HETEROCYCLES, Vol. 82, No. 2, 2011, pp.1685 - 1692. © The Japan Institute of Heterocyclic Chemistry
Received, 29th June, 2010, Accepted, 6th August, 2010, Published online, 10th August, 2010
DOI: 10.3987/COM-10-S(E)81

NEW OXIDATION PRODUCTS FROM (-)-EPIGALLOCATECHIN GALLATE IN NEUTRAL SOLUTION †

Takayo Ohyabu, Shoko Taniguchi, Hideyuki Ito, and Tsutomu Hatano*

Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Tsushima, Kita-ku, Okayama 700-8530, Japan

Abstract – Two new products, EGCG-MOx-D5 (**3**) and EGCG-MOx-D6 (**4**), on the oxidation of (-)-epigallocatechin gallate (EGCG) (**1**) in its aqueous solution were isolated and their structures were elucidated. These structures and those of previously established ones suggested the pathways of the changes in the oxidation. Rapid decrease of **1** accompanied by the formations of the oxidative products suggested the participation of these products in the biological and pharmacological effects reported to be those of **1**.

INTRODUCTION

(-)-Epigallocatechin gallate (EGCG) (**1**) is a major tea constituent and has been found to have various pharmacological effects such as inhibitory effects on tumor promotion,[1] and also on the antibiotic resistance shown by methicillin-resistant *Staphylococcus aureus* (MRSA).[2] However, this compound is labile in neutral solution. We investigated on its structural changes in solution, and identify the structures of some monomeric [(-)-gallocatechin gallate and products named EGCG-MOx-Ms] and dimeric products [theasinensins and EGCG-MOx-Ds].[3-5] The decrease of **1** in solution was suppressed in the presence of a metal-chelating agent, and the dependence of the changes on metal ions was also seen.[3] Further investigation on the structural changes of **1** lead to the isolation of two further new compounds, which is described in this paper. Rapid decrease of EGCG (**1)** accompanied by the formations of the oxidative products suggested the participation of these products in the biological and pharmacological effects reported to be those of EGCG (**1)**, and this means the importance of the structural studies on these products. Actually, the effectiveness of theasinensin A (**2**), the representative product from **1**, on MRSA was indicative for the participation of such products on the reported effect of **1**.[3]

† This paper is dedicated to Prof. Dr. Albert Eschenmoser, on the occasion of his 85th birthday.

RESULTS AND DISCUSSION

EGCG (**1**) was kept in an aqueous solution of pH 7.0 at 35 °C, and the products were separated by column chromatography on MCI-gel CHP-20P and then that on YMC-gel ODS AQ120-50S or on Sephadex LH-20, to give **3** and **4** (Figure 1), in addition to those described in previous reports.[3-5]

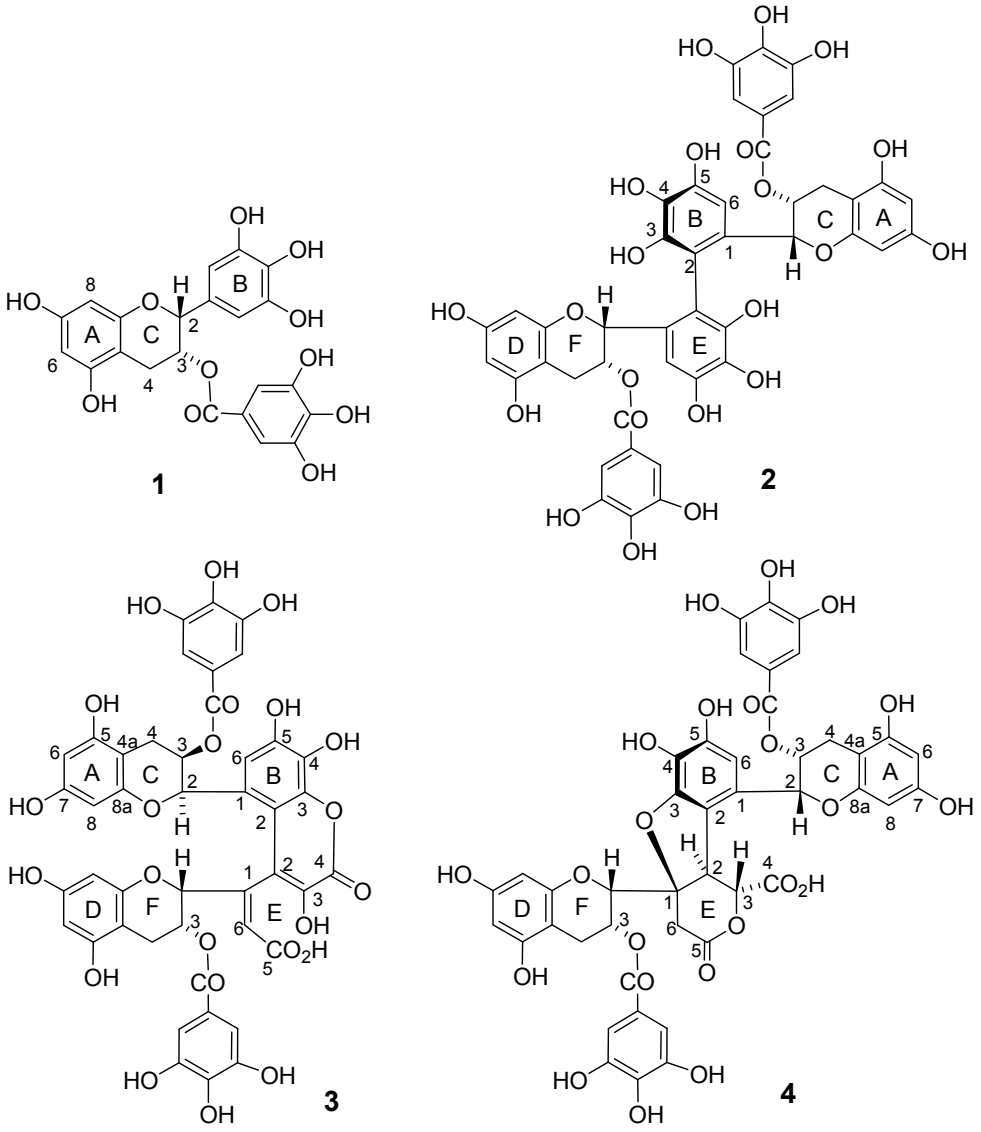


Figure 1. Structural formulas of **1**-**4**

Compound **3** was obtained as a light-brown amorphous powder. The high-resolution electrospray-ionization mass spectrometry (HR-ESI-MS) indicated its molecular formula $C_{44}H_{32}O_{23}$ by the $[M+H]^+$ ion peak. The ^1H NMR spectrum (600 MHz, in acetone-d_6 containing D_2O) showed two sets of *m*-coupled doublets [δ 5.87, 5.90 (1H each, d, J = 2.5 Hz; H-8 and H-6 of A-ring in **3**); δ 5.98, 6.01 (1H each, d, J = 2.5 Hz; H-6 and H-8 of D-ring in **3**)] derived from A-ring of **1**, two sets of four protons [δ 4.80 (1H, br s), 5.71 (1H, br m), 2.77 (1H, br d, J = 18 Hz), 2.82 (1H, dd, J = 18, 5 Hz) (H-2, H-3,

H-4a and H-4b of C-ring in **3**); δ 4.99 (1H, br s), 5.46 (1H, br m) , 3.07 (1H, br d, *J* = 18 Hz), 2.83 (1H, dd, *J* = 18, 5 Hz) (H-2, H-3, H-4a and H-4b of F-ring in **3**)] derived from C-ring of **1**, and two 2H singlets [δ 7.03 (galloyl at O-3 in C-ring of **3**) and 7.15 (galloyl at O-3 in F-ring of **3**)] due to galloyl groups of two molecules of **1**. In addition to these signals, **3** showed two 1H singlets at δ 6.82 and δ 7.74. The one at δ 6.82 is attributable to B-ring H-6 in **3** (which is derived from B-ring of **1**), while the remaining one appeared in the lower field suggested some structural change of B-ring of the other molecule of **1** (i.e., E-residue in **3**). The ^{1}H-^{1}H correlation spectroscopy (COSY) showed the long-range coupling between the signals of δ 4.80 and δ 6.82, substantiating the connectivity of B-C rings and respective assignments of C-ring H-2 and B-ring H-6. However, the signal at δ 7.74 (E-residue H-6) did not show such correlation on this COSY spectrum.

The ^{13}C NMR spectrum showed carbon signals of A-/D-rings, C-/F-rings, B-ring, and two galloyl groups, derived from two molecules of **1** (See Experimental). In addition to these signals, six carbon signals attributed to E-residue were observed at δ_C 109.5, 122.6, 154.8, 155.0, 163.5, and 164.4. Among them, one at δ_C 109.5 was attributed to C-6, and this assignment was supported by the corresponding cross peak (with E-residue H-6) in the HSQC spectrum. Two of the remaining five quaternary carbon signals at δ 163.5 and 164.4 were due to carbons of ester carbonyl/carboxyl groups (E-residue C-4 and C-5). The HMBC spectrum of **3** showed correlations of F-ring H-2 with three carbon signals at δ_C 154.8 (C-1), 122.6 (C-2), 109.5 (C-6) of E-residue, and also those of E-residue H-6 with four carbon signals at δ_C 75.7 (F-ring C-2), and at δ_C 154.8 (C-1), 122.6 (C-2), and 164.4 (C-5), indicating the sequence of the E-residue carbons (Figure 2).

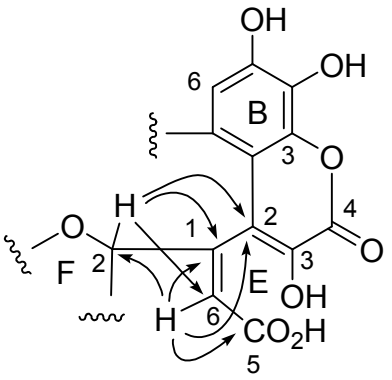


Figure 2. Partial structure of **3** and key HMBC correlations
Two and three bond H → C correlations were shown by the arrows

The molecular formula indicated by the mass spectrometry required one of the two carboxyl groups in E-residue is esterified with a hydroxyl group. Indeed, B-ring C-3 (δ_C 143.1) shifted upfield relative to the corresponding carbon of **2** (δ_C 144.8). Therefore, the structure where the carboxyl group C-4 forms a lactone with the hydroxyl group at B-ring C-3 (Figure 2) was assigned to this compound.

Compound **4** was obtained as a light-brown amorphous powder. The HR-ESI-MS indicated its molecular formula $C_{44}H_{34}O_{23}$ by the $[M + NH_4]^+$ ion peak, corresponding to the dimeric nature among the products from **1**. The ^{1}H NMR spectrum showed signals of A-/D-ring protons [δ 5.87 (1H, d, *J* = 2.5 Hz), 5.94-5.95 (3H in total, br s)], C-/F-ring protons [δ 5.41 (1H, br s, H-2), 5.91 (1H, br, H-3), 2.92 (1H, br d,

J = 17.5 Hz, H-4a), 3.36 (1H, dd, *J* = 17.5, 4 Hz, H-4b) (C-ring); δ 4.26 (1H, br s, H-2), 5.67 (1H, br, H-3), 2.81 (1H, dd, *J* = 18, 4 Hz, H-4a), 3.05 (1H, br d, *J* = 18 Hz, H-4b) (F-ring)], and protons of two galloyl groups [δ 7.11 and 7.13 (2H each, s)]. In addition to these signals, the signals of an aromatic methine proton at δ 6.92 (1H, s, B-ring H-6), two methine protons which coupled with each other [δ 4.68 (1H, d, *J* = 4 Hz, E-residue H-2) and 5.07 (1H, d, *J* = 4 Hz, E-residue H-3)], and a set of methylene proton signals [δ 3.19 (1H, br d, *J* = 16 Hz) and 3.5 (overlapped with the water signal) (E-residue H-6a and H-6b)] were also shown. The presence of the latter proton on the methylene carbon was supported by the cross peak between these two protons in the ^{1}H-^{1}H COSY. These signals, attributed to B-ring and E-residue in formula **4**, were regarded to be those derived from B-rings of two molecules of **1**. The COSY spectrum also showed the presence of the long-range coupling between B-ring H-6 (δ 6.92) and C-ring H-2 (δ 5.41), indicating the connectivity of the B-C rings. The ^{13}C NMR spectrum showed six carbon signals attributable to part E, in addition to the carbon signals of A-/D-rings, C-/F-rings, B-ring and two galloyl groups (see Experimental). The chemical shifts and the HSQC spectrum indicated that E-residue involves one methylene [δ_C 37.0 (bearing H-6a and H-6b) (C-6)], two methine [δ_C 48.5 (bearing H-2 at δ 4.68) (C-2), δ_C 79.8 (bearing H-3 at δ 5.07) (C-3)], one a quaternary bearing an oxygen [δ_C 90.7 (C-1)], and two of carboxyl/ester carbonyl [δ_C 171.5 (C-4) and 172.0 (C-5)] carbons. These assignments were substantiated by the following correlations observed by the HMBC measurement where the long-range J_{CH} value was set at 5 Hz (see Figure 3): F-ring H-2 with C-1, C-2, C-6 (E-residue) [and a four-bond correlation with C-3 (E-residue)]; E-residue H-2 with C-1, C-2, C-3 (B-ring), C-1, C-3 (E-residue), and C-2 (F-ring); E-residue H-3 with C-1, C-2, C-4 (E-residue), and C-2 (B-ring); E-residue H-6 with C-2 (F-ring), C-1, C-2, and C-5 (E-residue) [and a four-bond correlation with C-3 (E-residue)].

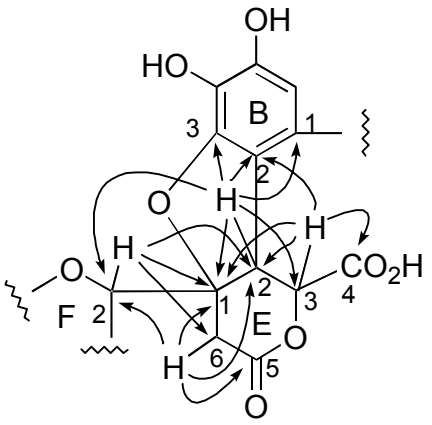
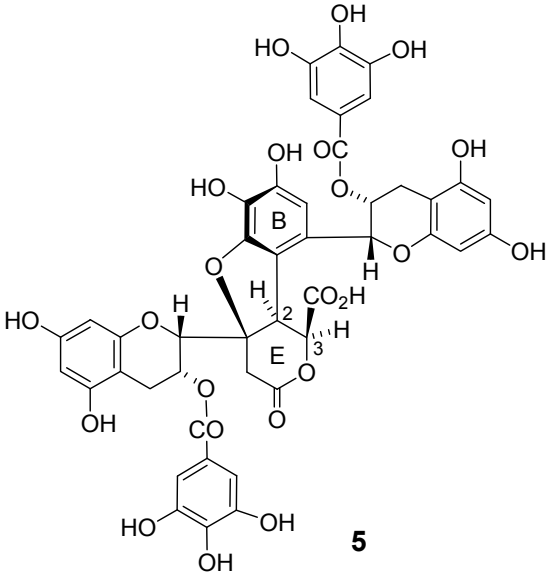


Figure 3. Partial structure of **4** and key HMBC correlations
Two and three bond H→C correlations were shown by the arrows



5

Figure 4. Structural formula of **5**

The molecular formula shown by the HR-ESI-MS was the same as that of EGCG-MOx-D1 (**5**)[4] (Figure 4), and the ^{13}C NMR spectral data was also closely similar to those for **5**.[4] Considering this, the similarity in the pattern of the short-wavelength region in the CD spectrum of **4** ($[\theta]_{224}$ –3.4 x 10^4, $[\theta]_{208}$ +1.9 x 10^4) to that of **5** ($[\theta]_{222}$ –2.2 x 10^4, $[\theta]_{208}$ +1.1 x 10^4)[5] suggested that the stereochemical relationship concerning the B/E moieties for **4** is the same as that in the case of **5**. While, the coupling constant H-2 – H-3 of the E-residue in **4** (4 Hz) was different from the corresponding one in **5** (6 Hz). The structure isomeric to **5** concerning E-residue C-3 was thus assigned for **4**.

These structures and those of previously reported ones indicated structural diversity of the products formed on the oxidation of the pyrogallol ring of **1** and its dimeric products, and suggested the pathway of the changes in the oxidation process.[5] The plausible relationship of compounds **3** and **4** with **1** may be shown by Figure 5.

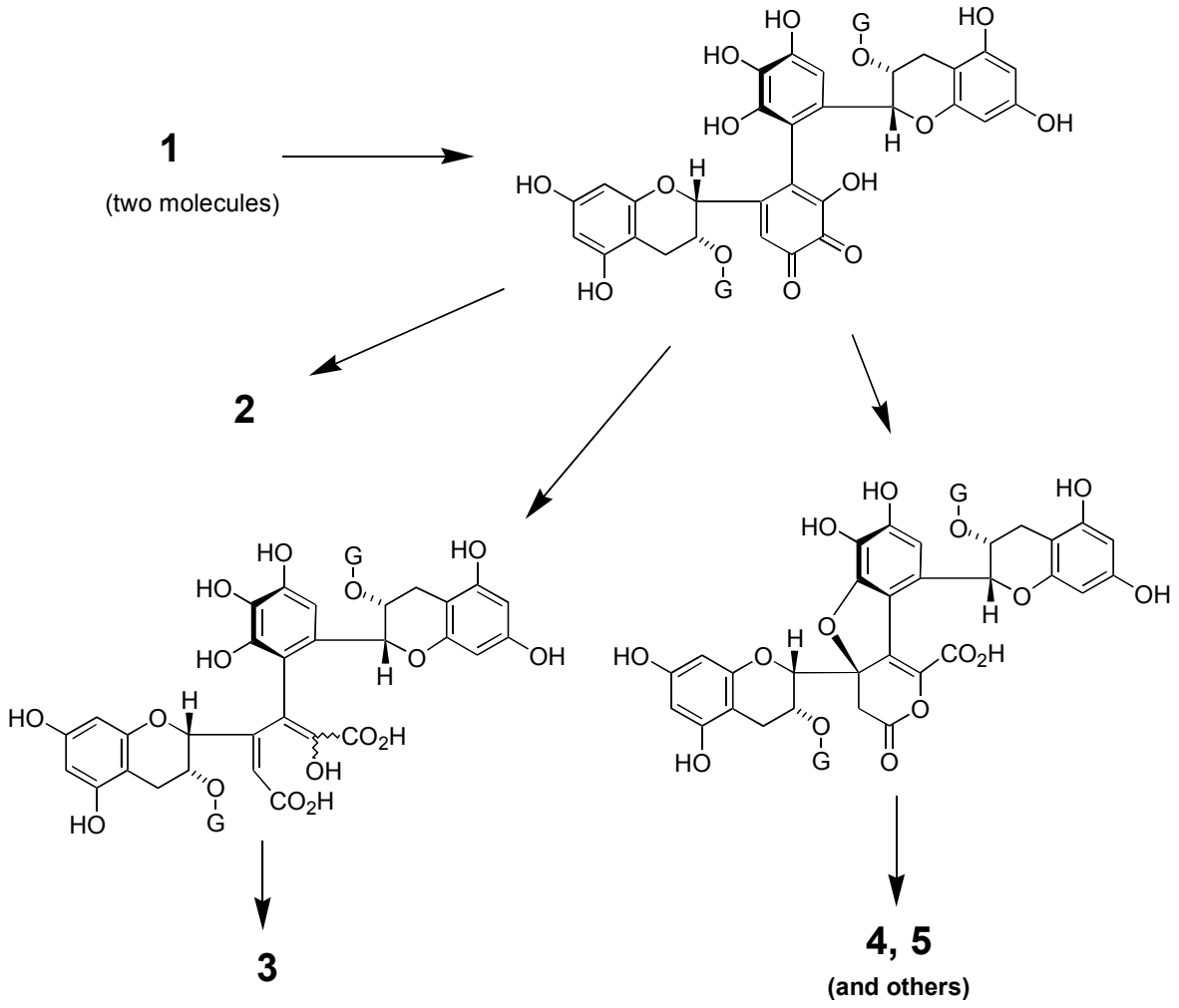


Figure 5. Plausible relationship of oxidative products from **1** (G = galloyl)

Occurrence of analogous structural changes was also suggested by structures of several hydrolyzable tannins such as chebulagic acid,[6] which were considered to be oxidative products of dehydroellagitannins.[7] Therefore, the findings on the changes due to oxidation of the pyrogallol moiety of EGCG (**1**) and its dimeric products may be applicable to the consideration of the oxidation pathway in the plants producing hydrolyzable tannins.

EXPERIMENTAL

Specific rotations were recorded on a JASCO DIP-1000 digital polarimeter. CD spectra were measured on a JASCO J-720W spectrophotometer. ESIMS were performed using Micromass Auto Spec OA-Tof spectrophotometer with positive-ion mode with a solvent of 50% MeOH + 0.1% $AcONH_4$. The ^1H and ^{13}C NMR spectra were recorded on a Varian INOVA AS 600 spectrophotometer (600 MHz for ^1H, and 150.7 MHz for ^{13}C) at 300 K. Chemical shifts are given in δ (ppm) values relative to that of the solvent signal [acetone-d_6 (δ_H 2.04; δ_C 29.8)] on the tetramethylsilane scale.

Materials

EGCG (**1**) used in this study was isolated from *Camellia japonica* leaves,[3] and kept in a sealed tube. The purity which was estimated using HPLC was over 99%.[3]

Isolation of products 3 and 4 from 1 at pH 7.0 in a phosphate buffer

EGCG (**1**) (1.0 g) dissolved in 0.1 M H_3PO_4 – 0.1 M KH_2PO_4 buffer (pH 7.0) (1.0 L) was kept at 35 °C for 20 h.[8] The solution was acidified to pH 4.0 with 1M HCl, and subjected to column chromatography on MCI-gel CHP-20P with increasing concentration of MeOH in water as eluants. The eluate with 20% MeOH (115 mg) was then chromatographed on YMC-gel AQ120-50S with aq. MeOH to give **3** (3.4 mg). The eluate with 30% MeOH (94 mg) from the MCI-gel column was chromatographed on Sephadex LH-20 with increasing concentrations of MeOH in EtOH to give **4** (6.8 mg).

EGCG-MOx-D5 (3). A light-brown amorphous powder, $[\alpha]_D^{20}$ –106.9 (*c* 0.29, MeOH). ESI-MS *m/z*: 929 $[M + H]^+$, 951 $[M + Na]^+$, 967 $[M + K]^+$. HRESIMS m/z: 929.1394 $[M + H]^+$ (calcd. for $C_{44}H_{32}O_{23}$ + H, 929.1413). UV λ_{max} (MeOH) nm (log ε): 275 (4.37). CD (in MeOH): $[\theta]_{280}$ –1.9 x 10^4, $[\theta]_{240}$ +1.1 x 10^3, $[\theta]_{219}$ –6.4 x 10^4). ^1H NMR: see text. ^{13}C NMR (acetone-d_6 + D_2O; 9:1): δ 26.1 (F-ring C-4), 27.2 (C-ring C-4), 68.3 (C-ring C-3), 69.1 (F-ring C-3), 75.7 (F-ring C-2), 75.8 (C-ring C-2), 95.46 (D-ring C-8), 95.53 (A-ring C-8), 96.4 (A-ring C-6), 96.7 (D-ring C-6), 97.9 (D-ring C-4a), 98.6 (A-ring C-4a), 108.3 (B-ring C-6), 109.5 (E-residue C-6), 110.0 (2 x C, C-2 and C-6 of galloyl at C-ring O-3), 110.1 (2 x C, C-2 and C-6 of galloyl at F-ring O-3), 111.8 (B-ring C-2), 120.9 (C-1 of galloyl at F-ring O-3), 121.5

(C-1 of galloyl at C-ring O-3), 122.6 (E-residue C-2), 130.5 (B-ring C-1), 133.4 (B-ring C-4), 138.8 (C-4 of galloyl at C-ring O-3), 138.9 (C-4 of galloyl at F-ring O-3), 143.1 (B-ring C-3), 145.8 (2 x C, C-3 and C-5 of galloyl at C-ring O-3), 145.9 (2 x C, C-3 and C-5 of galloyl at F-ring O-3), 146.4 (B-ring C-5), 154.8 (E-residue C-1), 155.0 (E-residue C-3), 155.8, 157.0, 157.1, 157.26, 157.30, 157.6 (A- and D-rings C-5, C-7, and C-8a), 163.5 (E-residue C-4), 164.4 (E-residue C-5), 166.4 (C-7 of galloyl at C-ring O-3), 167.0 (C-7 of galloyl at F-ring O-3).

EGCG-MOx-D6 (4). A light-brown amorphous powder, $[\alpha]_D^{20}$ –41.0 (*c* 0.61, MeOH). ESI-MS *m/z*: 931 $[M + H]^+$, 953 $[M + Na]^+$, 969 $[M + K]^+$. HRESIMS m/z: 948.1842 $[M + NH_4]^+$ (calcd. for $C_{44}H_{34}O_{23}$ + NH_4, 948.1835). UV λ_{max} (MeOH) nm (log ε): 277 (4.38); CD (MeOH): $[\theta]_{286}$ –1.0 x 10^4, $[\theta]_{242}$ +7.8 x 10^3, $[\theta]_{224}$ –3.4 x 10^4, $[\theta]_{208}$ +1.9 x 10^4. ^{1}H NMR: see text. ^{13}C NMR (acetone-d_6 + D_2O; 9:1): δ 26.4 (F-ring C-4), 26.8 (C-ring C-4), 37.0 (E-residue C-6), 48.6 (E-residue C-2), 66.0 (F-ring C-3), 70.0 (C-ring C-3), 75.1 (C-ring C-2), 78.6 (F-ring C-2), 79.8 (E-residue C-3), 90.7 (E-residue C-1), 95.5 (A-ring C-6), 95.6, 96.4, 97.8 (A-ring C-6; D-ring C-6 and C-8), 98.3 (D-ring C-4a), 99.3 (A-ring C-4a), 109.9 (2 x C, C-2 and C-6 of galloyl at F-ring O-3), 110.0 (2 x C, C-2 and C-6 of galloyl at C-ring O-3), 110.4 (B-ring C-6), 116.7 (B-ring C-2), 120.8 (C-1 of galloyl at F-ring O-3), 121.6 (C-1 of galloyl at C-ring O-3), 128.7 (B-ring C-1), 129.8 (B-ring C-4), 139.0 (C-4 of galloyl at C-ring O-3), 139.3 (C-4 of galloyl at F-ring O-3), 145.9 (2 x C, C-3 and C-5 of galloyl at C-ring O-3), 146.2 (2 x C, C-3 and C-5 of galloyl at F-ring O-3), 147.0 (B-ring C-5), 148.0 (B-ring C-3), 155.8, 157.33, 157.5 (D-ring C-5, C-7, and C-8a), 157.1, 157.30 (2 x C) (A-ring C-5, C-7, and C-8a), 166.4 (C-7 of galloyl at F-ring O-3), 167.2 (C-7 of galloyl at C-ring O-3), 171.5 (E-residue C-4), 172.0 (E-residue C-5).

ACKNOWLEDGMENTS

The NMR instrument used in this study is the property of the SC-NMR laboratory of Okayama University.

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8. Although the major products of this reaction with a shorter incubation time were theasinensins A and D as reported previously,[3] prolonged treatment gave various products including **3** and **4**.