Available online at www.sciencedirect.com
ScienceDirect
Phytochemistry 69 (2008) 3054–3061
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Enzymatic oxidation of gallocatechin and epigallocatechin: Effects of C-ring configuration on the reaction products

Yosuke Matsuo, Yuko Yamada, Takashi Tanaka *, Isao Kouno

Graduate School of Biomedical Sciences, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki 852-8521, Japan

Received 5 April 2007; received in revised form 20 July 2007
Available online 20 September 2007

Dedicated to the memory of Professor Yoshihiro Matsumura

Abstract

Tea leaf is rich in pyrogallol-type catechins, and their oxidation is important in the generation of black tea polyphenols. In the present study, the enzymatic oxidation of three pyrogallol-type catechins, (+)- and (−)-gallocatechins and (−)-epigallocatechin, was compared. The reactions yielded unstable quinone products, which were trapped as condensation products with *o*-phenylenediamine. The oxidation of (+)-gallocatechin proceeded very slowly compared to the reaction of (−)-epigallocatechin, and yielded a proepitheaflagallin-type dimer as the major product, though oxidation of (−)-epigallocatechin gave predominantly dehydrotheasinensin C. The *cis*-configuration of the C-3 hydroxyl group and the B-ring of (−)-epigallocatechin was apparently crucial for rapid and selective production of dehydrotheasinensin C. Oxidation of (−)-gallocatechin proceeded in a manner similar to that of (+)-gallocatechin, and produced an enantiomer of the (+)-gallocatechin product. The results suggest that enzymes catalyze oxidation of the pyrogallol B-ring to the *o*-quinone, with subsequent non-enzymatic coupling reactions proceed under highly steric control.

Keywords: Black tea; *Camellia sinensis*; Theaceae; Epigallocatechin; Gallocatechin; Oxidation; Polyphenol

1. Introduction

Flavan-3-ols and proanthocyanidins are widely distributed in the plant kingdom (Porter, 1998), and are thought to be defense substances (Feucht and Treutter, 1999). These polyphenols are oxidized by coexisting enzymes when the plants are damaged by predators. This chemistry is also important in food production, especially in the manufacture of black tea. However, the chemical details of the reactions and structures of the reaction products are not yet clearly understood. (−)-Epigallocatechin (**1**) and its galloyl ester are the major polyphenols in fresh leaves of the tea tree *Camellia sinensis*, and account for 50–76% of the total tea catechins (Saijo and Takeda, 1999). In black tea production, tea catechins are enzymatically oxidized to produce oxidation products characteristic of black tea (Hashimoto et al., 1992; Haslam, 2003). There are two major oxidation routes of **1**. One is condensation with coexisting epicatechin and its gallate, yielding theaflavins, pigments with a benzotropolone moiety (Takino et al., 1964, 1965). The other is production of some epigallocatechin dimers, such as theasinensins and oolongtheanins (Nonaka et al., 1983; Hashimoto et al., 1988). Our previous work has indicated that a highly stereoselective reaction of epigallocatechin quinone (**1a**) produces unstable quinone metabolites, dehydrotheasinensin C (**2**) and proepitheaflagallin (**3**), which are subsequently degraded to give theasinensins A and C, oolongtheanin and epitheaflagallin (Tanaka et al., 2002c, 2003; Matsuo et al., 2006). The stereoselectivity of the oxidative dimerization is presumed to be controlled by the configuration at the C-2 carbon, and the geometrical configuration between the C-2 and C-3 of

* Corresponding author. Tel.: +81 95 819 2433; fax: +81 95 819 2477.
E-mail address: t-tanaka@nagasaki-u.ac.jp (T. Tanaka).

0031-9422/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2007.08.007

flavan-3-ols, because the same selectivity was also observed in a non-enzymatic oxidation with potassium hexacyanoferrate(III) as an oxidant (Tanaka et al., 2003). In the present study, we compared the oxidation of (+)- (**4**) and (−)-gallocatechins (**7**) with that of (−)-epigallocatechin (**1**), and describe the reason for the stereoselective formation of dehydrotheasinensins and proepitheaflagallin. In addition, this study may contribute to the understanding of the production of minor black tea polyphenols, because a small amount (<2% of total catechins) of **4** is contained in fresh tea leaves (Saijo and Takeda, 1999).

2. Results and discussion

In the course of our study on catechin oxidation, Japanese pear homogenate was used as an enzyme source because it showed strong activity and gave oxidation products similar to those obtained by the reaction with tea leaf homogenate (Tanaka et al., 2002a,b). Furthermore, on HPLC analysis, it did not show interfering peaks in the background arising from the homogenate itself. The initial oxidation products of **1** were found to be unstable quinones or their dimers, which were difficult to isolate in their original forms (Tanaka et al., 2002c, 2003; Matsuo et al., 2006). Therefore, in this experiment, an ethanolic solution of *o*-phenylenediamine was added to the reaction mixtures to trap the unstable quinones as phenazine or quinoxaline derivatives. The reaction products were analyzed by reversed-phase HPLC equipped with a photodiode array detector. When **1** was treated with the pear homogenate, it was rapidly converted into dehydrotheasinensin C (**2**), which was detected as the phenazine derivatives **2a** and **2b** (Tanaka et al., 2002c; Matsuo et al., 2006) (Fig. 1a). The phenazine derivative **1b** of monomeric epigallocatechin quinone (**1a**) was also observed (Matsuo et al., 2006). The

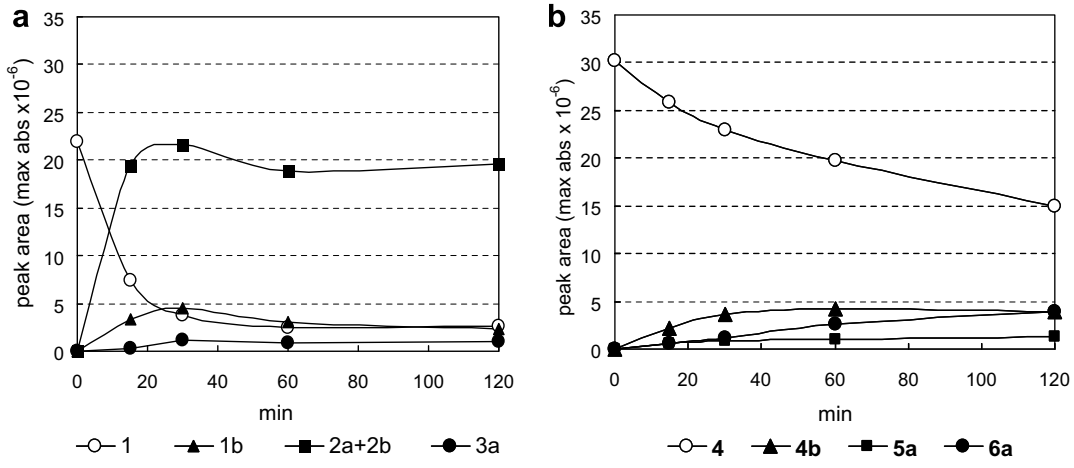
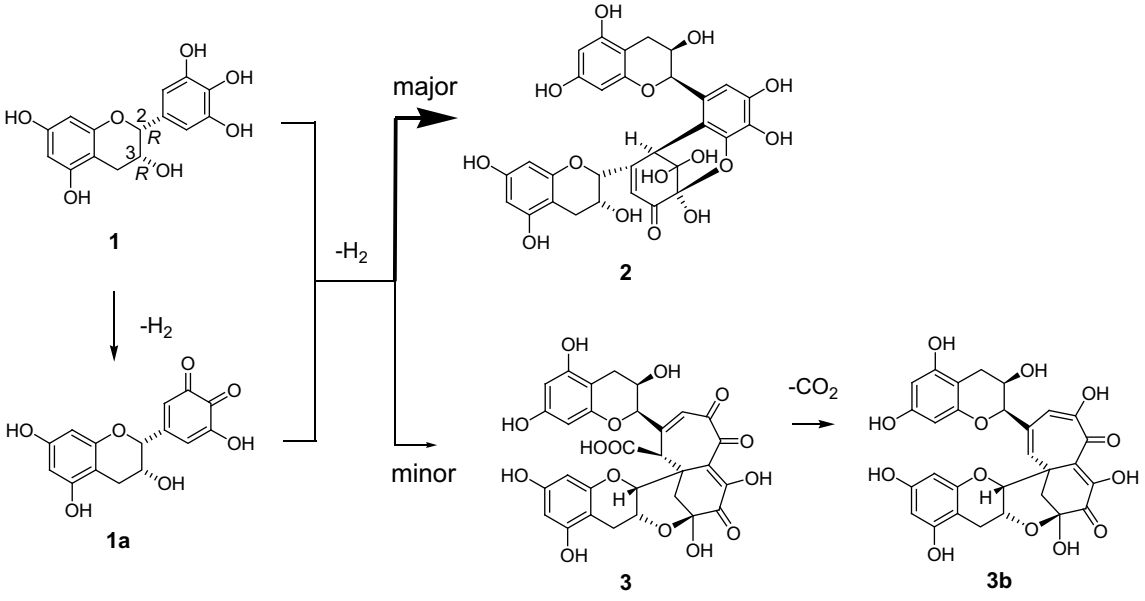
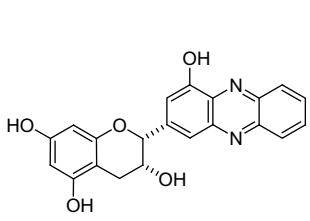


Fig. 1. Time course of oxidation of **1** (a) and **4** (b) and production of phenazine derivatives.

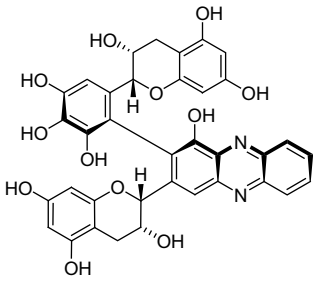


Scheme 1.

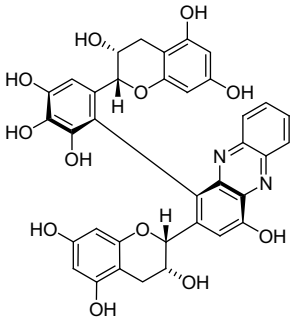
peaks of these products were identified by comparison of the retention time and UV absorption with those of authentic samples. Although dimer **2** was produced from **1** and **1a** (Scheme 1) (Li et al., 2007), the peak area of **1b** was much smaller than that of **2a** and **2b**, indicating that dimerization proceeded rapidly. Another small peak with UV absorption at 382 nm was identified as the bisquinoxaline derivative **3a**, based on the retention time and comparison of the UV spectrum (Matsuo et al., 2006). The derivative **3a** was originally obtained as the major product on treatment of proepitheaflagallin (**3**) with *o*-phenylenediamine. From these results, it was concluded that the oxidation of **1** predominantly yielded **2**, and **3** was a minor product (Scheme 1). The oxidation of **1** with potassium hexacyanoferrate(III), and subsequent treatment with *o*-phenylenediamine, also afforded **2a** and **2b** along with small amounts of **1a** and **3a**, indicating the stereoselectivity observed was non-enzymatic and controlled by configuration of the flavan-3-ol.



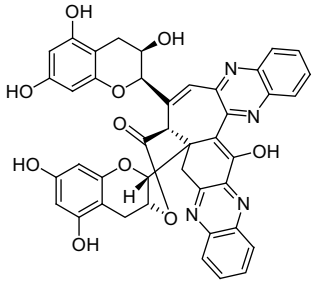
1b



2a



2b



3a

On the other hand, with oxidation of **4** under similar conditions, the decrease in **4** was significantly slower than that for **1**, and three derivatives **4b**, **5a** and **6a** were produced, which showed either UV absorption characteristic of phenazine (**4b**, 370 nm; **5a**, 374 nm) or quinoxaline (**6a**, 320 nm) derivatives (Fig. 1b). The three products were isolated in a preparative scale experiment. Product **4b** was readily identified as the phenazine derivative of the monomeric gallocatechin quinone (**4a**), by comparison of the spectroscopic data with that of **1b**. Except for the large coupling constant between H-2 and H-3 ($J_{2,3}$, 7.3 Hz), the ^{1}H NMR spectrum of **4b** was closely related to that of **1b**. The product **5a** exhibited an $[M+H]^+$ peak at *m/z* 681 in the FAB-MS spectrum, which coincided with that of **2a**. The ^{1}H NMR spectrum showed signals arising from two sets of flavan A C-rings along with aromatic resonances attributable to a tri-substituted phenazine ring (δ 8.12–8.20, 2H, m; 7.87–7.89, 2H, m; 7.85, 1H, s) and a penta-substituted pyrogallol ring (δ 6.69). These signals were also related to those of **2a**, except for the large $J_{2,3}$ values (7.8 and 5.9 Hz) of the C-rings. The ^{13}C NMR spectrum also resembled that of **2a**, with the difference in chemical shift of C-2 and C-3 indicating that the product **5a** was a 2,3-*trans* isomer of **2a**. The atropisomerism of the biphenyl bond was deduced to be in the *R* configuration by comparison of the CD spectrum with that of **2a**: both compounds showed positive Cotton effects at

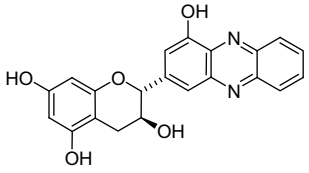
Table 1
^{1}H and ^{13}C NMR spectroscopic data for bisquinoxaline derivative **6a**[a]

Position	δ_C	δ_H	HMBC (H–C)
2	87.6	4.21 (*d*, 8.5)	3, 4, c, d, e
3	64.4	4.09 (*ddd*, 6.0, 8.5, 9.6)	2, d
4	29.0	3.01 (*dd*, 6.0, 15.6)	2, 3, 4a, 5, 8a
		2.41 (*dd*, 9.6, 15.8)	2, 3, 4a, 5, 8a
4a	100.5		
5	157.0[b]		
6	95.7[c]	5.97[b] (*d*, 2.3)	4a, 5, 7, 8
7	157.2[b]		
8	96.3[c]	5.98[b] (*d*, 2.3)	4a, 6, 7, 8a
8a	156.2[b]		
2′	81.3	4.27 (*d*, 9.8)	3′, 4′, e, f,j, k
3′	68.6	3.51 (*ddd*, 6.0, 9.8, 10.3)	2′, 4′, k
4′	28.1	2.83 (*dd*, 6.0, 15.3)	2′, 3′, 4a′, 5′, 8a′
		2.58 (*dd*, 10.3, 15.3)	2′, 3′, 4a′, 5′, 8a′
4a′	99.9		
5′	157.8[b]		
6′	95.7[c]	6.00[b] (*d*, 2.3)	4a′, 5′, 7′, 8′
7′	157.8[b]		
8′	96.8[c]	6.06[b] (*d*, 2.3)	4a′, 6′, 7′, 8a′
8a′	156.2[b]		
a	158.0		
b	153.4[d]		
c	34.5	4.63 (*dd*, 2.6, 15.6)	a, d, e
		3.70 (*d*, 15.6)	2, a, b, d, e
d	131.6		
e	132.6	6.19 (*d*, 2.5)	2, 2′, c, d, j, k
f	34.7	3.65 (*d*, 19.2)	2′, g, h, j, k
		2.85 (*d*, 19.2)	2′, e, g, h, k
g	153.0[d]		
h	152.8[d]		
i	95.3		
j	50.9	4.83 (*s*)	
k	43.5		
Quinoxaline	130.2, 130.1	6.87 (br *d*, 8.4)	[e]
CH	129.6, 129.5	7.40, 7.59 (each br *t*, 8.4)	[e]
	129.4 (3C)	7.84 (3H, *m*)	[e]
	129.1	7.97, 8.21 (br *d*, 8.0)	[e]
Quinoxaline	143.4, 141.8		
CN	141.4, 140.9		

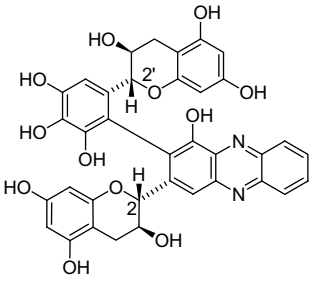
[a] ^{1}H NMR (500 MHz), ^{13}C NMR 125 MHz, measured in acetone-d_6.
[b–d] Assignments may be interchanged in each column.
[e] Complex cross-peaks in quinoxaline moieties were observed.

275 nm and a negative one at 252 nm. This result indicated that the atropisomerism reflected the configuration at the C-ring C-2 position, because **1** and **4** had the same *R* configuration at that position. The ^{1}H and ^{13}C NMR spectra of product **6a** showed signals arising from two sets of 2,3-*trans*-flavan A C-rings, along with aromatic resonances due to two quinoxaline moieties (Table 1). The remaining signals were assigned to be two aliphatic methylene (C-c and C-f), an aliphatic methine (C-j), an aliphatic quaternary (C-k), an acetal (C-i), a tri-substituted double bond (C-d and C-e), and four aromatic carbons connected to nitrogen atoms (C-a, C-b, C-g and C-h). The appearance of mutual ^{1}H–^{1}H long-range couplings between the H-e, H-2 and H-c in the ^{1}H-^{1}H COSY spectrum suggested an allylic relationship between these protons. This was supported by the HMBC correlations between these positions (Table 1). The H-e was also correlated with C-2′, and the H-2′ was correlated, in turn, with the C-e, C-f, C-j and C-k. Among the four nitrogen-bearing carbons, C-a and C-b were correlated to H-c, and C-g and C-h were coupled with H-f. Furthermore, correlation of H-j with C-2′, C-a, C-b, C-e, C-f, C-k, C-h, and C-i allowed us to construct the structure of the B-rings. The molecular formula of **6a** was deduced to be $C_{41}H_{32}N_4O_9$, based on the ^{13}C NMR signals and FAB-MS (*m*/*z*: 725, $[M+H]^+$) data, and finally confirmed by HR FAB-MS. The unsaturation index 28 and up- and low-field shifts of the C-2′ and C-3′, respectively, compared to the C-2 and C-3, suggested the formation of a hemiacetal ring at C-i with a C-3′ hydroxyl group. As for the stereochemistry, the strong NOESY correlations between H-2′ and H-j indicated that these protons are located on the same side of the molecule. In addition, since the configuration at C-2′ was *R*, the absolute configuration of **6a** was determined as shown in Fig. 2. Production of this derivative **6a** clearly indicated that compound **6** was produced as the major product of oxidation of **4** (Scheme 2). The quinone dimer **6** is an isomer of decarboxyl proepitheaflagallin (**3b**) (Matsuo et al., 2006), which has been shown to be a precursor of epitheaflagallin (Nonaka et al., 1986).

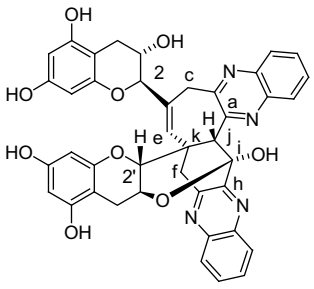
Next, we examined the oxidation of (−)-gallocatechin (**7**), which is an enantiomer of **4**. After treatment of the reaction mixture with *o*-phenylenediamine, HPLC analysis indicated that the reaction proceeded in a manner similar to that of **4**, and the retention time and UV absorption of the peaks of three major products coincided with those of **4b**, **5a** and **6a**. Although purification of the products corresponding to **5a** failed, **7a** and **8** were isolated and their ^{1}H NMR spectra were found to be identical to those of **4b** and **6a**, respectively. However, the optical rotations had the opposite sign. The result indicated that the oxidation reaction did not discriminate between the enantiomers **4** and **7**.



4b



5a



6a

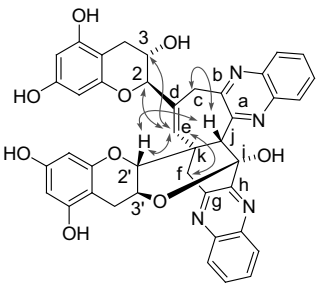
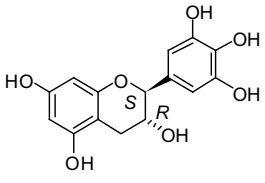
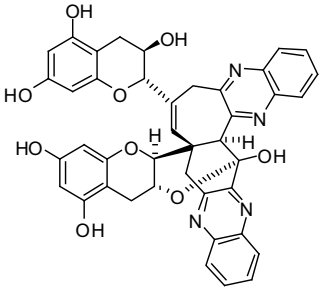


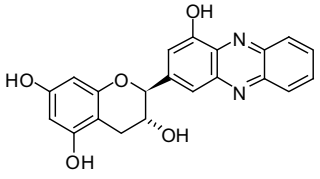
Fig. 2. Selected NOESY correlations for **6a**.



7

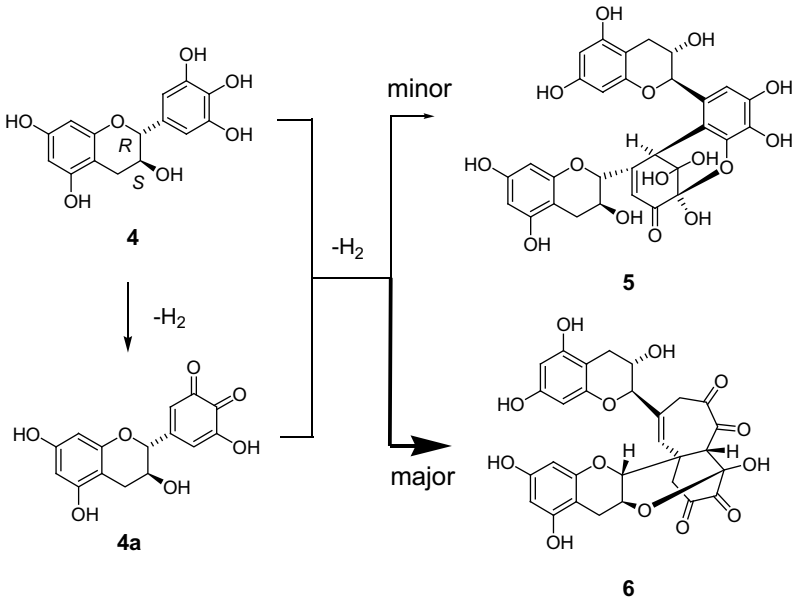


8



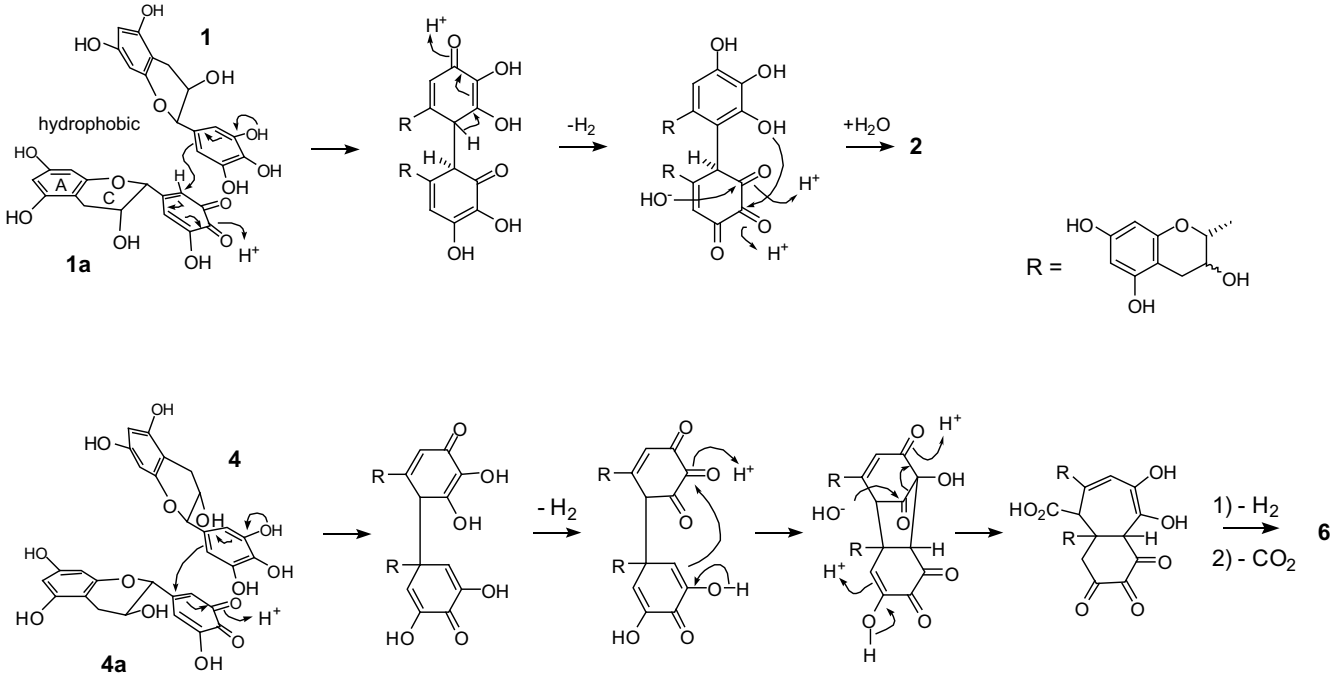
7a

The difference in the oxidation of **1** and **4** was very interesting because it implies that the reactions have a difference in



Scheme 2.

enzyme specificity and stereochemical features. As for enzyme specificity, it was obvious that enzymes preferentially oxidized **1** rather than **4**. Since the enzyme did not distinguish between **4** and **7**, it only oxidized the B-ring to *o*-quinone, and the subsequent dimerization reaction was probably non-enzymatic. Stereoselectivity in the production of **2**, which has previously been demonstrated (Tanaka et al., 2002c, 2003), was further confirmed in this experiment. The rapid and highly selective coupling between **1** and **1a** may be explained by their hydrophobic association at their sterically unhindered faces, which were on opposite sides of the C-3 hydroxyl group and B-rings (Scheme 3). In contrast, the C-3 hydroxyl group of **4** and **4a** probably hindered the intermolecular association; therefore dimerization proceeded slowly, despite the presence of monomeric quinone **4a** in the reaction mixture, which is shown as **4b** in Fig. 1. The difference in the position at which the coupling reaction occurred (between B-ring C-2 of **1a** and C-2′ of **1** for production of **2**; and between B-ring C-1 of **4a** and C-2′ of **4** for production of **6**) may also have been caused by the difference in the manner of molecular association. In order to evaluate the difference in hydrophobic



Scheme 3.

Table 2
$\Delta\delta$ ($\delta_{10\ mg/ml} - \delta_{0.5\ ros/ml}$) of **1** and **2** (500 MHz, 2% DMSO-d_6-D_2O)

	1	2
H-2	−0.128	−0.105
H-3	−0.047	−0.086
H-4	−0.026	−0.013
	−0.058	−0.052
H-6	−0.021	−0.002
H-8	−0.023	−0.003
B-ring H	−0.029	−0.003

self-association, the ^{1}H NMR chemical shift change ($\Delta\delta = \delta_{10\ mg/ml} - \delta_{0.5\ mg/ml}$) of **1** and **4** in deuterium oxide, containing 2% DMSO-d_6 at different concentrations, was compared (Table 2). Both **1** and **4** showed a large up-field shift of H-2 at the higher concentration, which was caused by the anisotropic effect of the aromatic rings of the other associating molecule. The $\Delta\delta$ value of the H-3 of **1** was smaller than that of **4** because it was located at the equatorial position. However, the remaining proton signals of **1** showed larger $\Delta\delta$ values compared to those of **4**, suggesting that the self-association of **1** in aqueous solution was stronger than that of **4**, and the association mainly occurred over the C-2 position.

Oxidation of catechins is a very important process during black tea production, and it also occurs in other plants when the tissues are injured. However, the chemical details are still ambiguous. In this study, we demonstrated some aspects of the stereochemical nature of the enzymatic oxidation of epigallocatechins. The results showed that the reaction strongly depended on the geometric configurations of the C-ring and the stereochemical restriction in the quinone–phenol association accounts for the high stereoselectivity in dimerization of epigallocatechin (**1**) and its gallate producing the major black tea polyphenol theasinensins (Tanaka et al., 2002c, 2003; Matsuo et al., 2006). Further studies on the oxidation mechanism of tea catechins are now in progress.

3. Experimental

3.1. General

Optical rotations were measured with a JASCO DIP-370 digital polarimeter. CD spectra were measured with a JASCO J-720w apparatus. The ^{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, Palo Alto, CA, USA) operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C NMR spectroscopy. The ^{1}H and ^{13}C NMR spectra were also measured using a JEOL JMN-AL400 (JEOL, Japan) operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C NMR. FAB and EIMS were recorded on a JMS DX-303 spectrometer (JEOL), and *m*-nitrobenzyl alcohol or glycerol was used as the matrix for FAB-MS. Elemental analysis was conducted with a Perkin–Elmer 2400 II analyzer (Perkin–Elmer, Waltham, MA, USA). CC was conducted on MCI-gel CHP 20P (Mitsubishi Chemical), Chromatorex ODS (Fuji Silysia Chemical, Japan), and Sephadex LH-20 (Pharmacia Fine Chemical), respectively. TLC was performed on 0.2 mm thick precoated Kieselgel 60 F_{254} plates (Merck) with benzene/HCO_2Et/HCO_2H (1:7:1, v/v) or $CHCl_3$/MeOH/H_2O (14:6:1, v/v), with spots detected by UV illumination, sprayed with 2% ethanolic $FeCl_3$ or 10% H_2SO_4 reagent, and heated. Analytical HPLC was performed on a Cosmosil 5C_{18}-AR II column (Nacalai Tesque; 250 × 4.6 mm i.d.), with gradient elution at 10–30% (30 min) and 30–75% (15 min) of CH_3CN in 50 mM H_3PO_4 (flow rate, 0.8 ml/min; detection, JASCO photodiode array detector MD-910). Preparative HPLC was performed on a COSMOSIL 5C_{18}-AR-II column (Nacalai Tesque; 10 mm i.d. × 250 mm) with 20–70% CH_3CN in 0.5% TFA (linear gradient elution). Epigallocatechin was isolated from commercial green tea and recrystallized from H_2O.

3.2. Oxidation of *1* and *4* and HPLC analysis

Japanese pear fruits (50 g) were homogenized with H_2O (50 ml) and filtered through four layers of gauze. The filtrate was then separately mixed with aqueous solutions of **1** and **4** (10 mg/2.5 ml) and vigorously stirred. Aliquots (0.1 ml) of the reaction mixture were taken at 10, 15, 30, 60 and 120 min, and mixed with 0.4 ml ethanolic *o*-phenylenediamine solution (0.5 mg/ml in 5% AcOH/EtOH). The mixtures were analyzed by HPLC after filtration by membrane filters (0.45 μm), with peak areas at maximum absorbance of each peak obtained by the JASCO-BORWIN chromatography software.

3.2.1. Non-enzymatic oxidation of *1*

An aqueous solution (0.4 ml) containing potassium hexacyanoferrate(III) (60 mg) and $NaHCO_3$ (16 mg) as added dropwise at 0 °C to an aqueous solution of **1** (10 mg/1 ml) with stirring for 10 min. The reaction was stopped by addition of 0.1 M HCl (0.6 ml). The mixture was passed through a Sep-Pak C_{18} cartridge with H_2O, and the phenolic compounds were eluted out with MeOH (2 ml). To the MeOH solution, *o*-phenylenediamine (2 mg) and AcOH (0.1 ml) were added, and the mixture was stirred for 1.5 h at 35 °C. The HPLC analysis of the mixture showed the peaks of **1** (t_R 17.51 min), **2b** (28.02 min), **2a** (30.51 min), **1a** (46.63 min), and **3a** (49.61 min).

3.3. Oxidation of *4* and isolation of phenazine derivatives

An aqueous solution of **4** (0.5 g/75 ml) was vigorously stirred with a Japanese pear homogenate (125 ml) for 180 min. To the mixture, a solution of *o*-phenylenediamine (250 mg) in 5% AcOH/EtOH (800 ml) was added and gently stirred for 1 h. After filtration, the filtrate was concentrated and applied to a column of MCI-gel CHP20P (3.0 cm i.d. × 25 cm), with 10% step-wise elution of H_2O containing increasing proportions of MeOH (0–100%, each 200 ml). Elution of the column with 10–30% MeOH in H_2O

(v/v) gave a fraction containing **4**, which was further purified by Sephadex LH-20 CC (2.0 cm i.d. × 20 cm) with MeOH–H_2O (4:6, v/v) to afford a recovery of **4** (171.5 mg). Further elution of the initial column with MeOH–H_2O (4:6–9:1) yielded three fractions containing phenazine derivatives: Fractions 1 (51.8 mg), 2 (42.7 mg) and 3 (44.9 mg), respectively. Fractions 1 and 2 were separately purified by Sephadex LH-20 CC (2.0 cm i.d. × 20 cm) eluted with MeOH–H_2O (4:1, v/v) to give **5a** (12.7 mg) from fraction 1, and **4b** (23.4 mg) from fraction 2. Fraction 3 was subjected to Sephadex LH-20 CC eluted with EtOH to yield **6a** (33.7 mg).

3.3.1. Phenazine derivative *4b*

Brown amorphous powder, $[\alpha]_D^{25}$ −27.3 ($c = 0.01$, MeOH); FAB-MS *m/z*: 377 $[M+H]^+$; IR ν_{max} cm^{-1}: 3288, 2921, 1631, 1607, 1566, 1519, 1470, 1468; UV (MeOH) λ_{max} nm (log ε): 269 (4.69), 370 (3.83); ^1H NMR (400 MHz, acetone-d_6) δ: 8.26–8.18 (2H, *m*, H-9′, 12′), 7.95–7.88 (2H, *m*, H-10′, 11′), 7.79 (1H, br *s*, H-6′), 7.32 (1H, *d*, $J = 1.5$, H-2′), 6.06, 5.98 (each 1H, *d*, $J = 2.0$, H-6, 8), 4.96 (1H, *d*, $J = 7.3$, H-2), 4.19 (1H, *m*, H-3), 2.94 (1H, *dd*, $J = 5.8$, 16.1, H-4), 2.63 (1H, *dd*, $J = 8.8$, 16.1, H-4); ^{13}C NMR (100 MHz, acetone-d_6) δ: 157.8, 157.2, 156.2 (C-5, 7, 8a), 153.4 (C-3′), 144.8, 144.5, 144.3, 142.1 (C-4′, 5′, 7′, 8′), 136.0 (C-1′), 131.7, 131.2, 130.2, 130.1 (C-9′, 10′, 11′, 12′), 118.8 (C-6′), 109.7 (C-2′), 100.3 (C-4a), 96.4, 95.2 (C-6, 8), 82.5 (C-2), 68.0 (C-3), 28.7 (C-4); HR FAB-MS 377.1152, $C_{21}H_{17}N_2O_5$ requires 377.1137.

3.3.2. Phenazine derivative *5a*

Brown amorphous powder; $[\alpha]_D^{25}$ +71.5 ($c = 0.05$, MeOH); FAB-MS *m/z*: 681 $[M+H]^+$; IR ν_{max} cm^{-1}: 3366, 2924, 2853, 1628, 1609, 1517, 1467; UV (MeOH) λ_{max} nm (log ε): 275(4.66), 374(3.75); CD (3.12×10^{-5} mol/l in MeOH) Δε (nm): +4.7 (275), −0.9 (252), +0.3 (238); ^1H NMR (400 MHz, acetone-d_6) δ: 8.20–8.12 (2H, *m*, H-9″, 12″), 7.89–7.87 (2H, *m*, H-10″, 11″), 7.85 (1H, *s*, H-6″), 6.69 (1H, *s*, H-6‴), 6.03, 6.02, 5.92, 5.89 (each 1H, *d*, $J = 2.4$, H-6, 6′, 8, 8′), 5.18 (1H, *d*, $J = 4.9$, H-2), 4.50 (1H, *d*, $J = 6.8$, H-2′), 4.29 (1H, *m*, H-3), 4.00 (1H, *m*, H-3′), 2.73 (1H, *dd*, $J = 5.4$, 16.1, H-4 or 4′), 2.62 (1H, *dd*, $J = 4.9$, 16.1, H-4 or 4′), 2.54 (1H, *dd*, $J = 5.9$, 16.1, H-4 or 4′), 2.22 (1H, *dd*, $J = 7.8$, 16.1, H-4 or 4′); ^{13}C NMR (100 MHz, acetone-d_6) δ: 157.6, 157.4, 157.3, 157.0, 156.7, 156.4 (C- 5, 7, 8, 5′, 7′, 8a′), 151.5 (C-3″), 146.8, 145.7, 144.7, 144.2, 144.1, 142.2 (C-1″, 5″, 7″, 8″, 3‴, 5‴), 135.4, 134.4 (C-4″, 4‴), 131.6, 131.2, 130.17, 130.11, 129.5 (C-9″, 10″, 11″, 12″, 1‴), 118.4, 117.8 (C-2″, 6″), 114.9 (C-2‴), 106.9 (C-6‴), 99.9, 99.5 (C-4a, 4a′), 96.2, 96.0, 95.4 (2C) (C-6, 6′, 8, 8′), 79.69, 79.67 (C-2, 2′), 67.2, 66.5 (C-3, 3′), 27.2, 26.3 (C-4, 4′); HR FAB-MS 681.1711, $C_{36}H_{29}N_2O_{12}$ requires 681.1720.

3.3.3. Phenazine derivative *6a*

Brown amorphous powder; $[\alpha]_D^{25}$ +133 ($c = 0.1$, MeOH); FAB-MS *m/z*: 725 $[M+H]^+$; IR ν_{max} cm^{-1}: 3359, 2919, 1627, 1606, 1517, 1491, 1468; UV (MeOH) λ_{max} nm (log ε): 235(4.76), 320(4.20); CD (3.12×10^{-5} mol/l in methanol) Δε (nm): +0.78 (318), −0.23 (268), +0.17 (255); ^1H and ^{13}C NMR see Table 1; HR FAB-MS 725.2247, $C_{41}H_{33}N_4O_9$ requires 725.2247.

3.4. Oxidation of 7 and isolation of phenazine derivatives

(−)-Gallocatechin (**7**) was prepared by isomerization of **1**. An aqueous solution of **1** (2 g/50 ml) was divided into five aliquots. Each aliquot was sealed in a screw-capped vial and heated in a microwave oven (600 W) for 2 min. The mixture was combined and separated by MCI-gel CHP20P (3.0 cm i.d. × 25 cm) with H_2O–MeOH (100:0 → 70:30, v/v, in a 5% step-wise elution) to give **7** (0.94 g), $[\alpha]_D^2 5$ −1.2 ($c = 1.0$, acetone). Oxidation of **7** and subsequent derivatization and separation were performed in a manner similar to that described for **4**, to give **7a** (46.7 mg) and **8** (40.0 mg). The ^1H NMR spectrum of **7a** was identical to that of **4b**.

3.4.1. Phenazine derivative *8*

Brown amorphous powder; $[\alpha]_D^{25}$ −89.7 ($c = 0.1$, MeOH); FAB-MS *m/z*: 725 $[M+H]^+$; IR ν_{max} cm^{-1}: 3359, 2919, 1627, 1606, 1517, 1491, 1468; UV (MeOH) λ_{max} nm (log ε): 235(4.76), 320(4.20); the ^1H NMR spectrum was identical to that of **6a**; HR FAB-MS 725.2273, $C_{41}H_{33}N_4O_9$ requires 725.2247.

3.5. ^1H NMR measurements of *1* and *4* in 2% DMSO-d_6/D_2O

The ^1H NMR spectra (500 MHz) of **1** and **4** in 2% DMSO-d_6/D_2O at the concentrations of 0.5 mg/ml and 10.0 mg/ml was measured and the chemical shift differences were compared (Table 2).

Acknowledgements

The authors are grateful to Mr. K. Inada and Mr. N. Yamaguchi for NMR and MS measurements. This work was supported by a Grant-in-aid for Scientific Research No. 18510189 from the Japan Society for the Promotion of Science.

References

Feucht, W., Treutter, D., 1999. The role of flavan-3-ols and proanthocyanidins in plant defense. In: Inderjit, Dakshini, K.M.M., Foy, C.L. (Eds.), Principles and Practices in Plant Ecology. CRC, Boca Raton, pp. 307–338.

Hashimoto, F., Nonaka, G., Nishioka, I., 1988. Tannins and related compounds. LXIX. Isolation and structure elucidation of B, B′-linked bisflavanoids, theasinensins D-G and oolongtheanin from oolong tea. (2). Chem. Pharm. Bull. 36, 1676–1684.

Hashimoto, F., Nonaka, G., Nishioka, I., 1992. Tannins and related compounds. CXIV. Structures of novel fermentation products,

theogallinin, theaflavonin and desgalloyl theaflavonin from black tea, and changes of tea leaf polyphenols during fermentation. Chem. Pharm. Bull. 40, 1383–1389.

Haslam, E., 2003. Thoughts on thearubigins. Phytochemistry 64, 61–73.

Li, Y., Tanaka, T., Kouno, I., 2007. Oxidative coupling of the pyrogallol B-ring with a galloyl group during enzymatic oxidation of epigallocatechin 3-*O*-gallate. Phytochemistry 68, 1081–1088.

Matsuo, Y., Tanaka, T., Kouno, I., 2006. A new mechanism for oxidation of epigallocatechin and production of benzotropolone pigments. Tetrahedron 62, 4774–4783.

Nonaka, G., Kawahara, O., Nishioka, I., 1983. Tannins and related compounds. XV. A new class of dimeric flavan-3-ol gallates, theasinensins A and B, and proanthocyanidin gallates from green tea leaf (**1**). Chem. Pharm. Bull. 31, 3906–3914.

Nonaka, G., Hashimoto, F., Nishioka, I., 1986. Tannins and related compounds. XXXVI. Isolation and structures of theaflagallins, new red pigments from black tea. Chem. Pharm. Bull. 34, 61–65.

Porter, L.J., 1998. Flavans and proanthocyanidins. In: Harborne, J.B. (Ed.), Flavonoids. Chapman and Hall, London, pp. 21–62.

Saijo, R., Takeda, Y., 1999. HPLC analysis of catechins in various kinds of green teas produced in Japan and abroad. Nippon Shokuhin Kagaku Kogaku Kaishi 46, 138–147, CAN 130:351456.

Takino, Y., Imagawa, H., Horikawa, H., Tanaka, A., 1964. Studies on the mechanism of the oxidation of tea leaf catechins. Part III. Formation of a reddish orange pigment and its spectral relationship to some benzotropolone derivatives. Agric. Biol. Chem. 28, 64–71.

Takino, Y., Ferretti, A., Flanagan, V., Gianturco, M., Vogel, M., 1965. The structure of theaflavin, a polyphenol of black tea. Tetrahedron Lett., 4019–4025.

Tanaka, T., Mine, C., Inoue, K., Matsuda, M., Kouno, I., 2002a. Synthesis of theaflavin from epicatechin and epigallocatechin by plant homogenates and role of epicatechin quinone in the synthesis and degradation of theaflavin. J. Agric. Food Chem. 50, 2142–2148.

Tanaka, T., Mine, C., Kouno, I., 2002b. Structures of two new oxidation products of green tea polyphenols generated by model tea fermentation. Tetrahedron 58, 8851–8856.

Tanaka, T., Mine, C., Watarumi, S., Fujioka, T., Mihashi, K., Zhang, Y.J., Kouno, I., 2002c. Accumulation of epigallocatechin quinone dimers during tea fermentation and formation of theasinensins. J. Nat. Prod. 65, 1582–1587.

Tanaka, T., Watarumi, S., Matsuo, Y., Kamei, M., Kouno, I., 2003. Production of theasinensins A and D, epigallocatechin gallate dimers of black tea, by oxidation-reduction dismutation of dehydrotheasinensin A. Tetrahedron 59, 7939–7947.