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Chemoenzymatic synthesis of hydroxytyrosol monoesters and their suppression effect on nitric oxide production stimulated by lipopolysaccharides

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ABSTRACT

Fatty acid monoesters of hydroxytyrosol [2-(3,4-dihydroxyphenyl)ethanol] were synthesized in two steps from tyrosol (4-hydroxyphenylethanol) by successive *Candida antarctica* lipase B-catalyzed chemoselective acylation on the primary aliphatic hydroxy group over phenolic hydroxy group in tyrosol, and 2-iodoxybenzoic acid (IBX)-mediated hydroxylation adjacent to the remaining free phenolic hydroxy group. Examination of their suppression effects on nitric oxide production stimulated by lipopolysaccharides in RAW264.7 cells showed that hydroxytyrosol butyrate exhibited the highest inhibition (IC_{50} 7.0 μM) among the tested compounds.

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Hydroxytyrosol; fatty acid monoesters; chemoselective acylation; site-selective hydroxylation; anti-inflammatory effect

Oleuropein (**1a**) and its hydrolysate, hydroxytyrosol (**1b**, Scheme 1) are representative antioxidative catechols in olives [1]. A variety of physiological activities of **1b** have been reported [2–15]. For example, **1b** exhibited anti-inflammatory activity by preventing nitric oxide (NO) production stimulated by lipopolysaccharides (LPS) in macrophages [15]. Recently, one of the authors (M. F.-T.) has investigated the protective effect of **1b** and its ester derivatives **1c** and **1d** against neuronal cells apoptosis induced by the Parkinson's disease-related neurotoxin 6-hydroxydopamine (6-OHDA) in SH-SY5 cells, with butyrate **1d** showing the strongest inhibition. Only **1d** among three compounds induced the expression of the transcription factor, NF-E2-related factor-2 (Nrf2) and enhanced its transcriptional activation in those cells [16]. Independently, they found that Nrf2 suppressed the LPS-induced transcriptional up-regulation of the proinflammatory cytokines and the inducible NO synthase (iNOS) [17]. We became interested in the inhibitory effect of esters **1c–f** in NO generation in the murine macrophage RAW 264.7 cell line. For longer chain fatty acid esters **1e** (stearate) and **1f** (oleate), which are known as antioxidants [18–22], the esterification with long chain fatty acids was expected to increase the effectiveness [23,24].

For the biological assay, the efficient preparation of fatty acid esters **1c–f** was demanded. In the previous study [16], butyrate **1d** was synthesized by lipase-mediated chemoselective acylation of primary aliphatic hydroxy group over phenolic hydroxy groups in commercially available **1b** [25,26] as shown in Scheme 1.

However, **1b** itself is an expensive starting material, although it can be supplied either by the extraction and purification of wastewater from the manufacture of olive oil [12,27–30]. Chemists' efforts have been devoted to the synthesis of **1b** [30–33]. Among them, the site-selective hydroxylation of tyrosol (**2a**) with 2-iodoxybenzoic acid (IBX) as shown in Scheme 1 is straightforward. The reported yield, however, was only 30% due to the difficulty in isolation of the desired product **1b**. Those authors insisted that either increased lipophilicity of substrates or use of the elaborated polymer-supported IBX derivative [33] is necessary, to acquire an enhanced yield.

To solve above-mentioned problems, we herein describe alternative routes for the synthesis of **1c–f** from readily available **2a**, by exchanging the order of hydroxylation and acylation.

Results and discussion

Candida antarctica lipase B-catalyzed acylation of **2a** has so far been reported [22,34]. In our case, the desired reaction proceeded smoothly by employing a short chain fatty acid vinyl ester to give the corresponding acetate **2b** and butyrate **2c** in a quantitative manner. Hydroxylation adjacent to the phenolic hydroxy group was performed by IBX-mediated oxidation [21] and subsequent reduction with sodium borohydride [35]. The desired products **1c** (at 88% conversion) and **1d** (at 91% conversion) were isolated in 62% and 58% yields, respectively (Scheme 2).

Due to limited availability of long chain fatty acid vinyl esters with high purity, we adopted free

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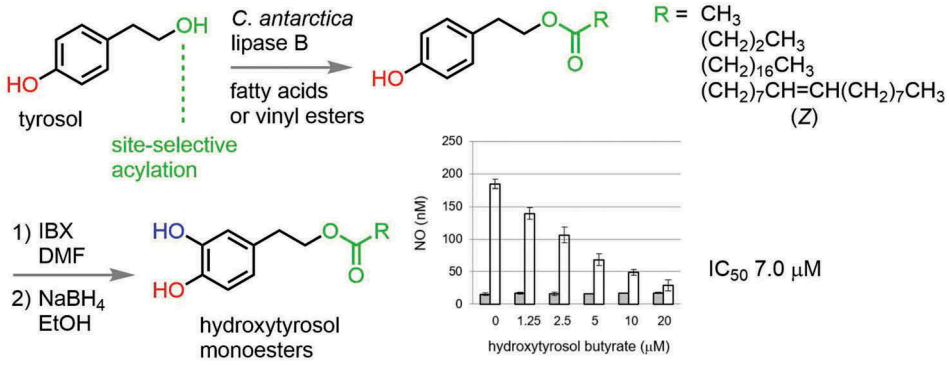


fatty acids for the lipase-catalyzed acylation [25] in the synthesis of stearate **2d** and oleate **2e**. Molecular sieves 4A were added to remove water that was concomitantly formed during the esterification. The reactions were slow even under such forced conditions and required prolonged reaction times (48 h) at room temperature, compared with the reactions of the short chain vinyl esters. At a conversion of around 50%, the reaction was worked up, and the desired products were purified by medium-pressure liquid chromatography. Monoesters **2c** and **2d** were isolated in 39% and 42% yields, respectively (Scheme 2). Although the hydroxylation yields were somewhat lower, stearate **1e** and oleate **1f** were isolated in 40% and 48% yields in sufficient amounts for the subsequent evaluation of inhibitory effects on NO production stimulated by LPS in RAW264.7 cells.

The results for the suppression effects of **1b-f** on NO production are summarized in Figure 1(a–e). Judging from the bar charts, butyrate **1d** exhibited the strongest inhibitory effect (Figure 1(c)) among the tested compounds. The estimated IC_{50} values (μM) for each compound were 18.5 for **1b**, 12.7 for **1c**, 7.0 for **1d**, 14.5 for **1e**, and 11.2 for **1f**, respectively. It should be noted that none of the compounds used in this study caused cell death, indicating that their inhibitory effects on NO production were not due to their cytotoxicity.

We state here only a brief comment for the proposed mechanism of the inhibition. As mentioned in the introduction [16,17], butyrate **1d** induced the expression of Nrf2 and enhanced its transcriptional activation in the cells. Also, Nrf2 interfered with the LPS-induced transcriptional up-regulation of the proinflammatory cytokines and inducible NO synthase (iNOS) in RAW264.7 cells. From these findings, we suppose that butyrate **1d** effectively inhibited NO production by activating Nrf2.

The action of intracellular short-chain fatty acid esterases on **1b–d** would promptly converge them into the identical free form **1b**. The difference in inhibitory effect among those of compounds is probably ascribable to the cell permeability depending upon the lipophilicity of compounds. The synthesis and evaluation of acylated forms with fatty acids of intermediate chain length between **1d** and **1e** may provide a clue to clarify this phenomenon.

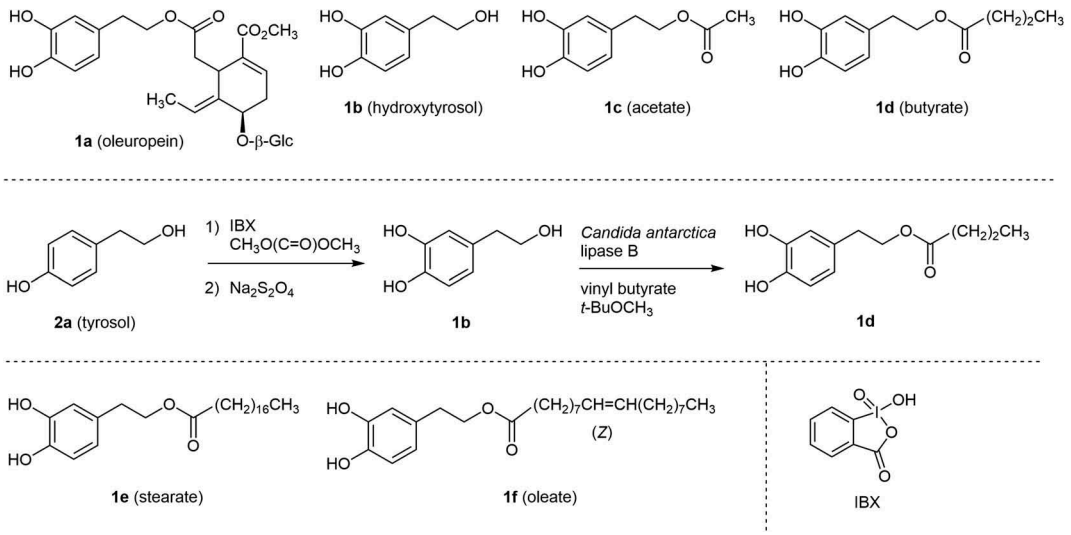
Conclusion

We synthesized four fatty acid monoesters (**1c-f**) of hydroxytyrosol (**1b**) from inexpensive tyrosol (**2a**) by combining lipase-catalyzed chemoselective acylation and subsequent hydroxylation. By arranging the order of these two reactions in this way, the total yield of the products and the handling of the intermediates were improved from those in the originally reported procedure [25,26]. All products showed inhibitory effects on NO production stimulated by LPS. Among them, butyrate **1d** was the most potent inhibitor with IC_{50} value of 7.0 μM. In this way, we found a new property of hydroxytyrosol monoesters other than so-far reported anti-apoptosis effect.

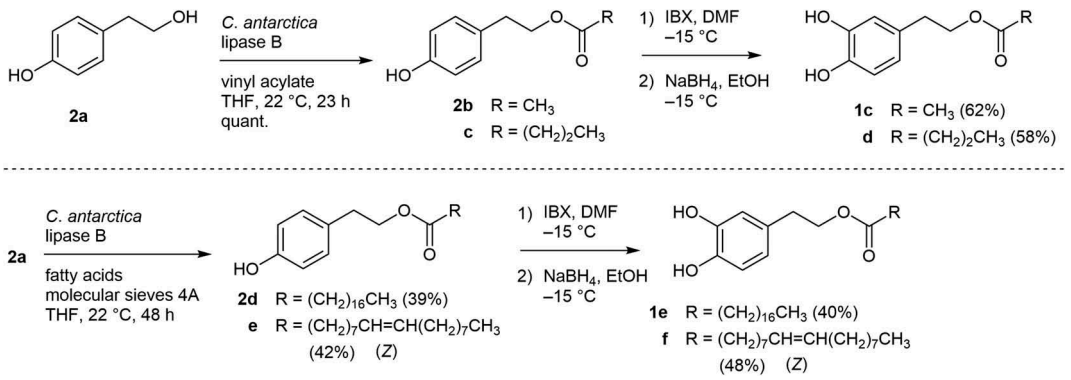
Experimental

General

Candida antarctica lipase B (Novozym 435) was purchased from Novozymes Japan. 2-(4-Hydroxyphenyl) ethanol (tyrosol, H0720) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). RAW264.7 cells were purchased from the Riken Cell Bank (Ibaraki, Japan). LPS (*E. coli* 055:B5) was purchased from Sigma-Aldrich Co. (St, Louis, MO, USA). Dulbecco's Modified Eagle's Medium (DMEM) and a penicillin (10,000 units/mL)-Streptomycin (1 mg/mL) mixed solution were purchased from Nacalai Tesque Inc. (Kyoto, Japan). Fetal bovine serum (FBS, Gibco®) was purchased from Thermo Fisher Scientific Inc. (Waltham MA, USA). (Nacalai Tesque). Column chromatography was performed with silica gel (Kanto Chemical Co. Silica Gel



Scheme 1. Catechols involved in olives **1a** and **1b**, examples of fatty acid monoesters **1c** to **1f** derived from **1a**, and the previous synthesis of **1d.**



Scheme 2. Chemoenzymatic synthesis of **1c** - **f** starting from readily available tyrosol **2a.**

60 N 37560-79, spherical and neutral, 40–50 μm). Medium-pressure liquid chromatography was performed using Silica Gel 60 (spherical and neutral; 100–210 μm, 37560-79, Kanto Chemical Co.) and the Isolera One flash purification system (Biotage, Sweden). Preparative TLC was performed with Merck Silica Gel 60 F_{254} plates (0.5 mm thickness, No. 1.05744.0001). ^{1}H NMR spectra were measured at 400 MHz on a VARIAN 400-MR or at 500 MHz on an Agilent INOVA-500 spectrometer and ^{13}C NMR spectra were measured at 100 MHz on a VARIAN 400-MR or at 125 MHz on an Agilent INOVA-500 spectrometer. DMSO-d_6 and $CDCl_3$ were used as a solvent and the residual peaks were used as an internal standard (^{1}H NMR: DMSO-d_6 2.48 ppm, $CHCl_3$ in $CDCl_3$ 7.26 ppm; ^{13}C NMR: DMSO-d_6 39.9 ppm, $CDCl_3$ 77.0 ppm). IR spectra were measured as ATR on a Jasco FT/IR-4700 FT-IR spectrometer. High resolution mass spectra (HRMS) were measured by a on Jeol JMS-T100LP AccuTOF. Microplate reader Infinite M1000 (Tecan Group Ltd. Tokyo Japan) was used for the measurement of absorbance.

2-(4-Hydroxylphenyl)ethyl acetate (2b)

To a solution of **2a** (2.8 g, 20 mmol) in tetrahydrofuran (THF, 37 mL) was added *C. antarctica* lipase B (409 mg) and vinyl acetate (2.8 mL, 30 mmol). The mixture was stirred for 4 h at room temperature. The mixture was diluted with chloroform and filtered with a pad of Celite. The combined filtrate and washings were dried *in vacuo* to give **2b** as colorless solid (3.84 g, quantitative). M.p. 59–60°C. ^{1}H-NMR (400 MHz, DMSO- d_6) δ: 1.96 (3H, s), 2.73 (2H, t, J = 7.0 Hz), 4.10 (2H, t, J = 7.0 Hz), 6.66 (2H, d, J = 8.4 Hz), 7.00 (2H, d, J = 8.4 Hz), 9.19 (s, OH). ^{13}C-NMR (100 MHz, DMSO-d_6) δ: 21.2, 34.0, 65.2, 115.6, 128.3, 130.2, 156.3, 170.8. IR ν_{max} cm^{-1}: 3361, 3022, 2957, 1705, 1614, 1596, 1515, 1444, 1388, 1364, 1227, 1173, 1107, 1030, 975, 830. HR-MS [ESI+, $(M+Na)^+$] calculated for $C_{10}H_{12}O_3Na$, 203.0684; found, 203.0707.

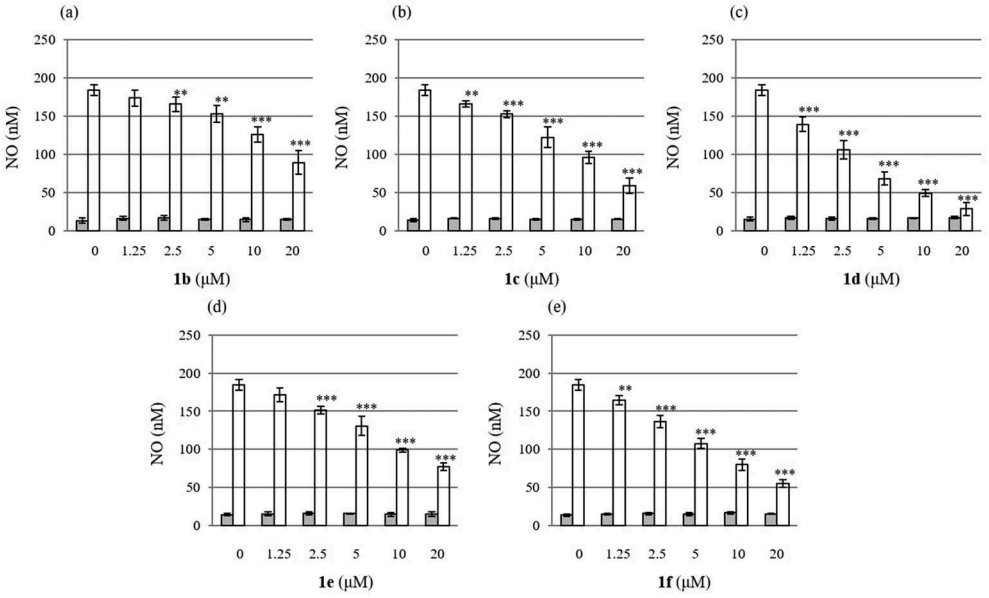


Figure 1. Suppression effect of **1b** - **f** on the NO production stimulated by LPS in RAW264.7 cells. Shaded (left) bars and open (right) bars represent concentration of NO without (control experiments) or with LPS, respectively. Values are given as the mean ± S.D. from four independent experiments. Multiple group comparisons were made using one- or two-way analysis of variance (ANOVA) followed by Tukey's test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ significantly different from the control group stimulated with LPS. For detail, see experimental section.

2-(4-Hydroxylphenyl)ethyl butyrate (2c)

In a similar manner as described for the synthesis of **2b** in the previous section, diol **2a** was treated with *C. antarctica* lipase B and vinyl butyrate. The workup and purification furnished **2c** as pale yellow oil (5.36 g, quantitative). ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 0.84 (3H, t, J = 7.4 Hz), 1.51 (2H, dq, J = 7.3, 7.4 Hz), 2.23 (2H, t, J = 7.3 Hz), 2.75 (2H, t, J = 7.0 Hz), 4.14 (2H, t, J = 7.0 Hz), 6.67 (2H, d, J = 8.6 Hz), 7.02 (2H, d, J = 8.6 Hz), 9.21 (OH, s). ^{13}C-NMR (125 MHz, DMSO-d_6) δ: 13.8, 18.4, 34.0, 35.8, 65.0, 115.6, 128.3, 130.2, 156.3, 173.1. IR ν_{max} cm^{-1} 3380, 2963, 2935, 2875, 1704, 1614, 1596, 1515, 1445, 1389, 1356, 1308, 1185, 1171, 1105, 1046, 985, 828. HR-MS [ESI+, (M+Na)$^+$]: calculated for $C_{12}H_{16}O_3Na$ 231.0997; found, 231.1001.

2-(4-Hydroxylphenyl)ethyl stearate (2d)

To a solution of **2a** (150 mg, 1.1 mmol) in THF (1.5 mL) was added *C. antarctica* lipase B (15 mg), molecular sieves 4A (50 mg), and stearic acid (460 mg, 1.6 mmol). The mixture was stirred for 48 h at room temperature. The mixture was diluted with chloroform and filtered with a pad of Celite. The combined filtrate and washings were dried *in vacuo*. The residue was purified by medium-pressure liquid chromatography. Elution with hexane-ethyl acetate (10:1) furnished **2d** as colorless amorphous solid (172 mg, 39%). ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 0.88 (3H, t, J = 7.0 Hz), 1.16–1.34 (28H, m), 1.51–1.65 (2H, m), 2.28 (2H, t, J = 7.6 Hz), 2.86 (2H, t, J = 7.1 Hz), 4.24 (2H, t, J = 7.1 Hz), 6.66 (2H, d, J = 8.4 Hz), 7.00 (2H, d, J = 8.4 Hz). Due to the broadening of signal, proton on phenolic hydroxy group was not detected. ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 14.1, 22.7, 24.9, 29.1, 29.3, 29.4, 29.5, 29.6, 29.7, 29.7, 31.9, 34.3, 34.4, 65.0, 115.3, 130.0, 154.2, 174.0. Some signals of methylene carbons were overlapped. IR ν_{max} cm^{-1}: 3252, 2915, 2847, 2359, 1731, 1615, 1519, 1464, 1392, 1251, 1234, 1214, 1193, 1163, 826, 719, 564, 521. HR-MS [ESI+, (M+Na)$^+$] calculated for $C_{26}H_{44}O_3Na$, 427.3188; found, 427.3171.

2-(4-Hydroxylphenyl)ethyl oleate (2e)

In a similar manner as described for the synthesis of **2d** in the previous section, diol **2a** was treated with *C. antarctica* lipase B and oleic acid. The workup and purification furnished **2e** as colorless amorphous solid (180 mg, 42%). ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 0.81 (3H, t, J = 6.9 Hz), 1.16–1.34 (20H), 1.43–1.63 (2H, m), 1.87–2.01 (4H, m), 2.21 (2H, t, J = 7.5 Hz), 2.78 (2H, t, J = 7.1 Hz), 4.17 (2H, t, J = 7.1 Hz), 5.10–5.44 (2H, m), 6.68 (2H, d, J = 8.4 Hz), 7.00 (2H, d, J = 8.4 Hz). Due to the broadening of signal, proton on phenolic hydroxy group was not detected. ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 14.1, 22.7, 24.9, 27.2, 27.2, 29.1, 29.2, 29.3, 29.5, 29.7, 29.8, 31.9, 34.3, 34.4, 65.0, 115.3, 129.8, 130.0, 130.0, 154.3, 174.0. Some signals of methylene carbons were overlapped. IR ν_{max} cm^{-1}: 3389, 3004, 2923, 2852, 2359, 2342, 1737, 1710, 1615, 1596, 1517, 1456, 1354, 1225, 1172, 1006, 830, 724. HR-MS [ESI+, (M+Na)$^+$] calculated for $C_{26}H_{42}O_3Na$, 425.3032; found, 425.3035.

2-(3,4-Dihydroxylphenyl)ethyl acetate (1c)

IBX (3.34 g, 45% purity, 5.4 mmol) was sonicated in *N,N*-dimethylformamide (DMF, 128 mL). The resulting suspension was stirred at – 15°C under Ar, and to that was added a solution of **2b** (724 mg, 4.0 mmol) in DMF (12 mL) in one portion. The mixture was further stirred for 22 h at – 15°C under Ar. To the mixture, a pre-cooled solution of sodium borohydride ($NaBH_4$, 120 mg) in ethanol (8.0 mL) was slowly added at – 15°C, and the color of the mixture turned from brown to yellow. The mixture was diluted with ethyl acetate (180 mL) and to that was added a solution of 0.1 M phosphate buffer with 3.5% of sodium dithionite and 35% of sodium chloride. The mixture was stirred at room temperature for 1.5 h. The organic layer was separated, and the aqueous layer was extracted with ethyl acetate twice. The combined extract was washed with aqueous solution including sodium hydrogen carbonate (10%) and sodium dithionite (5%) and brine, dried over anhydrous sodium sulfate and concentrated *in vacuo*. The residue was purified by silica gel column chromatography. Elution with hexane-ethyl acetate (5:1 to 3:1) furnished **1c** as a pale yellow oil (489 mg, conversion: 85%; yield: 62%). ^{1}H-NMR (500 MHz, DMSO-d_6) δ: 1.96 (3H, s), 2.67 (2H, t, J = 7.1 Hz), 4.08 (2H, t, J = 7.1 Hz), 6.45 (1H, dd, J = 2.2, 8.0 Hz), 6.59 (1H, d, J = 2.2 Hz), 6.62 (1H, d, J = 8.0 Hz), 8.67 (OH, s), 8.75 (OH, s). ^{13}C-NMR (100 MHz, DMSO-d_6) δ: 21.2, 34.2, 65.2, 116.0, 116.6, 119.9, 129.0, 144.2, 145.5, 170.8. IR ν_{max} cm^{-1} 3360, 3035, 2959, 1703, 1605, 1519, 1444, 1387, 1363, 1241, 1192, 1149, 1113, 1031, 978, 956, 864, 807, 782. HR-MS [ESI+, $(M+Na)^+$]: calculated for $C_{10}H_{12}O_4Na$, 219.0633; found, 219.0671.

2-(3,4-Dihydroxylphenyl)ethyl butyrate (1d)

In a similar manner as described for the synthesis of **1c** in the previous section, butyrate **2c** (418 mg, 2.0 mmol) was treated with IBX then $NaBH_4$. The workup and purification furnished **1d** as colorless oil (261 mg, conversion: 91%; yield: 58%). ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 0.85 (3H, t, J = 7.3 Hz), 1.51 (2H, tq, J = 7.3, 7.3 Hz), 2.24 (2H, t, J = 7.3 Hz), 2.69 (2H, t, J = 7.1 Hz), 4.12 (2H, t, J = 7.1 Hz), 6.46 (1H, dd, J = 2.2, 7.8 Hz), 6.60 (1H, d, J = 2.2 Hz), 6.63 (1H, d, J = 7.8 Hz), 8.69 (OH, s), 8.76 (OH, s). ^{13}C-NMR (125 MHz, DMSO-d_6) δ: 13.8, 18.4, 34.3, 35.8, 65.0, 115.9, 116.6, 119.9, 129.0, 144.2, 145.5, 173.2. IR ν_{max} cm^{-1} 3378, 3035, 2963, 2934, 2875, 1703, 1605, 1520, 1444, 1384, 1346, 1279, 1256, 1184, 1112, 1044, 986, 956, 923, 865, 807, 782. HR-MS [ESI+, $(M+Na)^+$]: calculated for $C_{12}H_{16}O_4Na$, 247.0946; found, 247.0938.

2-(3,4-Dihydroxylphenyl)ethyl stearate (1e)

In a similar manner as described for the synthesis of **1c** in the previous section, stearate **2d** (221 mg, 0.55 mmol) was treated with IBX then $NaBH_4$. The workup and purification furnished **1e** as colorless amorphous solid (92 mg, yield: 40%). ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 0.81 (3H, t, J = 7.0 Hz), 1.07–1.32 (28H, m), 1.51–1.65 (2H, m), 2.22 (2H, t, J = 7.6 Hz), 2.75 (2H, t, J = 7.1 Hz), 4.16 (2H, t, J = 7.1 Hz), 5.13 (1H, broad), 5.30 (1H, broad), 6.57 (1H, dd, J = 2.0, 8.0 Hz), 6.67 (1H, d, J = 2.0 Hz), 6.72 (1H, d, J = 8.0 Hz). ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 14.1, 22.7, 24.9, 29.1, 29.3, 29.4, 29.7, 29.7, 31.9, 34.4, 34.4, 64.8, 115.3, 116.0, 121.4, 130.9, 142.1, 143.5, 174.0. Some signals of methylene carbons were overlapped. IR ν_{max} cm^{-1}: 3321, 2954, 2915, 2848, 1737, 1598, 1520, 1462, 1330, 1275, 1254, 1234, 1213, 1192, 1173, 1119, 961, 822, 728. HR-MS [ESI+, $(M+Na)^+$] calculated for $C_{26}H_{44}O_4Na$, 443.3137; found, 443.3159.

2-(3,4-Dihydroxylphenyl)ethyl oleate (1f)

In a similar manner as described for the synthesis of **1c** in the previous section, oleate **2f** (120 mg, 0.3 mmol) was treated with IBX then $NaBH_4$. The workup and purification furnished **1f** as a colorless oil (60 mg, yield: 48%). ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 0.88 (3H, t, J = 7.0 Hz), 1.07–1.32 (20H, m), 1.51–1.63 (2H, m), 1.92–2.10 (4H, m), 2.29 (2H, t, J = 7.6 Hz), 2.81 (2H, t, J = 7.1 Hz), 4.24 (2H, t, J = 7.1 Hz), 5.23–5.45 (2H, m), 6.64 (1H, dd, J = 1.5, 8.0 Hz), 6.73 (1H, d, J = 1.5 Hz), 6.78 (1H, d, J = 8.0 Hz). Due to the broadening of signal, proton on phenolic hydroxy group was not detected. ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 14.1, 22.7, 24.9, 27.2, 29.1, 29.2, 29.3, 29.5, 29.7, 29.8, 31.9, 34.4, 34.4, 64.9, 115.3, 115.9, 121.3, 129.8, 130.0, 130.8, 142.1, 143.5, 174.1. Some signals of methylene carbons were overlapped. IR ν_{max} cm^{-1}: 3334, 3004, 2918, 2849, 2360, 2341, 1732, 1706, 1604, 1520, 1463, 1276, 1252, 1214, 1179, 1113, 813, 720, 504. HR-MS [ESI+, $(M+Na)^+$] calculated for $C_{26}H_{44}O_4Na$, 441.2981; found, 441.2990.

Examination of the effect of 1b–1f on the production of NO mediated by LPS

The murine macrophage cell line, RAW264.7 was cultured at 37°C under 5% CO_2/95% air in DMEM supplemented with 10% FBS and penicillin-streptomycin mixed solution. RAW264.7 cells (2×10^5 cells) were cultured in a 24-well plate and pre-incubated with various concentrations of hydroxytyrosol and its derivatives (**1b-f**) or a solution of dimethyl sulfoxide (0.1%) at 37°C for 1 h prior to the

stimulation with LPS (1 μg/mL) for 24 h. The concentration of nitrirte (NO_2^-) ion derived from NO in the culture supernatants was estimated with microplate reader at the absorbance at 540 nm, after coloration using Griess reagent, which contained sulfanilamide (1%), *N*-naphthylethylenediamine (0.1%), and H_3PO_4 (2.5%). IC_{50} values were calculated from the concentration of **1b-f** which was required for 50% inhibition of NO production in the experiments. The results were expressed as the bar charts in Figure 1 (a–e).

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Author contributions

T.S and M.F-T. designed this study; A.S. and M.P. mainly carried out the experiments in chemistry; A.N. mainly carried out the experiment in biology; T.S. and M.F.-T. wrote the manuscript with assistance from all authors; and K.H., S.H. and H.T. occasionally discussed on the results.

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