Biological Activities of 3,5-Dihydroxy-*N*-(4-hydroxyphenyl)benzamide: A Mimic Compound of *trans*-Resveratrol

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Photo-aging is mainly due to ultraviolet irradiation of sunlight, which overwhelmingly contributes to a premature aging.[1] Exposure to UV irradiation is known to cause excessive generation of reactive oxygen species (ROS), which is regarded to be an important contributor to a variety of skin damages.[2] Although skin possesses an elaborate antioxidant defense system, the excessive generation of ROS results in an oxidant-antioxidant imbalance in the skin.[3] Thus, exogenous supplementation of antioxidants may be an effective strategy to counteract the deleterious effects of the ROS generated from the excessive exposure to UV irradiation. Resveratrol (*trans*-3,4',5-trihydroxystilbene), a naturally occurring hydroxyl stilbene, is considered an essential antioxidative constituent of red wine.[4] It showed broad antioxidative activities such as scavenging of the free radicals,[5] inhibition of lipid peroxidation,[6] inhibition of the platelet aggregation[7] and anti-cancer activity.[8] However, because of its unfavorable photo stability, the application of *trans*-resveratrol has been limited. Various UV irradiation conditions showed that *trans*-resveratrol was easily transformed into *cis*-resveratrol.[9] Therefore, an attempt to search for photo stable alternative material of *trans*-resveratrol is desirable. In this study, a mimic compound of *trans*-resveratrol, 3,5-dihydroxy-*N*-(4-hydroxyphenyl)benzamide (**2**), containing an amide linker between two hydroxyl phenyl groups, is synthesized. We evaluated various biological activities of compound **2** compared with *trans*-resveratrol (Fig. 1).

Materials and Methods

Chemistry. The synthetic pathway of compound **2** is shown in Scheme 1.

The starting material, 3,5-dihydroxybenzoic acid **3** was reacted with acetic anhydride in the presence of TEA and a drop of DMAP in THF to afford 3,5-diacetoxybenzoic acid **4**. Compound **4** was converted into mixed anhydride **5** by the treatment with methansulfonyl chloride in the presence of TEA in THF, which was reacted immediately with 4-amino phenol to produce the corresponding amide derivative **6**. The hydrolysis of compound **6** afforded desired hydroxyl benzamide **2**.

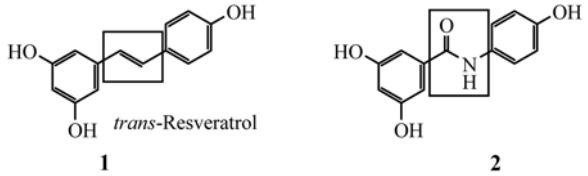
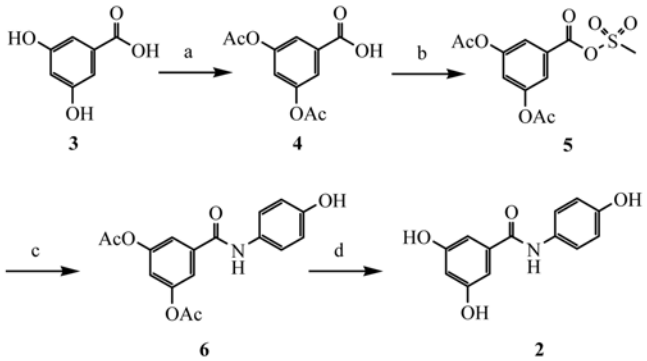


Figure 1. Structure of *trans*-resveratrol and compound **2**.

3,5-Diacetoxy benzoic acid (4). To a solution containing 3,5-dihydroxy benzoic acid (5.2 g, 0.03 mol) and triethyl amine (7.7 g, 0.076 mol), 4-(dimethylamino)pyridine (cat) in tetrahydrofuran (60 mL) was added acetic anhydride (7.8 g, 0.076 mol). The reaction mixture was refluxed for 3 h and then solvent was removed at reduced pressure. The residue was dissolved in dichloromethane and washed with HCl (1 M) solution. The organic layer was dried with anhydrous $MgSO_4$ and concentrated to give a crude product. The resultant was purified by crystallization from dichloromethane-hexane to give a 3,5-diacetoxy benzoic acid **4** (6.1 g) in 85% yields. TLC, SiO_2, EtOAc/hexanes 2:1, R_f = 0.43. ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.21 (s, 6H), 7.22 (s, 1H), 7.53 (s, 2H). Ms-FAB (m/e) 239 (M^++1).

***N*-(4-Hydroxyphenyl)-3,5-diacetoxy benzamide (6).** To a solution containing 3,5-diaceoxy benzoic acid (3.9 g, 0.017 mol) and triethyl amine (1.9 g, 0.019 mol) in tetrahydrofuran (60 mL) was added methanesulfonyl chloride (2.0 g, 0.018 mol). The reaction mixture was stirred for 40 min at room temperature and filtered to remove the resulting precipitate. To a filterate was added 4-amino phenol (2.0 g, 0.018 mol) in *N*,*N*-dimethylformamide (8 mL) and stirred 3 h at room temperature. The reaction mixture was concentrated *in vacuo* then dichloromethane was added followed by HCl (1 M) solution. The organic layer was dried with anhydrous $MgSO_4$ and concentrated to give a crude product. The resultant was purified by crystallization from ethyl



Scheme 1. Reagents and conditions: (a) acetic anhydride, TEA, DMAP (cat), THF; (b) $MeSO_2Cl$, TEA, THF; (c) 4-amino phenol, TEA, THF; (d) KOH, H_2O

acetate-hexane to afford 3,5-diacetoxy benzamide **6** (5.0 g) in 90% yields. TLC, SiO_2, EtOAc/hexanes 2:1, R_f = 0.54. ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.28 (s, 6H), 6.62 (d, 2H, J = 8.7 Hz), 7.20 (s, 1H), 7.42 (d, 2H, J = 8.7 Hz), 7.64 (s, 2H), 9.21 (s, 1H), 10.02 (s, 1H). Ms-FAB (m/e) 330 (M^++1).

3,5-Dihydroxy-*N*-(4-hydroxyphenyl)benzamide (2). *N*-(4-Hydroxypheny)-3,5-diacetoxy benzamide (3.3 g, 0.01 mol) was dissolved in KOH (0.5 M) solution and heated to 50 °C. After obtaining clear solution, the clear solution was acidified with HCl (1 M) solution and resulting precipitate was gathered by filteration to give *N*-(4-hydroxypheny)-3,5-dihydroxy benzamide **2** (2.0 g) in 83 yields. TLC, SiO_2, EtOAc/hexanes 2:1, R_f = 0.42. ^{1}H NMR (300 MHz, DMSO-d_6) δ 6.25 (s, 1H), 6.62 (m, 4H), 7.42 (d, 2H, J = 8.7 Hz), 9.19 (s, 1H), 9.42 (bs, 2H), 9.81 (s, 1H). Ms-FAB (m/e) 246 (M^++1).

DPPH radical scavenging assay. DPPH radical-scavenging assay was carried out using the following procedure. The reaction mixture containing various concentrations of the test samples and DPPH methanolic solution (0.2 mM) was incubated at room temperature for 30 min and the absorbance was measured at 517 nm. The scavenging activity was expressed as a percent compared to control DPPH solution (100%). The synthetic antioxidant trolox and L-ascorbic acid were included in experiments as a positive control.

Superoxide radical assay. Superoxide radicals were generated by the xanthine/xanthine oxidase (XO) system, following described procedure. The reaction mixture consisted of xanthine (0.5 mM), NBT (0.5 mM) and test samples, in a final volume of 100 mL. Xanthine and NBT were dissolved in phosphate buffer 200 mM with EDTA 0.25 mM, pH 7.5. The reaction was conducted at room temperature for 2 min, and initiated by the addition of 100 mL of XO (50 mU/mL). The mixture was allowed to stand for 30 min at room temperature, and the absorbance at 550 nm was measured. Different concentrations of compounds were analyzed and then the half-minimal inhibitory concentration (IC_{50}) was calculated by linear regression analysis.

Xanthine oxidase activity. The effect of compounds on xanthine oxidase activity was evaluated by measuring the formation of uric acid from xanthine. The reaction mixtures contained the same proportion of components as in assay for superoxide radical, except NBT. The absorbance was measured at 295 nm.

Cell culture. Human normal dermal fibroblasts (HNDF) were purchased from Sigma Co. Ltd. and cultured with Dulbecco's modified Eagle's medium (DMEM) containing 0.48 mg/mL glutamine, 100 IU/mL penicillin, 50 mg/mL streptomycin, and 10% fetal bovine serum.

Measurements of pro-collagen synthesis. Human normal dermal fibroblasts were seeded in 48-well plates. Cells were treated with medium containing various concentrations of samples and then further culture for 24 hours. Culture medium was used for the determination of collagen synthesis. The collagen content was determined by pro-collagen type I C-peptide ELISA kit (MK101 Takara, Japan).

Measurements of MMP-1 inhibition. Human normal dermal fibroblasts were seeded in 48-well plates. Confluent cultures of fibroblasts were irradiated with a high-intensity UVA source (Dermlight cube 401 equipped with UVA filters, Uvatec, Inc.) through the thin layer of PBS in the tissue culture plate. After irradiation, fibroblasts were re-fed with 0.5 mL of DMEM without serum and incubated for 48 hours. Interstitial collagenase was measured with the MMP-1 human ELISA system (Amersham Pharmacia Biotech, UK).

Results and Discussion

DPPH assay. Table 1 shows the DPPH radical scavenging of compound **2**. DPPH (Diphenylpicrylhydrazyl)[10] is widely used as a substrate to evaluate antioxidative activity of antioxidant. The method is based on the reduction of methanolic DPPH solution in the presence of a hydrogen donating antioxidant, due to the formation of non-radical form DPPH-H by the reaction. Compound **2** showed mild scavenging activity (IC_{50} = 99.4 ± 5.6 μM), which was similar to that of *trans*-resveratrol. However, scavenging activities of L-ascorbic acid and trolox, used as positive controls, were more potent than that of compound **2**.

Superoxide radicals scavenging assay. To evaluate scavenging of superoxide radicals, Xantine/Xanthine oxidase system[11] was used. Superoxide radicals can be generated by xantine oxidase in living cells. Table 2 shows that scavenging activities of compound **2**, *trans*-resveratrol and allopurinol.[12] Compound **2** showed moderate superoxide radical scavenging activity (IC_{50} = 227.4 ± 16.8 μM). Its activity was more potent than that of *trans*-resveratrol (IC_{50} = 388.0 ± 4.5 μM). A control experiment was performed to determine whether direct scavenging activity of superoxide radicals or inhibitory effect on the xanthine oxidase. Enzyme activity was measured by analyzing amount of uric acid. Compound **2** and *trans*-resveratrol showed similar inhibitory activities in xanthine oxidase.

Table 1. DPPH radical scavenging activity of compound **2**

Samples	IC_{50} (μM)*
Compound **2**	99.4 ± 5.6
trans-Resveratrol	124.8 ± 1.4
L-Ascorbic acid	48.8 ± 2.7
Trolox	51.3 ± 2.1

*IC_{50} was calculated from regression line using thirteen different concentrations in triplicate experiments

Table 2. Superoxide radical scavenging activity of compound **2**

Samples	Xanthine oxidase inhibitory activity (IC_{50}, μM)*	
	Superoxide generation inhibition	Uric acid generation inhibition
Compound **2**	227.4 ± 16.8	121.2 ± 10.2
trans-Resveratrol	388.0 ± 4.5	110.5 ± 10.8
Allopurinol	63.1 ± 1.2	19.2 ± 3.2

*IC_{50} was calculated from regression line using thirteen different concentrations in triplicate experiments

Table 3. Anti-aging activities of compound **2** in cultured human skin fibroblast

Sample	Concentration (μM)	Cell viability (%)	Expression rate (% of control)	
			Procollagen type I	MMP-1
Control		100.0	100.0	100.0
Compound **2**	10	91.4	138.0	59.4
trans-Resveratrol	10	85.88	152.0	105.2
L-Ascorbic acid	200	90.64	132.8	N.T
All-trans-RA	1	97.14	N.T	78.2

N.T: Not tested

Anti-aging activities. To evaluate anti-aging activities of compound **2**, we examined the stimulate effect on collagen synthesis of human fibroblast and the inhibitory effect on the expression of MMP-1 induced by UV irradiation. UV irradiation damages human skin and causes premature skin aging through the activation of matrix metalloproteinases (MMPs) which are responsible for the degradation of skin components such as collagen,[13] elastin[14] and fibronectin[15] etc. Of MMPs, MMP-1 (collagenase)[16] is mainly involved in skin aging due to its ability to degrade collagen. Table 3 shows anti-aging activities of compound **2**. The treated concentrations of compound **2** (10 μM), *trans*-resveratrol (10 μM), L-ascorbic acid[17] (200 μM) and *all-trans*-retinoic acid[18] (1 μM) did not affect significantly the cellular viabilities in cultured fibroblasts. The stimulate effect on collagen synthesis of human fibroblasts was evaluated by ELISA for type I procollagen. The procollagen protein level of cells, which were cultured with compound **2** at the concentration of 10 μM, was found to be 138%. The 10 μM *trans*-resveratrol and 200 μM L-ascorbic acid increased protein level by 152% and 132%, respectively. However, compound **2** decreased the expression of MMP-1 at the protein level by UV irradiation of cultured human skin fibroblasts whereas *trans*-resveratrol showed no activity. The MMP-1 expression level after the addition of compound **2** (10 μM) and RA (1 μM) was found to be 40.6% and 21.8% lower than UV irradiated control, respectively.

In conclusion, we synthesized 3,5-Dihydroxy-*N*-(4-hydroxyphenyl)benzamide (**2**) and analyzed whether this compound is suitable for a photo stable *trans*-resveratrol mimic compound. *trans*-Resveratrol possess two critical parts such as three phenolic hydroxyl groups (resorcinol and phenol) and unsaturated double bond. Compound **2** was designed by replacing the photo labile unsaturated group of *trans*-resveratrol with amide bond on the assumption that biological activities of *trans*-resveratrol may be originated from three phenolic hydroxyl groups. Compound **2** showed various radical scavenging activities similar to those of *trans*-resveratrol. Further, anti-aging activities of compound **2** were evaluated by analyzing effect on procollagen synthesis and down-regulation of MMP-1 induced by UV irradiation. Therefore, we suggest that compound **2** may be used as a candidate of photo stable *trans*-resveratrol mimic compound for the treatment of skin aging.

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