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O-Methylated Theaflavins Suppress the Intracellular Accumulation of Triglycerides from Terminally Differentiated Human Visceral Adipocytes

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ABSTRACT: A known *O*-methylated theaflavin, theaflavin 3-*O*-(3-*O*-methyl)gallate (3MeTF3G), and the new theaflavin 3-*O*-(3,5-di-*O*-methyl)gallate (3,5diMeTF3G) were synthesized via the *O*-methylation of theaflavin 3-*O*-gallate (TF3G). Both 3MeTF3G and 3,5diMeTF3G are more stable than TF3G at pH 7.5 in the order 3,5diMeTF3G > 3MeTF3G > TF3G. The inhibitory effects of these compounds on the intracellular accumulation of triglycerides from terminally differentiated human visceral adipocytes were investigated. Compound 3MeTF3G exhibited an inhibitory effect similar to that of TF3G at 3 μM and a slightly lower effect than that of TF3G at 10 μM. The result suggested that the degradants and oxidatively polymerized products of TF3G may also have inhibitory effects. For cells treated with 3,5diMeTF3G at 3 and 10 μM, intracellular triglyceride accumulation was dose dependent and significantly lower compared with that for other compounds. It was suggested that the higher effect of 3,5diMeTF3G was due to its higher stability and likely improved absorption owing to di-*O*-methylation.

KEYWORDS: *black tea, polyphenol, O-methylated theaflavin, inhibitory effect on intracellular triglyceride accumulation, pH stability*

INTRODUCTION

The biological activities of theaflavins (TFs), which are a type of black tea polyphenols, have been reported to include antioxidant activity,[1] lipase and amylase inhibitory activities,[2] inhibitory effects on lipid accumulation,[3] cholesterol-lowering effects,[4] etc. One of the TFs, theaflavin 3-*O*-gallate (TF3G), is known to be produced via oxidative coupling of the B-rings of epicatechin (EC) and epigallocatechin 3-*O*-gallate (EGCG) during the manufacture of black tea from green tea (*Camellia sinensis*) by fermentation.[5] It has been reported that *O*-methylated TFs, such as theaflavin 3-*O*-(3-*O*-methyl)gallate (3MeTF3G), which is formed via the oxidative coupling of EC and epigallocatechin 3-*O*-(3-*O*-methyl)gallate (EGCG3″Me), are also present in black tea.[6] However, the concentration of *O*-methylated TFs is lower than $^1/_{20}$ of that of the TFs, and their biological activities have not yet been revealed. On the other hand, *O*-methylated polyphenols have attracted significant attention because of their high stability and bioavailability.[7] Thus, *O*-methylated TFs are also expected to have a similar positive characteristics. We have therefore developed an efficient method described in a patent[8] for the synthesis of *O*-methylated TFs using an *O*-methyltransferase isolated from an edible mushroom. This method enables the preparation of large quantities of *O*-methylated TFs and the investigation of their biological activities. Herein, we describe the synthesis of two *O*-methylated TFs and the result of our investigation of their pH stability and inhibitory effects on the intracellular accumulation of triglycerides from terminally differentiated human visceral adipocytes.

MATERIALS AND METHODS

Chemicals and Plant Materials. All solvents (high or HPLC grade) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). TF3G was purchased from Funakoshi Co. (Tokyo, Japan). Commercially prepared black tea leaves and an edible mushroom (*Flammulina velutipes*) were obtained from a supermarket in Japan.

Apparatus. Time-of-flight mass spectrometry (TOFMS) was performed on a QSTAR Elite mass spectrometer (AB SCIEX, Tokyo, Japan) equipped with an electrospray ionization source. Nuclear magnetic resonance (NMR) spectra were recorded using a Bruker AV600 instrument (Bruker Biospin GmbH, Rheinstetten, Germany). Sep-Pak Vac C18 35 cm^3 (Waters Corp., Milford, MA, USA) and Inertsil ODS-3 (4.6 mm i.d. or 20 mm i.d. × 250 mm, GL Sciences Inc., Tokyo, Japan) columns were used for column chromatography. The purification of compounds was performed on a Hitachi HPLC system (Tokyo, Japan) with an L-7150 pump, an L-7420 UV−vis detector, and a D-2500 chromato-integrator. The analysis of compounds was performed on a Hitachi LaChrom Elite system with an L-2130 pump, an L-2450 diode array detector, an L-2350 column oven, and an L-2200 autosampler.

Preparation of Theaflavin 3-*O*-Gallate. TF3G (**1**) was prepared from black tea leaves by modifying a previous method.[6] Black tea leaves (200 g) were extracted three times with 50% EtOH containing 2% L-(+)-ascorbic acid (ASA) (1.5 l) by sonication for 20 min each time. The extracts were then centrifuged (3000 rpm, 4 °C, 5 min), and the 50% EtOH was evaporated. Next, the extracts were injected into a

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Sep-Pak Vac C18 35 cm^3 column. The column was rinsed with 15% EtOH (80 mL), and then the TFs were eluted with 40% EtOH (80 mL). After evaporation of the eluate, the residue (38.6 g) was dissolved in 40% EtOH and injected into an Inertsil ODS-3 column (20 mm i.d. × 250 mm) for fractionation. The mobile phase was acetonitrile/EtOAc/0.05% H_3PO_4 (21:3:76), the flow rate was 12 mL/min, and the compounds were detected at 280 nm. The fractions exhibiting the expected peak for TF3G were collected and evaporated, and the TF3G was purified on the Sep-Pak Vac C18 column by first rinsing with water and then eluting with EtOH to remove H_3PO_4. Finally, **1** (0.9 g) was obtained after lyophilization.

Synthesis and Purification of *O*-Methylated Theaflavins. The *O*-methyltransferase for the synthesis of *O*-methylated TFs was prepared from an edible mushroom (*F. velutipes*) according to the method described in a patent.[8] The stalk of an edible mushroom was cut, and the surface was disinfected with 0.5% hypochlorous acid for 15 min. The internal stalk was cut and washed with sterilized water. Next, a small piece of the stalk was cultured in a potato dextrose agar medium (Becton Dickinson, Sparks, MD, USA) at 25 °C. The mycelium was then isolated and cultured in a liquid culture medium (0.02% glucose, 0.01% peptone, 0.002% yeast extract, 0.002% KH_2PO_4, and 0.001% $MgSO_4$) at 28 °C with shaking. The cultured mycelium was harvested via filtration and homogenized with a buffer (pH 7.5) containing 20 mM Tris-HCl, 1 mM dithiothreitol, 1 mM EDTA, and 10% glycerol. After the homogenate was centrifuged (10000 rpm, 4 °C, 10 min), the crude enzyme was obtained. The *O*-methylated TFs were then synthesized via an enzymatic reaction using **1**, the crude enzyme, and *S*-adenosyl-L-methionine (SAM) as a methyl donor. A reaction solution (1000 mL) containing 20 mM phosphate buffer (pH 6.5), 2.5 mM $MgCl_2$, 0.05 mM TF3G, 0.5 mM SAM, 0.04% ASA, and a 3.3% (v/v) crude enzyme solution was incubated at 37 °C for 8 h, and the reaction was stopped with the addition of 1 N HCl (24 mL). Next, the enzymatic reaction solution was extracted three times with EtOAc. Removal of EtOAc by evaporation gave a solid mixture of *O*-methylated TFs. The solid was dissolved in 40% EtOH and injected into an Inertsil ODS-3 column (20 mm i.d. × 250 mm). Using a purification process similar to that described above for **1**, *O*-methylated TF3Gs were obtained and their yields were calculated.

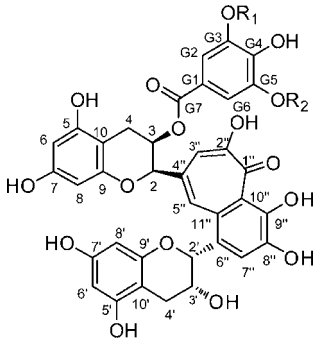
TOFMS Analysis and NMR Spectroscopy. TOFMS analysis was performed using Analyst QS 2.0 software. The TurboIonSpray source was set at 450 °C, and the instrument scanned in the range of m/z 100−1000. The optimized instrument settings were as follows: ion spray voltage, −4500 V; auxiliary gas, 50 psi; nebulizer gas, 50 psi; nitrogen curtain gas, 30 psi; declustering potential, −30 V; focusing potential, −250 V; declustering potential 2, −15 V; and accumulation time, 1 s. NMR acquisitions were carried out using TopSpin2 software. The ^{1}H NMR (600 MHz), ^{13}C NMR (150 MHz), heteronuclear single-quantum coherence, and heteronuclear multiple-bond correlation (HMBC) spectra were recorded at ambient temperature, and the chemical shifts are given in δ (ppm) relative to the solvent signal (acetone-d_6, δ_H 2.05; δ_C 29.8).

pH Stability of *O*-Methylated Theaflavins. The pH stability of **1** and the *O*-methylated TF3Gs was investigated by modifying a previous method.[9] First, the substrates (400 μM) were dissolved in an EtOH/0.3 M sodium acetate buffer (pH 5.0) (2:3) or an EtOH/0.3 M Tris-HCl buffer (pH 7.5) (2:3). Next, the substrate solutions (100 μL each) were incubated at room temperature for 2 h, and then a 1% ASA solution (100 μL) was added to prevent degradation; the solutions were injected into an Inertsil ODS-3 column (4.6 mm i.d. × 250 mm) for HPLC analysis. The mobile phase was the same as that used for purification of the substrates, the flow rate was 1.0 mL/min, and the column temperature was 50 °C. The peak areas of the substrates, which were not incubated, in HPLC analysis were set to 100%. The residual ratios of incubated substrates were analyzed.

Quantification of the Intracellular Accumulation of Triglycerides from Terminally Differentiated Human Visceral Adipocytes. Intracellular triglycerides are often used as a marker for adipocyte differentiation because the differentiation of human visceral preadipocytes is accompanied by intracellular triglyceride accumulation.[10] Human visceral preadipocytes (Lonza Walkersville, Inc., Walkersville, MD, USA) were cultured in a preadipocyte growth medium containing 10% fetal bovine serum and 1% L-glutamine (pH 7.0−7.3, Lonza Walkersville, Inc.). These cells were seeded into 96-well cell culture plates at 30000 cells/well and incubated with 5% CO_2 at 37 °C for 24 h. A preadipocyte growth medium (100 μL) was added to the cells designated undifferentiated controls, whereas a preadipocyte differentiation medium containing 10% fetal bovine serum, 1% L-glutamine, 2% human recombinant insulin, 0.2% dexamethasone, 0.4% indomethacin, and 0.2% 3-isobuthyl-1-methylxanthine (pH 7.0−7.3, Lonza Walkersville, Inc.) (100 μL) was added to all other cells. During differentiation, the cells were treated with the substrates, **1** and *O*-methylated TF3Gs (3 and 10 μM), once per day for 10 days. The control cells were treated with DMSO, which was the substrate vehicle. Intracellular triglyceride accumulation was determined using the AdipoRed Assay Reagent (Lonza Walkersville, Inc.). After the culture supernatants were removed, each well was rinsed with Dulbecco's phosphate-buffered saline (200 μL, pH 7.5, PBS; Funakoshi Co., Tokyo, Japan) and then filled with the same PBS. Next, the AdipoRed Assay Reagent (5 μL/well) was added, and the solutions were incubated at room temperature for 10 min. Intracellular triglyceride accumulation was measured by evaluating the fluorescence emission at 572 nm (excitation at 485 nm). The values were calculated as the percent of the control cells treated with DMSO and are shown as means ± standard error of the mean (SEM) for triplicate experiments. Comparisons between groups were performed using the Tukey test, and $p < 0.05$ was considered to be significant.

RESULTS AND DISCUSSION

Structures of the *O*-Methylated Theaflavins. MS and NMR analyses were conducted to investigate the number and sites of introduction of the methyl groups incorporated into **1**. After methylation and purification of **1**, compounds **2** (139.7 mg, 15.7% yield with respect to TF3G as a raw material) and **3** (1.0 mg, 1.1%) were obtained along with remaining **1** (388.9 mg, 43.7%) (Figure 2).



1 $R_1 = R_2 = H$
2 $R_1 = CH_3$, $R_2 = H$
3 $R_1 = R_2 = CH_3$

Figure 1. Structures of **1**−**3**.

Compound **2** was an orange amorphous powder. TOFMS analysis (m/z 729.1444 $[M - H]^-$, calculated for $C_{37}H_{29}O_{16}$, 729.1450) confirmed the molecular formula $C_{37}H_{30}O_{16}$, which was supported by the ^{13}C NMR analysis. The ^{1}H NMR spectrum of **2** was similar to that of **1**,[11] except for the presence of a methoxy signal (δ 3.79, 3H) and separate signals for H-G2 (δ 6.99) and H-G6 (δ 7.05) (Figure 3), indicating a 3-*O*-methylgalloyl group.[12] Because of the presence of an HMBC correlation from H−OCH_3 to C-G3, **2** was identified as the known *O*-methylated TF theaflavin 3-*O*-(3-*O*-methyl)gallate (3MeTF3G) (Figure 1; Table 1).[6]

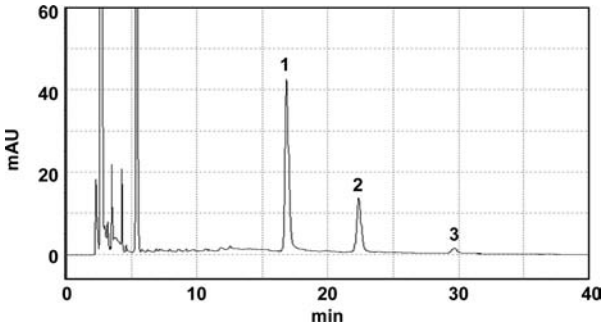


Figure 2. HPLC chromatogram of the enzymatic reaction solution including compounds **1**–**3**. Peaks: 1, TF3G; 2, 3MeTF3G; 3, 3,5diMeTF3G.

Compound **3** was also an orange amorphous powder. Its molecular formula was deduced to be $C_{38}H_{32}O_{16}$ on the basis of TOFMS analysis (*m*/*z* 743.1621 $[M - H]^-$, calculated for $C_{38}H_{31}O_{16}$, 743.1606), which indicated that it was dimethylated TF3G. The ^{1}H NMR spectrum of **3** was also similar to that of **1**, except for the presence of two methoxy proton signals (δ 3.81, 6H) and the downfield shift of H-G2 (H-G6) (δ 7.15) (Figure 3). Moreover, the ^{13}C NMR spectrum showed nine carbon signals at δ 55.6 (C-OCH_3 × 2), 119.9 (C-G1), 107.0 (C-G2 and C-G6), 147.3 (C-G3 and C-G5), 140.9 (C-G4), and 164.9 (C-G7), indicating a di-3,5-*O*-methylgalloyl group.[13] In the HMBC spectrum, the methoxy proton signals were correlated to C-G3 and C-G5. Therefore, **3** was established as theaflavin 3-*O*-(di-3,5-*O*-methyl)gallate (3,5diMeTF3G) (Figure1; Table 1). In this study, new *O*-methylated TF **3** was synthesized via the *O*-methylation of **1**. It is also expected that compound **3** can be synthesized via oxidative coupling of EC and epigallocatechin 3-*O*-(3,5-di-*O*-methyl)gallate (EGCG3″,5″diMe). However, it is very unlikely that **3** is contained in black tea because EGCG3″,5″diMe has been identified in *Stryphnodendron adstringens*[14] and *Bryophyllum pinnatum*[15] but not in *C. sinensis*. It was revealed that *O*-methyltransferase from *C. sinensis* transforms EGCG to EGCG3″,5″diMe in our previous study.[13] Therefore, EGCG3″,5″diMe in green tea and 3,5diMeTF3G in black tea may be identified by an analytical technique such as liquid chromatography–tandem mass spectrometry.

Improvement of the pH Stability with *O*-Methylation of the Galloyl Groups. In general, it has been known that most polyphenols are unstable under the near-neutral conditions commonly seen in biological environments. Therefore, the stability of the *O*-methylated TF3Gs at pH 5.0 and 7.5 was examined (Figure 4). In the pH 5.0 stability test, the residual ratios of all of the compounds were approximately 100% after 2 h of incubation, which is similar to previously reported results.[9] In the pH 7.5 stability test, it was found that *O*-methylated TF3Gs were more stable than TF3G, although the concentration of all of the compounds decreased. The order of stability was 3,5diMeTF3G (67.4%) > 3MeTF3G (58.5%) > TF3G (44.6%). This result suggested that *O*-methylation of the galloyl groups improved the pH stability of TF3G. In a previous paper,[9] the order of pH stability (pH 7.4, 2 h of incubation) was TF3G > EGCG. Thus, *O*-methylated TF3Gs should be more stable than EGCG, which is a typical polyphenol contained in green tea. This increased stability may be due to inhibition of the formation of *o*-quinones, which are known to be the initial intermediates in the oxidation of polyphenols such as TFs.[5] Oxidatively polymerized products such as thearubigins[16] were likely formed from all of the substrates in the pH 7.5 stability test because no notable peaks other than those for the analytes and a broad hump on the baseline were detected in the HPLC analysis.

Inhibitory Effects of TF3G, 3MeTF3G, and 3,5diMeTF3G on the Intracellular Accumulation of Triglycerides from Terminally Differentiated Human Visceral Adipocytes. Because numerous biological activities of TFs have been reported on lipid metabolism,[2−4] the inhibitory effects of TF3G, 3MeTF3G, and 3,5diMeTF3G on the intracellular accumulation of triglycerides from terminally differentiated human visceral adipocytes were investigated (Figure 5). In the cells treated with each compound at 3 and 10 μM, intracellular triglyceride accumulation was significantly decreased compared with that of the control cells ($p < 0.05$). The inhibitory effects of TF3G and 3MeTF3G at 3 μM were almost the same (93.7 and 91.9%, not regarded as significant). Previously, it was revealed that the inhibitory effect of EGCG3″Me on intracellular triglyceride accumulation is nearly the same as that of EGCG.[17] However, at 10 μM, TF3G exhibited a slightly lower value (86.1%) than that of 3MeTF3G (91.5%), and the results were significantly different ($p < 0.05$),

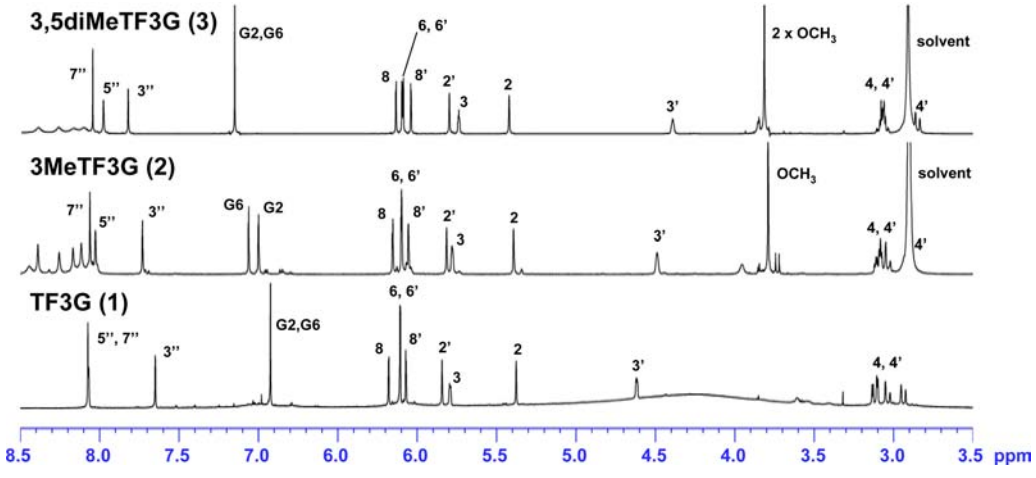


Figure 3. ^{1}H NMR spectra of **1**–**3**: (bottom) TF3G (**1**); (middle) 3MeTF3G (**2**); (top) 3,5diMeTF3G (**3**).

Table 1. ^{1}H and ^{13}C NMR Spectroscopic Data (δ_H, 600 MHz; δ_C, 150 MHz, Acetone-d_6) for 2 and 3

	2		3	
position	δ_H[a]	δ_C	δ_H[a]	δ_C
2	5.39 s	79.0	5.42 s	79.3
2′	5.81 s	75.6	5.80 s	75.5
3	5.77 br s	68.7	5.74 br s	69.4
3′	4.49 br s	64.5	4.39 br s	64.5
4α	3.09 dd (17.1, 4.2)	25.5	3.07 dd (16.2, 4.8)	25.4
4β	3.03 d (17.1)		3.06 d (16.2)	
4′α	3.09 dd (16.8, 4.2)	28.7	3.07 dd (16.2, 4.8)	28,7
4′β	2.90 d (16.8)		2.85 d (16.2)	
5		157.0[b]		157.0[c]
5′		156.8[b]		156.8[c]
6	6.09 d (2.1)	95.9	6.09 d (2.1)	95.9
6′	6.10 d (2.4)	95.6	6.09 d (2.4)	95.6
7		156.7[b]		156.7[c]
7′		156.5[b]		156.5[c]
8	6.15 d (2.1)	94.7	6.13 d (2.1)	94.7
8′	6.05 d (2.4)	94.9	6.04 d (2.4)	94.9
9		155.3		155.3
9′		156.1		156.2
10		97.7		97.7
10′		98.9		99.0
1″		184.1		184.1
2″		153.8		153.7
3″	7.72 s	116.8	7.82 s	116.9
4″		133.0		133.2
5″	8.02 s	125.7	7.97 s	126.0
6″		130.8[d]		130.9[e]
7″	8.05 s	123.0	8.04 s	123.0
8″		145.4[f]		145.5[g]
9″		149.8[f]		149.8[g]
9″-OH	14.90 s		14.90 s	
10″		120.6		120.6
11″		127.5[d]		127.5[e]
G1		120.2		119.9
G2	6.99 d (2.1)	104.9	7.15 s	107.0
G3		147.5		147.0
G4		139.0		140.9
G5		144.6		147.0[h]
G6	7.05 d (2.1)	110.5	7.15 s[i]	107.0[i]
G7		165.0		164.9
OCH_3	3.79 s	55.5	3.81 s	55.6

[a]Coupling constants (J in Hz) in parentheses. [b−g]Interchangeable. [h]Equivalent to G3 due to the symmetrical structure. [i]Equivalent to G2 due to the symmetrical structure.

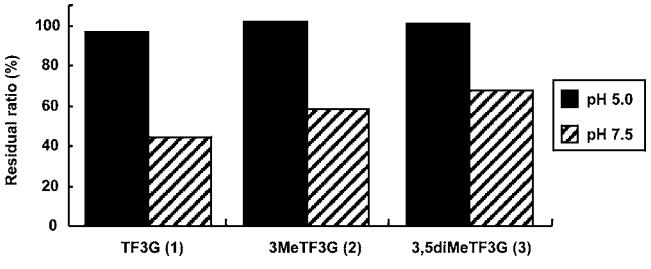


Figure 4. pH stability of **1**−**3**. pH stability was assayed in an EtOH/0.3 M sodium acetate buffer (pH 5.0) (2:3) or an EtOH/0.3 M Tris-HCl buffer (pH 7.5) (2:3). The values were calculated as the residual ratios of the substrates.

suggesting that the degradation products and oxidatively polymerized products of TF3G may also have inhibitory effects, because TF3G was more unstable and susceptible to oxidation than 3MeTF3G. For the cells treated with 3,5diMeTF3G at 3 and 10 μM (82.3 and 54.4%, respectively), intracellular triglyceride accumulation was dose dependent and significantly more decreased compared with that for TF3G and 3MeTF3G at the same concentrations ($p < 0.05$). It is known that TF3G migrates to tissues, such as the liver, after consumption[18] and that *O*-methylation of polyphenols improves pH and metabolic stability,[7] absorption, and accumulation of polyphenols in cells.[19] Therefore, it was suggested that the higher inhibitory effect of 3,5diMeTF3G observed in this study was due to its higher pH stability (shown in Figure 4) and likely improved absorption resulting from di-*O*-methylation. This study represents the first report of an increase in the inhibitory effect due to di-3,5-*O*-methylation of

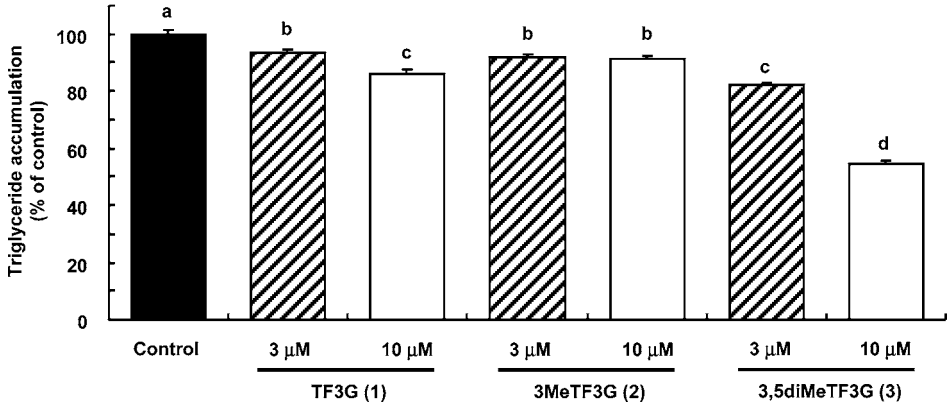


Figure 5. Inhibitory effects of **1**–**3** (3 or 10 μM) on the intracellular accumulation of triglycerides from terminally differentiated human visceral adipocytes. The values were calculated as the percent of the control cells treated with DMSO and are shown as means ± SEM for triplicate experiments. Different letters above the bars indicate significant difference ($p < 0.05$).

the galloyl group. It is possible that the mechanism of action is similar to that of catechins and *O*-methylated catechins.[10,17]

In conclusion, this study revealed that *O*-methylation of the galloyl group improves the stability of TF3G under near-neutral conditions and that 3,5diMeTF3G has a dose-dependent higher inhibitory effect than TF3G and 3MeTF3G on the accumulation of triglycerides from terminally differentiated human visceral adipocytes.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

TF3G, theaflavin 3-*O*-gallate; 3MeTF3G, theaflavin 3-*O*-(3-*O*-methyl)gallate; 3,5diMeTF3G, theaflavin 3-*O*-(3,5-di-*O*-methyl)gallate; TF, theaflavin; EGCG, epigallocatechin 3-*O*-gallate; EGCG3″Me, epigallocatechin 3-*O*-(3-*O*-methyl)gallate; EGCG3″,5″diMe, epigallocatechin 3-*O*-(3,5-di-*O*-methyl)-gallate; EtOH, ethanol; EtOAc, ethyl acetate; SAM, *S*-adenosyl-L-methionine; ASA, L(+)-ascorbic acid; PBS, phosphate-buffered saline

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