Identification of Oxidation Products of (−)-Epigallocatechin Gallate and (−)-Epigallocatechin with H_2O_2

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(−)-Epigallocatechin gallate (EGCG) and (−)-epigallocatechin (EGC) are two important antioxidants in tea. They also display some antitumor activities, and these activities are believed to be mainly due to their antioxidative effects. However, the specific mechanisms of antioxidant action of tea catechins remain unclear. In this study are isolated and identified two novel reaction products of EGCG and one product of EGC when they were reacted separately with H_2O_2. These products are formed by the oxidation and decarboxylation of the A ring in the catechin molecule. This study provides unequivocal proof that the A ring of EGCG and EGC may also be an antioxidant site. This study also indicates an additional reaction pathway for the oxidation chemistry of tea catechins.

Keywords: *Catechins; EGCG; EGC; antioxidants; H_2O_2*

INTRODUCTION

Tea (*Camellia sinensis*) is one of the most widely consumed beverages in the world. During the past decade numerous in vitro and in vivo studies have suggested the possible beneficial effects of tea and tea polyphenols in cancer and cardiovascular disease development (Yang and Wang, 1993; Dreosti et al., 1997; Tijburg et al., 1997; Wiseman et al., 1997). The beneficial effects of tea are believed to be mainly due to the antioxidative activity of polyphenolic compounds in green and black tea (Huang et al., 1992; Koketsu, 1997; Wiseman et al., 1997; Yang et al., 1998a,b). The major polyphenolic compounds in tea are catechins, which include (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECG), and (−)-epigallocatechin gallate (EGCG). Because the growing body of evidence suggests that tea catechins may act as potent antioxidants or modulate key biological pathways in vivo in mammals (Lunder, 1992; Wiseman, 1997), detailed studies of the antioxidation of catechins are of scientific and commercial interest.

It is generally accepted that the major pigments of black tea, theaflavins and thearubigins, are produced by enzymatic or chemical oxidation of catechins of green tea (Balentine, 1992; Finger et al., 1992). The first chemical analysis of the products formed from the free radical-initiated oxidation of catechin was reported by Hirose et al. (1991). The major step in the formation of products is the radical-initiated oxidative cleavage of C-3′ and C-4′ of the B-ring. Later, when studying the chemical oxidation of ECG with potassium ferricyanide, Wan et al. (1997) isolated and elucidated a new type of tea pigment. This compound was found to have a novel benzotropolone skeleton formed between the B-ring of one ECG molecule and the galloyl ester group of another. Most recently, the B-ring cleavage products of EGCG were identified when EGCG was reacted with peroxyl radicals (Valcic et al., 1999). All reactions reported so far suggested that the most likely site of oxidation for catechins occurs at the B-ring or gallate ester moiety.

As an important oxidant, hydrogen peroxide (H_2O_2), can be produced in vivo in a number of ways. For example, H_2O_2 is generated during NADH oxidation by cell wall peroxidase, a process that can be stimulated by monophenolic compounds. H_2O_2 is also generated during autoxidation and enzymic oxidation of catechins (Jiang and Miles, 1993). On the other hand, catechins exhibited a strong capacity for scavenging $^{\bullet}OH$ radicals (Hanasaki et al., 1994) and suppressed cytotoxicity induced by H_2O_2 (Nakayama, 1994). However, the specific mechanism of catechins oxidized by H_2O_2 remains unclear. In this paper, we report the oxidation products formed by EGC and EGCG with H_2O_2.

EXPERIMENTAL PROCEDURES

Materials. Silica gel F_{254} TLC plates (259 μm thickness, 2−25 μm particle size) and silica gel (130−270 mesh) were purchased from Aldrich Chemical Co. (Milwaukee, WI) and used for chromatography. All solvents and H_2O_2 (50%) were of analytical grade quality and purchased from Fisher Scientific (Springfield, NJ).

NMR and FAB-MS. ^{1}H NMR (Table 1) and ^{13}C NMR (Table 2) spectra were obtained on a Varian Gemini-200 instrument (Varian Inc., Melboune, Australia) at 200 and 50 MHz, respectively. ^{1}H−^{1}H COSY, NOESY, HMQC, and HMBC were performed on a U-500 instrument (Varian Inc.). Methanol-d_4 was used as solvent, and chemical shifts were expressed in parts per million (δ) using TMS as internal standard. FAB mass spectra were recorded on a Finnigan MAT-90 instrument (Finnigan Corp., Bremen, Germany). All TLC spots were visualized under UV (254 and 365 nm) and with 10% H_2SO_4 in EtOH followed by heating.

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Table 1. ^{1}H NMR Data of Compounds 1−3 (CD_3OD)

position	1	2	3
2	5.06 (1H, br s)	5.03 (1H, br s)	4.78 (1H, br s)
3	5.50 (1H, br s)	5.51 (1H, br s)	4.95 (1H, br s)
4	2.66 (1H, br d, J = 17.6 Hz)	2.74 (1H, br d, J = 17.6 Hz)	2.56 (1H, br d, J = 17.6 Hz)
	2.82 (1H, dd, J = 4.0, 17.6 Hz)	2.88 (1H, dd, J = 4.0, 17.6 Hz)	2.68 (1H, dd, J = 4.0, 17.6 Hz)
8	3.64 (1H, d, J = 16.0 Hz)		3.50 (1H, d, J = 15.2 Hz)
	3.86 (1H, d, J = 16.0 Hz)		3.86 (1H, d, J = 15.2 Hz)
2′,6′	6.47 (2H, s)	6.52 (2H, s)	6.48 (2H, s)
G-2,6	7.00 (2H, s)	7.00 (2H, s)	

Table 2. ^{13}C NMR Data of Compounds 1−3 (CD_3OD)

position	1	2	3	position	1	2	3
2	79.8	79.5	81.0	1′	129.8	129.7	130.7
3	69.2	69.3	67.0	2′,6′	107.1	107.2	107.2
4	29.9	31.7	32.6	3′,5′	147.0	146.9	147.0
5	103.1	105.6	104.0	4′	134.4	134.2	134.0
6	171.9	171.7	172.6	G-0	167.8	167.7	
7	175.0	169.8	176.0	G-1	121.7	121.6	
8	41.2	158.1	42.7	G-2,6	110.7	110.6	
9	162.1		161.9	G-3,5	146.6	146.7	
				G-4	140.2	140.1	

Isolation of EGC and EGCG. Green tea polyphenol extract (10 g, Lipton Co.) was dissolved in 95% ethanol solution and the solution loaded onto a Sephadex LH-20 column (38 mm i.d. × 457 mm). After elution with 95% ethanol and monitoring by TLC [chloroform/methanol/water (3:1:0.2) as eluent], 700 mg of EGC and 1300 mg of EGCG were obtained. The purity of isolated EGCG and EGC was determined to be >98% according to the HPLC method described previously (Chen and Ho, 1995).

Oxidation of EGC and EGCG. Two hundred millligrams of EGCG (EGC) in 5 mL of water were treated with 1 mL of H_2O_2 (50%). The reaction mixture was kept at room temperature for 48 h until no EGCG (EGC) was present as monitored by TLC.

Separation and Purification of the EGCG and EGC Oxidation Products. The crude EGCG reaction mixture was loaded directly onto a Sephadex LH-20 column (38 mm i.d. × 457 mm); the column was then eluted with 95% aqueous ethanol and monitored by TLC. Five fractions were collected and evaporated to dryness in vacuo. The major products were present in fraction 4.

Fraction 4 was purified on a silica gel column (25 mm i.d. × 305 mm) using a mixture of ethyl acetate, methanol, and water (7:1:1) as eluent. After repeated chromatography, 24 mg of compound **1** and 16 mg of **2** (R_f 0.25, compared to R_f 0.30 of **1** in the solvent system ethyl acetate/methanol/water 7:1:1) were collected.

Using the same procedure as described for the separation of EGCG products, the crude EGC reaction mixture was separated into four fractions. Fraction 3 containing the major product was further purified by repeated silica gel chromatography, using ethyl acetate/methanol/water (6.5:1:1) as eluent. After removal of solvent, the combined fractions gave 30 mg of compound **3** (R_f 0.25 in the solvent system ethyl acetate/methanol/water 6.5:1:1).

RESULTS

Oxidation of EGCG. Two compounds, **1** and **2**, were isolated from the H_2O_2 oxidation of EGCG in the yields of 12 and 8%, respectively. The other compounds formed were polymeric pigments and not possible to purify.

Compound **1** was a colorless, amorphous solid. The FAB-MS of **1** showed the molecular ion $[M + H]^+$ at m/z 479; combined with ^{13}C NMR spectra, **1** has a molecular formula of $C_{21}H_{18}O_{13}$.

The ^{1}H and ^{13}C NMR spectra of **1** were similar to those of EGCG (Valcic et al., 1999) except for signals due to the A-ring. The two signals at δ 6.47 (2H, s) and δ 7.00 (2H, s) were assigned to H-2′, H-6′ of ring B and H-2″, H-6″ of the galloyl moiety. The two broad singlets at δ 5.06 and 5.50, together with the signals at δ 2.66 (1H, br d, J = 17.6 Hz) and δ 2.82 (1H, dd, J = 4.0, 17.6 Hz), were very close to protons H-2, H-3, H-4a, and H-4b, respectively, of ring C of EGCG (Valcic et al., 1999). Supported by HMQC, HMBC, ^{1}H−^{1}H COSY, and NOESY correlations were observed, and these data prove that ring B, ring C, and the galloyl moiety of **1** are intact and the changes in the molecule had occurred only in ring A of EGCG.

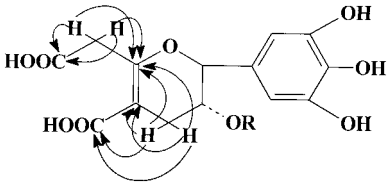

Figure 1. Observed correlations in the HMBC spectrum of compound **1**.

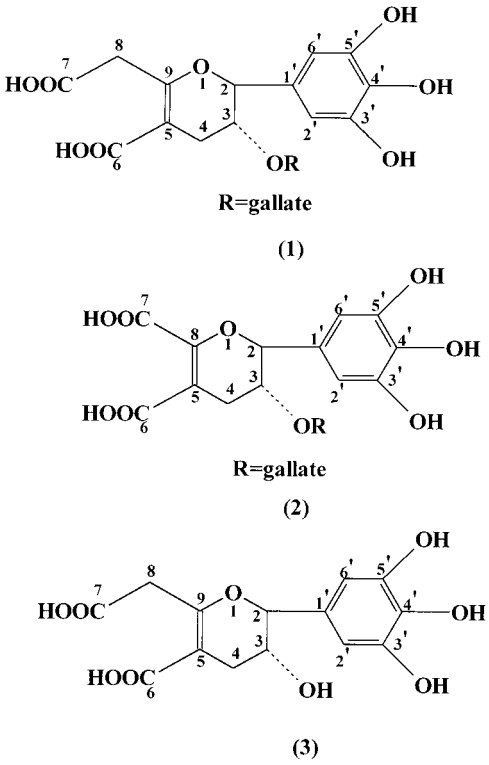

Figure 2. Structures of compounds **1**−**3**.

For the modified A-ring, the ^{1}H NMR spectrum exhibited two signals at δ 3.64 (1H, d, J = 16.0 Hz) and δ 3.86 (1H, d, J = 16.0 Hz), which had HMQC correlations with C-8 at δ 41.2. In addition, the ^{13}C NMR spectrum indicates two carbonyl carbons (δ 171.9 and 175.0). The chemical shifts of two carbons suggest the presence of two acid groups. Moreover, HMBC correlations (shown in Figure 1) were observed between H-8 with C-7 and C-9 and H-4 with C-5, C-6, and C-9; thus, we assigned the structure of **1** as shown in Figure 2.

Compound **2** was isolated as a colorless amorphous substance. As another oxidized product of EGCG, **2** was found to have a molecular weight of 464 (FAB-MS: *m/z* 465 $[M + H]^+$), which together with the ^{13}C NMR spectrum of **2** led to its formula as $C_{20}H_{16}O_{13}$. Compared with the ^1H and ^{13}C NMR spectra of **1**, the only significant difference is the disappearance of H-8 and C-8 of **1**. In addition, the ^{13}C NMR shift of C-7 was decreased to 169.8 because of conjugation with the double bond. Apparently, the methylene group was lost due to oxidation; therefore, the structure of **2** was elucidated as shown in Figure 2.

Oxidation of EGC. Only one compound (compound **3**) was isolated from the H_2O_2 oxidation of EGC, in a yield of 15%. Compound **3** was a colorless amorphous substance. It was found to have a molecular weight of 326 (FAB-MS: *m/z* 327 $[M + H]^+$), which together with the ^{13}C NMR spectrum of **3** led to its formula of $C_{14}H_{14}O_9$. Compared with the spectrum of **1**, the NMR and FAB-MS data are in good agreement with those of **1** except for the galloyl moiety. It is apparent that the difference between **1** and **3** is the lack of the galloyl moiety in **3**. Because of this, some small change occurs at the chemical shifts of H-2, H-3, and H-4 or C-2, C-3, and C-4. Thus, the structure of **3** was identified as shown in Figure 2.

DISCUSSION

The isolation and identification of products **1**−**3** are of great interest. It was previously considered that ring A is very insensitive to oxidation and, therefore, unlikely to participate directly in the antioxidant reaction. It has been reported (Jovanovic et al., 1994) that the phenoxyl radical of EGC exhibits a one-electron reduction potential of 0.43 V, whereas that of methyl gallate exhibits a one-electron reduction potential of 0.56 V. The work of Valcic et al. (1999) confirmed that the B-ring rather than the galloyl moiety is the principal site of antioxidant in EGCG. In addition, Nanjo et al. (1996) and Wan et al. (1997) suggested that the galloyl moieties of EGCG and ECG were involved in oxidative reactions. However, there are no reports on the chemical oxidation of the A-ring. Although further work is needed to understand the mechanism of this oxidation process, the observation that ring A can be oxidized to form acid provides unambiguous proof that oxidation can occur at the A-ring. Recently, it has also been reported that δ-(3,4-dihydroxyphenyl)-γ-valerolactone was formed as a major metabolite of catechins in the colon (Hollman et al., 1997). The exact mechanism for this type of transformation is not known, but a step involved in the breakdown of the A-ring has been proposed (Hollman et al., 1997).

From the result of our work as well as the reports of Hirose et al. (1991), Wan et al. (1997), Valcic et al. (1999), and Nanjo et al. (1996), it can be concluded that the use of different oxidants can result in distinctly different oxidation products from catechins and that the main site of antioxidant action of catechins seems to depend on the oxidant used.

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