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Synthesis of Feruloyl-*myo*-inositols and their Inhibitory Effects on Superoxide Generation

Asao Hosoda,[a] Eisaku Nomura,[a] Akira Murakami,[b] Koichi Koshimizu,[b] Hajime Ohigashi,[c] Kazuhiko Mizuno[d] and Hisaji Taniguchi[a,*]

[a]*Industrial Technology Center of Wakayama Prefecture, 60 Ogura, Wakayama 649-6261, Japan*
[b]*Department of Biotechnological Science, Faculty of Biology-Oriented Science and Technology, Kinki University, Iwade-Uchita, Wakayama 649-6493, Japan*
[c]*Division of Applied Science, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan*
[d]*Department of Applied Chemistry, College of Engineering, Osaka Prefecture University, 1-1 Gakuen-cho, Sakai, Osaka 599-8531, Japan*

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Abstract—Ester compounds consisting of ferulic acid and *myo*-inositol, obtained from rice bran, were synthesized. The inhibitory effects of these feruloyl-*myo*-inositols on 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced superoxide (O_2^-) generation were examined using differentiated HL-60 cells. Among the derivatives tested, only 3,4,5,6-tetra-*O*-acetyl-1,2-di-*O*-[3-(4′-acetoxy-3′-methoxyphenyl)-2-propenoyl]-*myo*-inositol (**3**) showed a distinct inhibitory activity.

We have recently developed a method for the mass production of ferulic acid from the oily component of rice bran.[1] Due to the phenolic hydroxy group and an extended side chain conjugation of ferulic acid, it readily forms a resonance stabilized phenoxy radical which acounts for its antioxidant potential.[2] Moreover, the potential health promotion and disease preventive effects of ferulic acid have been demonstrated in many animal models and in vitro assays.[3] The ferulic acid derivative of EGMP, in which the geranyl group is attached to the phenolic hydroxyl group of ethyl ferulate, notably showed a suppressive effect on the formation of colonic tumor marker in rats.[4] On the other hand, *myo*-inositol, also occurring in rice bran, binds to phosphoric acid to produce inositol 1,4,5-triphosphate and inositol hexaphosphate (IP_6), the latter of which has shown notable anti-cancer action in a variety of experimental tumor models.[5] Thus, we expected that feruloyl-*myo*-inositols, consisting of ferulic acid and inositol moieties, would have significant biological activities, including anti-oxidation and anti-carcinogenesis.

In the present study, we synthesized seven novel feruloyl-*myo*-inositols and evaluated their inhibitory effects on generation of the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced superoxide (O_2^-) in differentiated HL-60 cells, which contain the NADPH oxidase system generating O_2^- and have been used as a cellular system to search for anti-tumor promoters.[6]

3,4,5,6-Tetra-*O*-acetyl-*myo*-inositol (**1**) was prepared in three steps from *myo*-inositol.[7,8] Ferulic acid was converted into 3-(4′-acetoxy-3′-methoxyphenyl)-2-propenoyl chloride (**2**) by two step synthesis. The reaction of the acetyl-*myo*-inositol **1** with the acid chloride **2** was carried out in the presence of a mixture of triethylamine and 4-dimethylaminopyridine (DMAP) in dichloromethane. When the substrate molar ratio of **2** to **1** was 2.4, the reaction gave a mixture of 3,4,5,6-tetra-*O*-acetyl-1,2-di-*O*-[3-(4′-acetoxy-3′-methoxyphenyl)-2-propenoyl]-*myo*-inositol (**3**)[9] and 3,4,5,6-tetra-*O*-acetyl-1-*O*-[3-(4′-acetoxy-3′-methoxyphenyl)-2-propenoyl]-*myo*-inositol (**4**)[10] in 33 and 31% yields respectively. When the molar ratio of **2** to **1** was 4, the reaction afforded the product **3** in 79% yield. When a mixture of pyridine and DMAP was used as the reaction catalyst, only the product **4** was selectively obtained in 82% yield. Under these conditions, the hydroxyl group in the 2-position of *myo*-inositol did not react. These results are consistent with the fact that the hydroxyl group on the 2-position of *myo*-inositol has poor reactivity due to an axial bond.[11]

The compound **3** has a total of six acetyl esters (four on the cyclohexane ring and two on the benzene ring) and two

*Corresponding author. Tel.: +81-73-477-1271; fax: +81-73-477-2880; e-mail: taniguti@wakayama-kg.go.jp

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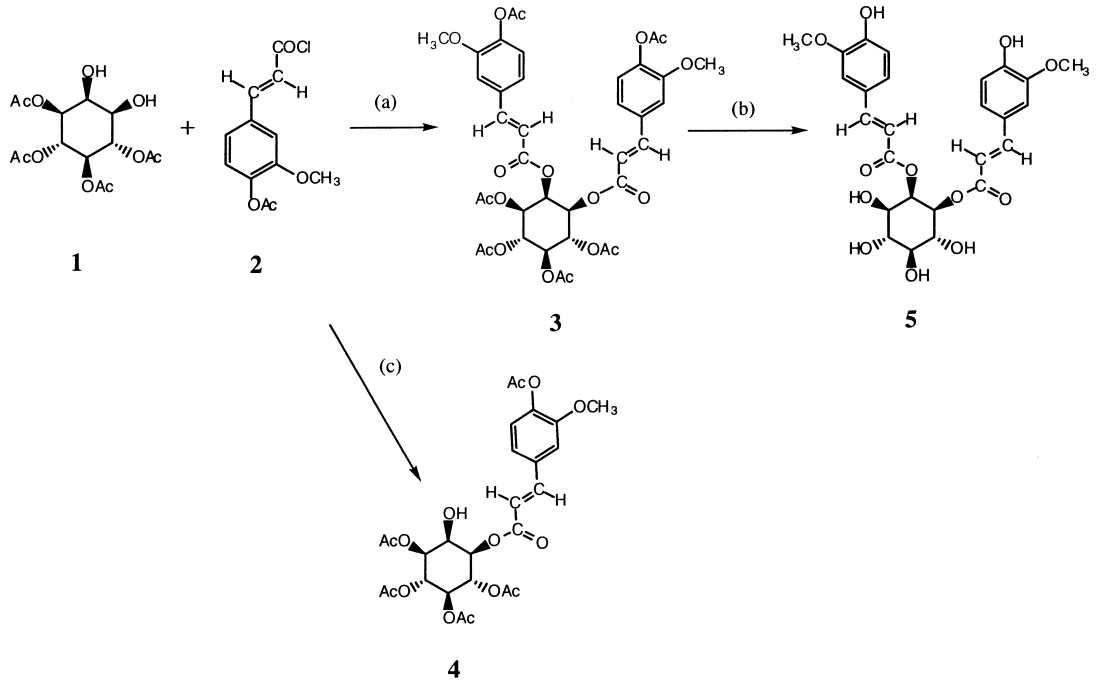


Scheme 1. Reagents and conditions: (a) Et_3N, DMAP, CH_2Cl_2, rt; (b) $NH_2NH_2 \cdot H_2O$, MeOH, rt; (c) Py, DMAP, CH_2Cl_2, rt.

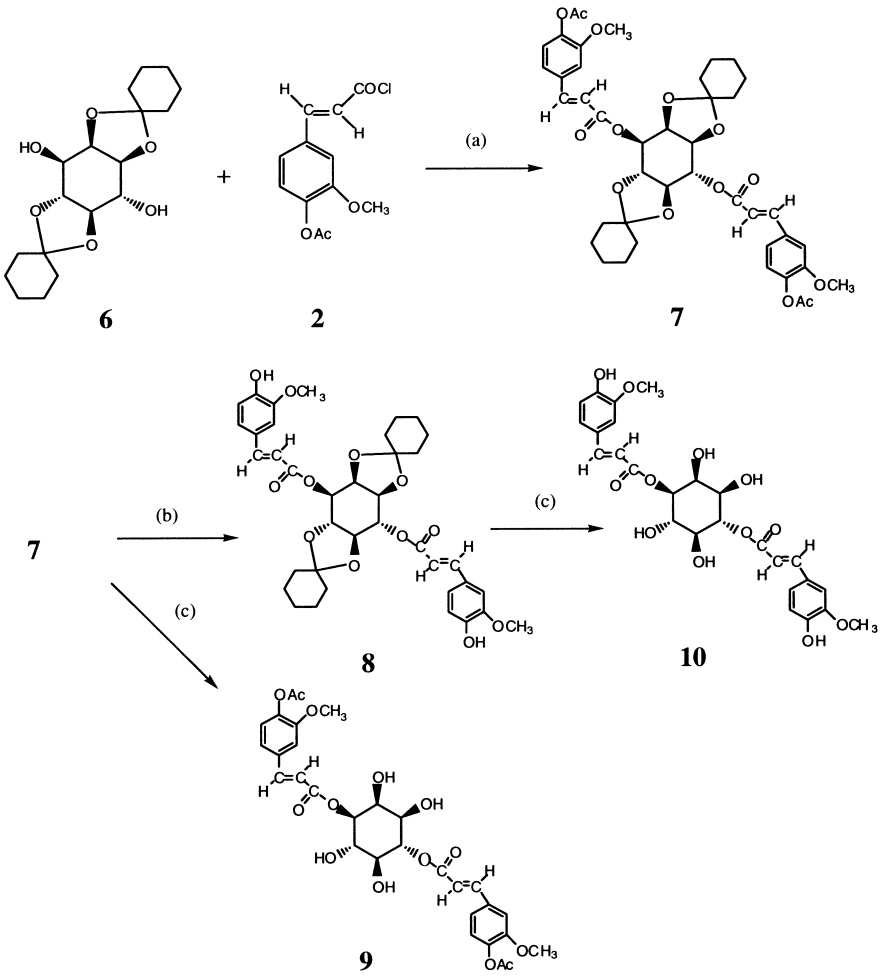
feruloyl esters. We investigated the hydrolysis conditions of the compound **3**, which cleaved only acetyl esters and left feruloyl esters. Hydrazine treatment of **3** in methanol yielded only the compound 1,2-di-*O*-[3-(4′-hydroxy-3′-methoxyphenyl)-2-propenoyl]-myo-inositol (**5**)[12] in 86% yield. Recrystallization of **5** from ethanol–chloroform gave pale yellow needles.

The reaction of 1,2:4,5-di-*O*-cyclohexylidene-*myo*-inositol (**6**)[7] with the acid chloride **2** in the presence of a mixture of triethylamine and DMAP in dichloromethane gave 1,2:4,5-di-*O*-cyclohexylidene-3,6-di-*O*-[3-(4′-acetoxy-3′-methoxyphenyl)-2-propenoyl]-*myo*-inositol (**7**)[13] in 74% yield. The compound **7** was deacetylated by the use of hydrazine to produce 1,2:4,5-di-*O*-cyclo-hexylidene-3,6-di-*O*-[3-(4′-hydoxy-3′-methoxyphenyl)-2-propenoyl]- *myo*-inositol (**8**) in 98% yield. The compound **7** was heated at 100–110 °C for 2 h in 80% aq acetic acid to produce 3,6-di-*O*-[3-(4′-acetoxy-3′-methoxyphenyl)-2-propenoyl]- *myo*-inositol (**9**) in 75% yield. The compound **8** was treated with 80% aq acetic acid at 100–110 °C for 1 h to afford 3,6 -di-*O*-[3-(4′-hydroxy-3′-methoxyphenyl)-2-pro-penoyl] -*myo*-inositol (**10**)[14] in 89% yield.

Inhibitory Test of TPA-Induced O_2^- Generation in Differentiated HL-60 Cells [15]

We examined the inhibitory activity of seven compounds, **3**, **4**, **5**, **7**, **8**, **9** and **10**, toward TPA-induced O_2^- generation in human promyelocytic leukemia HL-60 cells. For comparison, we also examined the inhibitory activity of ferulic acid (**11**) and its derivatives (**12**–**14**).[1] By treating the HL-60 cells with 1.25% (v/v) of DMSO for 6 days, they were differentiated into granulocytes generating O_2^-, when stimulated with TPA.[6] The O_2^- generation was detected by measuring the visible absorption at 550 nm due to the reduced form of cytochrome *c*.[16] As shown in Figure 1, only compound **3** at a concentration of 100 μM inhibited cytochrome *c* reduction by 100%, while **5** was much less active. The potent suppressive activity by **3** of O_2^--induced the cytochrome *c* reduction is attributable to suppression and/or inhibition of the NADPH oxidase system which localizes in differentiated HL-60 cells and is responsible for O_2^- generation because: (1) compounds **3** showed no significant O_2^- scavenging activity up to a concentration of 500 μM in the xanthine/xanthine oxidase system generating O_2^- (data not shown); (2) reaction of **3** with cytochrome *c* can be neglected since we performed a negative control experiment in which **3** and cytochrome *c*, without TPA, were incubated to estimated cytochrome *c* reduction, and found no interactions between them in the present experimental conditions; and (3) we wash out the extracellular test compounds including **3** before adding TPA. The structural difference of **3** from **5** is the presence of six acetoxyl groups in **3**, i.e., there is no acetoxyl group in **5**. Their contrasting activities may be due to their differences in molecular hydrophorbicity. Similarly, three compounds, **8**, **9** and **10**, bearing hydroxyl groups at the ferulic and/or inositol moiety(ies), were weak inhibitors (Fig. 1). It is interesting, however, that the derivative **7**, possessing no hydroxyl group and recognized as being relatively hydrophorbic, had little activity. The ferulic acid derivative (**12**) and methyl ferulate (**13**) showed no activity, and both ferulic acid (**11**) and ethyl ferulate (**14**) exhibited a very low activity (Fig. 1). Therefore, it is important that ferulic acid binds to *myo*-inositol to obtain such feruloyl-*myo*-inositol as the compound **3**.

In conclusion, we synthesized seven novel feruloyl-*myo*-inositol derivatives and examined their structure–activity



Scheme 2. Reagents and conditions: (a) Et_3N, DMAP, CH_2Cl_2, rt; (b) $NH_2NH_2 \cdot H_2O$, MeOH, rt; (c) 80% CH_3COOH aq, reflux.

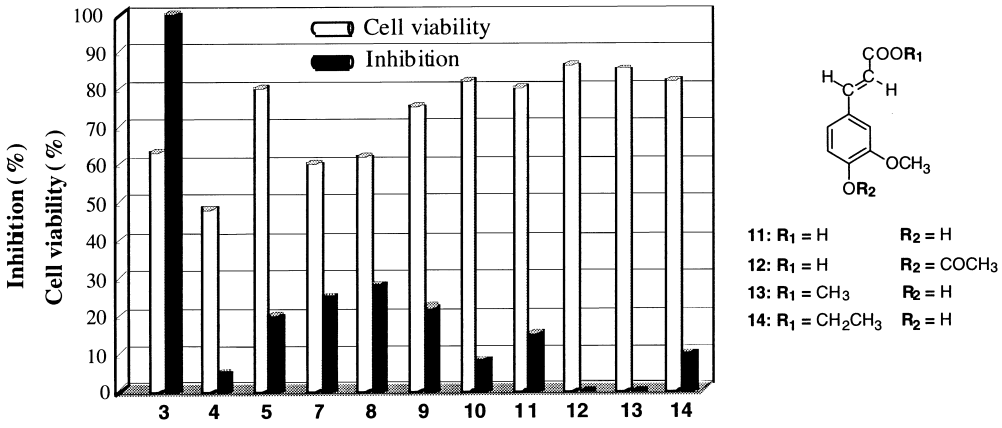


Figure 1. Inhibitory effects of feruloyl-*myo*-inositol compounds on TPA-induced O_2^- generation in differentiated HL-60 Cells (test compounds were added at a concentration of 100 μM). Data are shown as the mean value of duplicate experiments.

relationships in the suppression of O_2^- generation. The derivative **3** may warrant further evaluation in other biological assay systems with respect to its anti-oxidation and anti-carcinogenesis activities. Further chemical synthesis, directed to the location and number of feruloyl moiety(ies) in the inositol structure, is now in progress.

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9. Compound **3**: Recrystallization from AcOEt–hexane gave white powder. Mp 195–198 °C; IR (KBr) ν 2943, 1764, 1729, 1637, 1510, 1370, 1228, 1154, 1127, 1043, 908 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.71 (d, 1H, J = 16.0 Hz, CH=), 7.60 (d, 1H, J = 16.0 Hz, CH=), 7.00–7.23 (m, 6H, aromatic), 6.56 (d, 1H, J = 16.0 Hz, CH=), 6.27 (d, 1H, J = 16.0 Hz, CH=), 5.84 (t, 1H, J = 2.7 Hz, H-2), 5.69 (t, 1H, J = 10.3 Hz, H-6), 5.61 (t, 1H, J = 10.0 Hz, H-4), 5.32 (dd, 1H, J = 2.7 Hz, J = 10.0 Hz, H-3), 5.27 (t, 1H, J = 10.3 Hz, H-5), 5.22 (dd, 1H, J = 2.7 Hz, J = 10.3 Hz, H-1), 3.93 (s, 3H, OCH_3), 3.80 (s, 3H, OCH_3), 2.34 (s, 3H, acetyl), 2.30 (s, 3H, acetyl), 2.05 (s, 3H, acetyl), 2.04 (s, 3H, acetyl), 2.01 (s, 3H, acetyl), 2.00 (s,3H, acetyl) ppm; ^{13}C NMR ($CDCl_3$) δ 169.9, 169.8, 169.6, 169.5, 168.6, 165.5, 165.1, 151.5, 151.4, 146.1, 146.0, 141.9, 141.8, 132.8, 123.3, 123.2, 122.1, 121.6, 116.6, 116.3, 111.3, 111.0, 71.0, 69.6, 68.7, 68.6, 68.3, 56.1, 55.9, 20.7, 20.6, 20.5 ppm; Found: C, 57.90; H, 5.27. calcd for $C_{38}H_{40}O_{18}$: C, 58.16; H, 5.14.
10. Compound **4**: Recrystallization from acetone–EtOH gave white powder. Mp 229–231 °C; IR (KBr) ν 3500, 2945, 1760, 1640, 1600, 1515, 1420, 1365, 1330, 1260, 1225, 1170, 1155, 1130, 1070, 1040, 980, 920, 855, 835 cm^{-1}; ^1H NMR (400MHz, $CDCl_3$) δ 7.65 (d, 1H, J = 15.9 Hz, CH=), 7.03–7.24 (m, 3H, aromatic), 6.36 (d, 1H, J = 15.9 Hz, CH=), 5.69 (t, 1H, J = 10.1 Hz, H-6), 5.61 (t, 1H, J = 10.0 Hz, H-4), 5.20 (t, 1H, J = 10.0 Hz, H-5), 5.12 (dd, 1H, J = 2.4 Hz, J = 10.1 Hz, H-1), 5.07 (dd, 1H, J = 2.4 Hz, J = 10.1 Hz, H-3), 4.38 (t, 1H, J = 2.4 Hz, H-2), 3.85 (s, 3H, OCH_3), 2.30 (s, 3H, acetyl), 2.07 (s, 3H, acetyl), 1.99 (s, 6H, acetyl), 1.96 (s, 3H, acetyl), ppm; ^{13}C NMR ($CDCl_3$) δ 169.8, 169.77, 169.73, 169.5, 168.7, 165.3, 151.4, 146.1, 141.9, 132.9, 123.3, 121.7, 116.5, 111.2, 70.9, 70.8, 70.7, 69.6, 69.3, 68.6, 55.9, 20.7, 20.61, 20.58, 20.52 ppm; Found: C, 55.05; H, 5.37. calcd for $C_{26}H_{30}O_{16}$: C, 55.12; H, 5.34.
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12. Compound **5**: Recrystallization from EtOH–$CHCl_3$ gave pale yellow needles. Mp 118–120 °C; IR (KBr) ν 3400, 2940, 1695, 1630, 1600, 1590, 1520, 1460, 1450, 1425, 1270, 1160, 1120, 1030, 980, 840, 820 cm^{-1}; ^1H NMR (400MHz, DMSO-d_6) δ 9.65 (s, 2H, OH), 7.50 (d, 1H, J = 15.9 Hz, CH=), 7.49 (d, 1H, J = 15.9 Hz, CH=), 6.74–7.33 (m, 6H, aromatic), 6.50 (d, 1H, J = 15.9 Hz, CH=), 6.38 (d, 1H, J = 15.9 Hz, CH=), 5.46 (t, 1H, J = 2.6 Hz, H-2), 4.91–5.12 (m, 4H, OH), 4.77 (dd, 1H, J = 2.6 Hz, J = 10.2 Hz, H-1), 3.81 (s, 3H, OCH_3), 3.73 (s, 3H, OCH_3), 3.67 (m, 1H, H-6), 3.50 (m, 2H, H-3 & H-4), 3.14 (m, 1H, H-5) ppm; ^{13}C NMR (DMSO-d_6) δ 166.1, 166.0, 149.2, 149.2, 147.9, 147.8, 145.1, 144.9, 125.5, 125.4, 123.1, 123.0, 115.4, 114.6, 114.3, 110.9, 74.6, 72.5, 72.2, 71.6, 70.4, 69.3, 55.6, 55.5 ppm; Found: C, 55.79; H, 5.41. calcd for $C_{26}H_{28}O_{12} \cdot 3/2\ H_2O$: C, 55.81; H, 5.58.
13. Compound **7**: Recrystallization from acetone–AcOEt gave pale yellow powder. Mp 188–190 °C; IR (KBr) ν 2935, 2855, 1765, 1720, 1635, 1600, 1508, 1470, 1450, 1420, 1370, 1330, 1260, 1220, 1200, 1150, 1120, 1035, 1010, 905, 850, 825 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.74 (d, 1H, J = 15.9 Hz, CH=), 7.71 (d, 1H, J = 15.9 Hz, CH=), 7.04–7.15 (m, 6H, aromatic), 6.54 (d, 1H, J = 15.9 Hz, CH=), 6.45 (d, 1H, J = 15.9 Hz, CH=), 5.43 (dd, 1H, J = 6.8 Hz, J = 10.8 Hz, H-6), 5.26 (dd, 1H, J = 4.5 Hz, J = 10.7 Hz, H-3), 4.71 (t, 1H, J = 4.5 Hz, H-2), 4.24–4.31 (m, 2H, H-1 & H-4), 3.87 (s, 6H, OCH_3), 3.60 (dd, 1H, J = 10.8 Hz, J = 9.5 Hz, H-5), 2.33 (s, 6H, acetyl), 1.3-1.9 (m, 20H, cyclohexylidene) ppm; ^{13}C NMR ($CDCl_3$) δ 168.7, 166.2, 165.5, 151.4, 151.3, 145.2, 144.7, 141.6, 141.4, 133.3, 133.1, 123.2, 123.2, 121.6, 121.3, 118.0, 117.5, 113.7, 111.3, 111.2, 111.1, 79.1, 76.2, 74.8, 74.6, 70.9, 55.9, 37.3, 36.3, 36.2, 35.1, 24.8, $23._9$, $_2$3.6, 20.6 ppm; Found: C, 64.15; H, 6.29. calcd for $C_{42}H_{48}O_{14} \cdot 1/2\ H_2O$: C, 64.19; H, 6.28.
14. Compound **10**: Recrystallization from EtOH–H_2O gave pale yellow powder. Mp 199–202 °C; IR (KBr) (3450, 2940, 1700, 1635, 1600, 1520, 1430, 1380, 1330, 1270, 1180, 1160, 1025, 1035, 980, 840, 820 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6 + D_2O) δ 7.57 (d, 1H, J = 15.9 Hz, CH=), 7.51 (d, 1H, J = 15.7 Hz, CH=), 6.76–7.29 (m, 6H, aromatic), 6.48 (d, 1H, J = 15.9 Hz, CH=), 6.45 (d, 1H, J = 15.9 Hz, CH=), 5.11(t, 1H, J = 10.0 Hz, H-6), 4.60 (dd, 1H, J = 2.6 Hz, J = 10.0 Hz, H-3), 3.91 (t, 1H, J = 2.5 Hz, H-2), 3.80 (s, 6H, OCH_3), 3.74 (t, 1H, J = 9.5 Hz, H-4), 3.41 (m, 1H, H-1), 3.28 (t, 1H, J = 9.7 Hz, H-5) ppm; ^{13}C NMR (DMSO-d_6) δ 166.3, 166.2, 149.1, 148.9, 147.8, 144.7, 144.1, 125.7, 125.9, 122.9, 122.7, 115.6, 115.4, 115.0, 110.9, 74.4, 74.1, 72.6, 70.1, 69.3, 55.6 ppm; Found: C, 54.63; H, 5.67. calcd for $C_{26}H_{28}O_{12} \cdot 2H_2O$: C, 54.93; H, 5.67.
15. Inhibitory tests of TPA-induced O_2^- generation were performed as previously reported,[17] with modifications. Differentiated HL-60 cells, suspended in 1 mL of Hank's buffer, were treated with 100 μM of each test compound (10 μL of stock solution) or the vehicle. After preincubation at 37 °C for 15 min, the suspension was centrifuged and the extracellular compounds were removed by washing with 1% bovine serum albumin (BSA) twice. Then, the cells were suspended in 1 mL of Hank's buffer, and incubated with 100 nM TPA or vehicle and 1 mg/mL cytochrome c at 37 °C for 30 min The reaction was terminated by adding an O_2^- dismutase solution (10,000 units/mL) and being placed on ice. After centrifugation, the level of extracellular O_2^- was measured by the cytochrome c reduction method, in which reduced cytochrome c was quantified by measuring the visible absorption of the supernatant at 550 nm. Cells treated with the compound, cytochrome c, and vehicle without TPA, and cells with the vehicle without the compound, cytochrome c, or TPA were used as negative and positive controles, respectively. Cells treated with the vehicle without the compound, cytochrome c, or vehicle without TPA were used as a blanks. Inhibitory rates (IRs) were calculated by the following formula

$$\left(1 - \frac{\text{compound Abs}_{550} - \text{negative Abs550}}{\text{positive Abs}_{550} - \text{blank Abs550}}\right) \times 100(\%)$$

Cell viability was detemined by a Trypan Blue dye exclusion test. Each experiment wsa done independently in duplicate twice, and the data are shown as mean standard deviation (SD) values.
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