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Synthesis of Pyronyl Derivatives as Resveratrol Analogues and Their Inhibitory Effects on Nitric Oxide and PGE_2 Productions

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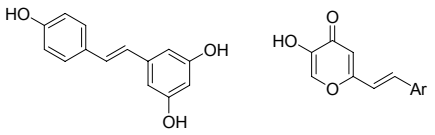
Inflammation is one of the most important aspects of host defense mechanisms against invading pathogens. However, inflammation may also aid microbial pathogenesis because the inflammatory response elicited by an invading microorganism can result in considerable host damage, making nutrients available and providing access to host tissues.[1]

Nitric oxide (NO) and prostaglandins (PGs) are two major mediators produced at inflammatory sites by the enzymes such as *i*NOS (inducible nitric oxide synthase) and COX-2. In inflammation, overproduction of NO is mainly caused by *i*NOS, which is up-regulated in macrophages by cytokine and/or bacterial lipopolysaccharide (LPS) stimulation.[2] Despite its beneficial role in host defense, sustained NO production can be deleterious to the host and has been implicated in the pathogenesis of various inflammatory diseases.[3] COX-2 is also one of enzymes that participate in inflammation, and induction of COX-2 is responsible for the production of PGE_2 at the site of inflammation.[4] Therefore, the regulations of NO and PGE_2 productions have been current research topics for the new anti-inflammatory drugs development.

Resveratrol (**1**), a polyphenolic phytochemical found in grapes,[5] blueberries,[6] and peanuts,[7] has been found to have a wide range of pharmacological activities, such as antioxidant, anti-inflammatory, antitumour and immunomodulatory activities. Previous studies have shown that resveratrol exhibit anti-inflammatory activities through the non-selective inhibition of COX-2 and COX-1.[8-9] Resveratrol has also been found to suppress macrophage activation and controls the expression of *i*NOS which accounts for its anti-inflammatory effects.[10-11] However, due to its low oral bioavailability[12] and the relatively high concentrations of resveratrol which are required to exert anti-inflammatory activity,[13] the modifications of the structure of resveratrol were desired to produce more efficient anti-inflammatory agents. In fact, there has been a number of reports on the synthesis of resveratrol analogues, such as hydroxystilbenes,[14-17] styrylthiophenes, styrylpyridines, and styrylquinolines.[18] We also reported the synthesis of styrylquinazolines as resveratrol analogues and their ability to inhibit the production of PGE_2 in LPS-activated macrophage cells.[19]

In the present study, pyronyl-vinyl derivatives **2** were prepared as resveratrol analogues and evaluated for their ability to inhibit both enzymes using *in vitro* assay for *i*NOS and COX-2 by measuring NO and PGE_2 productions, respectively, by LPS-activated macrophage cells. We envisioned that the substituted

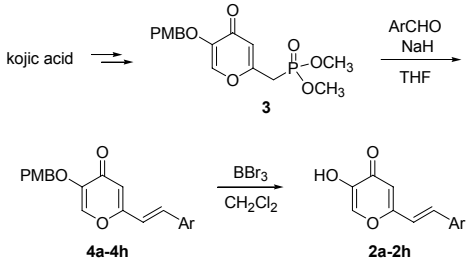


1, resveratrol **2**

pyran ring in **2** can be considered as hydroxylated benzene in resveratrol as was proven in our synthesis of tyrosinase inhibitory (4-oxo-4*H*-pyran-2-yl)acrylic acid as a caffeic or ferulic acid mimic.[20]

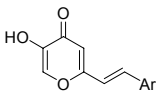
Chemistry. The synthesis of pyronyl-vinyl derivatives was accomplished as shown in Scheme 1. For the construction of the *trans*-olefins, Horner-Emmons-Wadsworth reaction was utilized.[21] Reaction of pyronyl phosphonate **3**[19] with aromatic carboxaldehydes in THF using NaH as the base afforded PMB-protected pyronyl-vinyl derivatives **4a-4j**. Several systems such as AcCl/ethanol,[22] trifluoroacetic acid/CH_2Cl_2,[23] cerium (IV) ammonium nitrate,[24] and DDQ[25] were tried for the deprotection of PMB group in **4a-4j** without success. Finally, it was found that the use of BBr_3 was effective for clean removal of PMB group. The PMB protecting group on compounds **4a-4j** was removed using BBr_3 in CH_2Cl_2 to give eight pyronylvinyl derivatives **2a-2j** in moderate to good yields.[26] For the synthesis of hydroxylated benzene-substituted pyronyl-vinyl derivatives **2b** and **2c**, acetoxybenzene carboxaldehydes were used in the Horner-Emmons-Wadsworth reaction. Acetyl protecting groups were simultaneously removed during the deprotection step of PMB group.

Biological evaluation. The synthesized pyronyl-vinyl derivatives **2a-2h** were evaluated for their ability to inhibit the LPS-activated production of the inflammatory mediators, NO and PGE_2 in RAW 264.7 cells. The screen for activity was performed



Scheme 1

Table 1. Chemical yields, NO and PGE_2 production inhibition, and cytotoxicity of pyronyl-vinyl derivatives **4a-4h** on LPS-activated RAW 264.7 macrophage cells



2a–2h

Compds	Ar	Overall yields (%) from **3**	IC_{50} (μM)[a]		
			NO	PGE_2	Cytotoxicity
2a	phenyl	52	84.29 ± 6.44	47.40 ± 2.76	157.69 ± 5.10
2b	4-hydroxyphenyl	33	45.01 ± 1.59	3.20 ± 0.00	81.62 ± 11.54
2c	3,4-dihydroxyphenyl	37	31.11 ± 2.51	10.27 ± 0.32	71.15 ± 6.26
2d	4-bromophenyl	45	23.23 ± 8.71	11.49 ± 2.82	25.97 ± 3.41
2e	4-nitrophenyl	77	33.19 ± 4.61	87.99 ± 8.46	73.62 ± 14.10
2f	2-pyridyl	48	52.05 ± 4.06	76.40 ± 2.02	153.38 ± 1.78
2g	2-furyl	13	37.94 ± 3.15	32.92 ± 1.33	95.02 ± 20.41
2h	2-thienyl	43	41.03 ± 1.79	49.10 ± 0.39	150.92 ± 17.51
1, resveratrol			45.65 ± 2.87	5.69 ± 1.82	21.81 ± 4.46
L-NIL			9.50 ± 5.89	-	-
NS-398			-	2.20 ± 1.25	-

[a]IC_{50} is defined as the concentration that results in a 50 % inhibition. Data are presented as the means ± S.D. of three independent experiments.

in a dose-response to determine the IC_{50} value. The results are summarized in Table 1. Resveratrol was used as a reference for comparisons. L-N^6-(1-iminoethyl) lysine (L-NIL, 10 μM) and NS-398 (5 μM) were also used as an inhibitor of NO accumulation and a COX-2 selective inhibitor for PGE_2 production, respectively. The cytotoxic effects of compounds were also evaluated in the presence or absence of LPS using the MTT assay to test whether the NO and PGE_2 production inhibitory effects observed were attributable to cytotoxic effects. Generally, pyronyl-vinyl derivatives exhibited similar level of NO production and enhanced PGE_2 production inhibitory activities on RAW264.7 cells when compared to those of the parent compound, resveratrol (**1**). On the other hands, every compound except **2c** showed 3 - 7 times less cytotoxicities on tested cells than **1** indicating that the change of phenyl ring in resveratrol into pyrone ring influenced on the cytotoxicity. Compounds **2b** and **2c**, which have a hydroxyl-substituted benzene ring showed the most potent PGE_2 production inhibitory activities with IC_{50} values of 3.2 and 10.27 μM with similar level of NO production inhibitory activities to that of resveratrol. Introduction of heterocycles (**2f-2h**) also retained NO production inhibition activity, but reduced PGE_2 production inhibition effects.

In conclusion, pyronyl-vinyl derivatives **2** were prepared as resveratrol analogues through the Horner-Emmons-Wadsworth reaction of pyronyl phosphonate **3** with various aromatic carboxaldehydes and evaluated for their ability to inhibit NO and PGE_2 productions on LPS-activated macrophage cells. Among synthesized, compound **2b** showed improved NO and PGE_2 production inhibitory activities on LPS-activated RAW264.7 cells with less cytotoxicity than parent compound, resveratrol.

Experimental Section

Materials and chemicals. Dulbecco's modified Eagle's minimum essential medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Life Technologies Inc. (Grand Island, NY, USA). The enzyme immunoassay (EIA) kit for PGE_2 was obtained from R&D Systems (Minneapolis, MN, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), L-N^6-(1-iminoethyl)lysine (L-NIL), NS-398, lipopolysaccharide (LPS) (*Escherichia coli*, serotype 0111:B4) and all other chemicals were purchased from the Sigma Chemical Co. (St. Louis, MO, USA). ^1H and ^{13}C NMR spectra were recorded on a Gemini Varian-400 (400 and 100 MHz, respectively). Analytical thin layer chromatographies (TLC) were carried out by pre-coated silica gel (E. Merck, Kiesegel 60F254 layer thickness 0.25 mm). All solvents used were purified according to standard procedures.

General procedure for the synthesis of 4a-4h. To a stirred suspension of NaH (1.5 eq.) in THF was added dropwise a solution of dimethyl [5-(4-methoxybenzyloxy)-4-oxo-4*H*-pyran-2-yl]methylphosphonate[19] (**3**, 1 eq.) in THF at 0 °C and stirred for 20 min. The mixture was treated with aromatic carboxaldehyde (1.2 eq.) at the same temperature, warmed up to room temperature, and further stirred for 2 h. The reaction mixture was diluted with EtOAc and washed with water. The organic layer was dried over $MgSO_4$, concentrated, and purified by crystallization or flash column chromatography to afford **4a-4h**.

(*E*)-5-(4-Methoxybenzyloxy)-2-styryl-4*H*-pyran-4-one (4a): The compound **4a** (251 mg) was obtained according to the above general procedure from the compound **3** (300 mg, 0.84 mmol) and benzaldehyde (0.1 mL, 0.93 mmol). Yield: 89%; ^1H NMR (CD_3OD) δ 8.01 (s, 1H, pyrone-*H6*), 7.64-7.59 (m, 2H, aromatic), 7.47 (d, 1H, *J* = 16.2 Hz, -C*H*=CH-), 7.40-7.35 (m, 4H, aromatic), 6.96 (d, 1H, *J* = 16.2 Hz, -CH=C*H*-), 6.93 (d, 2H, *J* = 8.6 Hz, aromatic), 6.51 (s, 1H, pyrone-*H3*), 4.98 (s, 2H, -OCH_2Ar), 3.79 (s, 3H, -OCH_3).

(*E*)-2-[2-(4-Acetoxyphenyl)vinyl]-5-(4-methoxybenzyloxy)-4*H*-pyran-4-one (4b): The compound **4b** (225 mg) was obtained according to the above general procedure from the compound **3** (300 mg, 0.84 mmol) and 4-acetoxybenzaldehyde (153 mg, 0.93 mmol). Yield: 67%; ^1H NMR ($CDCl_3$) δ 7.53 (s, 1H, pyrone-*H6*), 7.50 (d, 2H, *J* = 8.4 Hz, aromatic), 7.35-7.31 (m, 3H, aromatic, -C*H*=CH-), 7.12 (d, 2H, *J* = 10.2 Hz, aromatic), 6.89 (d, 2H, *J* = 8.4 Hz, aromatic), 6.60 (d, 1H, *J* = 16.2 Hz,

-CH=C*H*-), 6.41 (s, 1H, pyrone-*H3*), 5.04 (s, 2H, -OCH_2Ar), 3.80 (s, 3H, -OCH_3), 2.03 (s, 3H, CH_3CO_2-).

(*E*)-2-[2-(3,4-Diacetoxyphenyl)vinyl]-5-(4-methoxybenzyloxy)-4*H*-pyran-4-one (4c): The compound **4c** (1.61 g) was obtained according to the above general procedure from the compound **3** (2 g, 5.64 mmol) and 3,4-diacetoxybenzaldehyde (1.38 g, 6.2 mmol). Yield: 63%; ^{1}H NMR ($CDCl_3$) δ 7.52 (s, 1H, pyrone-*H6*), 7.35-7.21 (m, 6H, aromatic, -C*H*=CH-), 6.90 (d, 2H, *J* = 8.6 Hz, Ar-*H6*), 6.87 (d, 2H, aromatic), 6.58 (d, 1H, *J* = 15.9 Hz, -CH=C*H*-), 6.37 (s, 1H, pyrone-*H3*), 5.04(s, 2H, -OCH_2Ar), 3.80 (s, 3H, -OCH_3), 2.31 (s, 3H, CH_3CO_2-), 2.30 (s, 3H, CH_3CO_2-).

(*E*)-2-(4-Bromostyryl)-5-(4-methoxybenzyloxy)-4*H*-pyran-4-one (4d): The compound **4d** (257 mg) was obtained according to the above general procedure from the compound **3** (300 mg, 0.84 mmol) and 4-bromobenzaldehyde (172 mg, 0.93 mmol). Yield: 72%; ^{1}H NMR ($CDCl_3$) δ 7.53-7.21 (m, 7H, aromatic, -C*H*=CH-, pyrone-*H6*), 6.94 (d, 1H, *J* = 8.4 Hz, aromatic), 6.64 (d, 1H, *J* = 16.2 Hz, -CH=C*H*-), 6.64 (s, 1H, pyrone-*H3*), 5.04 (s, 2H, -OCH_2Ar), 3.80 (s, 3H, -OCH_3).

(*E*)-5-(4-Methoxybenzyloxy)-2-(4-nitrostyryl)-4*H*-pyran-4-one (4e): The compound **4e** (282 mg) was obtained according to the above general procedure from the compound **3** (300 mg, 0.84 mmol) and 4-nitrobenzaldehyde (140 mg, 0.93 mmol). Yield: 87%; ^{1}H NMR ($CDCl_3$) δ 8.28 (d, 2H, *J* = 8.7 Hz, aromatic), 8.26 (s, 1H, pyrone-*H6*), 8.21 (d, 2H, *J* = 8.7 Hz, aromatic), 7.55 (d, 1H, *J* = 16.5 Hz, -C*H*=CH-), 7.39-7.30 (m, 3H, aromatic, -CH=C*H*-), 6.98 (d, 2H, *J* = 8.7 Hz, aromatic), 6.60 (s, 1H, pyrone-*H3*), 4.91 (s, 2H, -OCH_2Ar), 3.77 (s, 3H, -OCH_3).

(*E*)-5-(4-Methoxybenzyloxy)-2-[2-(pyridin-2-yl)vinyl]-4*H*-pyran-4-one (4f): The compound **4f** (167 mg) was obtained according to the above general procedure from the compound **3** (300 mg, 0.84 mmol) and picolinaldehyde (0.08 mL, 0.93 mmol). Yield: 58%; ^{1}H NMR ($CDCl_3$) δ 8.64 (d, 1H, *J* = 6.0 Hz, pyridin-*H6*), 7.72 (m, 1H, pyridin-*H4*), 7.55 (s, 1H, pyrone-*H6*), 7.73-7.23 (m, 6H, aromatic, -C*H*=C*H*-), 6.89 (d, 2H, *J* = 8.8 Hz, aromatic), 6.46 (s, 1H, pyrone-*H3*), 5.04 (s, 2H, -OCH_2Ar), 3.80 (s, 3H, CH_3).

(*E*)-2-(2-(Furan-2-yl)vinyl)-5-(4-methoxybenzyloxy)-4*H*-pyran-4-one (4g): The compound **4g** (111 mg) was obtained according to the above general procedure from the compound **3** (300 mg, 0.84 mmol) and furan-2-carbaldehyde (89 mg, 0.93 mmol). Yield: 40%; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.13 (s, 1H, pyrone-*H6*), 7.83 (br s, 1H, furan-*H5*) 7.36 (d, 2H, *J* = 8.7 Hz, aromatic) 7.24 (d, 1H, *J* =16.2 Hz, -C*H*=CH-), 6.96 (d, 2H, *J* = 8.7 Hz, aromatic) 6.83 (d, 1H, *J* =3.2 Hz, furan-*H3*), 6.75 (d, 1H, *J* =16.2 Hz, -CH=C*H*-), 6.63-6.65 (m, 1H, furan-*H4),* 6.53 (s, 1H, pyrone-*H3*), 4.89 (s, 2H, -OCH_2Ar), 3.77 (s, 3H, -OCH_3).

(*E*)-5-(4-Methoxybenzyloxy)-2-(2-(thiophen-2-yl)vinyl)-4*H*-pyran-4-one (4h): The compound **4h** (256 mg) was obtained according to the above general procedure from the compound **3** (300 mg, 0.84 mmol) and thiophene-2-carbaldehyde (104 mg, 0.93 mmol). Yield: 80%; ^{1}H NMR (DMSO-d_6) δ 8.12 (s, 1H, pyrone-*H6*), 7.66 (d, 1H, *J* = 4.6 Hz, thiophene-*H5*), 7.57 (d, 1H, *J* = 16.2 Hz, -C*H*=CH-), 7.43 (d, 1H, *J* = 3.6 Hz, thiophene-*H3*), 7.35 (d, 2H, *J* = 8.4 Hz, Ph-*H2*, *H6*), 7.13 (m, 1H, thiophene-*H4*), 6.92-6.97 (m, 2H, aromatic), 6.76 (d, 1H, *J* = 16.2 Hz, -CH=C*H*-), 6.49 (s, 1*H*, pyrone-*H3*), 4.88 (s, 2H, -OCH_2Ar), 3.76 (s, 3H, -OCH_3).

General procedure for the synthesis of (2a-2h). To a solution of **4a-4h** (1.0 eq.) in CH_2Cl_2 (5 mL) was added BBr_3 (1 M in CH_2Cl_2, 10 eq.), and the resultant mixture was stirred for 12 h at room temperature. The mixture was treated with ice-cold methanol (10 mL) and stirred at 15 °C for 30 min. The solvent was removed in vacuo, and the addition of fresh methanol (3 × 5 mL) followed by evaporation was repeated. The residue was treated with ethanol and the resulting precipitate was filtered, washed with ethanol, and dried to provide **2a-2h**.

(*E*)-5-Hydroxy-2-styryl-4*H*-pyran-4-one (2a): The compound **2a** (73 mg) was obtained according to the above general procedure from the compound **3** (200 mg, 0.59 mmol). Yield: 57%; ^{1}H NMR (DMSO-d_6) δ 7.98 (s, 1H, pyrone-*H6*), 7.63-7.59 (m, 2H, aromatic), 7.47 (d, 1H, *J* = 16.2 Hz, -C*H*=CH-), 7.40-7.35 (m, 3H, aromatic), 6.96 (d, 1H, *J* = 16.2 Hz, -CH=C*H*-), 6.50 (s, 1H, pyrone-*H3*).

(*E*)-5-Hydroxy-2-(4-hydroxystyryl)-4*H*-pyran-4-one (2b): The compound **2b** (40 mg) was obtained according to the above general procedure from the compound **3** (200 mg, 0.50 mmol). Yield: 34%; ^{1}H NMR (DMSO-d_6) δ 8.01 (s, 1H, pyrone-*H6*), 7.49 (d, 2H, *J* = 8.6 Hz, aromatic), 7.29 (d, 1H, *J* = 16.4 Hz, -C*H*=CH-), 6.82 (d, 1H, *J* = 16.4 Hz, -CH=C*H*-), 6.79 (d, 2H, *J* = 8.6 Hz, aromatic), 6.41 (s, 1H, pyrone-*H3*); ^{13}C NMR (DMSO-d_6) δ 174.3, 162.1, 159.4, 146.2, 139.2, 135.5, 129.8, 126.5, 116.9, 111.2.

(*E*)-2-(3,4-Dihydroxystyryl)-5-hydroxy-4*H*-pyran-4-one (2c): The compound **2c** (67 mg) was obtained according to the above general procedure from the compound **3** (200 mg, 0.44 mmol). Yield: 61%; ^{1}H NMR (DMSO-d_6) δ 8.04 (s, 1H, pyrone-*H6*), 7.21 (d, 1H, *J* = 16.2 Hz, -C*H*=CH-), 7.02 (d, 1H, *J* = 0.8 Hz, Ar-*H2*), 6.93 (dd, 1H, *J* = 8.4, 0.8 Hz, Ar-*H6*), 6.77 (d, 1H, *J* = 8.4 Hz, Ar-*H5*), 6.20 (d, 1H, *J* = 16.2 Hz, -CH=C*H*-), 6.44 (s, 1H, pyrone-*H3*); ^{13}C NMR (DMSO-d_6) δ 174.3, 162.1, 147.9, 146.1, 146.0, 139.1, 135.8, 127.0, 120.9, 116.7, 116.2, 114.5, 111.2.

(*E*)-2-(4-Bromostyryl)-5-hydroxy-4*H*-pyran-4-one (2d): The compound **2d** (88 mg) was obtained according to the above general procedure from the compound **3** (200 mg, 0.48 mmol). Yield: 62%; ^{1}H NMR (DMSO-d_6) δ 8.06 (s, 1H, pyrone-*H6*), 7.62 (br s, 4H, aromatic), 7.37 (d, 1H, *J* = 16.4 Hz, -C*H*=CH-), 7.12 (d, 1H, *J* = 16.4 Hz, -CH=C*H*-), 6.51 (s, 1H, pyrone-*H3*); ^{13}C NMR ($CDCl_3$) δ 174.7, 161.4, 146.8, 139.8, 135.1, 134.2, 132.6, 130.2, 123.4, 121.7, 113.0.

(*E*)-5-Hydroxy-2-(4-nitrostyryl)-4*H*-pyran-4-one (2e): The compound **2e** (120 mg) was obtained according to the above general procedure from the compound **3** (200 mg, 0.44 mmol). Yield: 88%; ^{1}H NMR (DMSO-d_6) δ 8.26 (d, 2H, *J* = 8.6 Hz, aromatic), 8.10 (s, 1H, pyrone-*H6*), 7.92 (d, 2H, *J* = 8.6 Hz, aromatic), 7.51 (d, 1H, *J* = 16.4 Hz, -C*H*=CH-), 7.34 (d, 1H, *J* = 16.4 Hz, -CH=C*H*-), 6.59 (s, 1H, pyrone-*H3*); ^{13}C NMR (DMSO-d_6) δ 174.6, 160.8, 148.1, 147.0, 142.4, 140.1, 133.0, 129.2, 125.2, 124.8, 114.1.

(*E*)-5-Hydroxy-2-(2-(pyridin-2-yl)vinyl)-4*H*-pyran-4-one (2f): The compound **2f** (83 mg) was obtained according to the above general procedure from the compound **3** (200 mg, 0.47 mmol). Yield: 82%; ^{1}H NMR (DMSO-d_6) δ 8.78 (dd, 1H, *J* = 6.2, 0.8 Hz, pyridin-*H6*), 8.23 (m, 1H, pyridin-*H4*), 8.16 (s, 1H,

pyrone-*H3*), 8.03 (d, 1H, *J* = 8.0 Hz, pyridin-*H3*), 7.68 (m, 1H, pyridin-*H5*), 7.63 (d, 1H, *J* = 15.6 Hz, -C*H*=CH-), 7.46 (d, 1H, *J* = 15.6 Hz, -CH=C*H*-), 6.69 (s, 1H, pyrone-*H6*); ^{13}C NMR (DMSO-d_6) δ 174.6, 160.1, 151.2, 147.1, 142.1, 140.3, 130.2, 127.6, 125.8, 125.1, 115.1.

(*E*)-2-(2-(Furan-2-yl)vinyl)-5-hydroxy-4*H*-pyran-4-one (2g): The compound **2g** (20 mg) was obtained according to the above general procedure from the compound **3** (100 mg, 0.30 mmol). Yield: 32%; ^{1}H NMR (DMSO-d_6) δ 7.92 (s, 1H, pyrone-*H6*), 7.17 (d, 1H, *J* = 15.9 Hz, -C*H*=CH-), 7.09 (d, 1H, *J* = 8.4 Hz, furan-*H5*), 6.74 (m, 2H, furan-*H,34*), 6.63 (d, 1H, *J* = 15.9 Hz, -CH=C*H*-), 6.55 (s, 1H, pyrone-*H3*); ^{13}C NMR (DMSO-d_6) δ 174.3, 161.2, 158.2, 156.4, 139.2, 130.0, 127.9, 122.5, 116.3, 115.7, 112.0, 109.7.

(*E*)-5-Hydroxy-2-(2-(thiophen-2-yl)vinyl)-4*H*-pyran-4-one (2h): The compound **2h** (70 mg) was obtained according to the above general procedure from the compound **3** (200 mg, 0.58 mmol). Yield: 54%; ^{1}H NMR (DMSO-d_6) δ 8.07 (s, 1H, pyrone-*H6*), 7.70 (d, 1H, *J* = 5.1 Hz, Ar-*H5*), 7.60 (d, 1H, *J* = 16.1 Hz, -C*H*=CH-), 7.46 (d, 1H, *J* = 3.4 Hz, Ar-*H3*), 7.16 (m, 1H, Ar-*H4*), 6.80 (d, 1H, *J* = 16.1 Hz, -CH=C*H*-), 6.56 (s, 1H, pyrone-*H3*); ^{13}C NMR (DMSO-d_6) δ 174.3, 163.5, 161.0, 146.3, 140.5, 139.9, 139.4, 130.8, 128.9, 128.4, 127.4, 126.3, 119.0, 113.3, 112.1.

Cell culture and sample treatment. The RAW 264.7 macrophage cell line was obtained from the Korea Cell Line Bank (Seoul). Cells were grown at 37 °C in DMEM medium supplemented with 10% FBS, penicillin (100 units/mL), and streptomycin sulfate (100 μg/mL) in a humidified 5% CO_2 atmosphere. Cells were incubated with various concentrations of tested samples or with positive controls (L-NIL or NS-398) and then stimulated with LPS 1 μg/mL for the indicated time.

Nitrite determination. RAW 264.7 cells were plated at 4×10^5 cells/well in 24 well-plates and then incubated with or without LPS (1 μg/mL) in the absence or presence of various concentrations (3.15, 6.25, 12.5, 25, 50 and 100 μM) of tested samples for 24 h. Nitrite levels in culture media were determined using the Griess reaction and presumed to reflect NO levels (Won *et al*., 2006). Briefly, 100 μL of cell culture medium was mixed with 100 μL of Griess reagent [equal volumes of 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid and 0.1% (w/v) naphtylethylenediamine-HCl], and incubated at room temperature for 10 min. Absorbance was then measured at 540 nm using a microplate reader (Perkin Elmer Cetus, Foster City, CA, USA). Fresh culture media were used as blanks in all experiments. The amount of nitrite in the samples was measured with the sodium nitrite serial dilution standard curve and nitrite production was measured.

PGE_2 assay. RAW 264.7 cells were pretreated with tested samples for 1 h and then stimulated with LPS (1 μg/mL) for 24 h. Levels of PGE_2 in the culture media was quantified using EIA kits (R&D Systems, Minneapolis, MN. USA) according to the manufacturer′s instructions.

MTT assay for cell viability. RAW 264.7 cells were plated at a density of 10^5 cells/well in 96-well plates. To determine the appropriate concentration not toxic to cells, cytotoxicity studies were performed 24 h after treating cells with various concentrations of tested compounds. Viabilities were determined using colorimetric MTT assays, as described previously.[27]

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