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THE STRUCTURAL VARIATION IN THE INCUBATION PRODUCTS OF (-)-EPIGALLOCATECHIN GALLATE IN NEUTRAL SOLUTION SUGGESTS ITS BREAKDOWN PATHWAYS

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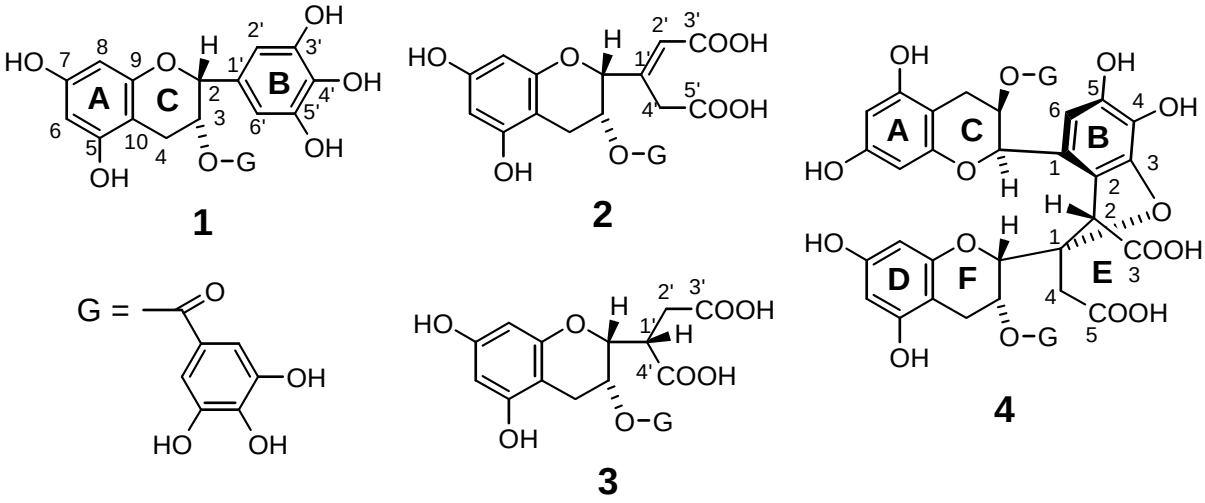
Abstract – Three new compounds, in addition to previously reported compounds, were isolated from a product mixture obtained on incubating (-)-epigallocatechin gallate (EGCG) in neutral solution. The products were two monomeric (EGCG-MOx-M3 and EGCG-MOx-M4) and one dimeric (EGCG-MOx-D4) structure involving the flavan skeleton. Possible oxidation-reduction pathways producing drastic changes in the structure of EGCG were presented based on the structures of the breakdown products.

INTRODUCTION

Although various biological and pharmacological effects of tea polyphenols, including their antioxidant, antimicrobial, and antitumor properties, have been revealed, (-)-epigallocatechin gallate (EGCG) (**1**), the main constituent of tea polyphenols, is unstable even in the neutral solution.[1,2] Therefore, some of the effects reported for EGCG may be attributed to such products of EGCG.[2] Previously, we reported the structures of five new compounds (**7** – **11**) produced from EGCG (**1**) treated with neutral solution.[1] The participation of trace amounts of metal ions, such as Fe^{3+} ions, in the breakdown of **1** was suggested.[2] Further investigation of the changes in the structure of EGCG after incubating it in neutral solution has led to the isolation of three additional products (**2**, **3**, and **4**). This paper describes the structures of these products, and variation in the breakdown pathways from EGCG, which was deduced from the structures of the products. The results of incubating EGCG dimers, theasinensins A (**5**) and D (**6**),[2,3] were also discussed in relation to the formation of **4**, along with EGCG-MOx-D1 (**7**), D2 (**8**), and D3 (**9**).[1]

RESULTS AND DISCUSSION

EGCG was incubated in a neutral solution, and the product mixture was subjected to column chromatography on MCI-gel CHP-20P and Sephadex LH-20. Further purification of the fractions with preparative HPLC gave three new compounds, EGCG-MOx-M3 (**2**), EGCG-MOx-M4 (**3**), and EGCG-MOx-D4 (**4**), in addition to compounds reported previously.[1]



EGCG-MOx-M3 (**2**) was obtained as a light-brown powder. High-resolution electrospray ionization mass spectrometry (HR-ESI-MS) indicated that it had the molecular formula $C_{21}H_{18}O_{12}$. The ^{1}H NMR spectrum (in acetone-d_6 + D_2O) showed a 2H singlet at δ 6.98 due to the galloyl group, two *meta*-coupled doublets at δ 6.03 and δ 5.99 (1H each, d, J = 2.5 Hz) attributed to the H-6 and H-8 protons, and four aliphatic protons: H-2 at δ 4.77 (1H, br s), H-3 at δ 5.69 (1H, br m), H-4a at δ 2.98 (dd, J = 4.0, 17.5 Hz), and H-4b at δ 2.92 (br d, J = 17.5 Hz). These signals indicated that this compound still has the galloyl, A-ring, and C-ring structures of the EGCG molecule. The spectrum also showed signals due to one proton on an sp^2 carbon at δ 6.32 (1H, m, H-2') and a pair of methylene protons at δ 4.09 (br d, J = 17.0 Hz, H-4'a) and δ 3.51 (br d, J = 17.0 Hz, H-4'b) instead of the 2H singlet signal of the B-ring of EGCG. The ^{13}C NMR spectrum of **2** (in acetone-d_6 + D_2O) showed signals of two sp^2 carbons at δ 148.8 (C-1') and δ 121.7 (C-2'), and one sp^3 carbon at δ 35.5 (C-4'), in addition to two carboxyl carbons at δ 168.6 (C-3') and δ 172.1 (C-5'), due to the structure derived from the EGCG B-ring. The ^{1}H-^{13}C heteronuclear multiple-bond correlation (HMBC) spectrum showed correlations of the C-ring H-2 with C-1', C-2', and C-4'. The HMBC spectrum also showed correlations of H-2' with C-1', C-3', C-4', and C-2, and of the H-4' protons with C-1', C-2', C-5', and C-2 (Figure 1). These correlations showed the connectivity C-2 – C-1' = C-2' – C-3' and that of a branch C-1' – C-4' – C-5'. The *E*-form of the isomerism at the C-1' – C-2' double bond was indicated by the correlation of

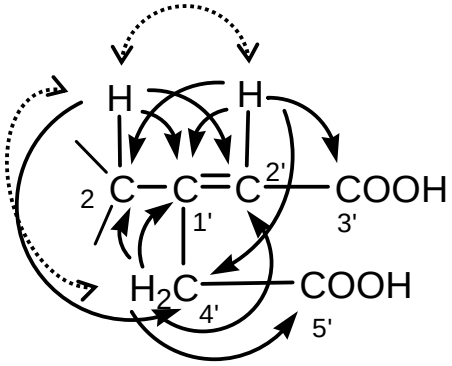


Figure 1 Observed HMBC (→) and ROE (⇠⇢) correlations involving the aliphatic side chain in **2**

H-2' with H-2 in rotating-frame Overhauser effect spectroscopy (ROESY). If the isomerism could be assigned as the *Z*-form, the H-2' – H-2 rotating-frame Overhauser effect (ROE) correlation would not be observed. Therefore, EGCG-MOx-M3 was assigned structure (**2**).

EGCG-MOx-M4 (**3**) was obtained as a light-brown powder. The molecular formula $C_{20}H_{18}O_{12}$ for this compound was determined using HR-ESI-MS. The signals of the galloyl, A-ring, and C-ring moieties in the ^1H and ^{13}C NMR spectra were similar to those of **1** and **2**. The remaining signals due to the structure derived from the EGCG B-ring indicated the presence of one methine [δ_C 43.8 (C-1'); δ_H 3.28 (dt, J = 4.0, 9.0 Hz, H-1')], one methylene [δ_C 33.7 (C-2'); δ_H 2.95 (dd, J = 4.0, 17.5 Hz, H-2'a) and δ_H 2.83 (dd, J = 9.0, 17.5 Hz, H-2'b)], and two carboxyl carbons [δ_C 173.8 (C-3' and C-4')]. The correlations seen on ^1H-^1H correlation spectroscopy (COSY) substantiated the connectivity C-2 – C-1' – C-2'. The carboxyl carbons showed correlations with H-1', H-2' (for C-3' and C-4'), and H-2 (for C-4') in the HMBC spectrum. The configuration at C-2 is the same as that of EGCG, since the coupling constant of H-2 – H-3 is smaller than 2.0 Hz for **3**, as with the 2,3-*cis* configuration of EGCG. The ROESY spectrum of **3** showed correlations of H-8 with H-2' protons, and H-1' with H-3 (as well as H-1' with H-2 and H-2'). The large coupling constant between H-2 and H-1' (9.0 Hz) indicates that the dihedral angle of the bonds C-2 – H-2 and C-1' – H-1' is ca. 0° or 180°. The *S*-configuration at C-1', rather than the *R*-configuration, satisfies all of these in a single conformation, as shown in Figure 2, where the dihedral angle involving H-2 and H-1' is *ca.* 0°. Based on these observations, EGCG-MOx-M4 was assigned structure (**3**).

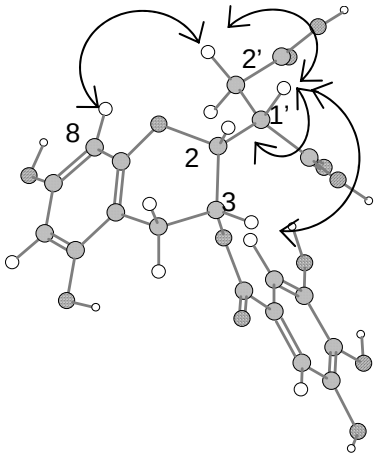
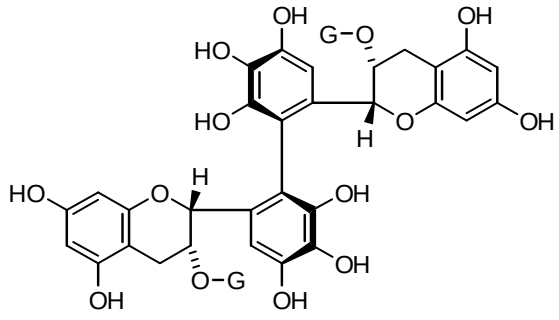


Figure 2. A possible conformation of **3** (the arrows indicate ROEs)

EGCG-MOx-D4 (**4**) was obtained as a brown powder. The molecular formula $C_{43}H_{34}O_{23}$ was determined using HR-ESI-MS. The ^1H NMR spectrum (in acetone-d_6 + D_2O) showed the presence of two galloyl groups [δ 7.02 and 7.14 (2H each, s)] and H-6 and H-8 protons on the A/D-rings {δ 6.01 [H-8(A)] and δ 5.980 [H-6(A)] (1H each d, J = 2.0 Hz, coupled with each other); δ 5.983 [H-6(D)] and δ 5.88 [H-8(D)] (1H each, d, J = 2.0 Hz, coupled with each other)}, and C/F-ring protons [δ 5.05 (1H, br s, H-2), δ 5.73 (1H, br m, H-3) and δ 2.91 (2H, m, H-4) (C-ring); δ 4.91 (1H, br s, H-2), δ 6.05 (1H, br m, H-3) and δ 2.88 (2H, m, H-4) (F-ring)]. The spectrum also showed an aromatic proton at δ 6.68 (1H, s, B-ring H-6) and three aliphatic protons at δ 4.84 (1H, br s, H-2), δ 3.05 (1H, br d, J = 15.0 Hz, H-4a), and δ 2.82 (1H, br d, J = 15.0 Hz; H-4b) due to part E in the structure of **4**. The ^{13}C NMR spectrum (in acetone-d_6 + D_2O) of **4** showed the

signals of one methine [δ 55.4, C-2(E)], one methylene [δ 42.5, C-4(E)], and one quaternary [δ 91.5, C-1(E)] carbon, along with two carboxyl carbons [δ 172.6 and δ 172.3; C-3(E) and C-5(E)]. These data suggested that this compound has a dimeric structure in which one of the aromatic rings derived from EGCG B-ring is cleaved, as observed in the structures of **7** - **9**. The HMBC spectrum of **4** indicated the connectivity C-2(F) – C-1(E) – C-4(E) – C-5(E) by the correlations H-2(F) – C-1(E), H-4(E) – C-2(F), H-4(E) – C-1(E), H-4(E) – C-2(E), and H-4(E) – C-5(E) [C-2(F) means C-2 of part F]. The connectivity C-2(F) – C-1(E) – C-2(E) – C-3(E) was also seen in the HMBC correlations of H-2(E) with C-2(F), C-1(E), C-4(E), and C-3(E). The H-2(E) signal also showed HMBC correlations with C-1(B), C-2(B), and C-3(B), indicating the connectivity of the C-2(E) carbon with the B-ring. This connectivity was also substantiated by a four-bond correlation H-6(B) – C-2(E). The connectivity C-3(B) – O – C-1(E) was indicated by the ^{13}C chemical shifts of C-3(B) (δ 148.3) and C-1(E) (δ 91.5), which were similar to those for the corresponding carbons of **9** [C-3(B), δ 147.3; C-1(E), δ 90.7]. Therefore, this compound was given the same plain structure as **9**. However, the CD spectral pattern in the short-wavelength region ($[\theta]_{205}$ -5.2×10^4 and $[\theta]_{215}$ $+7.4 \times 10^4$) of the spectrum of **4** differed from that of **9** in this region ($[\theta]_{206}$ $+2.4 \times 10^4$ and $[\theta]_{223}$ -6.2×10^4), while the spectral pattern of **9** in this region was similar to those of **7** ($[\theta]_{208}$ $+1.1 \times 10^4$ and $[\theta]_{222}$ -2.2×10^4) and **8** ($[\theta]_{206}$ $+2.4 \times 10^4$ and $[\theta]_{217}$ -9.4×10^4). Therefore, EGCG-MOx-D4 was assigned structure (**4**), which is isomeric to compound (**9**) with respect to the C-2(E) carbon. The four protons of H-4(C) and H-4(F) of **4** in the ^1H NMR spectrum were all near δ 2.9, while those of **9** showed larger differences in the chemical shifts: δ 2.85 and δ 2.92 [H-4(C)], 2.68 and 2.89 [H-4(F)]. Analogous changes in the chemical shifts were also



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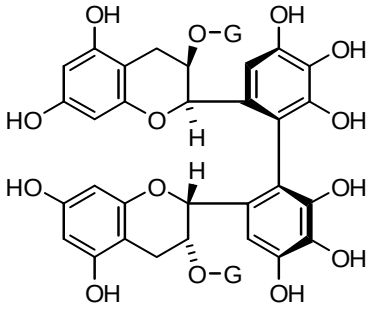

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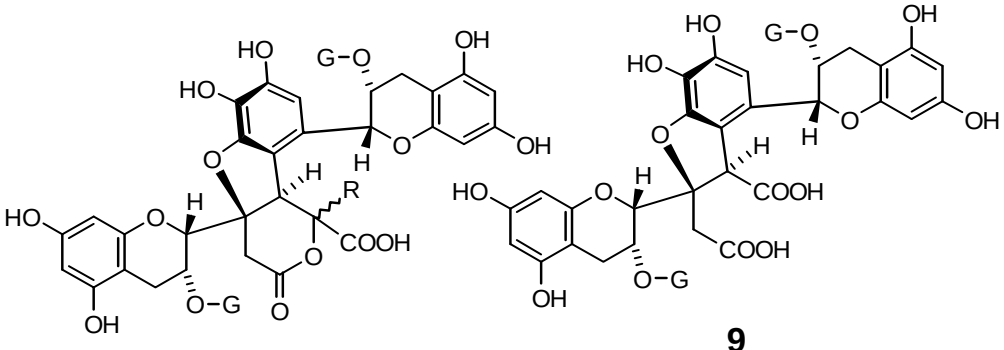


9

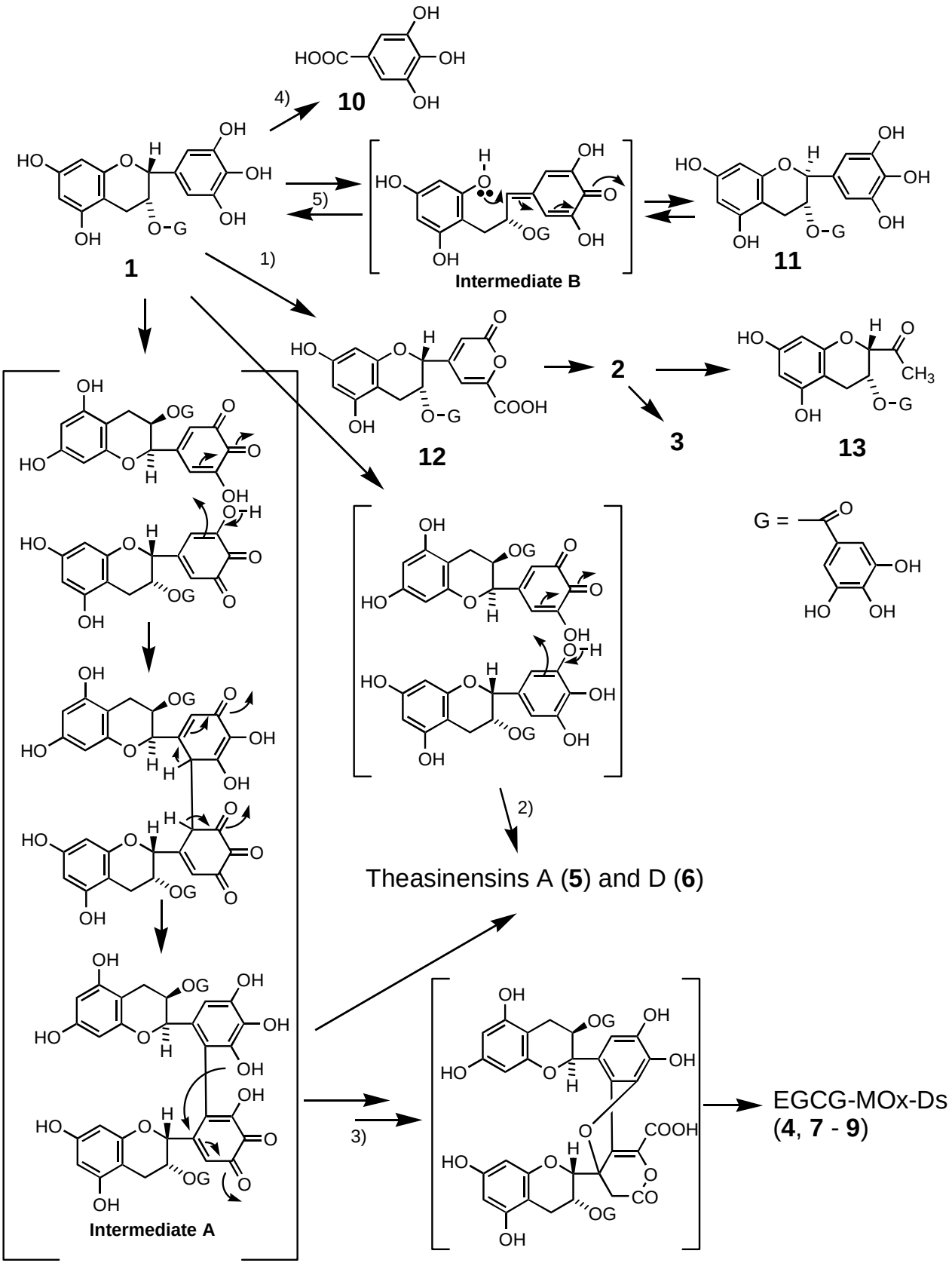
7: R = (α-)H
8: R = (α,β-)OH

observed for the H-4(C/F) protons of theasinensins A (**5**) [δ 2.53 (2H, dd, *J* = 4.5, 17.0 Hz) and 2.86 (2H, br d, *J* = 17.0 Hz)] and D (**6**) [δ 2.81 (4H, m)], which are stereoisomers with respect to the biphenyl bond. The similarity of the signal patterns for the combination of **5** and **9**, and that for the combination of **6** and **4**, suggested the resemblance of the spatial correlations of the two chromane structures (the A-C and D-F moieties). These changes in the signal pattern of the H-4 of the C/F rings were probably due to spatial overlap between the two chromane structures for one of the combinations.

Since the dimers EGCG-MOx-Ds (**4** and **7** – **9**) were presumed to be formed *via* the oxidative cleavage of ring E in compounds (**6**) and (**5**), in a manner analogous to the pathway observed for the monomeric compounds, **5** and **6** were treated in neutral solution, and the reaction mixture was analyzed to see whether EGCG-MOx-Ds formed. The dimers were not observed in the mixture after incubating solutions of **5** and **6**. This result may be explained by some stability of the biphenyl moiety[4] against the oxidation (ring fission). Therefore, EGCG-MOx-Ds are not formed from **5** or **6** directly, but form *via* other intermediates, such as an intermediate (**A**) shown in Scheme 1,[5] which would give **5** or **6** by reduction, and EGCG-MOx-Ds *via* oxidative ring-cleavage. The oxidation of the *ortho*-trihydroxy B-ring would be initiated with the chelation of some metal ion at the hydroxyl groups,[6,7] followed by autoxidation in the presence of oxygen.[8]

The pathways that give the products of the incubation of EGCG are summarized in Scheme 1: 1) ring cleavage of EGCG, 2) dimerization of EGCG, and 3) ring cleavage of a dimeric intermediate (**A**). 4) Hydrolysis of the ester linkage to give gallic acid (**10**) and 5) epimerization at C-2(C) in EGCG to give (-)-gallocatechin gallate (**11**) [through an intermediate (**B**) shown in Scheme 1] also accompany these reactions. Among those products, the major ones were **5**, **6** and **11**, as shown in a previous paper.[2] The production of gallic acid (**10**) from B-ring of **1** may also be assumed. The structures of compounds (**2**) and (**3**) suggested the pathway concerning the oxidative cleavage of the B-ring of EGCG (**1**), **1** → EGCG-MOx-M1 (**12**)[1] → **2** (→ **3**) → EGCG-MOx-M2 (**13**)[1], although the pathway is not experimentally substantiated by formation of **13** from **12** and **2** (because of the insufficient amounts of the available samples).

Present investigation suggested participation of various products such as those discussed here in the pharmacological or biological effects reported for EGCG, and further studies on the activity of the products of EGCG are required.



Scheme 1. Plausible pathways to give the products that form during the incubation of EGCG in neutral solution [For an explanation of reactions 1 to 5, see the text].

EXPERIMENTAL

The ^{1}H and ^{13}C NMR spectra were measured on an INOVA-600 NMR (600 MHz for ^{1}H and 151 MHz for ^{13}C NMR) instrument, and a mixture of acetone-d_6 and D_2O was used as the solvent. The chemical shifts were based on those of the solvent signals (δ_H 2.04; δ_C 29.8) and given in δ (ppm) from TMS. ESI-MS spectra were measured on a Micromass Autospec OA-Tof instrument with 50% MeOH aq. containing 0.1% ammonium acetate as the solvent, and the flow rate was set at 0.02 – 0.03 ml/min. Analytical and preparative HPLC was conducted on YMC A302 and 324 columns, respectively, using combinations of 0.01M H_3PO_4 aq. – KH_2PO_4 aq. (1:1) and acetonitrile as solvents. The (-)-epigallocatechin gallate used in this study was isolated from tea (*Camellia sinensis*) leaves.[9]

Incubation of 1 in solution to form products (2, 3, and 4)

(-)-Epigallocatechin gallate (**1**) (1.0 g) in 0.1 M phosphate (K_2HPO_4 – KH_2PO_4) buffer (pH 7) (1.0 l) was kept at 35°C for 22 h. The mixture was then acidified with 1 M HCl to pH 4, and chromatographed on a column of MCI-gel CHP-20P (Mitsubishi Chemical Industries) with increasing concentrations of MeOH in water as the eluant. Fractions containing polyphenolic products were further purified with column chromatography on Sephadex LH-20 (EtOH), and preparative HPLC, to give **2**, **3**, and **4** (Yield: **2**, 7.0 mg; **3**, 3.3 mg; **4**, 5.0 mg), together with products reported previously.

EGCG-MOx-M3 (2). A light-brown powder. $[\alpha]_D$ -50.8° (*c* 0.13, MeOH). UV λ_{max} (MeOH): 275 nm (log ε 3.87). ESI-MS: *m/z* 480 ($[M + NH_4]^+$). HR-ESI-MS: *m/z* 485.0595 ($[M + Na]^+$) (Calcd for $C_{21}H_{18}O_{12}$ + Na, 485.0696). ^{1}H NMR, see text. ^{13}C NMR: δ 26.4 [C-4(C)], 35.5 (C-4'), 66.3 [C-3(C)], 78.3 [C-2(C)], 95.5, 96.8 [C-6(A), C-8(A)], 98.6 [C-10(A)], 109.9 [C-2(G), C-6(G)], 121.2 [C-1(G)], 121.7 (C-2'), 139.0 [C-4(G)], 145.9 [C-3(G), C-5(G)], 148.8 (C-1'), 155.8, 157.3 [C-5(A), C-9(A)], 157.7 [C-7(A)], 166.6 [C-7(G)], 168.6 (C-3'), 172.1 (C-5'). The assignments with (A) and (C) mean the carbon numbers on the A and C-rings, respectively, and (G) means the galloyl group.

EGCG-MOx-M4 (3). A light-brown powder. $[\alpha]_D$ -28.6° (*c* 0.10, MeOH). UV λ_{max} (MeOH): 275 nm (log ε 3.89). ESI-MS: *m/z* 473 ($[M + Na]^+$). HR-ESI-MS: *m/z* 473.0666 ($[M + Na]^+$) (Calcd for $C_{20}H_{18}O_{12}$ + Na, 473.0696). ^{1}H NMR, see text. ^{13}C NMR: δ 26.1 [C-4(C)], 33.7 (C-2'), 43.8 (C-1'), 67.1 [C-3(C)], 76.1 [C-2(C)], 95.3 [C-8(A)], 96.6 [C-6(A)], 98.5 [C-10(A)], 109.8 [C-2(G), C-6(G)], 121.4 [C-1(G)], 139.0 [C-4(G)], 145.9 [C-3(G), C-5(G)], 155.8, 157.3 [C-5(A), C-9(A)], 157.5 [C-7(A)], 166.5 [C-7(G)], 173.8 (2C, C-3' and C-4').

EGCG-MOx-D4 (4). A brown powder. $[\alpha]_D$ -1.4° (*c* 0.10, MeOH). UV λ_{max} (MeOH): 276 nm (log ε 4.35). HR-ESI-MS: *m/z* 936.1793 $[M + NH_4]^+$ (Calcd for $C_{43}H_{34}O_{23} + NH_4$, 936.1835). ^{1}H NMR: see text. ^{13}C NMR: δ 26.9 [C-4(C)], 28.0 [C-4(F)], 42.5 [C-4(E)], 55.4 [C-2(E)], 64.9 [C-3(F)], 68.2 [C-3(C)], 76.3 [C-2(F)], 76.4 [C-2(C)], 91.5 [C-1(E)], 95.4 [C-8(D)], 95.6 [C-8(A)], 96.5 [C-6(A)], 96.7 [C-6(D)], 98.7 [C-10(A)], 98.9 [C-10(D)], 107.9 [C-6(B)], 109.8 (2C), 110.2 (2C) [C-2(G) × 2, C-6(G) × 2], 116.1 [C-2(B)], 121.3, 121.5 [C-1(G) × 2], 127.5 [C-1(B)], 129.6 [C-4(B)], 138.9 [2C, C-4(G) × 2], 145.79 (2C), 145.83 (2C) [C-3(G) × 2, C-5(G) × 2], 147.1 [C-5(B)], 148.3 [C-3(B)], 156.5, 156.7, 157.2, 157.32, 157.36, 157.43 [C-5(A), C-5(D), C-7(A), C-7(D), C-9(A), C-9(D)], 166.8, 167.0 [C-7(G) × 2], 172.3 [C-5(E)], 172.6 [C-3(E)].

Treatment of dimers (5) and (6) in neutral solution. Solutions of **5** and **6** in 0.1 M phosphate buffer (pH 7.4) (0.25 mg/mL) were kept at 37°C, and the reactions in the respective solutions were monitored using HPLC. EGCG-MOx-Ds (**4** and **7** – **9**) were not detected in either solution during the incubation until **5** and **6** decreased to 13% and 2.5%, respectively.

ACKNOWLEDGMENTS

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