Bioorganic & Medicinal Chemistry 16 (2008) 7975–7982

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Antiproliferative and apoptosis-inducing activities of alkyl gallate and gallamide derivatives related to (−)-epigallocatechin gallate

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ARTICLE INFO

Article history:
Received 23 June 2008
Revised 22 July 2008
Accepted 23 July 2008
Available online 29 July 2008

Keywords:
Gallate
Gallamide
Catechin
Apoptosis
Caspase

ABSTRACT

Green tea and (−)-epigallocatechin gallate (EGCG: one of the main components of green tea) are reported to have cancer-preventive activity in humans. A previous SAR study of EGCG and derivatives indicated that a galloyl group is essential for the activity. To test this hypothesis, we synthesized various alkyl gallate and gallamide derivatives and evaluated their antiproliferative effects on human leukemia HL-60 cells. Dodecyl 3,4,5-trihydroxybenzoate (**6c**) showed the most potent activity, being more potent than EGCG. To clarify the molecular mechanism of the antiproliferative action, we investigated the effects of **6c** on various factors. Compound **6c** was found to induce apoptosis mediated by endoplasmic reticulum (ER)-stress-related caspase-12. Upregulation of *gadd-153*, an ER-stress marker protein, was also observed. These results indicate that **6c** induced apoptosis via the ER-stress-related pathway.

1. Introduction

Green tea is a Japanese traditional beverage made from the leaves of *Camellia sinensis* (*Theaceae*). Numerous studies have shown that green tea has preventive activity against cancers in various organs in humans,[1,2] but the molecular mechanism of these activities remains unclear. Green tea contains several polyphenolic components, such as catechin, (−)-epicatechin, (−)-epicatechin gallate (ECG), (−)-epigallocatechin (EGC), and (−)-epigallocatechin gallate (EGCG).[3] EGCG, the main constituent of green tea polyphenols,[4] was reported to inhibit the growth of various cancer cell lines.[5] A study on growth inhibition of human lung cancer cell line PC-9 by EGCG and related polyphenolic compounds (Fig. 1) revealed that both ECG and EGCG have strong growth-inhibitory activity.[6] Because ECG and EGCG commonly contain a galloyl group in their structures, we considered that a galloyl group might be important for the antiproliferative activity.

Gallic acid (3,4,5-trihydroxybenzoic acid), obtained by alkaline or acid hydrolysis of tannins, is used in the manufacture of alkyl gallates, which are widely used as antioxidant additives in foods. Thus, gallic acid and its derivatives are consumed by many people. Recent studies have shown that several phenolic food antioxidant additives can accelerate oxidative damage to biomolecules, including DNA, proteins, and carbohydrates, in vitro, despite their antioxidant action toward lipids.[7–9] Aruoma et al. reported a comprehensive analysis of the antioxidant (protection against reactive oxygen species) and prooxidant effects of alkyl gallates.[10] Antimicrobial activities of alkyl gallates have been reported by Kubo

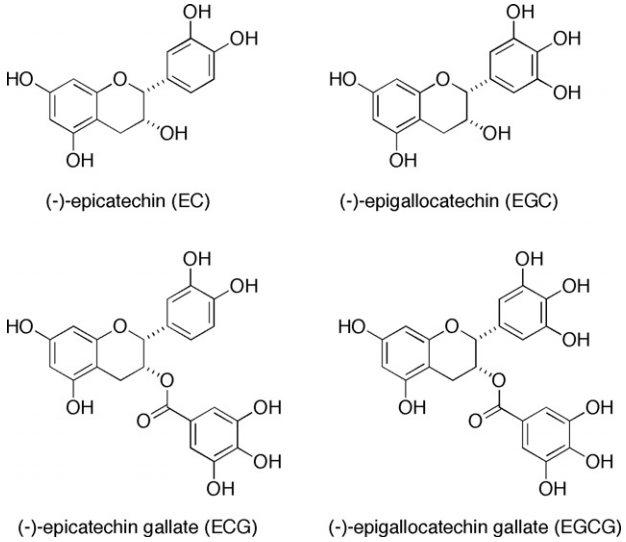

Figure 1. Structures of catechins derived from green tea.

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doi:10.1016/j.bmc.2008.07.063

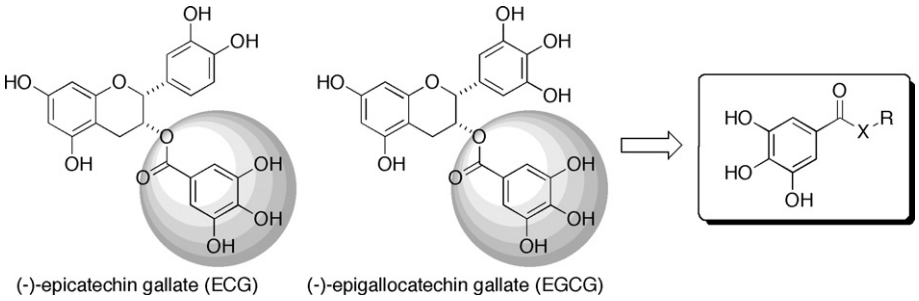
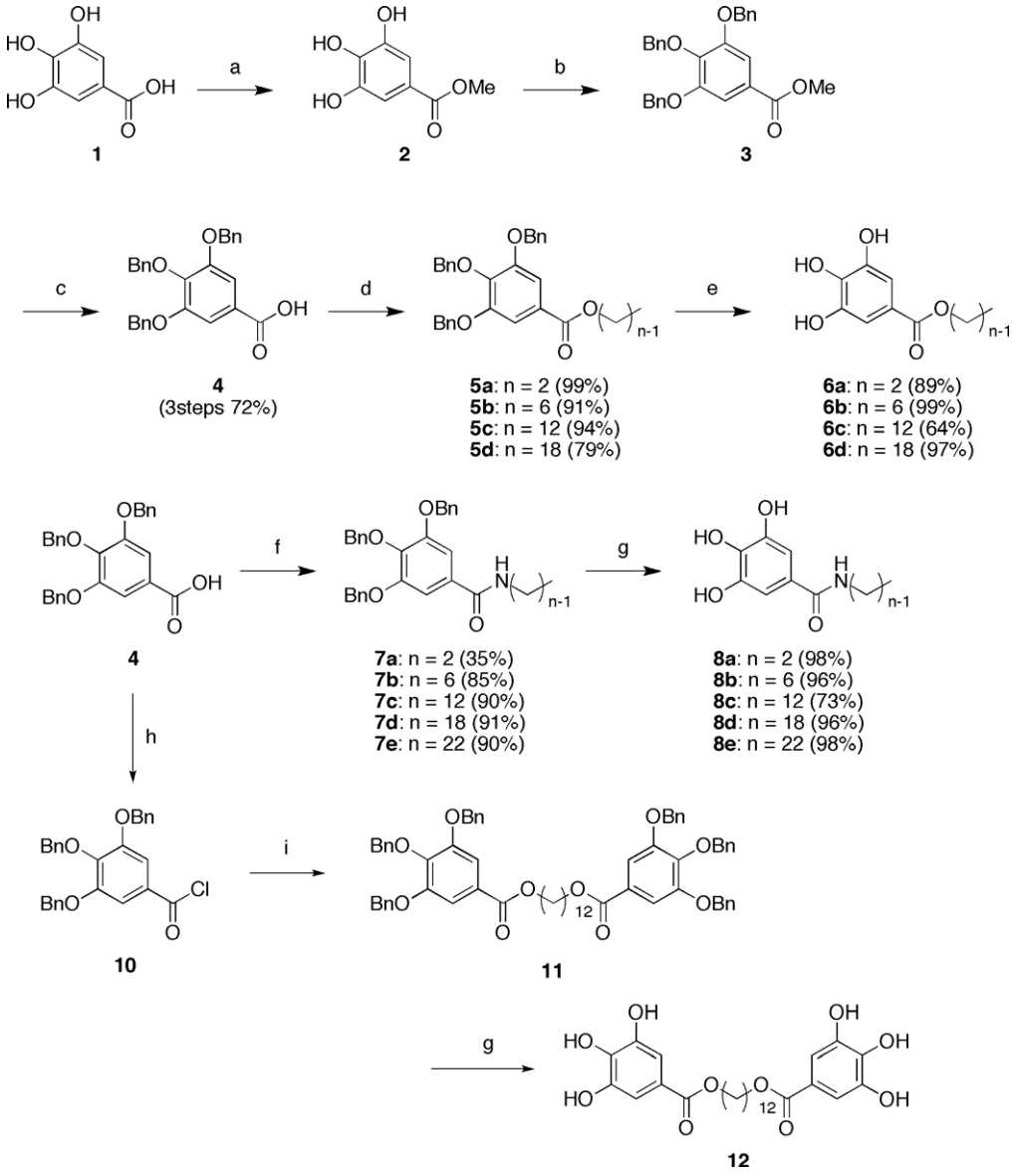


Figure 2. Design of galloyl derivatives.



Scheme 1. Synthesis of alkyl gallate and gallamide derivatives. Reagents and conditions: (a) MeOH, H_2SO_4, 100 °C, 2 h; (b) BnCl, K_2CO_3, DMF, 80 °C, 15 h; (c) NaOH, MeOH/H_2O/dioxane, 120 °C, 6 h; (d) $CH_3(CH_2)_{n-1}X$, K_2CO_3, DMF, rt, overnight; (e) 10% Pd/C, H_2, AcOEt, rt, overnight; (f) $CH_3(CH_2)_{n-1}NH_2$, EDCI, DMAP, CH_2Cl_2 or THF, rt, 1 h; (g) 10% Pd/C, H_2, AcOEt or THF, rt, overnight; (h) ClCOCOCl, DMF, toluene, rt, 0.5 h; (i) $HOC_{12}H_{24}OH$, K_2CO_3, DMF, rt, overnight.

et al., and appear to be related at least in part to the effect of alkyl gallate on redox biofunctions.[11,12] In addition, alkyl gallates influence secretion of various cytokines, including interleukin 4 and interferon γ.[13] Thus, alkyl gallates appear to have a wide range of biological activities. In order to find candidate cancer-preventive compounds containing galloyl moieties, we designed and synthesized various galloyl derivatives (Fig. 2) and evaluated their antiproliferative and apoptosis-inducing activities.

2. Results and discussion

2.1. Chemistry

First, we synthesized alkyl gallates **6a**–**6d** having various lengths of alkyl chain according to Scheme 1. Briefly, gallic acid (**1**) was converted to methyl ester (**2**), and the phenol groups were protected by benzylation (**3**), followed by hydrolysis of the ester group to afford protected gallic acid (**4**). This compound (**4**) was subjected to alkylation with alkyl halides having various lengths of alkyl chain in the presence of K_2CO_3 to give protected alkyl gallates (**5a–5d**), which were deprotected by debenzylation using H_2/Pd–C to afford alkyl gallates **6a**–**6d**. Considering the possibility of intracellular hydrolysis of the ester group, alkyl gallamides **8a**–**8e** having an amide bond instead of the ester bond were also synthesized. As described in Scheme 1, alkyl gallamides having various lengths of alkyl chain were synthesized by condensation of alkyl amine and protected gallic acid (**4**) in the presence of EDC and DMAP to give protected alkyl gallamides (**7a–7e**), which were deprotected by debenzylation using H_2/Pd–C. Dehydroxylated derivatives of **6c** (**9a–9f**) were similarly synthesized. The bis-gallate analog (**12**) was synthesized by condensation of protected gallic acid chloride (**10**: prepared from **4** by treatment with oxalyl chloride in a mixture of toluene and DMF) with 1,12-dodecanediol. The structures of the synthesized compounds were confirmed by NMR and mass spectroscopy.

2.2. Antiproliferative activity toward HL-60 cells

The alkyl gallates **6a**–**6d** and gallamides **8a**–**8e** were tested for antiproliferative activity by calculating the IC_{50} values from the viability data of human leukemia HL-60 cells. As shown in Table 1, alkyl gallate and alkyl gallamide derivatives both showed potent activity compared with EGCG, as expected. Conversion from ester bond to amide bond, which is not cleavable in cells, did not decrease the antiproliferative activity, suggesting that alkyl gallate derivatives showed the activity without hydrolysis by esterase. Interestingly, the optimal length of the alkyl chain was different between gallate and gallamide derivatives. The C_{12} (**6c**) and C_{18} (**8d**) alkyl chain analogs are best for maximizing antiproliferative activity of gallate and gallamide derivatives, respectively.

Recently, Locatelli et al. reported the synthesis of various alkyl gallate derivatives and examined the relationship between the C log*P* value and cytotoxic effect.[14] From the viewpoint of C log*P* value, there are some differences between ester and amide bonds. Therefore, we supposed that the difference in optimal length of the alkyl chain might be related to the lipophilicity of the ester and amide bonds. To examine this hypothesis, we calculated C log*P* values of alkyl gallate and gallamide derivatives and compared them with the antiproliferative activities. As shown in Figure 3, C log*P* values were indeed correlated with antiproliferative activity for both gallate and gallamide derivatives. The difference in the optimal length of the alkyl chain thus appeared to be due to the difference of C log*P* values between ester and amide bonds. These results indicated that the hydrophobicity of the alkyl chain of galloyl derivatives is important for the antiproliferative activity, and the alkyl chain might contribute to improving the cell permeability or the interaction with hydrophobic pockets in the target molecule.

Next, taking **6c** as a lead compound, its dehydroxylated analogs (**9a–9f**) were prepared and assayed to investigate the role of hydroxyl groups. As shown in Table 2, non-hydroxylated (**9a**) and mono-hydroxylated (**9b–9d**) derivatives showed no antiproliferative activity toward HL-60 cells, suggesting that the polyphenolic moiety is mandatory for the activity. Among dihydroxylated derivatives (**9d–9f**), 2,3-dihydroxylated (**9e**) and 3,4-dihydroxylated (**9f**) derivatives were active, while the 2,5-dihydroxylated analog (**9d**) was inactive. The antiproliferative activity of dihydroxylated derivatives decreased in the order of **9f** > **9e** ≫ **9d**, where the activ-

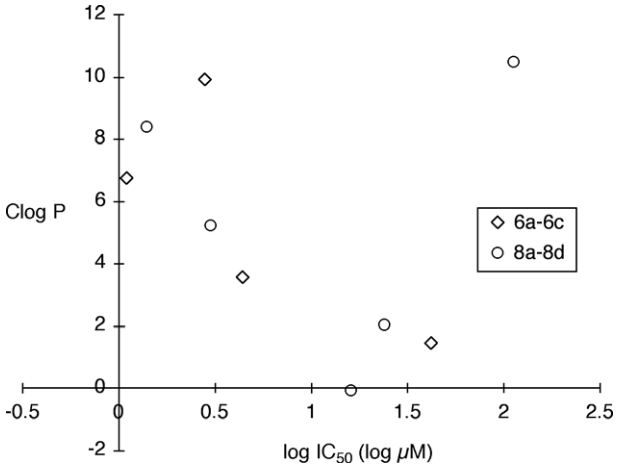
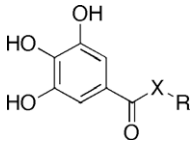


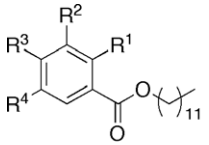
Figure 3. Relationship between C log*P* values and log IC_{50} of alkyl gallate and gallamide derivatives.

Table 1
Antiproliferative activity of alkyl gallate and gallamide derivatives

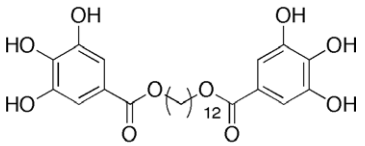


	X	R	IC_{50} (μM)
6a	O	C_2H_5	42
6b	O	C_6H_{13}	4.4
6c	O	$C_{12}H_{25}$	1.1
6d	O	$C_{18}H_{37}$	2.8
8a	NH	C_2H_5	16
8b	NH	C_6H_{13}	24
8c	NH	$C_{12}H_{25}$	3.0
8d	NH	$C_{18}H_{37}$	1.4
8e	NH	$C_{22}H_{45}$	112
	EGCG		9.4

Table 2
Substituent effect of hydroxyl group on antiproliferative activity



6c, 9a-f



12

	R^1	R^2	R^3	R^4	Cell viability[a] (%)
6c	H	OH	OH	OH	2.5
9a	H	H	H	H	ca.100
9b	H	OH	H	H	ca. 100
9c	H	H	OH	H	ca.100
9d	H	OH	H	OH	99
9e	OH	OH	H	H	29
9f	H	OH	OH	H	7.2
		12			2.6
		EGCG			45

[a] Compounds are added at the concentration of 10 μM.

ity of **9f** is slightly weaker than that of **6c**. These results suggest that at least two hydroxyl groups located adjacent to each other are mandatory for the activity, and one of the hydroxyl groups should be located at the *para* (4-)position. This substituent effect(s) prompted us to design and synthesize the bis-gallate analog (**12**). Though compound **12** showed potent antiproliferative activity toward HL-60 cells, its activity did not exceed that of **6c** (Table 2).

2.3. Mechanism of the antiproliferative activity

In the course of the cell viability assay, compound **6c** was found to induce typical apoptotic morphology, such as membrane blebbing and formation of apoptotic bodies, after 4 h treatment, as shown in Figure 4. EGCG, which was previously reported to induce apoptosis in some cancer cells,[15] induced similar morphological changes in HL-60 cells. Based on this observation, we considered that galloyl derivatives showed antiproliferative activity by inducing apoptosis. To test this hypothesis, we looked for typical hallmarks of apoptosis, such as DNA fragmentation and activation of caspase-3/7, executioner caspase. After 6 h treatment with EGCG (100 μM) or compound **6c** (10 μM), cellular DNA was extracted and electrophoresed in 1.7% agarose gel. A typical DNA ladder was observed in both cases (Fig. 4). Moreover, the activity of caspase-3/7 was monitored based on cleavage of a fluorescent substrate by activated caspase-3/7, and strong activation of caspase-3/7 was detected in HL-60 cells treated with **6c** (data not shown). These results confirm that galloyl derivatives inhibit the proliferation of HL-60 cells by inducing apoptosis.

Next, to clarify the molecular mechanism of apoptosis, we planned to identify the target site of compound **6c**. Various caspases are sequentially activated in the signaling pathway of apoptosis.[16,17] Therefore, the use of subtype-selective caspase inhibitors is effective for the identification of the major pathway contributing to apoptosis induction. Upstream of caspase-3/7, three pathways are thought to be involved[18,19]: (i) the death-ligand-induced pathway mediated by caspase-8, (ii) the mitochondrial pathway mediated by caspase-9, and (iii) the endoplasmic reticulum (ER)-stress-related pathway mediated by caspase-12. Therefore, Z-VAD-FMK (a general caspase inhibitor), Z-IETD-FMK (caspase-8 inhibitor), Z-LEHD-FMK (caspase-9 inhibitor), and Z-ATAD-FMK (caspase-12 inhibitor) were tested for inhibitory activity against **6c**-induced apoptosis, as shown in Figure 5. Z-ATAD-FMK, an inhibitor of caspase-12, showed a cytoprotective effect, as did Z-VAD-FMK, indicating that caspase-12 was a major mediator of the apoptosis induced by compound **6c**. Caspase-12 is thought to be an endoplasmic reticulum (ER)-stress-related caspase.[20,21] Therefore, to confirm the involvement of ER stress in apoptosis induced by **6c**, the expression of the growth arrest and DNA damage-inducible gene (*gadd-153*), which encodes an ER-stress-related protein,[22] was examined. As shown in Figure 6, strong expression of *gadd-153* was induced by compound **6c**, as expected, indicating the involvement of ER stress. Moreover, the induction of *gadd-153* was correlated with the antiproliferative activity of various gallate and gallamide derivatives.

There have been various cohort studies to examine the relationship between the intake of green tea and the risk of cancer, and it is widely appreciated that green tea and its main component, EGCG, have a preventive effect against cancer in various organs in humans.[23] Although some EGCG-interacting molecules have been identified, the molecular mechanism of antitumor activity is not fully clarified. Considering the multi-targeting character of catechins, green tea is thought to exert its antitumor activity via multiple mechanisms. Therefore, to elucidate the molecular mechanism of antitumor effects of green tea, molecular probes having minimum essential structure for activity would be useful. In this study, we have developed alkyl gallates and gallamides having a galloyl group as an essential structure for antiproliferative activity. We further found that the galloyl derivatives induced apoptosis related to ER-stress. Recently, Kuzuhara et al. reported that EGCG and related derivatives having a galloyl group interact with DNA and RNA directly, while in contrast, catechin and related derivatives lacking a galloyl group did not interact with DNA or RNA.[24] Therefore, we assumed that a direct target of galloyl derivatives might be a DNA- or RNA-related molecule related to ER, such as RNA incorporated in ribosomes. Based on this hypothesis, we have developed some galloyl derivatives having nitrogen(s) in the alkyl chain of compound **6c**, with the aim of increasing the cationic character to strengthen the interaction with DNA or RNA. But, disappointingly, the activity of these derivatives was very weak, and their IC_{50} values were about 20–30 μM (data not shown). However, considering the involvement of cell permeability, examination of direct interaction between galloyl derivatives and DNA/RNA will be

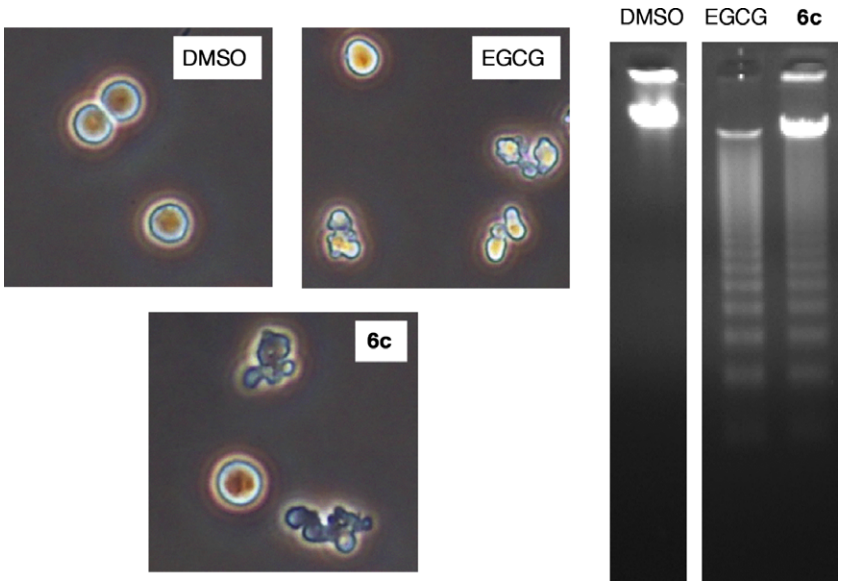


Figure 4. Typical apoptotic morphological changes and DMA fragmentation induced by EGCG (100 μM) or **6c** (10 μM).

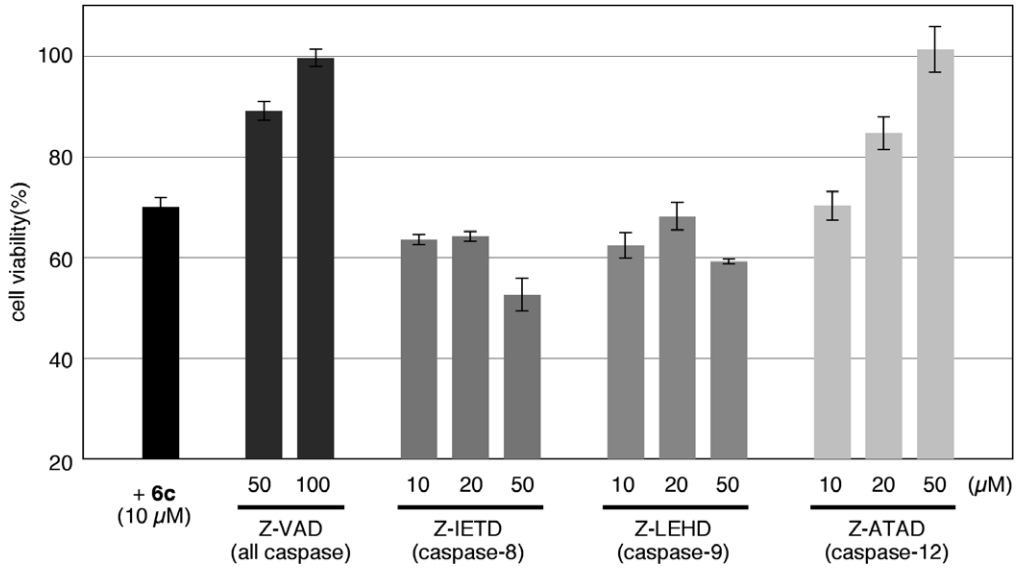


Figure 5. Effects of various caspase inhibitors on apoptosis induced by **6c**.

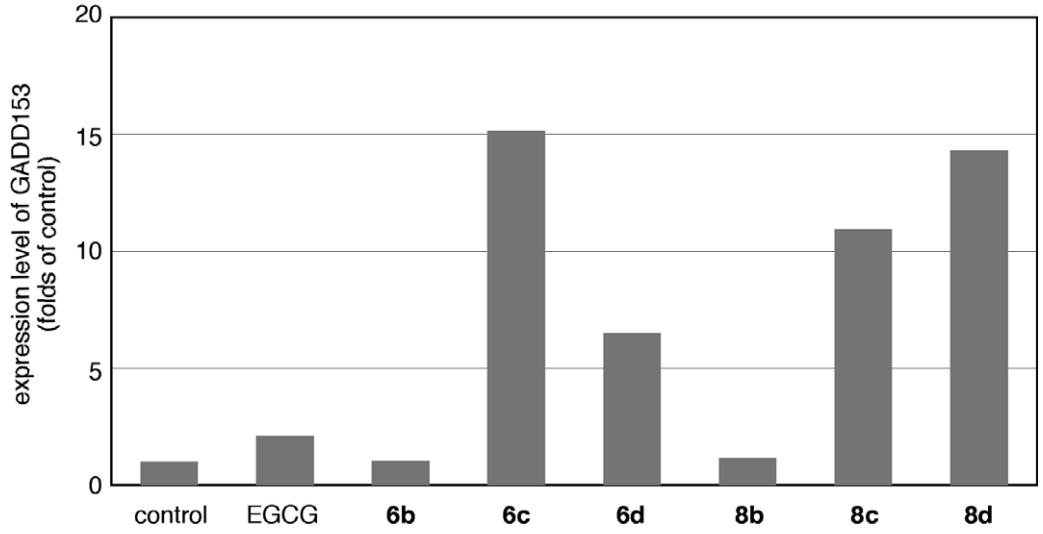


Figure 6. Expression of *gadd-153* in HL-60 treated with various gallate and gallamide derivatives.

necessary to exclude DNA/RNA as candidates for the target molecule of galloyl derivatives. On the other hand, Ermakova et al. reported that glucose-regulating protein 78 (GRP78), a molecular chaperone in ER, is an EGCG-binding protein.[25] They also described that EGCG and ECG, catechins having a galloyl group, showed stronger inhibition of GRP78 than EC and EGC, catechins lacking a galloyl group. Thus, there may be some interaction between the galloyl group and GRP78. Therefore, not only DNA/RNA, but also proteins in ER could be considered as candidates for the target molecule of galloyl derivatives.

3. Conclusion

In conclusion, we have developed alkyl gallate and gallamide derivatives based on the structure of EGCG and EGC, which are major components of green tea. Among them, some compounds showed stronger antiproliferative activity than EGCG. Biological studies indicated that compound **6c** induced apoptosis through the ER-stress-related pathway. Further structure development and biological studies on the molecular mechanisms are in progress.

4. Experimental

4.1. Biology

4.1.1. Cell culture

HL-60 cells were maintained in RPMI 1640 medium supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% heat-inactivated fetal bovine serum (FBS). Cells were grown in a humidified incubator at 37 °C under 5% CO_2/95% air.

4.1.2. Cell viability assay

HL-60 cells (1×10^5 cells/well) suspended in fresh medium in a 96-well plate (100 μL/well) were treated with test compounds (DMSO solution, 0.5 μL/well). After 3-day incubation, 10 μL of Cell

Counting Kit (Dojindo) was added to each well. The cell viability was determined based on the increase of absorbance (450 nm) during 4 h incubation.

4.1.3. DNA fragmentation analysis

HL-60 cells (6×10^5 cells) treated with compound **6c** (10 μM) or EGGC (100 μM) for 6 h were collected, washed with PBS, and lysed with 500 μL of cell lysis buffer (1% Triton X-100, 10 mM Tris–HCl, 10 mM EDTA, RNase A 1 mg/mL, pH 8.0) for 60 min at 50 °C. The lysate was incubated with 200 μg/mL proteinase K at 50 °C overnight, and phenol/chloroform-extracted DNA was electrophoresed on a 1.7% agarose gel and visualized by ethidium bromide staining.

4.1.4. Quantitative real-time PCR of *gadd-153* gene

HL-60 cells (6×10^5 cells/well) suspended in fresh medium in a 12-well plate (2 mL/well) were treated with test compounds (DMSO solution, 10 μL/well). After 24 h incubation, the cells were collected and washed with PBS twice. Total RNA was isolated by using ISOGEN reagent (Nippon Gene), and then subjected to reverse-transcription. The expression level of the *gadd-153* gene was quantitatively measured by real-time PCR as described previously.[26]

4.2. Chemistry

4.2.1. General

^{1}H NMR (500 MHz), spectra were recorded on a JEOL JNM-a 500 spectrometer. The ^{1}H NMR chemical shifts were reported in parts par million (ppm) relative to the singlet at 7.26 ppm for chloroform in deuteriochloroform or 2.49 ppm for dimethyl sulfoxide (DMSO) in DMSO-d_6 and coupling constants were given in hertz (Hz). The following abbreviations are used for spin multiplicity: s, singlet; d, doublet; t, triplet; q, quartet; quint, quintet; sept, septet; m, multiplet; br, broad. Mass spectra were recorded on JEOL JMA-HX110 spectrometer with *m*-nitrobenzyl alcohol. Routine thin layer chromatography (TLC) was performed on silica gel 60 F254 plates (Merck, Germany). Flash column chromatography was performed on Silica gel (spherical, particle size 40–100 μm, Kanto).

4.2.2. Methyl 3,4,5-trihydroxybenzoate (2)

To a stirred solution of **1** (6.60 g, 38.8 mmol) in MeOH (40 mL), H_2SO_4 (2 mL) was added. The reaction mixture was refluxed for 2 h at 100 °C, then evaporated. The residue was taken up in water and extracted with ethyl acetate three times. The organic layer was washed with brine, dried over $MgSO_4$, filtered, and concentrated in vacuo to afford **2**, which was all used for the next synthesis. ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.25 (s, 2H), 8.97 (s, 1H), 6.93 (s, 2H), 3.73 (s, 3H) 3.51 (br, 3H). MS (FAB, MH^+) *m*/*z* 185.

4.2.3. Methyl 3,4,5-tribenzyloxybenzoate (3)

To a stirred solution of **2** and K_2CO_3 (16.1 g, 0.116 mol) in DMF (50 mL), BnCl (14.3 g, 0.113 mol) was added. The reaction mixture was stirred at 80 °C under Ar atmosphere. After 15 h, water was added, and the whole was extracted with dichloromethane. The organic layer was washed with brine, dried over $MgSO_4$, and concentrated in vacuo to afford **3**, which was all used for the next synthesis. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.43–7.25 (m, 17H), 5.28 (s, 2H), 5.13 (s, 4H), 5.12 (s, 3H). MS (FAB, MH^+) *m*/*z* 455.

4.2.4. 3,4,5-Tribenzyloxybenzoic acid (4)

To a stirred solution of **3** in MeOH (50 mL), dioxane (100 mL), and H_2O (40 mL), NaOH (11.9 g, 2.98 mol) was added. The reaction mixture was refluxed for 6 h at 120 °C, then evaporated. The residue was taken up in water and extracted with dichloromethane three times. The organic layer was washed with brine, and dried over $MgSO_4$. The organic layer was filtered and concentrated in vacuo. The residue was purified by recrystallization with CH_2Cl_2 to afford **4** (12,571 mg, 28.2 mmol, three steps 72%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.43–7.25 (m, 17H), 5.28 (s, 2H), 5.13 (s, 4H). MS (FAB, MH^+) *m*/*z* 441.

4.2.5. Methyl 3,4,5-tribenzyloxybenzoate (5a)

To a stirred solution of **4** (70 mg, 0.159 mmol) and K_2CO_3 and (54 mg, 0.391 mmol), in DMF (5 mL) was added bromoethane (30 μL, 0.402 mmol). The reaction mixture was stirred at room temperature under an N_2 atmosphere overnight, then water was added, and the whole was extracted with ethyl acetate three times. The organic layer was washed with water and brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. The residue was purified by column chromatography on silica gel (hexane/ethyl acetate = 5:1) to afford **5a** (74 mg, 0.158 mmol, 99%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.45–7.30 (m, 15H), 7.23 (m, 2H), 5.12 (s, 4H), 5.09 (s, 2H), 4.33 (q, *J* = 6.7 Hz, 2H), 1.36 (t, *J* = 6.7 Hz, 2H). MS (FAB, MH^+) *m*/*z* 469.

4.2.6. Hexyl 3,4,5-tribenzyloxybenzoate (5b)

According to the same procedure used for **5a**, starting from **4** (305 mg, 0.692 mmol), **5b** (332 mg, 0.633 mmol, 91%) was obtained. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.42–7.31 (m, 17H), 5.12 (s, 4H), 5.10 (s, 2H), 4.25 (t, *J* = 6.5 Hz, 2H), 1.72 (quint, *J* = 6.5 Hz, 2H), 1.38 (quint, *J* = 6.5 Hz, 2H), 1.34–1.30 (m, 4H), 0.90 (t, *J* = 6.5 Hz, 3H). MS (FAB, MH^+) *m*/*z* 525.

4.2.7. Dodecyl 3,4,5-tribenzyloxybenzoate (5c)

According to the same procedure used for **5a**, starting from **4** (293 mg, 0.665 mmol), **5c** (379 mg, 0.623 mmol, 94%) was obtained. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.42–7.25 (m, 17H), 5.12 (s, 4H), 5.09 (s, 2H), 4.25 (t, *J* = 7.0 Hz, 2H), 1.72 (quint, *J* = 7.0 Hz, 2H), 1.42–1.22 (m, 18H), 0.86 (t, *J* = 7.0 Hz, 3H). MS (FAB, MH^+) *m*/*z* 609.

4.2.8. Octadecyl 3,4,5-tribenzyloxybenzoate (5d)

According to the same procedure used for **5a**, starting from **4** (312 mg, 0.708 mmol), **5d** (390 mg, 0.563 mmol, 79%) was obtained. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.42–7.25 (m, 17H), 5.12 (s, 4H), 5.09 (s, 2H), 4.25 (t, *J* = 7.0 Hz, 2H), 1.72 (br, 2H), 1.42–1.22 (m, 30H), 0.86 (t, *J* = 7.0 Hz, 3H). MS (FAB, MH^+) *m*/*z* 693.

4.2.9. Ethyl 3,4,5-trihydroxybenzoate (6a)

To a stirred solution of **5a** (72 mg, 0.154 mmol) in ethyl acetate (5 mL) was added 10% Pd/C (23 mg). The reaction mixture was stirred at room temperature under an H_2 atmosphere overnight, filtered, and concentrated in vacuo. The residue was purified by column chromatography on silica gel (hexane/ethyl acetate = 1:2) to afford **6a** (27 mg, 0.136 mmol, 89%). ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.23 (s, 2H), 8.89 (s, 1H), 4.19 (q, *J* = 6.5 Hz, 2H), 1.26 (t, *J* = 6.5 Hz, 3H).

4.2.10. Hexyl 3,4,5-trihydroxybenzoate (6b)

According to the same procedure used for **6a**, starting from **5b** (330 mg, 0.629 mmol), **6b** (160 mg, 0.629 mmol, 99%) was obtained. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.27 (br, 3H), 5.70 (s, 2H), 4.25 (t, *J* = 6.0 Hz, 2H), 1.72 (quint, *J* = 6.0 Hz, 2H), 1.41 (br, 2H), 1.35–1.26 (m, 4H), 0.88 (t, *J* = 6.0 Hz, 3H).

4.2.11. Dodecyl 3,4,5-trihydroxybenzoate (6c)

According to the same procedure used for **6a**, starting from **5c** (427 mg, 0.702 mmol), **6c** (153 mg, 0.453 mmol, 64%) was obtained. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.22 (s, 2H), 8.90 (s, 1H), 6.92 (s, 2H), 4.13 (t, *J* = 6.4 Hz, 2H), 1.63 (quint, *J* = 6.4 Hz, 2H), 1.18–1.36 (m, 18H) 0.84 (t, *J* = 6.4 Hz, 3H).

4.2.12. Octadecyl 3,4,5-trihydroxybenzoate (6d)

According to the same procedure used for **6a**, starting from **5d** (388 mg, 0.560 mmol), **6d** (230 mg, 0.544 mmol, 97%) was obtained. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.24 (s, 2H), 8.92 (s, 1H), 6.92 (s, 2H), 4.13 (t, J = 6.5 Hz, 2H), 1.63 (quint, J = 6.5 Hz, 2H), 1.38-1.16 (m, 30H), 0.84 (t, J = 6.5 Hz, 3H).

4.2.13. Ethyl 3,4,5-tribenzyloxybenzamide (7a)

To a stirred solution of **4** (196 mg, 0.445 mmol), EDCI (137 mg, 0.715 mmol) and DMAP (14 mg, 0.115 mmol) in CH_2Cl_2 (10 mL) was added ethylamine (78 mg, 1.32 mmol). The reaction mixture was stirred at room temperature under an N_2 atmosphere for 1 h, washed with water and brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. The residue was purified by column chromatography on silica gel (hexane/ethyl acetate = 2:3) to afford **7a** (73 mg, 0.156 mmol, 35%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.41–7.25 (m, 15H), 7.03 (s, 2H), 5.88 (br, 1H), 5.12 (s, 4H), 5.07 (s, 2H), 3.44 (quint, J = 6.5 Hz, 2H), 1.21 (t, J = 6.5 Hz, 3H). MS (FAB, MH$^+$) m/z 468.

4.2.14. Hexyl 3,4,5-tribenzyloxybenzamide (7b)

According to the same procedure used for **7a**, starting from **4** (187 mg, 0.425 mmol), **7b** (189 mg, 0.361 mmol, 85%) was obtained. ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.41–7.25 (m, 15H), 7.02 (s, 2H), 5.85 (br, 1H), 5.12 (s, 4H), 5.07 (s, 2H), 3.37 (q, J = 6.4 Hz, 2H), 1.54 (br, 2H), 1.42–1.26 (m, 6H), 0.89 (t, J = 6.4 Hz, 3H). MS (FAB, MH$^+$) m/z 524.

4.2.15. Dodecyl 3,4,5-tribenzyloxybenzamide (7c)

According to the same procedure used for **7a**, starting from **4** (203 mg, 0.461 mmol), **7c** (252 mg, 0.415 mmol, 90%) was obtained. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.41–7.25 (m, 15H), 7.02 (s, 2H), 5.84 (t, J = 6.5Hz, 1H), 5.12 (s, 4H), 5.07 (s, 2H), 3.37 (q, J = 6.5 Hz, 2H), 1.31–1.24 (m, 20H), 0.86 (t, J = 6.5 Hz, 3H). MS (FAB, MH$^+$) m/z 608.

4.2.16. Octadecyl 3,4,5-tribenzyloxybenzamide (7d)

According to the same procedure used for **7a**, starting from **4** (201 mg, 0.456 mmol), **7d** (287 mg, 0.415 mmol, 91%) was obtained. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.41–7.25 (m, 15H), 7.02 (s, 2H), 5.84 (t, J = 6.5 Hz, 1H), 5.12 (s, 4H), 5.07 (s, 2H), 3.38 (q, J = 6.5 Hz, 2H), 1.34–1.21 (m, 30H), 0.86 (t, J = 6.5 Hz, 3H). MS (FAB, MH$^+$) m/z 692.

4.2.17. Docosyl 3,4,5-tribenzyloxybenzamide (7e)

According to the same procedure used for **7a** but with THF as a solvent instead of CH_2Cl_2, starting from **4** (357 mg, 0.810 mmol), **7e** (547 mg, 0.731 mmol, 90%) was obtained. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.42–7.25 (m, 15H), 7.02 (s, 2H), 5.84 (t, J = 6.5 Hz, 1H), 5.12 (s, 4H), 5.07 (s, 2H), 3.38 (q, J = 6.5 Hz, 2H), 1.34-1.21 (m, 40H), 0.86 (t, J = 6.5 Hz, 3H).

4.2.18. Ethyl 3,4,5-trihydroxybenzamide (8a)

According to the same procedure used for **6a**, starting from **7a** (71 mg, 0.152 mmol), **8a** (29 mg, 0.147 mmol, 98%) was obtained. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.96 (s, 2H), 8.58 (s, 1H), 8.01 (t, J = 6.4 Hz, 1H), 6.79 (s, 2H), 3.18 (quint, J = 6.4 Hz, 2H), 1.05 (t, J = 6.4 Hz, 3H).

4.2.19. Hexyl 3,4,5-trihydroxybenzamide (8b)

According to the same procedure used for **6a**, starting from **7b** (184 mg, 0.351 mmol), **8b** (85 mg, 0.336 mmol, 96%) was obtained. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.97 (s, 2H), 8.59 (s, 1H), 8.00 (t, J = 6.4 Hz, 1H), 6.78 (s, 2H), 3.14 (q, J = 6.4 Hz, 2H), 1.44 (quint, J = 6.4 Hz, 2H), 1.30-1.20 (m, H), 0.85 (t, J = 6.4 Hz, 3H).

4.2.20. Dodecyl 3,4,5-trihydroxybenzamide (8c)

According to the same procedure used for **6a**, starting from **7c** (248 mg, 0.408 mmol), **8d** (100 mg, 0.296 mmol, 73%) was obtained. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.97 (s, 2H), 8.60 (s, 1H), 8.00 (t, J = 6.5 Hz, 1H), 6.78 (s, 2H), 3.13 (q, J = 6.5 Hz, 2H), 1.45–1.42 (m, 2H), 1.28-1.18 (m, 18H), 0.84 (t, J = 6.5 Hz, 3H).

4.2.21. Octadecyl 3,4,5-trihydroxybenzamide (8d)

According to the same procedure used for **6a**, starting from **7d** (284 mg, 0.410 mmol), **8d** (166 mg, 0.394 mmol, 96%) was obtained. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.97 (s, 2H), 8.59 (s, 1H), 8.00 (t, J = 6.5 Hz, 1H), 6.78 (s, 2H), 3.13 (q, J = 6.5 Hz, 2H), 1.45–1.42 (m, 2H), 1.27–1.18 (m, 30H), 0.84 (t, J = 6.5 Hz, 3H).

4.2.22. Docosyl 3,4,5-trihydroxybenzamide (8e)

According to the same procedure used for **6a** but with THF as a solvent instead of AcOEt, starting from **7e** (527 mg, 0.704 mmol), **7e** (331 mg, 0.693 mmol, 98%) was obtained. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.95 (s, 2H), 8.58 (s, 1H), 7.99 (t, J = 6.5 Hz, 1H), 6.78 (s, 2H), 3.13 (q, J = 6.5 Hz, 2H), 1.47–1.41 (m, 2H), 1.27–1.18 (m, 38H), 0.84 (t, J = 6.5 Hz, 3H).

4.2.23. Benzoic acid dodecyl ester (9a)

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.94 (d, J = 7.9 Hz, 2H), 7.66–7.63 (br, 1H), 7.52 (t, J = 7.9 Hz, 2H), 4.25 (t, J = 6.7 Hz, 2H), 1.69 (quint, J = 6.7 Hz, 2H), 1.38 (quint, J = 6.7 Hz, 2H), 1.30–1.22 (m, 16H), 0.83 (t, J = 6.7 Hz, 3H). MS (FAB, MH$^+$) m/z 229.

4.2.24. 3-Hydroxybenzoic acid dodecyl ester (9b)

^{1}H NMR (500 MHz, DMSO-d_6) δ 9.79 (s, 1H) 7.38–7.28 (m, 3H), 7.01 (d, J = 7.9 Hz, 1H), 4.22 (t, J = 6.7 Hz, 2H), 1.67 (quint, J = 6.7 Hz, 2H), 1.37 (quint, J = 6.7 Hz, 2H), 1.29–1.22 (m, 16H), 0.84 (t, J = 6.7 Hz, 3H). MS (FAB, MH$^+$) m/z 307.

4.2.25. 4-Hydroxybenzoic acid dodecyl ester (9c)

^{1}H NMR (500 MHz, $CDCl_3$) δ 10.30 (s, 1H), 7.79 (d, J = 1.2, 7.3 Hz, 2H), 6.83 (dd, J = 1.2, 7.3 Hz, 2H), 4,18 (t, J = 6.7 Hz, 2H), 1.65 (quint, J = 6.7 Hz, 2H) 1.36 (quint, J = 6.7 Hz, 2H) 1.33–1.22 (m, 16H), 0.84 (t, J = 6.7 Hz, 3H). MS (FAB, MH$^+$) m/z 307.

4.2.26. 2,4-Dihydroxybenzoic acid dodecyl ester (9d)

^{1}H NMR (500 MHz, $CDCl_3$) δ 11.04 (s, 1H), 7.72 (d, J = 9.2 Hz, 1H), 6.38–6.34 (m, 2H), 5.35 (s, 1H), 4.28 (t, J = 6.7 Hz, 2H), 1.74 (quint, J = 6.7 Hz, 2H) 1.41 (quint, J = 6.7 Hz, 2H), 1.31 (quint, J = 6.7 Hz, 2H), 1.28–1.24 (m, 14H), 0.86 (t, J = 6.7 Hz, 3H).

4.2.27. 2,3-Dihydroxybenzoic acid dodecyl ester (9e)

^{1}H NMR (500 MHz, $CDCl_3$) δ 10.98 (s, 1H), 7.36 (d, J = 7.9 Hz, 1H), 7.08 (d, J = 7.9 Hz, 1H), 6.78 (t, J = 7.9 Hz, 1H), 5.61 (s, 1H) 4.32 (t, J = 6.7 Hz, 2H), 1.76 (quint, J = 6.7 Hz, 2H), 1.41 (quint, J = 6.7 Hz, 2H), 1.32 (quint, J = 6.7 Hz, 2H), 1.28–1.24 (m, 14H), 0.86 (t, J = 6.7 Hz, 3H). MS (FAB, MH$^+$) m/z 323.

4.2.28. 3,4-Dihydroxybenzoic acid dodecyl ester (9f)

^{1}H NMR (500 MHz, DMSO-d_6) δ 9.75 (s, 1H), 9.33 (s, 1H), 7.34–7.30 (m, 2H), 6.79 (s, 1H), 4.16 (m, 2H), 1.65 (m, 2H), 1.24–1.19 (m, 23H). MS (FAB, MH$^+$) m/z 323.

4.2.29. 3,4,5-Tribenzyloxybenzoyl chloride (10)

To a stirred solution of **4** (18.0 g, 40.8 mmol) and DMF (0.4 mL) in toluene (200 mL), oxalyl chloride (7.40 g, 58.3 mmol) was added slowly. The reaction mixture was stirred at room temperature under an Ar atmosphere, then concentrated in vacuo. The residue was taken up in 70 volumes of toluene and the solution was filtered. Cyclohexane (70 mL) was added, and the mixture was cooled to

room temperature. Filtration afforded **9** (1.64 g, 35.7 mmol, 89%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.48–7.01 (m, 17H), 5.14 (s, 4H), 4.98 (s, 2H).

4.2.30. 12-(3,4,5-Trihydroxybenzoate dodecan-1-yl)-3,4,5-trihydroxybenzoate (12)

To a stirred solution of **10** (646 mg, 1.41 mmol) and K_2CO_3 (392 mg, 2.84 mmol) in DMF (30 mL) was added 1,12-dodecanediol (171 mg, 0.846 mmol). The reaction mixture was stirred overnight, then extracted with ethyl acetate three times. The organic layer was washed with sat $NaHCO_3$ aq and brine, dried over $MgSO_4$, and filtered. The residue was purified by column chromatography (toluene/ethyl acetate = 7:1) to afford 12-(3,4,5-tribenzyloxybenzoate dodecan-1-yl)-3,4,5-tribenzyloxybenzoate (**11**: 327 mg, 0.321 mmol, 44%, MS (FAB, MH^+) m/z 1048). Then, to a stirred solution of **11** (279 mg, 0.290 mmol) in THF was added Pd/C (92 mg). The reaction mixture was stirred at room temperature under a H_2 atmosphere for 1 h, filtered, and concentrated. The residue was recrystallized from ethanol and hexane to afford **12** (31 mg, 0.085 mmol, 78%). ^{1}H NMR (500 MHz, DMSO-d_6) δ 6.92 (s, 4H), 4.13 (t, J = 6.5 Hz, 4H), 1.63 (quint, J = 6.5 Hz, 4H), 1.35 (br, 4H), 1.32–1.18 (m, 12H).

Acknowledgments

The authors are grateful to Prof. Hirota Fujiki and Prof. Takashi Kuzuhara (Tokushima Bunri University) for their helpful discussion. The work described in this paper was partially supported by Grants-in-Aid for Scientific Research from The Ministry of Education, Culture, Sports, Science and Technology, Japan, and the Japan Society for the Promotion of Science.

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