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Identification of reaction products of (−)-epigallocatechin, (−)-epigallocatechin gallate and pyrogallol with 2,2-diphenyl-1-picrylhydrazyl radical

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Abstract

Catechins, the major constituents of tea, have been shown to have powerful antioxidant and antitumor activity. In this study, the radical scavenging behavior of catechins on 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) was studied. Three reaction products of (−)-epigallocatechin, (−)-epigallocatechin gallate and pyrogallol with DPPH radical were purified and identified. This study provides some insight into the specific mechanism of the antioxidant reaction of catechins. A possible mechanism for the formation of reaction products is suggested.

Keywords: Tea catechin; EGCG; EGC; Pyrogallol; Free radical; DPPH; Antioxidant

1. Introduction

Tea (*Camellia sinensis*) is one of the most widely consumed beverages in the world. During the last 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 (Dreosti, Wargovich & Yang, 1997; Tijburg, Mattern, Folts, Weisgerber & Katan, 1997; Wiseman, Balentine & Frei, 1997; Yang & Wang, 1993). The beneficial effects 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, Balentine & Frei; Yang, Chen, Lee, Balentine, Kuo & Schantz, 1998; Yang, Liao, Kim, Yurkow & Yang, 1998). The major polyphenolic compounds in tea are catechins which include (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECG) and (−)-epigallocatechin gallate (EGCG; for structures see Fig. 1). Since a 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, Balentine & Frei), detailed studies of antioxidation of catechins are of scientific interest (Suzuki, Mori, Nanjo & Hara, 1999; Valcic, Muders, Jacobsen, Liebler & Timmermann, 1999; Wan, Nursten, Cai, Davis, Wilkins & Davis, 1997). It is generally accepted that the major pigments of black tea, theaflavins and thearubigins, are produced by enzymatic and/or coupled chemical oxidation of catechins of green tea (Balentine, Wiseman & Bouwns, 1997; Finger, Kuhr & Engelhardt, 1992).

Free-radicals are very important, both in food systems and in biological systems. In food, the process of lipid autoxidation and development of rancidity involves a free radical chain mechanism, proceeding via initiation, propagation and termination steps (Shahidi, 1997). This lipid peroxidation process is responsible for the development of off-flavors and undesirable chemical compounds in food (Aruoma, 1994). In vivo, free-radical-initiated autoxidation of cellular membrane lipids can lead to cellular necrosis and this is an accepted important factor for a variety of pathological conditions, such as cancer, cardiovascular disease and even aging (Slater, 1984; Yagi, 1987).

Since free-radical generation occurs normally in the human body, the importance of tea catechins as free-

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radical scavengers is of great interest. There are three suggested mechanisms for tea catechins as tumor inhibitors: the first is that tea catechins are strong metal ion chelators because of the catechol structure. They can bind and thus decrease the level of free cellular ferric and ferrous ions, which are required for the generation of reactive oxygen radicals by the Fenton reaction. Secondly, tea catechins are strong scavengers against superoxide and hydroxyl radicals, which can damage DNA and other cellular molecules and initiate lipid peroxidation reactions. Finally, catechins can trap peroxyl radicals and thus suppress radical chain reactions and terminate the lipid peroxidation (Yang & Wang, 1993). Thus, two mechanisms are related to the free-radical-scavenging activity of tea catechins. Recently, a relatively stable radical, 2,2-diphenyl-1-picrylhydrazyl (DPPH) was used to evaluate the antioxidative activity of tea catechin (Chen & Ho, 1995). The DPPH radical scavenging ability of catechins was in the oreer:- EGCG > ECG > EGC > EC. However, EGCG and EGC displayed much stronger growth inhibitory effects against tumor cells than ECG and EC (Yang et al., 1998); hence, in the present study, EGCG and EGC were chosen to investigate the antioxidant mechanism of catechins. In addition, pyrogallol (Fig. 1) was also included because of its structural characteristics. Here we report, for the first time, the identification of these three dimerized products as free-radical-initiated oxidation products of EGCG, EGC and pyrogallol. This work provides some insight into the suggested oxidation pathway of tea catechins during fermentation (Hashimoto, Nonaka & Nishioka, 1988). A possible mechanism for the formation of these products is also suggested.

2. Materials and methods

2.1. Instruments and chemicals

^{1}H NMR and ^{13}C NMR spectra were obtained on a Varian Gemini-200 instrument (Varian Inc., Melbourne, Australia) at 200 and 50 MHz, respectively. Methanol-d_4 and acetone-d_6 were used as solvents, and chemical shifts were expressed in parts per million (δ). APCI-MS was obtained on a Fiscon/VG Platform II mass spectrometer. Thin-layer chromatography was performed on Fisher Scientific (Morris Plain, NJ) TLC plates (250 μm thickness, 2–25 μm particle size), with compounds visualized by spraying with 5% (v/v) H_2SO_4 in ethanol solution. Pyrogallol was purchased from Fisher Scientific (Springfield, NJ). DPPH and silica gel (130–270 mesh) was purchased from Aldrich Chemicals Co. (Milwaukee, WI). Green tea polyphenol powder was a gift from the Lipton Company.

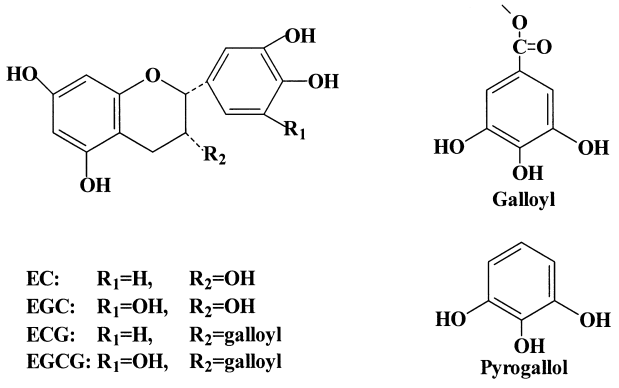

Fig. 1. Structures of four major tea catechins and pyrogallol.

2.2. Isolation of EGC and EGCG

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

2.3. Oxidation of EGCG and isolation of reaction product 1

DPPH (550 mg) and 300 mg EGCG were dissolved in 10 ml of acetonitrile and the mixture was kept in the dark for 2 days. After being dried in air, the resulting mixture was subjected to silica gel chromatography, using ethyl acetate:methanol:water (10:1:0.8) as the eluent, which yielded three crude fractions. Fraction 2 was purified by column chromatography on Sephadex LH-20 with acetone:water (6:4) as the eluent to yield 20 mg of 1.

2.4. Oxidation of EGC and isolation of reaction product 2

Using the same procedure as above, with a mixture of 450 mg DPPH and 250 mg EGC and 10 ml of acetonitrile, three fractions were obtained after silica gel chromatography, using ethyl acetate:methanol:water (12:1:0.8) as the eluent. One apparent oxidation product was present in Fraction 2. Repeated chromatography on a Sephadex LH-20 column with acetone:water (6:4) as the eluent, gave reaction product 2 (8mg).

2.5. Oxidation of pyrogallol and isolation of reaction product 3

After the same procedure as above, the final reaction of the mixture of pyrogallol (150 mg), DPPH (350 mg) and 10 ml of acetonitrile yielded 8 mg of product 3.

3. Results

3.1. Identification of three reaction products

The reaction of 300 mg of EGCG with DPPH radical produced 20 mg of compound 1 (6.7% yield). The other compounds formed were dark-colored polymeric material and not purifiable by column chromatography. Similarly, the reaction of 250 mg of EGC with DPPH yielded only 8 mg of compound 2 (3.2%) and the reaction of 150 mg of pyrogallol with DPPH also yielded only 8 mg of compound 3 (5.3%).

The structure of compounds 1 and 2 were confirmed by comparisons of their ^{1}H and ^{13}C NMR data with those in the literature (Hashimoto et al., 1988), respectively. The structure of product 3 was determined by ^{1}H and ^{13}C NMR data together with its CI-MS spectrum.

Product 1: an off-white amorphous powder. ^{1}H NMR (acetone-d_6, δ ppm): 2.59 (2H, dd, J = 18.0, 3.8 Hz, 4a-H), 2.92 (2H, d, J = 18.0 Hz, 4b-H), 4.81 (2H, s, 2-H), 5.37 (2H, m, 3-H), 5.96 (2H, d, J = 2.2 Hz, 6-H), 6.05 (2H, d, J = 2.2 Hz, 8-H), 6.90 (2H, s, 6′-H), 7.01 (4H, s, 2″, 6″-H). ^{13}C NMR data are listed in Table 1. By comparison with the literature data (Nonaka, Kawahara & Nishioka, 1983), compound 1 was confirmed as theasinensin A (Fig. 2).

Product 2: an off-white amorphous powder. ^{1}H NMR (acetone-d_6, δ ppm): 2.39 (2H, dd, J = 18.0, 3.8 Hz, 4a-H), 2.75 (2H, d, J = 18.0 Hz, 4b-H), 4.05 (2H, m, 3-H), 4.63 (2H, s, 2-H), 5.83 (2H, d, J = 2.2 Hz, 6-H), 5.96 (2H, d, J = 2.2 Hz, 8-H), 6.90 (2H, s, 6″-H). ^{13}C NMR data are listed in Table 1. According to the literature (Hashimoto et al, 1988), product 2 was determined as theasinensin C (Fig. 2).

Table 1
^{13}C NMR spectral data of products 1 and 2

Carbon No.	Product 1	Product 2
2	75.86	77.02
3	68.40	64.66
4	26.71	28.30
5	157.08	157.00
6	95.75	95.45
7	157.19	157.17
8	96.37	95.78
9	157.58	157.17
10	98.34	95.78
1′	128.92	130.14
2′	111.56	120.16
3′	144.68[a]	144.73[b]
4′	133.58	132.89
5′	146.42[a]	145.77[b]
6′	107.98	108.21
Gallate:-COO-	166.45	
1″	121.37	
2″, 6″	109.79	
3″, 5″	145.73	
4″	138.83	

[a] Assignments may be interchanged in each column.
[b] Assignments may be interchanged in each column.

Both dimers have been reported to show atrop-isomerism due to restricted rotation of the biphenyl bond; however, products 1 and 2 could be determined, confidently, as theasinensin A and C, which are in the R series, since there were big differences in the ^{1}H NMR data compared with theasinensin D and E, which are in the S sereies (Hashimoto, Nonaka, & Nishioka, 1988).

Product 3 was obtained as an amorphous powder. Quasi-molecular peaks were exhibited in the CI-MS spectrum at m/z 250 $[M]^+$ and 268 $[M+NH_4]^+$, which suggested that the molecular weight of product 3 was 250, about twice of that of pyrogallol. In the ^{1}H NMR (methanol-d_4) spectrum, two signals showed at δ 6.37 (2H, d, J = 8.2 Hz, 6-H) and 6.56 (2H, d, J = 8.2 Hz, 5-H), whereas the ^{13}C NMR (Table 1) spectrum, it exhibited only six signals at δ 108.38 (d, 6-C), 110.07 (s, 4-C), 119.15 (d, 5-C), 131.89 (s, 2-C), 146.10 (s, 1-C) and 146.90 (s, 3-C). Together with the CI-MS data, apparently the structure of product 3 (Fig. 2) should be a 4-4 dimer of pyrogallol.

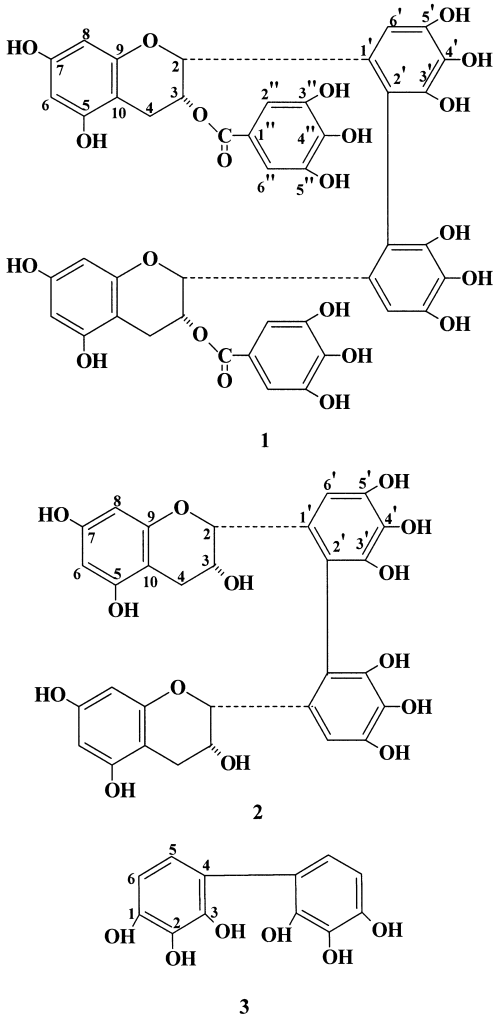

Fig. 2. Structures of reaction products 1, 2 and 3.

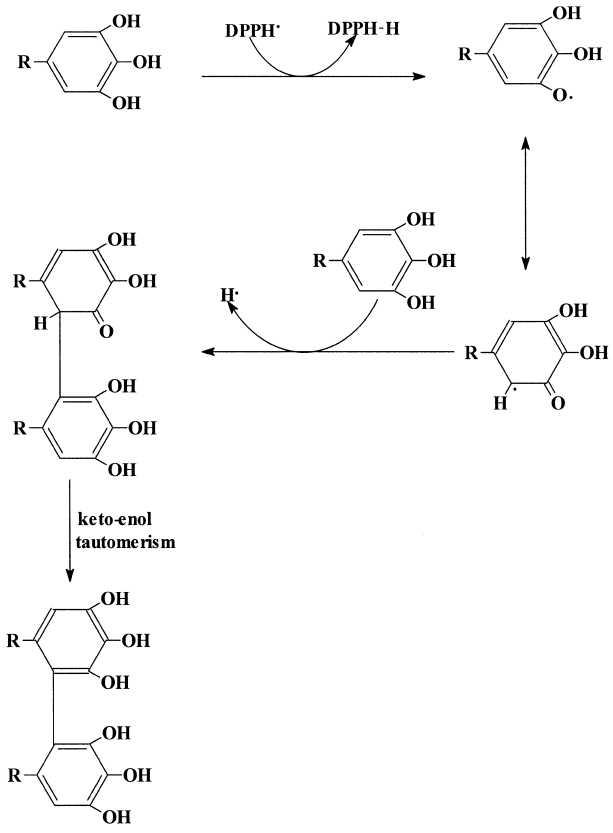

Fig. 3. Proposed mechanism for the formation of three products.

4. Discussion and conclusion

The study of Jovanovic et al. (Jovanovic, Steenken, Tosic, Marjanovic & Simic, 1996) indicated that the phenoxyl radical of EGC exhibits a 1-electron reduction potential of 0.43 V, whereas that of methyl gallate exhibits the potential of 0.56 V. Very recently, two products, resulting from the oxidation of the B ring, have also been isolated from the reaction between EGCG and peroxy radicals (Valcic, Muders, Jacobsen, Liebler & Timmermann, 1999). In the present study, the identification of compounds 1 and 2 as the radical-initiated reaction products of EGCG and EGC, further confirms the view that the trihydroxyphenyl B ring, rather than the gallate moiety, is the active site of antioxidant reaction in catechins. Indeed, we have observed the occurrence of product 3 when the mixture of pyrogallol and gallic acid was reacted with DPPH, no similar dimer of gallic acid could be found. In addition, during the chromatography on silica gel, compounds 1, 2 and 3 were all very unstable. It was observed that the color of the column became gradually darker, and these dimerized products quickly decomposed. The presence of these dimeric catechins in fresh and oolong tea has already been reported (Hashimoto et al., 1988), and this suggests that these catechin dimers may be the intermediate precursors for the pigments in oolong tea and black tea. The proposed mechanism for the formation of these dimeric theasinensins is shown in Fig. 3.

More importantly, compound 1, identified as theasinensin A, was recently reported to be one of the two major metabolites during the incubation of EGCG with either bile or plasma of rat (Tomita, Sano, Sasaki & Miyase, 1998). It is interesting to note that the IC_{50} for TBARS (using rat brain homogenates) of compound 1 was found to be smaller than that of EGCG. Because in vivo antioxidant activity of tea might not solely be due to the intact catechin itself, our study may provide some additional insight of the antioxidant actions of tea catechins in biological systems.

Acknowledgements

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