Bioorganic & Medicinal Chemistry Letters 24 (2014) 571–575

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Synthesis of tricyclic fused coumarin sulfonates and their inhibitory effects on LPS-induced nitric oxide and PGE_2 productions in RAW 264.7 macrophages

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ARTICLE INFO

Article history:
Received 29 August 2013
Revised 25 November 2013
Accepted 4 December 2013
Available online 10 December 2013

Keywords:
Antiinflammatory
Coumarin
Nitric oxide
PGE_2
Sulfonate
Tricyclic fused coumarin

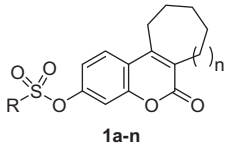
ABSTRACT

The regulations of NO and PGE_2 productions are research topics of interest in the field of antiinflammatory drug development. In the present study, a series of tricyclic fused coumarin sulfonate derivatives was synthesized and evaluated for their abilities to inhibit NO and PGE_2 productions in LPS-induced RAW 264.7 macrophages. Among all the target compounds, compound **1g** possessing *p*-(trifluoromethyl)phenyl and fused cycloheptane moieties showed the highest inhibitory effects on NO and PGE_2 productions. Compound **1g** not only inhibited COX-2 activity but also reduced expressions of COX-2 and iNOS. Furthermore, ADME profiling showed that compounds **1g**, **1j**, **1m**, and **1n** are estimated to be orally bioavailable.

Inflammation is a complex physiological and pathological process accompanied by the activation of the immune system, local vascular system, and various cells within the damaged tissue.[1] It is a normal protective response to tissue injury caused by physical trauma (cut, burn or bruise), noxious chemicals, microbiologic agents, or even autoimmune disease. Acute inflammation is a part of the defense response by organisms to remove injurious stimuli, such as pathogens, irritants, or physical injury, from tissues and to initiate the healing process. However, persistent and excessive immune response can promote tissue damage, resulting in chronic inflammation. This chronic inflammation is a part of many human diseases including arteriosclerosis,[2] inflammatory bowel disease,[3] arthritis,[4] cancer,[5] and Alzheimer's disease.[6]

In the inflammatory state, activated immune cells, such as macrophages secrete large amounts of proinflammatory cytokines, nitric oxide (NO), and prostaglandin E_2 (PGE_2). However, high levels of NO and PGE_2 in a chronic inflammation state can result in various pathological conditions.[7,8] Accordingly, control of the production of NO and PGE_2 in macrophages are current research topics for the development of new antiinflammatory agents.

Coumarins and their derivatives have acquired much attention from the pharmacological and pharmaceutical arena due to their broad range of therapeutic qualities. Several coumarin analogs have been reported as potential antiinflammatory agents.[9–11] In the present study, various fused tricyclic coumarin sulfonate analogs **1a–n** (Fig. 1) were synthesized with the aim to study structure–activity relationships, and thereby provide new lead compounds possessing enhanced antiinflammatory activity and reduced cytotoxicity. The antiinflammatory activities of the target compounds



1a-n

n = 1, 2
R = Me, Et, *n*-Pr, *c*-Pr, Ph, *p*-tolyl, *p*-$(CF_3)C_6H_4$

Figure 1. Structures of the target compounds.

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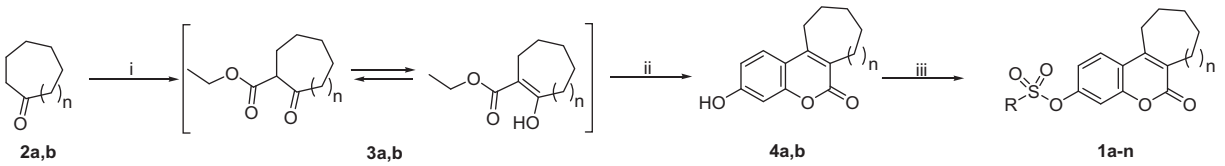
http://dx.doi.org/10.1016/j.bmcl.2013.12.018



Scheme 1. Reagents and conditions: (i) diethyl carbonate, NaH, benzene, reflux, 90% (**3a**, $n = 1$), 85% (**3b**, $n = 2$); (ii) resorcinol, CF_3COOH, concd H_2SO_4, 0 °C; rt, 3 h; (iii) appropriate sulfonyl chloride derivative, triethylamine, CH_2Cl_2, 0 °C; rt, 1 h.

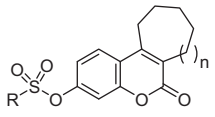
were evaluated by measuring the inhibitions of the productions of lipopolysaccharide (LPS)-induced NO and PGE_2 productions in RAW 264.7 macrophages.

The target compounds were synthesized by the pathway illustrated in Scheme 1. Cycloheptanone (**2a**) or cyclooctanone (**2b**) was reacted with diethyl carbonate in refluxing benzene in the presence of sodium hydride to produce the corresponding ethoxycarbonate derivatives **3a**,**b** which exist in keto–enol tautomers.[12] Cyclization to the phenolic tricyclic intermediates **4a**,**b** was achieved by reaction of compounds **3a**,**b** with resorcinol in the presence of concentrated sulfuric acid and trifluoroacetic acid.[13] Treatment of the phenolic compounds **4a**,**b** with the appropriate sulfonyl chloride derivatives in the presence of triethylamine yielded the target sulfonates **1a**–**n**. Table 1 illustrates structures of the final compounds, their yield percentages, and their melting points.

Inflammation is a protective immune response against tissue damage induced by external stimulants in the body.[2] However, the inflammatory response can also result in considerable damage to the host, because microbial components such as LPS stimulate macrophages to produce pro-inflammatory cytokines, iNOS, and COX-2. In particular, the large amounts of NO and PGE_2 secreted by activated immune cells in the inflammatory state can induce various pathological conditions. Thus, inhibition of the production of inflammatory mediators is a potential strategy for the treatment of many acute and chronic inflammatory disorders. The synthesized coumarin sulfonates **1a**–**n** were assessed in terms of their ability to inhibit the production of inflammatory mediators, NO and PGE_2, in LPS-induced RAW 264.7 macrophages.[14–16] *N*6-(1-Iminoethyl)-L-lysine (L-NIL) and *N*-(2-cyclohexyloxy-4-nitrophenyl)methanesulfonamide (NS398) were used as reference compounds for screening of NO and PGE_2 production inhibitions, respectively. The cytotoxic effects of the coumarins on RAW 264.7 macrophages were also evaluated using the MTT assay[17] to test whether the inhibitory effects on the productions of NO and PGE_2 were caused by nonspecific cytotoxicity (Table 2). All the target compounds showed IC_{50} values $\geqslant$9.2 μM. Compounds **1e**–**g** and **1l**–**n** with aromatic sulfonate moieties did not inhibit 50% of RAW 264.7 macrophages growth up to 100 μM. After that, the target compounds were tested for NO and PGE_2 production inhibitions at 5 μM.

The target coumarin compounds **1a**–**n** expressed varying inhibitory activities on the LPS-induced NO and PGE_2 productions (Figs. 2 and 3). Compounds **1f** and **1g** possessing *p*-toluenesulfonate and *p*-(trifluoromethyl)benzenesulfonate moieties, respectively, showed higher inhibitory effect on NO production than compounds **1a**–**e**. Among cyclooctane-fused derivatives, compound **1j** with *n*-propylsulfonate terminal moiety demonstrated the strongest inhibitory effect on NO production. Compounds **1b**, **1d**, and **1g** with fused cycloheptane ring were more active as NO production

Table 1
Structures of the target compounds, and their yield percentages and melting points



Compound no.	R	*n*	Yield%	Melting point (°C)
1a	Me	1	95	172–3
1b	Et	1	92	169–72
1c	*n*-Pr	1	93	133–5
1d	Cyclo-Pr	1	85	128–30
1e	Ph	1	93	125–8
1f	*p*-Tolyl	1	95	149–52
1g	*p*-$(CF_3)C_6H_4$	1	90	137–40
1h	Me	2	94	146–8
1i	Et	2	90	157–60
1j	*n*-Pr	2	88	114–7
1k	Cyclo-Pr	2	87	133–6
1l	Ph	2	92	108–11
1m	*p*-Tolyl	2	95	107–10
1n	*p*-$(CF_3)C_6H_4$	2	90	154–7

Table 2
Cytotoxicty (IC_{50}, MTT assay) of the target compounds **1a**–**n** over RAW 264.7 cells

Compound no.	IC_{50}[a] (μM)
1a	90.80 ± 3.25
1b	23.40 ± 2.36
1c	23.90 ± 1.25
1d	20.30 ± 2.56
1e	>100
1f	>100
1g	>100
1h	24.50 ± 2.89
1i	21.50 ± 3.69
1j	9.20 ± 1.20
1k	10.50 ± 3.22
1l	>100
1m	>100
1n	>100

[a] Data are presented as the means ± SD of three independent experiments.

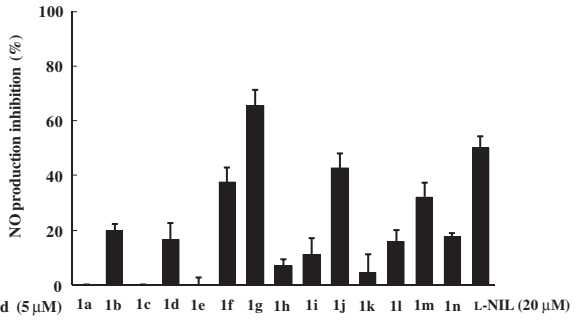


Figure 2. Inhibitory effect of the target compounds **1a**–**n** on LPS-induced NO production in RAW 264.7 cells. Compounds **1a**–**n** were tested at 5 μM concentration, while the reference compound L-NIL was tested at 20 μM concentration. Data are presented as the means ± SD of three independent experiments.

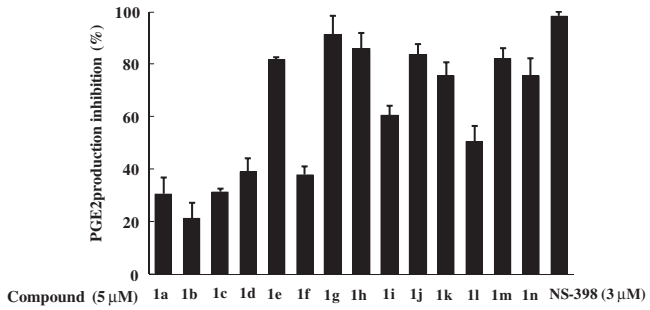


Figure 3. Inhibitory effect of the target compounds **1a**–**n** on LPS-induced PGE_2 production in RAW 264.7 cells. Compounds **1a**–**n** were tested at 5 μM concentration, while the reference compound NS398 was tested at 3 μM concentration. Data are presented as the means ± SD of three independent experiments.

inhibitors than the corresponding cyclooctane-containing compounds **1i**, **1k**, and **1n**. On the other hand, cyclooctane-possessing compounds **1h**, **1j**, and **1l** showed higher inhibitory effects on NO production compared to the corresponding compounds **1a**, **1c**, and **1e** with cycloheptane ring. Compounds **1f** and **1m** possessing *p*-toluenesulfonate moiety were more active than compounds **1e** and **1l** with benzenesulfonate group.

Regarding PGE_2 production inhibition results, compounds **1h**, **1i**–**k**, and **1m** with cyclooctane ring showed higher inhibitory effect than the corresponding compounds **1a**, **1b**–**d**, and **1f** possessing fused cycloheptane ring. This may be rationalized that the cyclooctane ring may perform stronger hydrophobic interaction at the receptor site. Or the steric factor may influence the affinity with the receptor and therefore the inhibitory effect of the compounds. The results of compounds **1e** (cycloheptane, benzenesulfonate),

Table 3
NO and PGE_2 production inhibitory potency (IC_{50}) and calculated Lipinski's rule of five for the most active target compounds

Compd. no.	NO production inhibition[a] (IC_{50}, μM)	PGE_2 production inhibition[a] (IC_{50}, nM)	Log *P*[b]	TPSA[c]	MW[d]	*n*ON[e]	*n*OHNH[f]	*n*Violations
1g	3.84 ± 0.26	6.45 ± 1.02	5.11	73.59	438.42	5	0	1
1j	10.00 ± 1.84	4.02 ± 1.09	3.42	73.59	350.43	5	0	0
1m	40.69 ± 5.30	6.75 ± 1.10	5.09	73.59	398.47	5	0	1
1n	75.32 ± 5.01	6.04 ± 0.92	5.53	73.59	452.44	5	0	1
NS398	—	7.01 ± 0.66	2.79	101.23	314.36	7	1	0
L-NIL	20.14 ± 2.32	—	—	—	—	—	—	—

[a] Data are presented as the means ± SD of three independent experiments.
[b] Calculated lipophilicity.
[c] Total polar surface area (Å^2).
[d] Molecular weight.
[e] Number of hydrogen bond acceptors.
[f] Number of hydrogen bond donors.

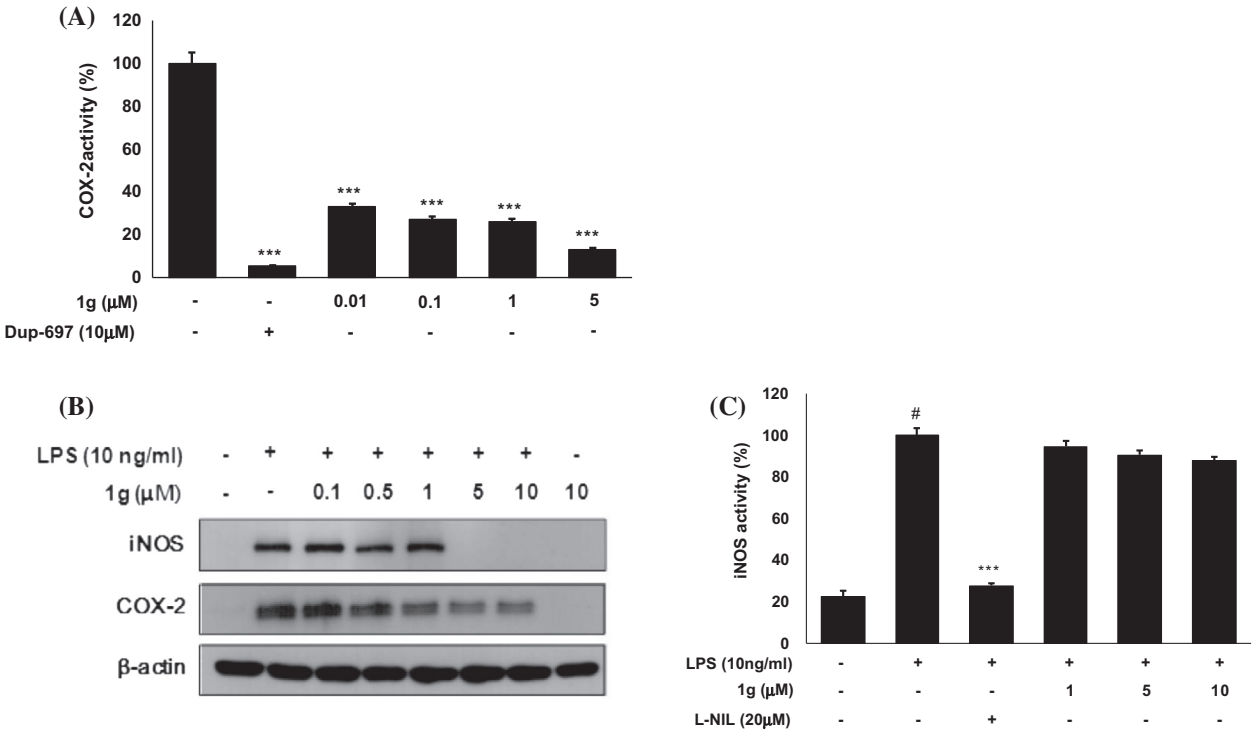


Figure 4. Inhibitory effects of compound **1g** on LPS-induced COX-2 and iNOS enzyme activity and protein expressions in RAW 264.7 macrophages. (A) Recombinant COX-2 enzyme was in vitro treated with the indicated concentrations of **1g** for 10 min. As a negative control, these enzymes were inactivated by boiling for 3 min. Dup-697 (10 μM) were used as a positive COX-2 inhibitor controls. (B) Cellular lysates were prepared from the pretreated with/without **1g** (0.010.1, 0.5, 1, 5 or 10 μM) for 1 h and then with LPS (10 ng/mL) for 24 h. Total cellular proteins were resolved by SDS–PAGE, transferred to PVDF membranes, and detected with specific iNOS and COX-2 antibody. β-actin was used as an internal control. (C) Following pretreatment with LPS (10 ng/ml) for 12 h and wash PBS, cells were treated with **1g** (1, 5, or 10 μM) for 12 h. L-NIL (20 μM) was used as positive control in the assay. Levels of NO in culture media were quantified using Griess reaction assay. Data are presented as the means ± SDs of three independent experiments. $^{\#}p$ <0.05 versus the control cells; $^{***}p$ <0.001 versus LPS-stimulated cells; statistical significances were compared using ANOVA and Dunnett's post hoc test.

1g (cycloheptane, *p*-(trifluoromethyl)benzenesulfonate), **1h** (cyclooctane, methanesulfonate), **1j** (cyclooctane, *n*-propanesulfonate), and **1m** (cyclooctane, *p*-toluenesulfonate) were near the inhibitory effect expressed by the reference compound, NS398.

Among all the target compounds, compound **1g** possessing fused cycloheptane ring and *p*-(trifluoromethyl)benzenesulfonate moieties showed the highest inhibitory effects on NO and PGE_2 productions. It showed higher efficacy and potency than the reference NO production inhibitor, L-NIL. It inhibited NO production by 65.76% at 5 μM concentration, while L-NIL inhibited it by 50% at 20 μM. It also inhibited the PGE_2 production in RAW 264.7 macrophages by 91.48%, which is very near to the inhibition effect produced by NS398.

Based on NO and PGE_2 production inhibition% data, compounds **1g**, **1j**, **1m**, and **1n** with the most promising results were selected for further screening of their potencies. Their IC_{50} values were calculated and are summarized in Table 3. Compounds **1g** and **1j** were more potent than L-NIL as NO production inhibitor. Compound **1g** possessing fused cycloheptane ring showed higher potency than the corresponding analog with fused cyclooctanone ring, compound **1n**. Among the four tested compounds, compound **1g** demonstrated the highest potency. It is 5.24 times more potent than L-NIL. Regarding PGE_2 inhibitory potency, the four compounds **1g**, **1j**, **1m**, and **1n** showed higher potency than NS398. Compound **1j** possessing fused cyclooctanone and *n*-propanesulfonate moieties was the most potent, and it was 1.74-fold more potent than NS398. We further investigated whether the inhibitory effects of compound **1g** (0.1, 1 or 5 μM) on PGE_2 production are related to COX-2 enzyme activity and protein expression. We observed that compound **1g** significantly inhibited COX-2 enzyme activity (Fig. 4A). Because iNOS expression induced by LPS produce NO, the protein expression of iNOS was determined. As shown in Figure 4B, COX-2 protein levels were found to be markedly up-regulated in response to LPS, and compound **1g** dose-dependently inhibited LPS-induced COX-2 protein expression. Next, we investigated whether inhibitory effects of compound **1g** on LPS-induced NO production are related to iNOS enzyme activity or iNOS expression. We found that **1g** exhibited no effect on the iNOS enzyme activity (Fig. 4C). However, **1g** suppressed the LPS-induced iNOS expression (Fig. 4B). Thus, we suggest that the inhibition of PGE_2 and NO production by **1g** are due to its inhibition of the COX-2 enzyme activity and protein expressions of COX-2 and iNOS. Thus, the marked inhibitory effect of **1g** on PGE_2 production can be caused by simultaneously suppressed COX-2 enzyme activity and protein synthesis. However, **1g** did not affect iNOS enzyme activity. Although the present study does not clarify the mechanism responsible for the inhibition of iNOS and COX-2 expression by **1g**, we suggest that **1g** could interferes the LPS-induced activation of TAK1/IKK-α/β/NF-κB through TLR4.

Since STX-64 (Irosustat, BN83495), the first steroid sulfatase (STS) inhibitor to enter diverse clinical trials for patients with advanced hormone-dependent cancer is closely related to the structures of tricyclic coumarin sulfonates in this manuscript, we investigated whether STX-64 has inhibitory effects on the LPS-induced NO and PGE_2 production in RAW 264.7 macrophages. As shown in Figure 5A and B, STX-64 inhibited LPS-induced NO and PGE_2 production in a dose-dependent manner. However, STX-64 more potently inhibited LPS-induced PGE_2 production (IC_{50}: 81.04 nM) than NO production (IC_{50}: 85.76 μM). These results suggest that STX-64 showed similar inhibitory effects of four most active target compounds (Table 3) via a selective PGE_2 inhibition in LPS-stimulated macrophages.

The bioavailability of compounds **1g**, **1j**, **1m**, and **1n** with promising biological results was assessed using ADME (absorption, distribution, metabolism, and excretion) prediction methods. In particular, we calculated the compliance of compounds to the

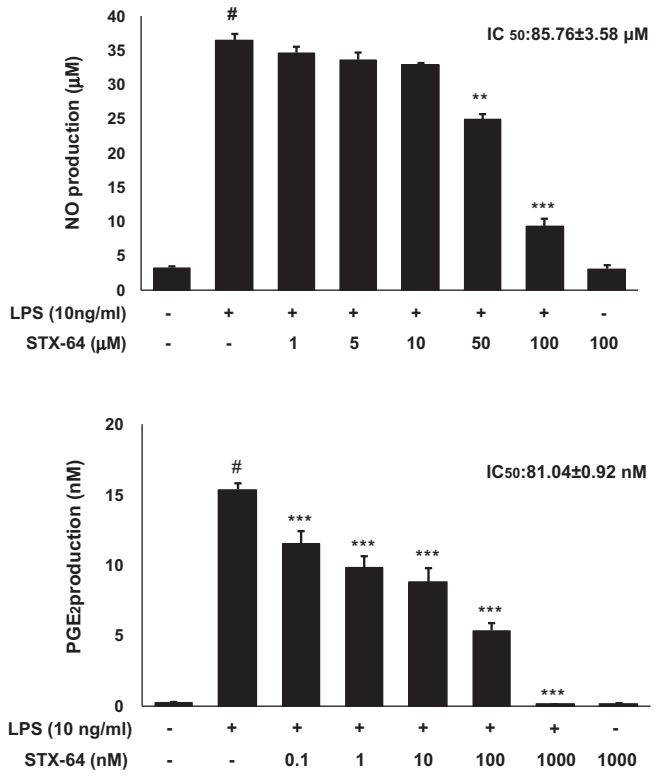


Figure 5. Inhibitory effects of STX-64 on LPS-induced NO and PGE_2 production in RAW 264.7 macrophages. Following pretreatment with STX-64 (1, 5, 10, 50 or 100 μM) for 1 h, cells were treated with LPS (10 ng/mL) for 24 h. Levels of NO in culture media were quantified using Griess reaction assay. Following pretreatment with STX-64 (0.1, 1, 10, 100 or 1000 nM) for 1 h, cells were treated with LPS (10 ng/mL) for 24 h. Levels of PGE_2 in culture media were quantified using EIAs. Data are presented as the means ± SDs of three independent experiments. $^{\#}p$ <0.05 versus the control cells; $^{**}p$ <0.01, $^{***}p$ <0.001 versus LPS-stimulated cells; statistical significances were compared using ANOVA and Dunnett's post hoc test.

Lipinski's rule of five.[19] This approach has been widely used as a filter for substances that would likely be further developed in drug design programs. In addition, we calculated the total polar surface area (TPSA) since it is another key property that has been linked to drug bioavailability. Thus, passively absorbed molecules with a TPSA >140 are thought to suffer from low oral bioavailability.[20] Molecules violating more than one of these rules may have problems with bioavailability. Predictions of ADME properties for the studied compounds are summarized in Table 3. The results showed that all the four tested compounds and the reference compound, NS398, comply with these rules. Theoretically, compounds **1g**, **1j**, **1m**, and **1n** should present good passive oral absorption and differences in their bioactivity cannot be attributed to this property.

In conclusion, we synthesized a series of tricyclic coumarin sulfonates in order to explore the relationship between their structures and their antiinflammatory activities. The compounds showed varying cytotoxicities and inhibitory activities on the LPS-induced productions of NO and PGE_2. The biological screening of this series of coumarins led to discovery of a new potential lead compound **1g**. This compound with fused cycloheptane and *p*-(trifluoromethyl)benzenesulfonate moieties exhibited the highest inhibitory activities on both NO and PGE_2 productions in LPS-induced RAW 264.7 macrophages with low cytotoxicity. It showed superior potencies to the reference compounds, L-NIL and NS398, respectively. In silico ADME prediction showed that compound **1g** complies with Lipinski's rule of five and it can be passively absorbed orally. Compound **1g** can be utilized as a promising lead

compound for further development of potential antiinflammatory coumarin compounds.

Acknowledgment

This work was supported by Korea Institute of Science and Technology (KIST), KIST Project (2E22360).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.12.018.

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14. Cell culture and sample treatment: the RAW 264.7 macrophage cell line was obtained from the Korea Cell Line Bank (Seoul, Republic of Korea). Cells were grown at 37 °C in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (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 and then stimulated with LPS (1 μg/mL) for the indicated time.
15. Nitrite determination: RAW 264.7 macrophages 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 (1.60, 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. 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.
16. PGE_2 assay: RAW 264.7 macrophages were pretreated with tested samples for 1 h and then stimulated with LPS (1 μg/mL) for 24 h. The 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.
17. MTT assay for cell viability: RAW 264.7 macrophages 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. Cell viabilities were determined using colorimetric MTT assays, as described previously.[18]
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