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Enhanced anti-influenza A virus activity of (−)-epigallocatechin-3-*O*-gallate fatty acid monoester derivatives: Effect of alkyl chain length

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Abstract—A series of fatty acid monoester derivatives of (−)-epigallocatechin-3-*O*-gallate (EGCG) were prepared by one-pot lipase-catalyzed transesterification. The introduction of long alkyl chains enhanced anti-influenza A/PR8/34 (H1N1) virus activity 24-fold relative to native EGCG.

A major polyphenol component of green tea, (−)-epigallocatechin-3-*O*-gallate (EGCG; **1**), has received much attention due to its various biological activities such as antiviral,[1] antimicrobial,[2] and anticancer.[3] In particular, the anti-influenza activity of **1** has been investigated by several groups;[1a–c] the results show that **1** has the most potent antiviral activity among tea polyphenols. However, relatively high concentrations of **1** were required to observe significant antiviral activity, probably due to the compound's poor lipid membrane permeability,[4] low chemical stability,[5] and rapid metabolism.[5]

Several strategies for increasing these biological properties have been investigated. For example, the introduction of alcoxyl groups to **1** improved lipid-membrane permeability[4] and metabolic stability,[6] and peracetylation of **1** (Fig. 1) increased its chemical stability under physiological conditions.[7] Furthermore, the elimination of hydroxyl groups from the A-ring of **1** moderately enhanced anti-influenza virus activity.[8]

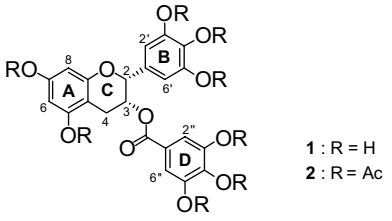

Figure 1. Structure of EGCG (**1**) and EGCG-peracetate (**2**).

We hypothesized that the introduction of straight-chain fatty acids to the phenolic hydroxyl groups of **1** would further enhance its anti-influenza virus activity.

A series of EGCG fatty acid monoester derivatives (**3**–**7**) were prepared by lipase-catalyzed transesterification (Scheme 1). EGCG-monoesters modified with butanoyl, octanoyl, lauroyl, palmitoyl, and eicosanoyl groups are represented as EGCG-C4, EGCG-C8, EGCG-C12, EGCG-C16, and EGCG-C20, respectively. Although **1** has been reported to interfere with the catalytic activity of lipases,[9] we succeeded in preparing **3**–**7** by lipase-catalyzed transesterification in polar organic solvents such as *N*,*N*-dimethylformamide or acetonitrile.[10,11] In contrast, the transesterification did not proceed in nonpolar organic solvents such as tetrahydrofuran or diisopropylether. The key to success with this approach might be

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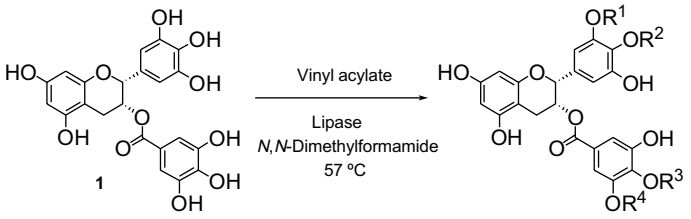

3a : $R^2=R^3=R^4=H$, $R^1=CO(CH)_2CH_3$
3b : $R^1=R^3=R^4=H$, $R^2=CO(CH)_2CH_3$
3c : $R^1=R^2=R^4=H$, $R^3=CO(CH)_2CH_3$
3d : $R^1=R^2=R^3=H$, $R^4=CO(CH)_2CH_3$

4a : $R^2=R^3=R^4=H$, $R^1=CO(CH)_6CH_3$
4b : $R^1=R^3=R^4=H$, $R^2=CO(CH)_6CH_3$
4c : $R^1=R^2=R^4=H$, $R^3=CO(CH)_6CH_3$
4d : $R^1=R^2=R^3=H$, $R^4=CO(CH)_6CH_3$

5a : $R^2=R^3=R^4=H$, $R^1=CO(CH)_{10}CH_3$
5b : $R^1=R^3=R^4=H$, $R^2=CO(CH)_{10}CH_3$
5c : $R^1=R^2=R^4=H$, $R^3=CO(CH)_{10}CH_3$
5d : $R^1=R^2=R^3=H$, $R^4=CO(CH)_{10}CH_3$

6a : $R^2=R^3=R^4=H$, $R^1=CO(CH)_{14}CH_3$
6b : $R^1=R^3=R^4=H$, $R^2=CO(CH)_{14}CH_3$
6c : $R^1=R^2=R^4=H$, $R^3=CO(CH)_{14}CH_3$
6d : $R^1=R^2=R^3=H$, $R^4=CO(CH)_{14}CH_3$

7a : $R^2=R^3=R^4=H$, $R^1=CO(CH)_{18}CH_3$
7b : $R^1=R^3=R^4=H$, $R^2=CO(CH)_{18}CH_3$
7c : $R^1=R^2=R^4=H$, $R^3=CO(CH)_{18}CH_3$
7d : $R^1=R^2=R^3=H$, $R^4=CO(CH)_{18}CH_3$

Scheme 1. Preparation of EGCG monoester derivatives by lipase-catalyzed transesterifications.

enhancement of the conformational flexibility of the lipase and elimination of nonspecific interactions between the lipase and **1** in polar organic solvents. Our lipase method affords **3**–**7** in 35–39% yield, whereas a conventional chemical method[12] affords them in less than 23% yield. As shown in Scheme 1, EGCG-monoesters (**3**–**7**) are composed of a mixture of four regioisomers, each with acyl groups at either the *meta*-position or *para*-position of the B- or D-ring. We confirmed the ratio of each EGCG-monoester regioisomer by ^1H NMR spectroscopy; the results are summarized in Table 1. Interestingly, this lipase-catalyzed method afforded B-ring modified esters as the major products, whereas the conventional chemical method afforded D-ring modified esters as the major products. The purification of each regioisomer was not accomplished as they have very similar chemical properties. However, the lipase method afforded EGCG-monoesters in a consistent ratio of regioisomers, regardless of the alkyl chain length (Table 1).

We investigated the antiviral protective effects of **1**, EGCG-peracetate (**2**),[13] and EGCG-monoesters (**3**–**7**) against influenza A/PR8/34 (H1N1) infection in MDCK cells. Briefly, a monolayer of MDCK cells was transfected with **1**–**7** 2 h prior to infection with the virus. The cell monolayer was rinsed to remove remaining EGCG derivative in the cell culture medium, then the virus was introduced. The antiviral activities of each sample were assessed by the plaque formation assay.[14]

Table 1. Mixture ratio of regioisomers in EGCG monoester derivatives prepared by lipase-catalyzed transesterifications

Compound	Regioisomer ratio		B/D[a]
EGCG-C4	3a:3b:3c:3d	35:44:8:12	79/21
EGCG-C8	4a:4b:4c:4d	35:39:6:20	74/26
EGCG-C12	5a:5b:5c:5d	30:39:9:22	69/31
EGCG-C16	6a:6b:6c:6d	38:35:7:20	73/27
EGCG-C20	7a:7b:7c:7d	38:36:8:19	73/27
$EGCG\text{-}C16_{Chem}$[b]	6a:6b:6c:6d	20:18:18:44	38/62

[a] Ratio of B- and D-ring modified EGCG monoester derivatives.
[b] Prepared by a conventional chemical method.

All compounds (**1**–**7**) inhibited virus infection in a dose-dependent manner (Fig. 2). The EC_{50} values of each compound are summarized in Table 2 (cell-based antiviral effect) and show that the antiviral activities of EGCG-monoesters were enhanced in an alkyl chain length-dependent manner. In particular, the EC_{50} of EGCG-C16 was approximately 4 μM and its inhibitory effect was 24-fold higher than **1**. This remarkable enhancement in antiviral activity can be attributed to the high efficiency of cellar uptake of EGCG-C16 as a result of its improved cell membrane permeability. In contrast, EGCG-C20 and EGCG-peracetate exhibited lower antiviral activities compared to EGCG-C16, presumably due to decreased water solubility resulting from their increased hydrophobicity.

The antiviral activity of EGCG-monoesters may vary depending on the position of the acyl groups. Therefore we prepared mixtures of EGCG-monopalmitate, composed of four regioisomers (**6a**–**d**) in different proportions, by both the lipase-catalyzed (EGCG-C16) and

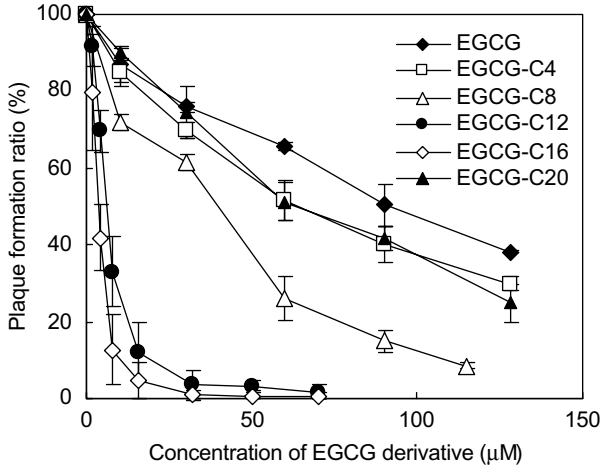

Figure 2. Inhibitory effects of EGCG monoester derivatives prepared by lipase-catalyzed transesterification on plaque formation in the cell based inhibition assay. Each data point represents the mean ± SD from at least three independent experiments.

Table 2. Anti-influenza effects and cytotoxicities of EGCG derivatives

Entry	CC_{50}[a] (μM)	Cell-based antiviral effect		Direct antiviral effect	
		EC_{50}[b] (μM)	SI[c]	EC_{50}[b] (μM)	SI[c]
EGCG	275.0 (±6.0)	94.60 (±11.10)	2.91	0.3910 (±0.0560)	703
EGCG-C4	309.0 (±4.0)	63.70 (±10.10)	4.85	0.8320 (±0.0910)	363
EGCG-C8	195.0 (±9.0)	39.00 (±3.10)	5.01	0.6200 (±0.0910)	276
EGCG-C12	42.0 (±3.9)	5.81 (±0.87)	7.23	0.1180 (±0.0230)	353
EGCG-C16	86.2 (±12.5)	4.02 (±0.48)	21.40	0.0204 (±0.0069)	4230
EGCG-C20	318.0 (±55.0)	66.90 (±8.30)	4.75	2.2500 (±0.1900)	127
EGCG-peracetate	ND[d]	77.00 (±12.60)	—	8.4900 (±3.3500)	—

[a] CC_{50} represents the concentration of compound required to reduce cell viability by 50% relative to the control well without test compound.
[b] EC_{50} represents the concentration of compound required to reduce plaque number by 50% relative to the control well without test compound.
[c] SI (Selectivity index) is the ratio of CC_{50} to EC_{50}.
[d] CC_{50} of EGCG-peracetate was not determined due to its low water solubility.

conventional chemical methods (EGCG-$C16_{Chem}$). The antiviral activities of these two sets of products were examined. However, no distinct difference in antiviral activity was apparent (EC_{50} of EGCG-$C16_{Chem}$ = 4.12 μM). This result suggested that the position of the acyl groups of EGCG derivatives does not affect their antiviral activities.

The cytotoxicities of each EGCG derivative to MDCK cells, given as CC_{50}, are summarized in Table 2.[15] EGCG-C12 and EGCG-C16 exhibited relatively higher cytotoxicities than other derivatives. However, their antiviral activities were significantly enhanced and thereby resulted in increased selectivity index values.

In addition, we studied the direct interaction between EGCG derivatives and influenza viral particles.[16] Each EGCG derivative was incubated with influenza virus solution for 30 min prior to infection, followed by the post-inoculation procedure described above. All compounds inhibited virus infection at much lower concentrations compared to the CC_{50} and the EC_{50} obtained from the cell-based antiviral assay (Table 2, direct antiviral effect). Interestingly, EGCG-C12 and -C16 also exhibited remarkable enhancement of their direct antiviral effect in a manner similar to their cell-based antiviral effect. To clarify if these enhanced antiviral effects were simply brought about by their detergent effects, we investigated the antiviral effect of conventional non-ionic detergents, *n*-dodecyl-β-D-maltoside and sorbitan monopalmitate. However, both detergents did not exhibit apparent direct antiviral effects up to 10 μM (data not shown). A possible mechanism to explain the enhanced antiviral effect of EGCG derivatives is that the acyl portion increases the accessibility of EGCG to the viral membrane as well as the cell membrane.

In conclusion, we prepared a series of EGCG fatty acid monoester derivatives using lipase-catalyzed transesterification and demonstrated that the introduction of long acyl groups, such as lauroyl or palmitoyl, to EGCG drastically enhanced its anti-influenza virus activity. Our simple and robust methodology should expand the utility of EGCG, an abundant natural tea ingredient, as a novel anti-influenza agent.

Acknowledgment

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10. Synthesis of EGCG monoester derivatives by lipase-catalyzed transesterification: as an example, experimental

procedure for synthesis of EGCG-C16 is described here. Lipase PL from *Alcaligenes* sp. (500 mg) was added to 100 mL *N*,*N*-dimethylformamide solution including **1** (1 g, 2.18 mmol) and vinyl palmitate (0.925 g, 3.27 mmol). This mixture was stirred for 1.5 h at 57 °C. After filtration of reaction mixture, the solvent was removed by vacuum pump and the residue was purified by silica gel column chromatography to give the mixture of EGCG-monopalmitate regioisomers (EGCG-C16). ^{1}H NMR (400 MHz, CD_3CN) δ 0.88 (3H, t, J = 6.8 Hz, –$COCH_2CH_2(CH_2)_{12}CH_3$ of **6a**–**6d**), 1.27 (26H, m, –$COCH_2CH_2(CH_2)_{12}CH_3$ of **6a**–**6d**), 1.67 (2H, tt, J = 14.6, 7.9 Hz, –$COCH_2CH_2(CH_2)_{12}CH_3$ of **6a**–**6d**), 2.58 (2H, m, –$COCH_2CH_2(CH_2)_{12}CH_3$ of **6a**–**6d**), 2.82 (1H, m, H-4 of **6a**–**6d**), 2.97 (1H, m, H-4 of **6a**–**6d**), 5.03 (1H, m, H-2 of **6a**–**6d**), 5.49 (1H, m, H-3 of **6a**–**6d**), 5.98 (2H, m, H-6 and H-8 of **6a**–**6d**), 6.49 (0.4H, s, 2′ and 6′ of **6d**), 6.50 (0.14H, s, 2′ and 6′ of **6c**), 6.56 (0.7H, s, 2′ and 6′ of **6b**), 6.74 (0.38H, d, J = 2.04 Hz, 2′ or 6′ of **6a**), 6.82 (0.38H, d, J = 1.96 Hz, 2′ or 6′ of **6a**), 6.82–6.6.70 (7H, br s, –O*H*), 6.88 (0.76H, s, 2″ and 6″ of **6a**), 6.90 (0.7H, s, 2″ and 6″ of **6b**), 6.92 (0.14H, s, 2″ and 6″ of **6c**), 7.09 (0.2H, d, J = 1.96 Hz, 2″ or 6″ of **6d**), 7.21 (0.2H, d, J = 2.00 Hz, 2″ or 6″ of **6d**); ESI-MS *m*/*z* 719 $C_{38}H_{48}O_{12}Na$ $(M+Na)^+$.

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12. Synthesis of EGCG-C16 by a conventional chemical method: Palmitoyl chloride (216 mg, 0.785 mmol) was dropped in 50 mL tetrahydrofuran including **1** (300 mg, 0.654 mmol) and triethylamine (0.850 mmol). Reaction mixture was stirred for 24 h at 0 °C to room temperature. After removed the solvent, the residue was purified by silica gel column chromatography to give the mixture of EGCG-monopalmitate regioisomers (EGCG-C16).
13. *Synthesis of* **2**: EGCG-peracetate (**2**) was synthesized by pyridine-catalyzed acetylation of EGCG using acetic anhydride according to the following literature: Kohri, T.; Nanjo, F.; Suzuki, M.; Seto, R.; Matsumoto, N.; Yamakawa, M.; Hojo, H.; Hara, Y.; Desai, D.; Amin, S.; Conaway, C. C.; Chung, F.-L. *J. Agric. Food Chem.* **2001**, *49*, 1042.
14. *Evaluation of cell-based antiviral inhibitory effects of EGCG derivatives on influenza A/PR8/34 (H1N1):* Opti-MEM containing 0.2% dimethyl sulfoxide and each sample at different concentration was added to a confluent monolayer Madin–Darby canine kidney (MDCK) cells in a 6-well plate, followed by incubation for 2 h at 37 °C in 5% CO_2. Then, after the solution was removed from each well, the cell sheets were washed by D-PBS and infected with influenza virus (MOI = 2.5×10^{-4}) in DMEM containing 0.2% BSA. After 1 h for virus adsorption at room temperature, the solution was removed and the cell sheets were washed twice with D-PBS. Then, overlay medium (DMEM containing 0.8% Oxoid agar No. 1, 6.0×10^{-4}% trypsin, and 0.2% BSA) was added to each well. After incubating for about 2 days at 37 °C in 5% CO_2, the cell sheets were fixed with 5% glutaraldehyde and the overlay agarose was removed. Then, the cell sheets were stained with methylene blue solution. Plaques formed on each well were counted and the inhibitory effects of each sample on virus infection were evaluated.
15. *Evaluation of cytotoxicities of EGCG derivatives on MDCK cells:* MDCK cells (1.5×10^4 cells/well) were cultured in a 96-well plate for 4–5 h. Opti-MEM containing each sample at different concentration was added to each well and the cell cultures were incubated for 2 h. After sample solutions were removed and the cell cultures were washed with D-PBS, DMEM containing 0.2% BSA was added to each well. The cell cultures were incubated for 24 h at 37 °C in 5% CO_2. Then, after the solution was removed and the cell cultures were washed with D-PBS, 100 μL MTT solution was added to each well. After incubation for 1 h, the optical densities at 490 nm of each well were measured by 96-well plate reader.
16. *Evaluation of direct antiviral inhibitory effects of EGCG derivatives on influenza A/PR8/34 (H1N1):* Each EGCG derivative was mixed with influenza virus in Opti-MEM containing 0.2% DMSO. After incubating for 30 min at room temperature, the mixed solution was applied to confluent monolayer MDCK cells in a 6-well plate (MOI = 2.5×10^{-4}). The following procedure was the same as the cell-based inhibition assay described above.