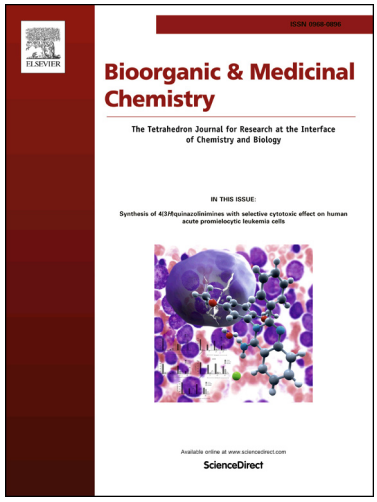

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Synthesis and biological activity of new phthalimides as potential anti-inflammatory agents

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ABSTRACT

The overproduction of nitric oxide (NO) plays an important role in a variety of pathophysiological processes, including inflammation. Therefore, the suppression of NO production is a promising target in the design of anti-inflammatory agents. In the present study, a series of phthalimide analogs was synthesized, and their anti-inflammatory activities were evaluated using lipopolysaccharide (LPS)-stimulated NO production in cultured murine macrophage RAW264.7 cells. A structure-activity relationship study showed that the free hydroxyl group at C-4 and C-6 and the bulkiness of the N-substituted alkyl chain are associated with biological activity. Among the series of phthalimide derivatives, compound **IIh** exhibited potent inhibitory activity, with an IC_{50} value of 8.7 μg/mL. Further study revealed that the inhibitory activity of compound **IIh** was correlated with the down-regulation of the mRNA and protein expression of LPS-stimulated inducible nitric oxide synthase (iNOS). Compound **IIh** also suppressed the induction of the pro-inflammatory cytokines tumor necrosis factor-α and interleukin-1β in LPS-stimulated RAW 264.7 cells. The anti-inflammatory activity of compound **IIh** was also found to be associated with the suppression of the Toll-like receptor (TLR)4 signaling pathway by down-regulating the activation of interferon regulatory factor 3 (IRF-3) and interferon-β and signal transducer expression. These findings demonstrate that novel phthalimides might be potential candidates for the development of anti-inflammatory agents.

Keywords: phthalimides, anti-inflammation, iNOS, Toll-like receptor (TLR) 4

1. INTRODUCTION

Inflammation is a complex biological response of the vascular tissue to harmful stimuli, such as pathogens, damaged cells, or irritants [1]. Consequently, the initiation and progression of many diseases, such as cardiovascular disease, cancer, obesity and insulin resistance, are highly associated with inflammatory responses [2,3]. Macrophages are one of the most dominant and widely distributed inflammatory cells and are involved in the initiation and maintenance of the acute inflammatory response [4]. Lipopolysaccharide (LPS), an endotoxin localized in the outer membrane of Gram-negative bacteria, can elicit significant immune and inflammatory responses in macrophages [5]. In the process of LPS-mediated inflammation, Toll-like receptors (TLRs) are key components. In particular, TLR4 is activated by the interaction with LPS, and activated TLR4 leads to the expression of several pro-inflammatory cytokines and mediators of inflammation, such as nitric oxide (NO) [6,7]. LPS/TLR4 signaling has been divided into two major downstream pathways: the myeloid differentiation factor 88 (MyD88)-dependent pathway and the MyD88-independent pathway. The activation of the MyD88-dependent pathway contributes to the early phase of NF-κB activation and pro-inflammatory cytokine expression, while the MyD88-independent pathway mediates the late phase of NF-κB activation and the induction of interferon (IFN)-inducible genes [8].

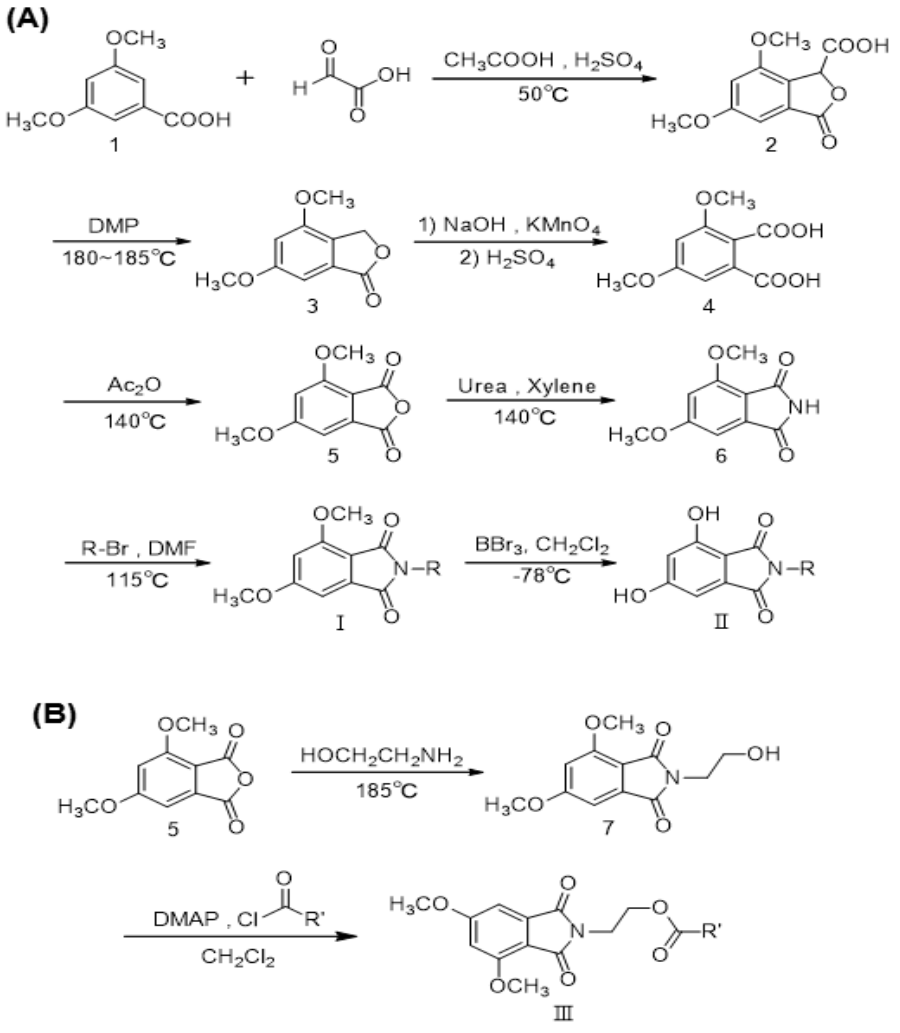
A variety of classes of compounds from natural or synthetic sources have been shown to be potential inhibitors of LPS-stimulated NO production in macrophages [1-3]. Phthalimides have been reported to have antifungal, anticonvulsant, analgesic, hypolipidemic, antitumor and anti-inflammatory activities [4, 5]. In the present study, we synthesized a series of novel phthalimide derivatives and evaluated the biological activity of LPS-induced NO production. We also further explored the underlying molecular mechanism responsible for the anti-

inflammatory activity using an active phthalimide analog.

2. RESULTS AND DISCUSSION

2.1. Chemistry

The synthesis of a series of novel phthalimide derivatives is outlined in **Scheme 1**.



Scheme 1. The synthesis of *N*-substituted phthalimide analogs. (**A**) Procedure for the synthesis of type I and II *N*-substituted phthalimide analogs. (**B**) Procedure for the synthesis of type III *N*-substituted phthalimide analogs.

Intermediate **2** was prepared using 3,5-dimethoxybenzoic acid and glyoxylic acid. In this

procedure, sulfuric acid was diluted with ten times amount of acetic acid to avoid the carbonization of product induced by concentrated sulfuric acid, and **2** was yielded as 64.2%. Decarboxylation was then performed in dimethyl phthalate (DMP) at 180~185 °C to afford **3** with the yield of 95.7%. **3** was oxidated to be substituted phthalic acid (**4**, 93.6%) and then dehydrated to afford anhydride **5** (90.2%). The intermediate **6** was prepared by ammonolysis and condensation of **5** and urea.

Target compounds **Ia**-**Im** were synthesized using compound **5** and different halohydrocarbons with yields ranging from 62% to 80%. **IIa**-**IIm** were synthesized by demethylation with **Ia**-**Im**, respectively, with yields from 60% - 70%.

Compound **5** was reacted with ethanolamine at 185°C to afford the intermediate **7** (52.2%) and target compounds **IIIa**-**IIIc** were synthesized using different acyl chloride as acylation reagent to yield from 60% to 73%.

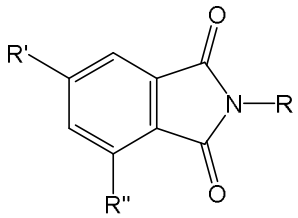
The data of NMR spectroscopy and ESI-MS spectra analysis of the target compounds (Supplementary data) reflected the assigned structures of the compounds.

2.2. Inhibitory activity of NO production by phthalimide derivatives and structure-activity relationship

The overproduction of NO by inducible nitric oxide synthase (iNOS) is significant in inflammatory processes. Therefore, high levels of NO are considered to be a biomarker for inflammatory disorders and a useful target for the procurement of anti-inflammatory agents. The synthetic phthalimide analogs were initially evaluated for inhibitory effects on the production of NO in LPS-stimulated RAW264.7 macrophage cells. When the cells were treated with LPS (1 μg/mL) for 22 h, the NO production was markedly stimulated from the basal level of 0.46 ± 0.2 to 25.3 ± 0.3 μM. The positive control, AMT, showed 98.0 % inhibition at the test concentration of 50 μM under the same experimental conditions. The inhibitory activity of the synthetic phthalimide analogs (**Ia**-**Im, IIa-IIm, IIIa-IIIc**) is

summarized in Table 1.

Table 1. Structures and inhibitory effects on nitric oxide production of compounds **Ia-Im**, **IIa-IIm** and **IIIa-IIIc** in LPS-induced RAW 264.7 macrophages.



Comp [a]	R	R′	R″	Nitrate (μM)	% control of NO production
LPS -	-	-	-	0.46	-
LPS + [b]	-	-	-	25.31	-
(+) [c]	-	-	-	0.5	2.0
Ia	$-C_2H_5$	$-OCH_3$	$-OCH_3$	10.81	43.5
Ib	$-C_3H_7$	$-OCH_3$	$-OCH_3$	23.24	93.5
Ic	$-CH(CH_3)_2$	$-OCH_3$	$-OCH_3$	24.34	97.9
Id	$-C_4H_9$	$-OCH_3$	$-OCH_3$	19.70	79.3
Ie	$-CH_2CH(CH_3)_2$	$-OCH_3$	$-OCH_3$	21.45	86.3
If	$-C_5H_{11}$	$-OCH_3$	$-OCH_3$	18.60	74.8
Ig	$-C_6H_{13}$	$-OCH_3$	$-OCH_3$	23.01	92.6
Ih	$-C_7H_{15}$	$-OCH_3$	$-OCH_3$	20.99	84.5
Ii	$-CH_2-C_6H_5$-F(*o*)	$-OCH_3$	$-OCH_3$	18.87	75.9
Ij	$-CH_2-C_6H_5$-F(*m*)	$-OCH_3$	$-OCH_3$	18.65	75.1
Ik	$-CH_2-C_6H_5$-Br(*o*)	$-OCH_3$	$-OCH_3$	20.79	83.7
Il	$-CH_2-C_6H_5$-Br(*p*)	$-OCH_3$	$-OCH_3$	20.16	81.1
Im	$-CH_2-C_6H_5-CH_3$(*m*)	$-OCH_3$	$-OCH_3$	18.43	74.2
IIa	$-C_2H_5$	-OH	-OH	21.12	85.0
IIb	$-C_3H_7$	-OH	-OH	20.71	83.3
IIc	$-CH(CH_3)_2$	-OH	-OH	20.47	82.4
IId	$-C_4H_9$	-OH	-OH	18.24	73.4
IIe	$-CH_2CH(CH_3)_2$	-OH	-OH	21.07	84.8
IIf	$-C_5H_{11}$	-OH	-OH	11.30	45.5
IIg	$-C_6H_{13}$	-OH	-OH	1.98	8.0
IIh	$-C_7H_{15}$	-OH	-OH	0.72	2.9
IIi	$-CH_2-C_6H_5$-F(*o*)	-OH	-OH	11.76	47.3
IIj	$-CH_2-C_6H_5$-F(*m*)	-OH	-OH	10.46	42.1
IIk	$-CH_2-C_6H_5$-Br(*o*)	-OH	-OH	9.65	38.8
IIl	$-CH_2-C_6H_5$-Br(*p*)	-OH	-OH	8.04	32.4
IIm	$-CH_2-C_6H_5-CH_3$(*m*)	-OH	-OH	10.36	41.7
IIIa	$-C_2H_4$-O-CO-CH_3	$-OCH_3$	$-OCH_3$	24.13	97.1
IIIb	$-C_2H_4$-O-CO-C_2H_5	$-OCH_3$	$-OCH_3$	25.31	101.9
IIIc	$-C_2H_4$-O-CO-CH_2Cl	$-OCH_3$	$-OCH_3$	12.14	48.9
7	$-CH_2-CH_2$-OH	$-OCH_3$	$-OCH_3$	24.80	99.8

[a] Comp: compound 20 μg/mL, [b] LPS +: 1 μg/mL, [c] (+): 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine hydrochloride (AMT: positive control, 50 μM)

Among the tested compounds, 12 phthalimides inhibited the LPS-stimulated NO production by at least 50 % at the test concentration of 20 μg/mL. Regarding the N-alkyl chain, the biological activity varied depending on the length of the carbon chain and the substitution patterns. Activity decreased when the number of carbon atoms exceeded 8 in the N-alkyl chain. The introduction of halides or ester groups into the alkyl chain also decreased the activity. The substitution of a methoxy group in the R′ and R″ positions decreased the activity compared to the free hydroxyl group in the position. The C6- to C7-alkyl chains maintained the biological activity without any substitutions in the alkyl chain and the R′ and R″ groups. Among the tested compounds, the C7-alkyl chain with a free hydroxyl group in the R′ and R″ , compound **IIh**, was the most active in the inhibition of NO production. Therefore, the inhibitory activity of NO production by phthalimides is dependent on the size of the carbon chain, the spatial geometry of the *N*-alkyl chain and the substitutions at the aromatic R′ and R″ positions.

2.3. Mechanism of action studies with compound IIh

Based on the SAR study of phthalimide analogs, compound **IIh** (Figure 1), which showed the highest activity (Table 1), was selected for further detailed mechanism of action studies in RAW264.7 cells.

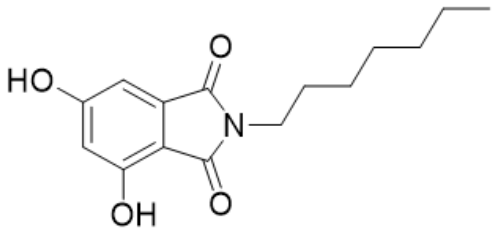


Figure 1. The chemical structure of compound **IIh.**

Compound **IIh** exhibited concentration-dependent inhibition of LPS-induced NO production in RAW264.7 cells, with an IC_{50} value of 8.7 μg/mL (Figure 2A).

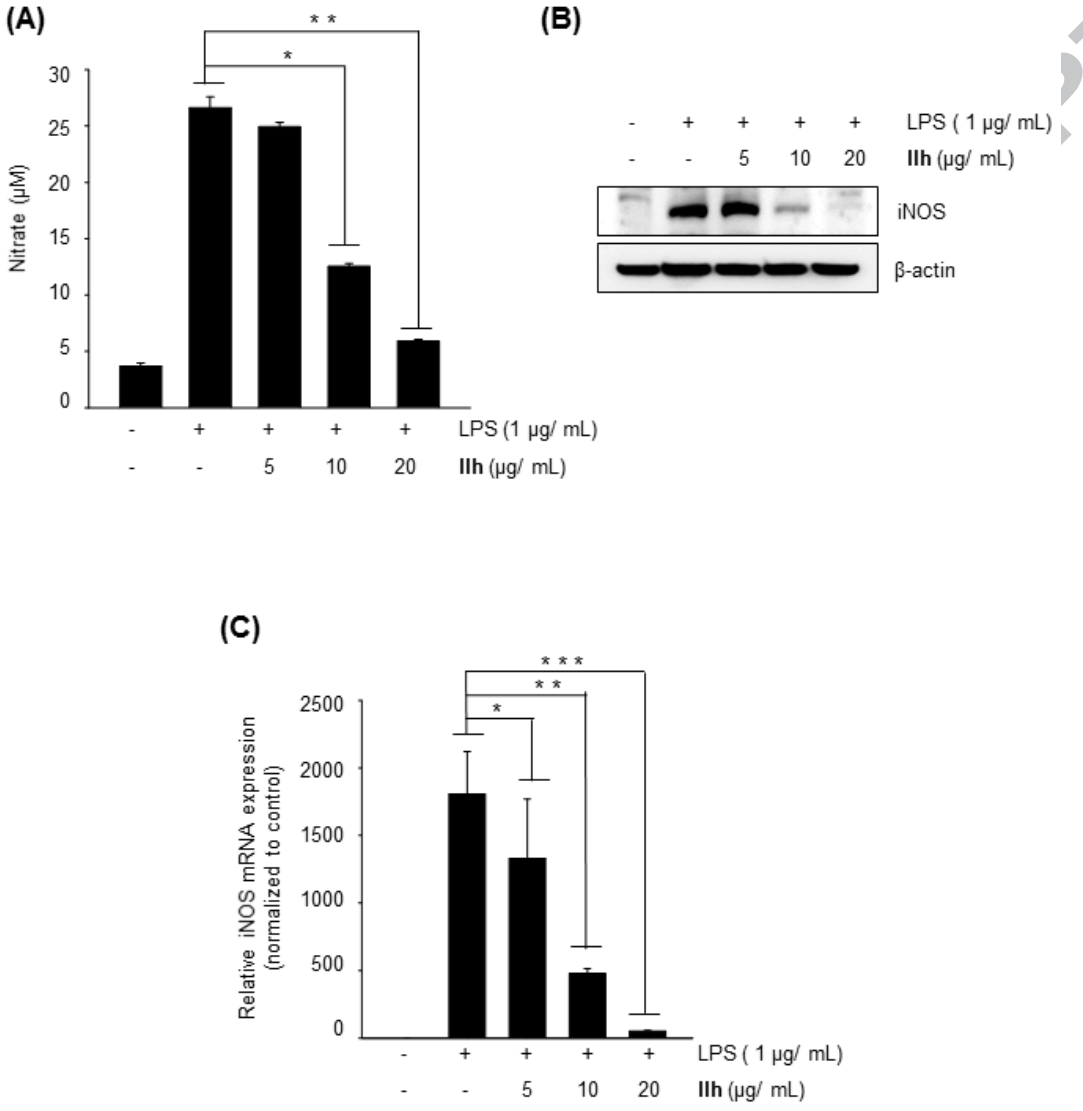


Figure 2. The effects of compound **IIh** on NO production and iNOS expression in RAW 264.7 cells. (A) Cells were treated with LPS (1 μg/mL) in the presence or absence of various concentrations of compound **IIh** for 22 h. Nitrite was measured using the Griess reaction. Data are presented as the means ± SD of three independent experiments. (B) RAW 264.7 cells were pretreated with various concentrations of compound **IIh** for 30 min and then stimulated with 1 μg/mL LPS for 16 h. The iNOS protein level was analyzed using western blotting. (C) RAW 264.7 cells were pretreated with different concentrations of compound **IIh** for 30 min and

then stimulated with 1 μg/mL LPS for 4 h. The iNOS mRNA expression was analyzed using real-time PCR. The values are expressed as the means ± SD of triplicate tests.

Employing MTT assay the cytotoxic effect of compound **IIh** was also analyzed at the test concentrations of up to 40 μg/mL. The compound did not show any significant cell viability at the concentrations of up to 20 μg/mL (> 95% survival), but the compound **IIh** at the test concentration of 40 μg/mL affected to the cell viability (approximately 17% survival). Therefore, further study for the mechanism of action in the anti-inflammatory activity with the compound **IIh** was observed at the test concentrations of up to 20 μg/mL. Thereby, the inhibition of NO production by compound **IIh** was not attributable to cytotoxic effects at the test concentrations. To further elucidate the possible mechanisms of active phthalimides, the suppression of NO production by compound **IIh** was investigated in relation to the levels of iNOS protein and mRNA expression in the LPS-stimulated RAW 264.7 cells. The RAW264.7 cells were incubated with LPS (1 μg/mL) in the presence or absence of various concentrations of compound **IIh**. After 4 h and 12 h of treatment, total RNA and protein were isolated for real-time PCR and western blot analysis, respectively. The iNOS protein level was markedly increased in LPS-induced RAW264.7 cells, but co-treatment with compound **II h** significantly suppressed the iNOS protein level in a concentration-dependent manner (Figure 2B). In addition, treatment with compound **IIh** significantly suppressed iNOS mRNA expression in LPS-stimulated RAW264.7 cells in a concentration-dependent manner (Figure 2C). These findings suggest that the inhibition of NO production by compound **IIh** might be correlated with its suppressive effect on LPS-induced iNOS protein and mRNA expression.

Because inflammation involves the activation of pro-inflammatory cytokines, such as IL-1β and TNF-α [6], the effects of compound **IIh** on cytokine expression were also investigated

in LPS-stimulated RAW264.7 cells. As shown in Figure 3A, treatment with compound **IIh** for 4 h significantly suppressed the LPS-stimulated expression of TNF-α and IL-1β.

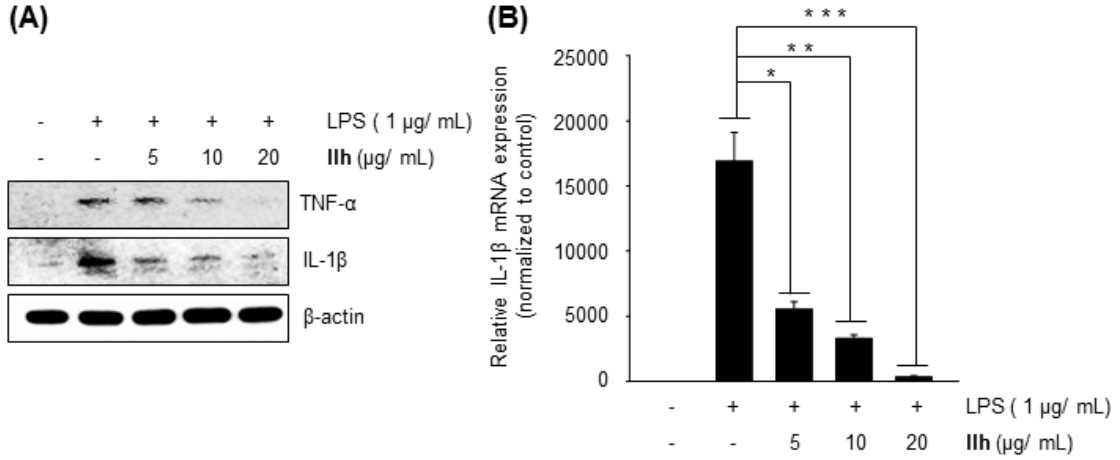


Figure 3. The effect of compound **IIh** on LPS-stimulated pro-inflammatory cytokine protein and mRNA expression in RAW 264.7 cells. (**A**) & (**B**) RAW 264.7 cells were pretreated with various concentrations of compound **IIh** for 30 min and then stimulated with LPS (1 μg/mL) for 4 h. After incubation, total protein (**A**) and mRNA (**B**) were extracted and then further analyzed using western blotting and real-time PCR, respectively. The values are expressed as the means ± SD of triplicate tests.

In addition, the overexpression of IL-1β mRNA by LPS (1 μg/mL) was significantly down-regulated by treatment with compound **IIh** in a concentration-dependent manner (Figure 3B). These findings suggest that the anti-inflammatory activity of compound **IIh** is associated in part with the suppression of pro-inflammatory cytokine expression.

NF-κB is a major transcription factor that modulates the expression of pro-inflammatory proteins (e.g., iNOS) and pro-inflammatory cytokines (e.g., TNF-α and IL-1β) induced by LPS [7]. To further investigate whether compound **IIh** is able to affect the transcriptional activity of NF-κB, a reporter gene assay (SEAP) was performed in LPS-stimulated RAW 264.7 cells. As shown in Figure 4A, in response to the stimulation with LPS for 16 h, the NF-κB transcriptional activity increased by approximately 5.5-fold, but co-treatment with

compound **IIh** effectively suppressed NF-κB transcriptional activity.

The translocation of NF-κB to the nucleus is preceded by the phosphorylation and proteolytic degradation of IκBα by IκB kinases [8]. In general, in unstimulated cells, NF-κB subunits (p50 and/or p65) form a complex with an inhibitory factor, IκBα, in the cytosol and thus are inactivated [5]. However, when stimulated by pro-inflammatory signaling, IκBα is phosphorylated by IκB kinases (IKKs) and then subject to ubiquitin-mediated degradation [6]. These events lead to the translocation of free NF-κB into the nucleus, where it serves as a transcription factor. Accordingly, the higher levels of phosphorylated IκBα induced by LPS were reduced by treatment with compound **IIh**, but the lower level of total IκBα recovered with compound **IIh** treatment for 30 min (Figure 4B).

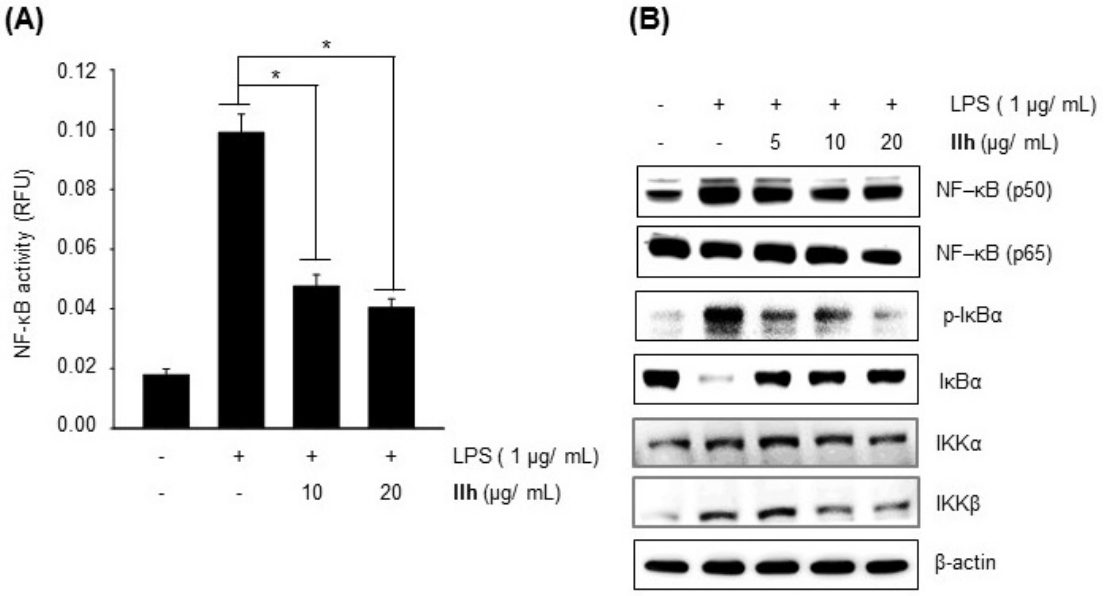


Figure 4. The effect of compound **IIh** on the MyD88-dependent signaling pathway in RAW 264.7 cells. (**A**) The effect of compound **IIh** on LPS-stimulated NF-κB transcriptional activity. RAW-SEAP cells were incubated in a 12-well plate for 24 h and pretreated with compound **IIh** for 2 h. Subsequently, the cells were incubated with LPS (1 μg/mL) for 16 h. For secreted alkaline phosphatase activity (SEAP), relative fluorescence units (RFU) were measured using a 96-well fluorometer. (**B**) The effect of compound **IIh** on NF-

κB and its related proteins. RAW 264.7 cells were pretreated with various concentrations of compound **IIh** and then stimulated with LPS (1 μg/mL) for different lengths of time NF-κB (p50 and p65) and IKK (α and β) for 16 h, and IκBα and p-IκBα for 30 min. Proteins were analyzed using western blotting.

In addition, the increased level of IKKβ protein caused by LPS was reduced by treatment with compound **IIh** for 16 h. These data suggest that compound **IIh** effectively suppresses LPS-induced NF-κB activation by blocking the transcriptional activity of NF-κB, up-regulating IκBα, and inhibiting IKKβ.

In an additional regulation of iNOS expression, the TRIF-mediated signal transduction pathway is highly involved in inflammatory responses. The adaptor protein, TRIF-related adaptor molecule (TRAM), together with TIR domain-containing adaptor-inducing IFN-β (TRIF), contributes to the activation of interferon regulatory factor 3 (IRF3) and subsequently induces the IRF3-dependent expression of genes such as interferon-β (IFN-β) [9]. Therefore, the effects of compound **IIh** on TRIF-mediated signaling pathways were also examined in LPS-stimulated RAW264.7 cells. Compound **IIh** significantly suppressed the LPS-stimulated activation of IRF3 (p-Ser^{396}) (Figure 5A) and suppressed the mRNA expression of IFN-β (Figure 5B), suggesting the involvement of the inhibition of the TRIF-mediated pathway in the regulation of iNOS expression by compound **IIh**.

Recent studies have demonstrated that heme-oxygenase 1 (HO-1), an anti-oxidative protein, acts as a major protective factor through its antioxidant, anti-apoptotic, and anti-inflammatory properties [10-12]. The induction of HO-1 also enables its anti-inflammatory function via the down-regulation of iNOS and NO production in macrophages [13-17]. Therefore, we also investigated whether the anti-inflammatory activity of compound **IIh** is in part associated with the up-regulation of HO-1 expression. At the test concentration of 20 μg/mL, compound **IIh** significantly up-regulated HO-1 protein expression (Figure 5C).

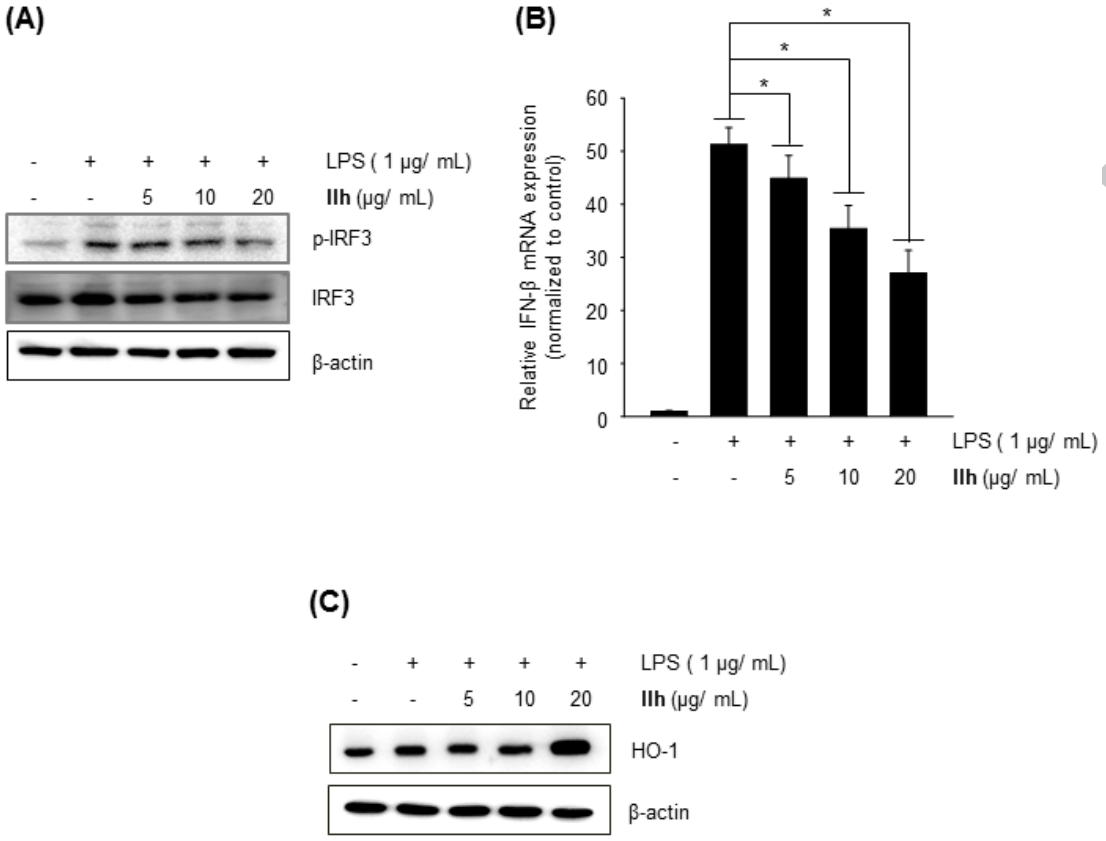


Figure 5. The effect of compound **IIh** on MyD88-independent signaling pathway in RAW 264.7 cells. (**A**) and (**B**) RAW 264.7 cells were pretreated with various concentrations of compound **IIh** for 30 min and then stimulated with LPS (1 μg/mL) for 30 min (**A**) and 4 h (**B**). Subsequently, the expression of proteins and mRNA were further analyzed by western blot and real-time PCR, respectively. (**C**) The effect of compound **IIh** on heme oxygenase 1 (HO-1) expression in RAW 264.7 cells. RAW 264.7 cells were pretreated with compound **IIh** for 30 min and subsequently stimulated with LPS (1 μg/mL) for 16 h. The level of HO-1 protein was further analyzed using western blotting.

These findings suggest the involvement of HO-1 in the regulation of iNOS expression and NO production by compound **IIh** in LPS-stimulated macrophage cells.

3. EXPERIMENTAL SECTION

Bruker ARX-300 and AV-600 spectrophotometers (Bruker Corporation, Germany) at 300 MHz were used to obtain ^{1}H- and ^{13}C-NMR spectra. Mass spectra were obtained using a 6890 GC-5975 spectrometer (Agilent Technologies, US) using positive electrospray ionization. High Performance Liquid Chromatography (HPLC) was performed on a JAI LC9103 Recycling preparative HPLC (Japan Analytical Industries, Japan) equipped with a JAIGEL-ODS-AP-P column and a JAIGEL-GS310 column using a JAI refractive index detector and a JAI UV-3702 detector with a Multichro 2000 workstation. TLC was performed on pre-coated GF_{254} plates (Merk, Germany) and detected by spraying with 10 % H_2SO_4 followed by heating. The purity of all compounds screened in the biological assays was examined using HPLC analysis and was found to be not less than 95 %.

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), sodium pyruvate, L-glutamine, antibiotic-antimycotic solution, and trypsin-EDTA were purchased from Invitrogen Co. (Grand Island, NY, USA). Lipopolysaccharide (*E. coli* 0111: B4), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise indicated. iNOS, COX-2, IL-1β, IKKα/β, p50, p65, IRF3, IκB-α, and p-IκB-α ($Ser^{32/36}$) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibodies against TNF-α, and p-IRF3 (Ser^{396}) were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-(HO)-1(ADI-SPA-895) antibody was obtained from Enzo Life Sciences. Gene-specific primers were synthesized by Bioneer (Daejeon, Korea). AMV reverse transcriptase, the dNTP mixture, random primers, RNasin, and Taq polymerase were purchased from Promega (Madison, WI, USA).

3.1. General procedure for the synthesis of *N*-substituted phthalimideanalogs (Ia-Im, IIa-IIm, and IIIa-IIIc)

3.1.1. Procedure for the synthesis of type I *N*-substituted phthalimide analogs (Ia-Im)

Procedures for the synthesis of intermediates **2**-**6** were using previously published protocol with slight modifications [18]. Briefly, a total of 1.82 g (0.01 mol) of 3,5-dimethoxybenzoic acid and 1.48 g (0.02 mol) of glyoxylic acid were dissolved in acetic acid (10 mL) under N_2, and 1.0 mL (0.01 mol) H_2SO_4 (diluted with acetic acid beforehand) was added carefully. The solution was then stirred for 5 h at 50°C. Then intermediate **2** (1.38 g, 64.2%) was afforded after recrystallization with EtOAc.

Then, 2.38 g (0.01 mol) of compound **2** was suspended in 7 mL dimethyl phthalate (DMP) and was stirred at 180~185°C until the solid was dissolved successfully. The solution was then cooled, and 5 mL MeOH was added. After stirring for 20 min, the residue was washed with Et_2O three times. The intermediate **3** (1.84 g, 95.7%) was afforded as yellow needles after recrystallization with CH_2Cl_2.

A total of 1.94 g (0.01 mol) of compound **3** was dissolved in 50 mL NaOH (8%, m/m). Then, 1.59 g $KMnO_4$ was added, and the mixture was stirred at ambient temperature for 24 h. Next, 2 mL EtOH was added to remove surplus $KMnO_4$, and white crystals of **4** (2.10 g, 93.6%) were afforded after acidification with HCl and washing with water.

A total of 2.26 g (0.01 mol) of compound **4** and 7.14 g (0.07 mol) of acetic anhydride were mixed and reacted for 2 h at 140°C. The intermediate **5** (1.91 g, 90.2%) was afforded as white needles after recrystallization with EtOAc.

Then, 2.08 g (0.01 mol) of compound **5**, 0.30 g (0.005 mol) of urea and 2.0 mL of dimethylbenzene were mixed and reacted for 2 h at 140 °C. The mixture was dissolved in EtOH and refluxed for 1.5 h at 78 °C. The intermediate **6** (2.04 g, 91.5%) was afforded as white needles after recrystallization with EtOAc.

Finally, 2.07 g (0.01 mol) of compound **6** was heated and dissolved in 15 mL anhydrous *N,N*-dimethylformamide, and then 6.21 g of anhydrous K_2CO_3 was added. After stirring for 20 min, halohydrocarbon (0.01 mol) was added and stirred for 0.5 h at 115°C. The products **Ia**-**Im** were afforded after recrystallization with EtOAc.

Ia *N*-ethyl-3,5-dimethoxyphthalimide

m.p.: 147~148 °C; MS m/z: 235.1 $[M]^+$; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 6.98(1H, J=2.1 Hz, H-6), 6.64(1H, d, J=1.8 Hz, H-4), 3.99(3H, s, OCH_3), 3.93(3H,s, OCH_3), 3.70(2H,q, J=7.2 Hz, N-CH_2), 1.26(3H, t, J=7.2 Hz, CH_3).

Ib *N*-propyl-3,5-dimethoxyphthalimide

m.p.: 119~120 °C;MS m/z: 249.1 $[M]^+$; ^{1}H-NMR (600 MHz, $CDCl_3$) δ: 6.97(1H, d, J=0.9 Hz, H-6), 6.63(1H, d, J=0.9 Hz, H-4), 3.97(3H, s, OCH_3), 3.92(3H, s, OCH_3), 3.60(2H, t, J=3.6 Hz, N-CH_2), 1.68(2H, m, CH_2), 0.93(3H, t, J=3.6 Hz, CH_3). 13C-NMR (600 MHz, $CDCl_3$) δ: 168.11, 166.95, 166.45, 157.94, 136.41, 110.49, 103.55, 100.26, 56.28, 56.18, 39.36, 21.88, 11.30.

Ic *N*-isopropyl-3,5-dimethoxyphthalimide

m.p.: 197~198 °C; MS m/z: 249.2 $[M]^+$; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 6.90(1H, d, J=1.8 Hz, H-6), 6.59(1H, d, J=1.8 Hz, H-4), 3.94(3H, s, OCH_3), 3.88(3H, s, OCH_3), 4.45(1H, m, N-CH), 1.43(6H, d, J=6.9 Hz, 2×CH_3). ^{13}C-NMR (600 MHz, $CDCl_3$) δ: 167.92, 166.91, 166.37, 157.89, 136.41, 110.50, 103.49, 99.98, 56.24, 56.16, 42.73, 20.10.

Id *N*-butyl-3,5-dimethoxyphthalimide

m.p.: 117~118 °C; MS m/z: 263.1 $[M]^+$; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 6.93(1H, d, J=2.1 Hz, H-6), 6.60(1H, d, J=2.1 Hz, H-4), 3.95(3H, s, OCH_3), 3.90(3H, s, OCH_3), 3.60(2H, q, J=7.2 Hz, N-CH_2), 1.61(2H, m), 1.32(2H, m), 0.91(3H, t, J=7.2 Hz, CH_3). ^{13}C-NMR (600 MHz, $CDCl_3$) δ: 168.07, 166.93, 166.43, 157.92, 136.41, 110.50, 103.52, 100.24, 56.27, 56.17, 37.52, 30.63, 20.01, 13.65.

Ie *N*-isobutyl-3,5-dimethoxyphthalimide

m.p.: 140~141 °C; MS m/z: 263.2 $[M]^+$; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 6.96(1H, d, J=1.5 Hz, H-6), 6.63(1H, d, J=1.5 Hz, H-4), 3.98(3H, s, OCH_3), 3.92(3H, s, OCH_3), 3.45(2H, q, J=7.2 Hz, N-CH_2), 2.10(1H, m, CH), 0.92(6H, d, J=6.9 Hz, 2×CH_3). ^{13}C-NMR (600 MHz, $CDCl_3$) δ: 168.27, 167.09, 166.46, 157.95, 136.29, 110.40, 103.58, 100.25, 56.28, 56.18, 45.06, 27.78, 20.09.

If *N*-amyl-3,5-dimethoxyphthalimide

m.p.: 110~112 °C; MS m/z: 277.2 $[M]^+$; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 6.94(1H, d, J=1.8 Hz, H-6), 6.60(1H, d, J=1.8 Hz, H-4), 3.95(3H, s, OCH_3), 3.89(3H, s, OCH_3), 3.59(2H, q, J=7.2 Hz, N-CH_2), 1.62(2H, m), 1.24~1.35(4H, m), 0.85(3H, t, J=6.9 Hz, CH_3). ^{13}C-NMR (600 MHz, $CDCl_3$) δ: 168.07, 166.92, 166.43, 157.92, 136.42, 110.50, 103.52, 100.24, 56.27, 56.17, 37.78, 28.92, 28.25, 22.28, 13.93.

Ig *N*-hexyl -3,5-dimethoxyphthalimide

m.p.: 104~105 °C; MS m/z: 291.2 $[M]^+$; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 6.94(1H, d, J=1.8 Hz, H-6), 6.60(1H, d, J=1.8 Hz, H-4), 3.99(3H, s, OCH_3), 3.93(3H, s, OCH_3), 3.63(2H, q, J=7.2 Hz, N-CH_2), 1.65(2H, m), 1.31(6H, s), 0.88(3H, t, J=6.6 Hz, CH_3). ^{13}C-NMR (600 MHz, CDCl3) δ: 168.08, 166.93, 166.44, 157.93, 136.44, 110.53, 103.54, 100.25, 56.29, 56.18, 37.83, 31.40, 28.54, 26.48, 22.50, 14.02.

Ih *N*-heptyl -3,5-dimethoxyphthalimide

m.p.: 89~91 °C; MS m/z: 305.2 $[M]^+$; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 6.93(1H, d, J=1.8 Hz, H-6), 6.60(1H, d, J=1.8 Hz, H-4), 3.94(3H, s, OCH_3), 3.89(3H, s, OCH_3), 3.59(2H, q, J=7.2 Hz, N-CH_2), 1.61(2H, m), 1.21~1.31(6H, m), 0.84(3H, t, J=6.6 Hz, CH_3). ^{13}C-NMR (600 MHz, $CDCl_3$) δ: 168.06, 166.92, 166.43, 157.92, 136.43, 110.51, 103.53, 100.24, 56.27, 56.17, 37.83, 31.68, 28.88, 28.38, 26.77, 22.57, 14.04.

Ii *N*-(2-fluorobenzyl)-3,5-dimethoxyphthalimide

m.p.: 181~183 °C; MS m/z: 316.1 $[M+H]^+$, 338.0 $[M+Na]^+$, 653.1 $[2M+Na]^+$; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 7.16~7.33(2H, m, Ar-H), 6.96~7.05 (2H, m, Ar-H), 6.95(1H, d, J=1.8 Hz, H-6), 6.60(1H, d, J=1.8 Hz, H-6), 4.85(2H, s, CH_2), 3.94(3H, s, OCH_3), 3.89(3H, s, OCH_3). ^{13}C-NMR (600 MHz, $CDCl_3$) δ: 167.44, 166.65, 166.32, 160.58, 158.17, 136.27, 130.09, 129.33, 124.10, 123.43, 115.44, 110.41, 103.73, 100.53, 56.32, 56.22, 35.03.

Ij *N*-(3-fluorobenzyl)-3,5-dimethoxyphthalimide

m.p.: 144~145 °C; MS m/z: 316.0 $[M+H]^+$, 338.0 $[M+Na]^+$, 653.1 $[2M+Na]^+$; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 7.07~7.24(3H, m, Ar-H), 6.90(1H, m, Ar-H), 6.94(1H, d, J=1.8 Hz, H-6), 6.60(1H, d, J=1.8 Hz, H-4), 4.74(2H, s, CH_2), 3.94(3H, s, OCH_3), 3.89(3H, s, OCH_3). ^{13}C-NMR (600 MHz, $CDCl_3$) δ: 167.51, 166.68, 166.35, 162.28, 158.18, 138.97, 136.23, 130.10, 124.18, 115.53, 114.64, 110.33, 103.69, 100.58, 56.32, 56.23, 40.83.

Ik *N*-(2-bromobenzyl)-3,5-dimethoxyphthalimide

m.p.: 205~206 °C; MS m/z: 376.0 $[M+H]^+$; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 7.52(1H, dd, J=0.9, 6.9 Hz, Ar-H), 7.05~7.22(3H, m, Ar-H), 6.97(1H, d, J=1.8 Hz, H-6), 6.63(1H, d, J=1.8 Hz, H-4), 4.89(2H, s, CH_2), 3.95(3H, s, OCH_3), 3.90(3H, s, OCH_3). ^{13}C-NMR (600 MHz, $CDCl_3$) δ: 167.55, 166.74, 166.33, 158.25, 136.22, 135.29, 132.86, 128.89, 128.35, 127.47, 122.77, 110.37, 103.83, 100.62, 56.35, 56.26, 41.60.

Il *N*-(4-bromobenzyl)-3,5-dimethoxyphthalimide

m.p.: 186~187 °C; MS m/z: 376.0 $[M+H]^+$; 1H-NMR (300 MHz, $CDCl_3$) δ: 7.40(2H, d, J=1.8 Hz, Ar-H), 7.38(2H, d, J=2.1 Hz, Ar-H), 6.93(1H, d, J=1.8 Hz, H-6), 6.59(1H, d, J=2.1 Hz, H-4), 4.70(2H, s, CH_2), 3.93(3H, s, OCH_3), 3.88(3H, s, OCH_3). ^{13}C-NMR (600 MHz, $CDCl_3$) δ: 167.52, 166.68, 166.39, 158.17, 136.24, 135.63, 131.70, 130.50, 121.75, 110.33, 103.67, 100.58, 56.32, 56.23, 40.75.

Im *N*-(4-methybenzyl)-3,5-dimethoxyphthalimide

m.p.: 148~149 °C; MS m/z: 311.1 $[M]^+$; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 7.12~7.21(3H, m,

Ar-H), 7.03(1H, d, J=7.2 Hz, Ar-H), 6.93(1H, d, J=1.8 Hz, H-6), 6.59(1H, d, J=2.1 Hz, H-4), 4.72(2H, s, CH_2), 3.94(3H, s, OCH_3), 3.88(3H, s, OCH_3), 2.28(3H, s, CH_3). ^{13}C-NMR (600 MHz, $CDCl_3$) δ: 167.66, 166.54, 166.54, 158.08, 138.25, 136.57, 136.40, 129.33, 128.46, 128.41, 125.74, 110.50, 103.61, 100.45, 56.31, 56.20, 41.35, 21.35.

3.1.2. Procedure for the synthesis of type II *N*-substituted phthalimide analogs (IIa-IIm)

A total of 0.01 mol of **Ia-Im** was dissolved in CH_2Cl_2. Then, 50 mL (0.05 mol) of aqueous borontribromide (1.0 mol/L) was added dropwise at -78°C. The reaction was kept at ambient temperature for 2 h. The reaction was carefully quenched with water at low temperature, and the mixture was then extracted with CH_2Cl_2. After the solvent was removed, the products **IIa-IIm** were afforded after recrystallization with MeOH.

IIa *N*-ethyl-3,5-dihydroxyphthalimide

m.p.: 250~252 °C; MS m/z: 208.1 $[M+H]^+$, 230.1 $[M+Na]^+$; ^{1}H-NMR (300 MHz, C_5D_5N) δ: 12.66(2H, brs, 2×OH), 7.19(1H, d, J=1.8 Hz, H-6), 6.93 (1H, d, J=2.1 Hz, H-4), 3.68(2H, q, J=7.2 Hz, N-CH_2), 1.16(3H, t, J=7.2 Hz, CH_3). ^{13}C-NMR (600 MHz, C_5D_5N) δ:168.14, 167.43, 165.93, 158.06, 136.76, 108.59, 107.97, 103.89, 32.28, 13.97.

IIb *N*-propyl-3,5-dihydroxyphthalimide

m.p.: 194~197 °C; MS m/z: 222.1 $[M+H]^+$, 244.0 $[M+Na]^+$; ^{1}H-NMR (600 MHz, C_5D_5N) δ: 13.04(2H, brs, 2×OH), 7.26(1H, d, J=1.8 Hz, H-6), 6.98(1H, d, J=1.2 Hz, H-4), 3.67(2H, t, J=7.2 Hz, N-CH_2), 1.70(2H, m, CH_2), 0.84(3H, t, J=7.2 Hz, CH_3). ^{13}C-NMR (600 MHz, C_5D_5N) δ: 168.42, 167.69, 165.96, 158.09, 136.67, 108.64, 107.89, 103.91, 39.00, 22.16, 11.21.

IIc *N*-isopropyl-3,5-dihydroxyphthalimide

m.p.: 190~193 °C; MS m/z: 222.1 $[M+H]^+$, 244.1 $[M+Na]^+$; ^{1}H-NMR (300 MHz, DMSO) δ: 10.68(2H, brs,2×OH), 6.56(1H, d, J=1.8 Hz, H-6), 6.47(1H, d, J=1.8 Hz, H-4), 4.24(1H, m,

N-CH), 1.30(6H, d, J=6.9 Hz, 2×CH_3). ^{13}C-NMR (600 MHz, DMSO) δ: 167.93, 166.73, 164.68, 157.24, 136.06, 107.78, 106.83, 103.08, 42.01, 20.36.

IId *N*-butyl-3,5-dihydroxyphthalimide

m.p.: 175~176 °C; MS m/z: 236.1 $[M+H]^+$， 258.0 $[M+Na]^+$; 1H-NMR (300 MHz, C_5D_5N) δ: 12.80(2H, brs, 2×OH), 7.26(1H, d, J=2.1 Hz, H-6), 6.98(1H, d, J=2.1 Hz, H-4), 3.71(2H, t, J=7.2 Hz, N-CH_2), 1.65(2H, m, CH_2), 1.26(2H, m, CH_2), 0.81(3H, t, J=7.5 Hz, CH_3). ^{13}C-NMR (600 MHz, C_5D_5N) δ: 168.40, 167.68, 165.96, 158.09, 136.70, 108.63, 107.91, 103.91, 37.14, 30.87, 20.06, 13.53.

IIe *N*-isobutyl-3,5-dihydroxyphthalimide

m.p.: 222~224 °C; MS m/z: 236.1 $[M+H]^+$, 258.0 $[M+Na]^+$; ^{1}H-NMR (300 MHz, DMSO) δ: 10.75(1H, s, OH-3), 10.66(1H, s, OH-5), 6.60(1H, d, J=1.8 Hz, H-6), 6.49(1H, d, J=1.8 Hz, H-4), 3.22(2H, d, J=7.2 Hz, N-CH_2), 1.89(1H, m, CH), 0.78(6H, d, J=6.6 Hz, 2×CH_3). ^{13}C-NMR (600 MHz, DMSO) δ: 168.25, 166.98, 164.75, 157.28, 135.95, 107.82, 106.77, 103.33, 44.67, 27.82, 20.37.

IIf *N*-amyl-3,5-dihydroxyphthalimide

m.p.: 194~196 °C; MS m/z: 250.1 $[M+H]^+$, 272.1 $[M+Na]^+$, 521.2 $[2M+H]^+$; ^{1}H-NMR (300 MHz, C_5D_5N) δ: 13.04(2H, brs, 2×OH), 7.27(1H, d, J=1.8 Hz, H-6), 6.99(1H, d, J=1.8 Hz, H-4), 3.71(2H, t, J=7.2 Hz, N-CH_2), 1.68(2H, m, CH_2), 1.20~1.25(4H, m, 2×CH_2), 0.77(3H, t, J=7.2 Hz, CH_3). ^{13}C-NMR (600 MHz, C_5D_5N) δ: 169.91, 169.18, 167.46, 159.59, 138.20, 110.13, 109.41, 105.41, 38.91, 30.48, 30.05, 23.76, 15.31.

IIg *N*-hexyl-3,5-dihydroxyphthalimide

m.p.: 185~188 °C; MS m/z: 264.1 $[M+H]^+$, 286.0 $[M+Na]^+$, 549.2 $[2M+Na]^+$; ^{1}H-NMR (600 MHz, C_5D_5N) δ: 12.99(2H, brs, 2×OH), 7.22(1H, d, J=1.8 Hz, H-6), 6.94 (1H, d, J=1.8 Hz, H-4), 3.68(2H, t, J=6.6 Hz, N-CH_2), 1.65(2H, m, CH_2), 1.21(2H, m, CH_2), 1.11~1.13(4H, m, 2×CH_2), 0.73(3H, t, J=6.6 Hz, CH_3). ^{13}C-NMR (600 MHz, C_5D_5N) δ: 168.42, 167.70, 165.97,

158.10, 136.72, 108.64, 107.92, 103.92, 37.46, 31.34, 28.84, 26.57, 22.51, 13.88.

IIh *N*-heptyl-3,5-dihydroxyphthalimide

m.p.: 175~176 °C; MS m/z: 278.1 $[M+H]^+$, 300.1 $[M+Na]^+$, 577.2 $[2M+Na]^+$; ^{1}H-NMR (600 MHz, C_5D_5N) δ: 12.95(2H, brs, 2×OH), 7.20(1H, s, H-6), 6.92(1H, s, H-4), 3.68(2H, t, J=7.2 Hz, N-CH_2), 1.65(2H, m, CH_2), 1.08~1.22(8H, m, 4×CH_2), 0.73(3H, t, J=6.9 Hz, CH_3). ^{13}C-NMR (600 MHz, C_5D_5N) δ: 168.43, 167.70, 165.97, 158.11, 136.72, 108.64, 107.92, 103.92, 37.48, 31.67, 28.89, 28.88, 26.89, 22.55, 13.96.

IIi *N*-(2-fluorobenzyl)-3,5-dihydroxyphthalimide

m.p.: 217~219 °C; MS m/z: 288.0 $[M+H]^+$, 310.0$[M+Na]^+$; ^{1}H-NMR (600 MHz, DMSO) δ: 10.87(1H, s, OH-3), 10.75(1H, s, OH-5), 7.32~7.07 (4H, m, Ar-H), 6.63(1H, d, J=1.8 Hz, H-6), 6.51(1H, d, J=1.8 Hz, H-4), 4.66(2H, s, CH_2). ^{13}C-NMR (600 MHz, DMSO) δ: 167.67, 166.28, 164.96, 160.25, 157.54, 135.95, 129.87, 129.83, 124.97, 124.28, 115.78, 107.98, 106.84, 103.60, 39.99.

IIj *N*-(3-fluorobenzyl)-3,5-dihydroxyphthalimide

m.p.: 196~197 °C; MS m/z: 288.0 $[M+H]^+$, 310.0 $[M+Na]^+$; ^{1}H-NMR (600 MHz, DMSO) δ: 10.86(1H, s, OH-3), 10.74(1H, s, OH-5), 7.33(1H, q, J=7.2 Hz, Ar-H), 7.08~7.02(3H, m, Ar-H), 6.64(1H, d, J=1.5 Hz, H-6), 6.51(1H, d, J=1.5 Hz, H-4), 4.63(2H, s, CH_2). ^{13}C-NMR (600 MHz, DMSO) δ: 167.81, 166.44, 164.97, 162.60, 157.57, 140.4, 135.97, 131.05, 123.64, 114.61, 114.47, 107.98, 106.81, 103.66, 40.31.

IIk *N*-(2-bromobenzyl)-3,5-dihydroxyphthalimide

m.p.: 231~233 °C; MS m/z: 348.0 $[M+H]^+$, 369.9 $[M+Na]^+$; ^{1}H-NMR (600 MHz, DMSO) δ: 10.89(1H, s, OH-3), 10.76(1H, s, OH-5), 7.61(1H, dd, J=0.9, 6.9 Hz, Ar-H), 7.16~7.31(2H, m, Ar-H), 7.03(1H, dd, J=1.2, 6.3 Hz, Ar-H), 6.66(1H, d, J=1.8 Hz, H-6), 6.54(1H, d, J=2.1 Hz, H-4), 4.64(2H, s, CH_2). ^{13}C-NMR (600 MHz, DMSO) δ: 167.79, 166.35, 165.02, 157.63, 136.00, 135.83, 133.07, 129.70, 128.45, 128.19, 122.05, 108.03, 106.90, 103.71, 41.26.

IIl *N*-(4-bromobenzyl)-3,5-dihydroxyphthalimide

m.p.: 233~235 °C; MS m/z: 348.0 $[M+H]^+$, 369.9 $[M+Na]^+$; ^{1}H-NMR (600 MHz, DMSO) δ: 10.86(1H, s, OH-3), 10.74(1H, s, OH-5), 7.61(2H, d, J=8.4 Hz, Ar-H), 7.03(2H, d, J=8.7 Hz, Ar-H), 6.63(1H, d, J=1.8 Hz, H-6), 6.51(1H, d, J=2.1 Hz, H-4), 4.59(2H, s, CH_2). ^{13}C-NMR (600 MHz, DMSO) δ: 167.78, 166.41, 164.96, 157.56, 137.01, 135.96, 131.89, 130.01, 120.86, 107.97, 106.79, 103.63, 40.22.

IIm *N*-(3-methybenzyl)-3,5-dihydroxyphthalimide

m.p.: 226~289 °C; MS m/z: 284.0 $[M+H]^+$, 306.0 $[M+Na]^+$; ^{1}H-NMR (600 MHz, DMSO) δ: 10.85(1H, s, OH-3), 10.73(1H, s, OH-5), 7.15(1H, t, J=7.8 Hz, Ar-H), 7.03~6.97(3H, m, Ar-H), 6.62(1H, d, J=1.8 Hz, H-6), 6.50(1H, d, J=1.8 Hz, H-4), 4.56(2H, s, CH_2), 2.22(3H, s, CH_3). ^{13}C-NMR (600 MHz, DMSO) δ: 167.83, 166.52, 164.92, 157.49, 138.17, 137.54, 136.01, 128.90, 128.38, 128.25, 124.83, 107.95, 106.84, 103.58, 40.75, 21.40.

3.1.3. Procedure for the synthesis of type III *N*-substituted phthalimide analogs (IIIa-IIIc)

A total of 2.08 g (0.01 mol) of compound **5** and 0.61 g (0.01 mol) of ethanolamine were mixed and reacted for 2 h at 185°C. The yellow solid was then chromatographed (silica gel; CH_2Cl_2: MeOH, 9:1) to afford the intermediate **7** (1.31 g, 52.2%) as a white solid.

Then, 2.51 g (0.01 mol) of compound **7** and 4-dimethylaminopyridine (DMAP) were dissolved in 20 mL of CH_2Cl_2, and 0.01 mol of acyl chloride was added dropwise in an ice bath and stirred for 6 h at ambient temperature. After the reaction, saturated $NaHCO_3$ was added, and the organic layer was evaporated to afford the compounds **IIIa-IIIc**.

IIIa *N*-(2-acetyloxyethyl)-3,5-dimethyoxyphthalimide

m.p.: 184~185 °C; MS m/z: 293.1 $[M]^+$; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 6.93(1H, d, J=1.8 Hz, H-6), 6.60(1H, d, J=1.8 Hz, H-4), 4.25(2H, t, J=5.4 Hz, OCH_2), 3.94(3H, s, OCH_3), 3.89(3H,

s, OCH_3), 3.86(2H, t, J=5.4 Hz, N-CH_2), 1.99(3H, s, CH_3). ^{13}C-NMR (600 MHz, $CDCl_3$) δ: 170.88, 167.80, 166.64, 166.44, 158.13, 136.22, 110.37, 103.75, 100.46, 61.62, 56.33, 56.23, 36.80, 20.83.

IIIb *N*-(2-propionyloxyethyl)-3,5-dimethyoxyphthalimide

m.p.: 124~125 °C; MS m/z: 307.1 $[M]^+$; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 6.93(1H, s, H-6), 6.60 (1H, s, H-4), 4.26(2H, t, J=4.8 Hz, OCH_2), 3.94(3H, s, OCH_3), 3.89(3H, s, OCH_3), 3.85(2H, m, N-CH_2), 2.26(2H, q, J=7.5 Hz, CH_2), 1.06(3H, t, J=7.5 Hz, CH_3). ^{13}C-NMR (600 MHz, CDCl3) δ: 174.24, 167.78, 166.62, 166.43, 158.12, 136.23, 110.38, 103.74, 100.43, 61.46, 56.33, 56.22, 36.85, 27.38, 8.93.

IIIc *N*-(2-chloroacetyloyethyl)-3,5-dimethyoxyphthalimide

m.p.: 143~144 °C; MS m/z: 328.0 $[M+H]^+$, 350.0 $[M+Na]^+$, 677.0 $[2M+Na]^+$; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 6.94(1H, d, J=1.8 Hz, H-6), 6.61(1H, d, J=2.1 Hz, H-4), 4.38(2H, t, J=5.1 Hz, OCH_2), 4.02(2H, s, CH_2-Cl), 3.95(3H, s, OCH_3), 3.92(3H, s, OCH_3), 3.89(2H, m, N-CH_2). ^{13}C-NMR (600 MHz, CDCl3) δ: 167.76, 167.25, 166.73, 166.37, 158.19, 136.11, 110.24, 103.80, 100.57, 63.38, 56.35, 56.25, 40.78, 36.52.

3.2 Methods

3.2.1. Cell culture

Mouse macrophage RAW 264.7 cells, obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA), were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % heat-inactivated fetal bovine serum (FBS), 100 units/mL penicillin, 100 µg/mL streptomycin, and antibiotics-antimycotics (0.25 µg/mL amphotericin B). The cells were incubated at 37 °C with 5 % CO_2 in humidified air.

3.2.2. Measurement of nitrite production

Nitric oxide (NO) has a short half-life and is oxidized to stable nitrite. Consequently, to evaluate the inhibitory activity of the test compounds on LPS-induced NO production, RAW 264.7 cells were plated at a density of 2×10^5 per well in a 24-well culture plate and incubated in a 37°C humidified incubator with 5% CO_2 in air for 24 h. The cells were washed twice with PBS, fresh medium was added, and the cells were then incubated with 1 μg/mL LPS in the presence or absence of the test compounds for 22 h. The amount of nitrite released in the culture media was measured using the Griess reaction (180 μL of Griess reagent [1:1 mixture (v/v) of 1% sulfanilamide in 5% H_3PO_4 and 0.1% *N*-(1-naphthyl) ethylenediaminedihydrochloride solution]). Serially diluted sodium nitrite was used as a standard, and the absorbance was measured at 540 nm. The IC_{50} value, which is the sample concentration resulting in 50% inhibition of NO production, was determined using nonlinear regression analysis (% inhibition vs concentration).

3.2.3. Cell viability assay (MTT)

After the Griess reaction, MTT solution (final concentration of 500 μg/mL) was added to each well and further incubated for 3 h at 37°C. The medium was discarded, and dimethyl sulfoxide (DMSO) was added to each well to dissolve the generated formazan. The absorbance was measured at 570 nm, and the percent survival was determined by comparison to a control (CTL) group.

3.2.4. Reporter gene (SEAP; Secreted Embryonic Alkaline Phosphatase) assay

To determine the effect of the test compounds on the activation of NF-κB, a reporter gene assay was performed. Briefly, the cells were pre-treated with the test compounds for 2 h and then further induced with LPS for an additional 16 h. Cell culture supernatants were heated at

65°C for 5 min and reacted with SEAP assay buffer [2 M diethanolamine, 1 mM $MgCl_2$, 500 μM 4-methylumbelliferyl phosphate (MUP)] in the dark at 37°C for 1 h. In addition, fluorescence from the product of SEAP/MUP reaction was measured in relative fluorescence units using a 96-well plate fluorometer with excitation at 360 nm and emission at 449 nm and normalized to protein concentration. Data are presented as the proportion relative to vehicle-treated control cells without LPS.

3.2.5. Western blot analysis

RAW 264.7 cells (3×10^5 cells/mL in a 60 mm dish) were treated with various concentrations of QSN-19 for the indicated times. Western blot analysis was carried out as described previously [19].

3.2.6. Real time-polymerase chain reaction (RT-PCR)

RAW 264.7 cells were stimulated with 1 μg/mL LPS in the presence or absence of QSN-19 for 4 h. Then, RT-PCR analysis was carried out as described previously[19]. The following sequences were used: iNOS F5' -ATGTCCGAAGCAAACATCAC-3'; iNOS R5' -TAATGTCCAGGAAGTAGGTG-3'; IL-1β F5′- TGCAGAGTTCCCCAACTGG TACATC-3′; IL-1β R5′- GTGCTGCCTAATGTCCCCTTGAATC-3′; IFN-β F5' -CATTT CCGAATGTTCGTCCT-3'; IFN-β R5' -CAC AGCCCTCTCCATCAACTA-3'; β-actin F5 ′-TGTGATGGTGGGAATGGGTCAG-3′; β-actin R5 ′-TTTGATGTCACGCACGATT TCC-3′. β-actin was used as an internal standard.

3.2.7. Statistical Analysis

All experiments were repeated at least three times. Data are presented as the means ± standard deviation(SD) for the indicated number of independently performed experiments. Student's t-test (SigmaStat 3.1, Systat Software Inc.) was used for the determination of statistical significance. The difference was considered statistically significant when p (*) <0.05, (**) < 0.005, (***) < 0.0001.

4. CONCLUSIONS

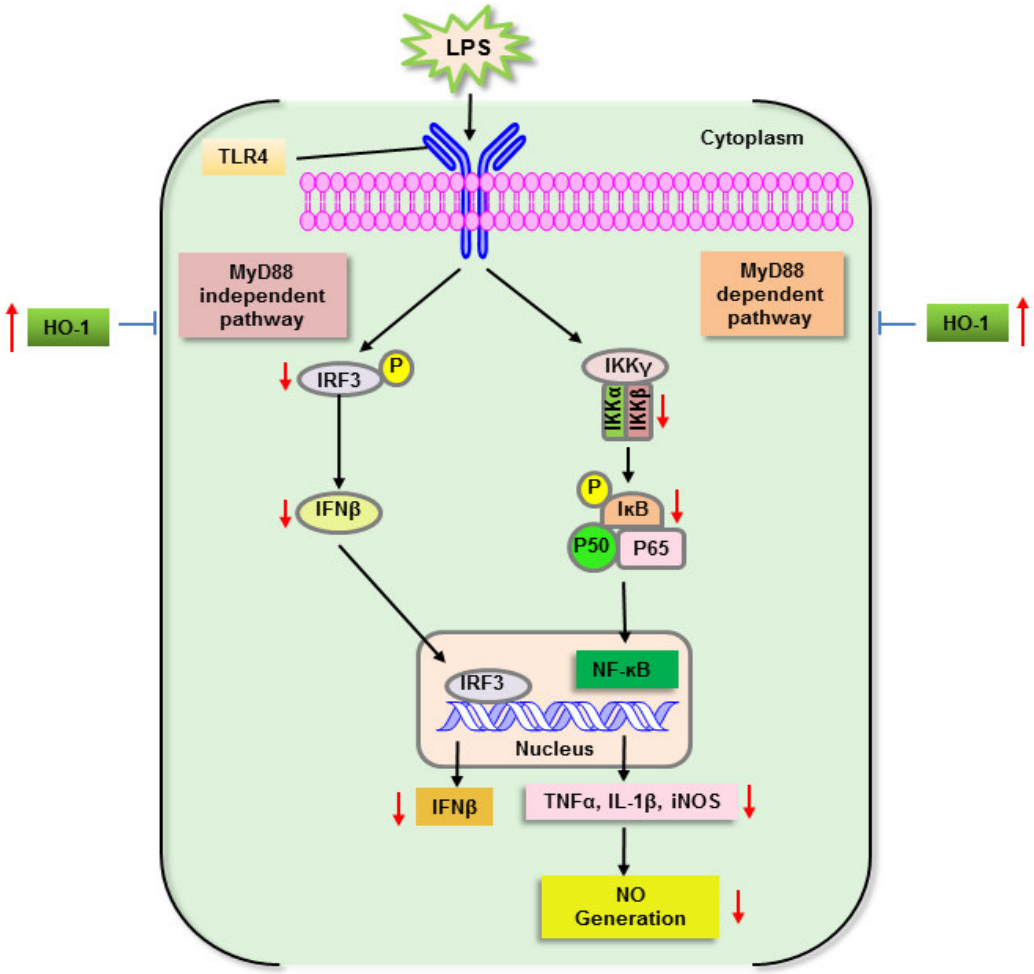


Figure 6. A possible mechanism by which compound **IIh** suppresses nitric oxide (NO) production in the lipopolysaccharide (LPS)/TLR4 signaling pathway. (Red arrow: the effect of compound **IIh**)

For treating inflammation, a variety of phytochemicals have been evaluated for their potential efficacy as anti-inflammatory agents, and several compounds have been identified as selective inhibitors of iNOS. In the present study, we designed and evaluated a series of phthalimides as inhibitors of NO production by iNOS. Several compounds showed potent inhibitory activity towards LPS-induced NO production. The present study suggests that the suppression of iNOS mRNA transcription is, in part, related to the inhibitory activity of phthalimides. Additionally, the details mechanism of **IIh** also is investigated (Figure 6). The inhibitors in this new series of are suggested as lead compounds for the development of potent and selective inhibitors for potential therapeutic use. Based on these findings, future studies aimed at producing more potent inhibitors are currently in progress in our laboratory.

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CONFLICT OF INTEREST

The authors declare no conflict of interest

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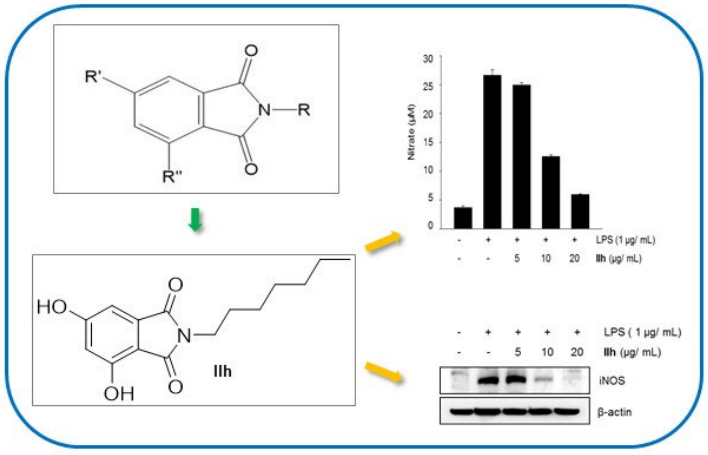

New series of phthalimides were synthesized including the C7-alkyl chain as a key finding and structure-activity study with the inhibition of nitric oxide production was investigated.