Biosci. Biotechnol. Biochem., **63** (2), 430–432, 1999

Note

Methylation of Tea Catechins by Rat Liver Homogenates

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Received August 26, 1998; Accepted October 16, 1998

Methylation of (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECg), and (−)-epigallocatechin gallate (EGCg) was carried out with a rat liver homogenate and S-adenosyl-L-methionine. A structural analysis of the reaction products by MS and NMR showed that 4′-*O*-methyl EGC, 4″-*O*-methyl ECg, and 4″-*O*-methyl EGCg had been formed from EGC, ECg, and EGCg, respectively. These results suggest that methylation may be one of the metabolic pathways to the catechins.

Key words: metabolism; methylation; (−)-epigallocatechin; (−)-epicatechin gallate; (−)-epigallocatechin gallate

Recent epidemiological studies have revealed that the intake of flavonoids is inversely associated with the risk of coronary heart disease and stroke.[1-3] These studies have suggested that tea catechins, (−)-epicatechin, EGC, ECg and EGCg, as the major ingestible flavonoids, may be responsible for this inverse association. Imai *et al.* have reported that a negative association between green tea consumption and cancer incidence was found in a prospective cohort study of a Japanese population.[4] They also suggested that the presence of tea catechins, especially EGCg, may be an important factor for this negative association. With the increasing significance of the potentially beneficial role of tea catechins in human health, the metabolic fate of tea catechins in the body has recently become a subject of considerable interest. Up until now, there have been some reports with respect to the absorption, distribution, and excretion of tea catechins.[5-13] However, little attention has been given to determining the chemical structures of their metabolites formed in the body, in spite of the evidence that 3′-*O*-methyl-(+)-catechin glucuronide is the major metabolite of orally administered (+)-catechin in rats and humans.[14-16] The present study was conducted to determine the structures of methylated EGC, ECg and EGCg that had been formed by treating with a rat liver homogenate as the source of methyltransferase in the presence of S-adenosyl-L-methionine as the methyl donor.

EGC, ECg, EGCg and S-adenosyl-L-methionine were obtained from Sigma Chemical Co. (St. Louis, MO, USA.). All other chemicals were products of analytical or HPLC grade.

Male Wistar rats (6 weeks of age) were maintained on a special diet free from naturally occurring polyphenols for a week as described previously.[5] Methylation of EGC, ECg and EGCg was performed with a rat liver homogenate and S-adenosyl-L-methionine according to the method of Shaw and Griffiths.[17] However, our preliminary experiments showed that EGC, ECg and EGCg were hardly methylated under the conditions for (+)-catechin described by Shaw and Griffiths,[17] suggesting that the catechins probably inhibited methyltransferase in the rat liver homogenate. Hence, we re-determined the respective methylation conditions for EGC, ECg and EGCg as will be described next. The reaction time for methylating each catechin was determined according to its stability in the reaction mixture. Rat liver tissue from freshly killed rats was homogenized with 20 volumes of 10 mM sodium phosphate buffer (pH 7.4) containing 10 mM $MgCl_2$ at 0–4°C. To methylate EGC, EGC (40 mg) and S-adenosyl-L-methionine (60 mg) separately dissolved in 10 ml of the above-mentioned buffer were added to 100 ml of the rat liver homogenate. The reaction mixture was incubated for 3 h at 37°C. Methylation of ECg was performed by using ECg (50 mg), S-adenosyl-L-methionine (60 mg), and 200 ml of the liver homogenate at 37°C for 6 h. To methylate EGCg, the reaction was done with EGCg (68 mg), S-adenosyl-L-methionine (120 mg), and 500 ml of the liver homogenate at 37°C for 1 h. Each reaction mixture was centrifuged at 3500 rpm for 10 min to remove the insoluble materials. The supernatant was extracted 3 times with the same volume of ethyl acetate, and each organic phase was evaporated to dryness under reduced pressure. The resulting residue was dissolved in 5 ml of chloroform and then extracted 3 times with 5 ml of distilled water. After removing the water, the residue was dissolved in 3 ml of acetonitrile:ethyl acetate:0.05% phosphoric acid (12:2:86 by volume) and isolated by preparative HPLC. Preparative HPLC was carried out in a Capcell-pak C-18 AG120 column (ϕ20 × 250 mm, Shiseido Co. Ltd.) with JASCO liquid chromatographic apparatus equipped with a JASCO 870 UV detector (UV 280 nm). Elution was done with acetonitrile:ethyl acetate:0.05% phosphoric acid (12:0.6:90 by volume) for EGC and with acetonitrile:ethyl acetate:0.05% phosphoric acid (12:2:86 by volume) for ECg and EGCg at a flow rate of 8 ml/min. The HPLC analysis was performed in a Capcell-pak C-18 AG column (ϕ4.6 × 250 mm, Shiseido Co.) with a Waters liquid chromatograph

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Abbreviations: EGC, epigallocatechin; ECg, epicatechin gallate; EGCg, epigallocatechin gallate; HMBC, heteronuclear multiple-bond connectivity

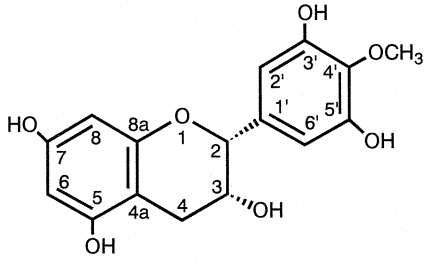
equipped with a Waters model M996 photodiode array detector. Elution was done with the same solvent as that for preparative HPLC at a flow rate of 1.2 ml/min. The liquid chromatography-MS (Frit-FAB ionization) analysis was done with an HPLC system (Hitachi L-6000 series) coupled to a JEOL JMS SX-120 mass spectrometer. The conditions for the HPLC were similar to those for the HPLC analysis, except for using acetonitrile:ethyl acetate:water (12:0.6:90 by volume) as the solvent at a flow rate of 1 ml/min. NMR spectra were recorded on a JEOL Lambda-500 system. A sample (about 5 mg) was dissolved in 0.5 ml of acetone-d_6. Chemical shifts are expressed in ppm relative to tetramethylsilane (TMS) as an internal standard.

After the reaction of EGC with the rat liver homogenate in the presence of S-adenosyl-L-methionine, a major reaction product with a retention time later than that of EGC was observed on the HPLC chromatogram. The UV spectrum of the product was found to be similar to that of EGC. Similar results were also obtained with ECg and EGCg.

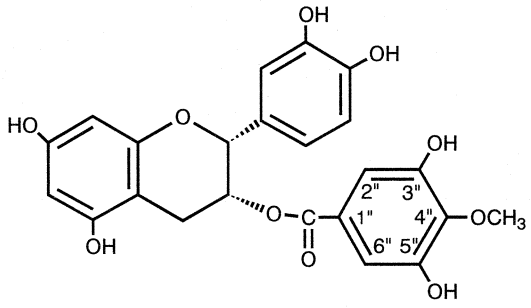
The negative FAB mass spectrum of the reaction product formed from EGC showed a deprotonated molecular ion peak (m/z=319), in agreement with the molecular weight of monomethylated EGC. ^{1}H- and ^{13}C-NMR spectra of the product from EGC were similar to those of EGC, except for the observation of proton (δ 3.80) and carbon (δ 60.6) signals derived from a methyl group and the lower-field shift (about 5 ppm) of C-1′. Further, a cross peak between C-4′ and the methyl protons was observed by the heteronuclear multiple-bond connectivity (HMBC) experiment. The assignments of the ^{1}H- and ^{13}C-NMR spectra were as follows: ^{1}H-NMR (acetone-d_6, 500 MHz) δ: 2.74 (dd, J=16.6, 3.4 Hz, H-4α), 2.78 (dd, J=16.6, 4.4 Hz, H-4β), 3.80 (s, methoxy protons), 4.23 (m, H-3), 4.85 (s, H-2), 5.93 (d, J=2.3 Hz, H-8), 6.03 (d, 2.3 Hz, H-6), 6.59 (d, J=0.6 Hz, H-2′, 6′); ^{13}C-NMR (acetone-d_6, 125 MHz) δ: 28.9 (C-4), 60.6 (methoxy carbon), 66.9 (C-3), 79.3 (C-2), 95.7 (C-8), 96.2 (C-6), 99.9 (C-4a), 107.1 (C-2′, 6′), 135.5 (C-4′), 136.3 (C-1′), 150.9 (C-3′, 5′), 157.0 (C-8a), 157.6 (C-5, 7). Thus, the product from EGC was identified as 4′-*O*-methyl-EGC.

Negative FAB-MS data of a major product from ECg showed [M-H]$^-$ ion peaks at m/z 455, in agreement with the monomethylated form of ECg. ^{1}H- and ^{13}C-NMR spectra of the monomethylated products from ECg were similar to those of ECg, except for methyl protons and carbon, and the lower-field shift (about 5 ppm) of C-1″ being observed. The HMBC experiment indicated that the methyl group was attached to the hydroxyl group at C-4″. The assignments of the ^{1}H- and ^{13}C-NMR spectra were as follows: ^{1}H-NMR (acetone-d_6, 500 MHz) δ: 2.94 (dd, J=17.5, 2.1 Hz, H-4α), 3.06 (dd, J=17.5, 4.6 Hz, H-4β), 3.82 (s, methoxy protons), 5.15 (s, H-2), 5.55 (m, H-3), 6.03 (d, J=2.3 Hz, H-8), 6.06 (d, 2.3 Hz, H-6), 6.77 (d, J=8.4 Hz, H-5′), 6.88 (dd, J=8.4, 1.9 Hz, H-6′), 7.01 (s, H-2″, 6″), 7.10 (d, J=1.9 Hz, H-2′); ^{13}C-NMR (acetone-d_6, 125 MHz) δ: 28.9 (C-4), 60.6 (methoxy carbon), 66.8 (C-3), 78.0 (C-2), 95.7 (C-8), 96.3 (C-6), 98.8 (C-4a), 109.8 (C-2″, 6″), 114.8 (C-2′), 115.6 (C-5′), 119.1 (C-6′), 126.5 (C-1″), 131.3 (C-1′), 140.2 (C-4″), 145.4 (C-3′), 145.5 (C-4′), 151.1 (C-3″, 5″), 157.1 (C-8a), 157.4 (C-7), 157.8 (C-5), 165.8 (C=O). From these results, the product from ECg was determined as 4″-*O*-methyl-ECg.

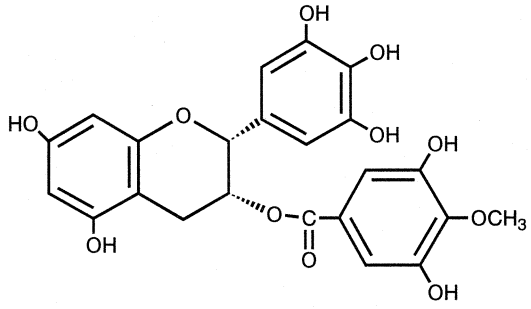
In the case of the product from EGCg, a pseudomolecular ion peak at m/z 471 [M-H]$^-$ by negative FAB-MS was observed, corresponding to monomethylated EGCg. The same NMR and HMBC experiments as those just described showed that monomethylated EGCg was 4″-O-methyl-EGCg. The assignments of the ^{1}H- and ^{13}C-NMR spectra were as follows: ^{1}H-NMR (acetone-d_6, 500 MHz) δ: 2.92 (dd, J=17.0, 2.0 Hz, H-4α), 3.05 (dd, J=17.0, 4.4 Hz, H-4β), 3.82 (s, methoxy protons), 5.09 (s, H-2), 5.58 (m, H-3), 6.04 (d, J=2.5 Hz, H-8), 6.06 (d, 2.5 Hz, H-6), 6.63 (s, H-2′, 6′), 6.99 (s, H-2″, 6″); ^{13}C-NMR (acetone-d_6, 125 MHz) δ: 26.5 (C-4), 60.5 (methoxy carbon), 69.7 (C-3), 78.0 (C-2), 95.8 (C-8), 96.3 (C-6), 99.2 (C-4a), 106.6 (C-2′, 6′), 109.8 (C-2″, 6″), 126.5 (C-1″), 130.6



4'-O-Methyl-(-)-epigallocatechin



4"-O-Methyl-(-)-epicatechin gallate



4"-O-Methyl-(-)-epigallocatechin gallate

Figure. Structures of Methylated (−)-Epigallocatechin, (−)-Epicatechin Gallate, and (−)-Epigallocatechin Gallate formed by a Rat Liver Homogenate.

(C-1′), 133.1 (C-4′), 140.4 (C-4″), 146.3 (C-3′, 5′), 151.1 (C-3″, 5″), 157.1 (C-8a), 157.4 (C-7), 157.7 (C-5), 165.9 (C=O). The Figure shows the chemical structures of the methylated catechins that were identified in this study.

In previous studies on the absorption, distribution and excretion of tea catechins,[5-11] attention has been directed to the detection of intact tea catechins in circulating blood and urine. More recently, conjugated forms of tea catechins have been examined by an enzymatic treatment with glucuronidase and sulfatase.[12,13] To date, while it is well documented that the conjugated forms of methylated (+)-catechin are the major metabolites of (+)-catechin in a wide range of species including rats and humans,[14-17] no reports have appeared concerning the detection or determination of methylated catechins, particularly EGC, ECg and EGCg, as metabolites. In this study, we have demonstrated that methylation of the tea catechins, EGC, ECg and EGCg, took place in *in vitro* experiments with a rat liver homogenate. Hence, it is likely that methylation may be one of the major metabolic pathways to tea catechins. Although further work is required to clarify these points, detection and determination of the methylated forms of catechins *in vivo* may be important for understanding their metabolism.

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