 cm^{-1}; UV (EtOH) λ_{max} 276 (ε 21450) nm; ^1H and ^{13}C NMR data, see Table 1 for major form; ^1H NMR (minor form, 500 MHz, d_6-acetone) δ 4.927 (s, H-2′), 5.229 (br s, H-2), 5.752 (br s, H-2″), 6.570 (s, H-6′), 7.123 (s, H-6‴); ^{13}C NMR (minor form, 125 MHz, d_6-acetone) δ 49.16 (C-2′), 74.50 (C-2″), 75.75 (C-2), 112.18 (C-6‴), 124.34 (C-6‴), 189.09 (C-5′); FABMS *m/z* 931 $[M+H]^+$, 913 $[M-H_2O+H]^+$, 760 [M−gallic acid]$^+$. Anal. calcd for $C_{44}H_{34}O_{23}$ $3H_2O$: C, 53.67; H, 4.09. Found: C, 53.76; H, 4.38.

4.5. Treatment of 3 with *o*-phenylenediamine

To a solution of **3** (10 mg) in EtOH (1.0 mL) was added *o*-phenylenediamine (5 mg) and AcOH (0.3 mL), and the mixture was stirred at room temperature for 15 min. The resulting mixture was subjected to Sephadex LH-20 column chromatography (1.0 cm×15 cm) with EtOH to give a phenazine derivative **3b** (10.7 mg, 97.2%). The product was identified by comparison of the ^1H and ^{13}C spectra and $[\alpha]_D$ value.

4.6. Reduction of 3 with ascorbic acid

To a solution of **3** (100 mg) in H_2O (10 mL) was added L-ascorbic acid (200 mg) and stirred for 12 h at room temperature. The mixture was applied to a column of MCI-gel CHP20P (2.0 cm×21 cm) with H_2O containing increasing proportions of MeOH to give **5** (46.7 mg).

4.7. Reduction of 3 with thiol compounds

Aqueous solutions of **3** (1 mg/1 mL) were separately treated with 10% aqueous solutions of cysteine methyl ester, dithiothreitol, and mercaptoacetic acid at room temperature for 30 min. Reversed phase HPLC analysis of each reaction mixture showed a peak corresponding to **5** (t_R=21.8 min), while peaks arising from **3** (t_R=21.4 min, broad) and **6** (t_R=22.9 min) were not observed.

4.8. Dismutation of 3 on heating

A solution of **3** (96.7 mg) in H_2O (80 mL) was heated at 80°C for 15 min. HPLC analysis of the mixture showed peaks corresponding to **6** (t_R=22.9 min) and **7** (t_R=29.7 min, broad), while peaks arising from **3** and **5** were not observed. Separation of the mixture by MCI-gel CHP20P column chromatography (2.0 cm×21 cm) with H_2O–MeOH afforded **6** (34.6 mg) and **7** (7.5 mg).

4.9. Dismutation of 3 in phosphate buffer

A solution of **3** (350 mg) in 6.5 mM phosphate buffer (pH 6.8, 150 mL) was stirred at room temperature for 15 h. HPLC analysis of the mixture showed a peak due to unknown product **8a** (t_R=20.0 min) as well as peaks corresponding to **5**, **6** and **7**. The reaction mixture was acidified by addition of TFA and subjected to MCI-gel CHP20P column chromatography (3.0 cm×21 cm) with H_2O–MeOH to give three fractions. The first fraction (120.7 mg) was repeatedly chromatographed over Chromatorex ODS (2.0 cm×25 cm) with H_2O–CH_3CN containing 0.1% TFA to give **5** (10.9 mg), **6** (19.7 mg) and **8** (17 mg). During the separation procedure, compound **8a** was completely converted to **8**. The second fraction (81.0 mg) was determined to be a mixture of **5** and **6**, and the third fraction (44.0 mg) was identified as **7** by ^1H NMR and HPLC analysis, respectively.

4.9.1. Compound 8. Off-white amorphous powder, $[\alpha]_D^{20}=-64.7°$ (*c* 0.2, acetone); IR (KBr) ν_{max} 3367, 1737, 1697, 1607 cm^{-1}; UV (EtOH) λ_{max} 277 (ε 25580) nm; ^1H and ^{13}C NMR data, see Table 1; NOESY, H-2/H-3, H-2/H-4 (δ 2.849), H-2/H-3′, H-2′/H-3′, H-2′/H-5′ (δ 3.802), H-2″/H-3′, H-2″/H-3″, H-2″/H-4″ (δ 3.159), H-2″/H-6‴, H-3″/H-6‴; FABMS *m/z* 931 $[M+H]^+$, 761 [M−gallic acid+H]$^+$. Anal. calcd for $C_{44}H_{34}O_{23}$ $7/2H_2O$: C, 53.18; H, 4.16. Found: C, 53.27; H, 4.44.

4.10. Production of 3 from 1 in phosphate buffer

A solution of **1** (1.0 g) in 20 mM phosphate buffer (pH 7.4, 250 mL) was stirred at room temperature for 30 min. To the mixture was added a solution of *o*-phenylenediamine (100 mg) in AcOH (25 mL) and stirred for 30 min. The mixture was applied to a column of MCI gel CHP20P (2.0 cm×16 cm) and washed with H_2O and then eluted with H_2O containing increasing proportions of MeOH to give **1** (980 mg), **5** (8.9 mg) and **3b** (8.8 mg).

4.11. Production of 3 from 1 by potassium ferricyanide

To a solution of **1** (400 mg) in H_2O (20 mL) an a aqueous solution (8 mL) containing $K_3Fe(CN)_6$ (1.2 g) and $NaHCO_3$ (320 mg) at 0°C was added dropwise with stirring. HPLC analysis of the reaction mixture showed a peak corresponding to **3**. After 15 min, the mixture was acidified by addition of HCl and applied to a column of MCI gel CHP20P (2.0 cm×10 cm), and reagents were washed out with H_2O. A solution of *o*-phenylenediamine (100 mg) in 20% AcOH–EtOH (2 mL) was added to the top of column gel and then the products were eluted out with EtOH. The fractions containing organic substances were concentrated and the residue was separated by MCI gel CHP20P column chromatography with H_2O–MeOH to give gallic acid (34.7 mg), **1** (14.8 mg), **1b** (24.8 mg) and **3b** (6.9 mg).

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