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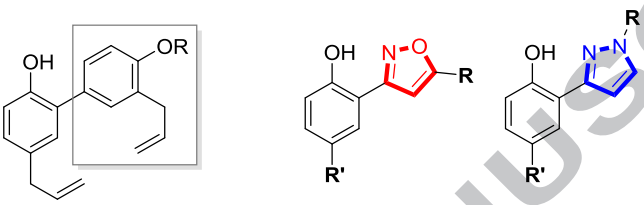

Honokiol (R = H)
4'-*O*-Methylhonokiol (R = CH_3)

17 isoxazoles

18 pyrazoles

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Synthesis and anti-neuroinflammatory activity of *N*-heterocyclic analogs based on natural biphenyl-neolignan honokiol

Yue Yuan[a,†], Lalita Subedi[a,†], Daesung Lim[a], Jae-Kyung Jung[b], Sun Yeou Kim[a,*], Seung-Yong Seo[a,*]

[a]*College of Pharmacy, and Gachon Institute of Pharmaceutical Sciences, Gachon University, Incheon 21936, Republic of Korea*
[b]*College of Pharmacy, Chungbuk National University, Cheongju, Chungbuk 28644, Republic of Korea*

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ABSTRACT

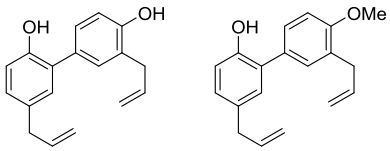
Novel isoxazole and pyrazole analogues based on natural biphenyl-neolignan honokiol were synthesized and evaluated for their inhibitory activities against nitric oxide production in lipopolysaccharide-activated BV-2 microglial cells. The isoxazole skeleton was constructed via nitrile oxide cycloaddition from oxime **3** and pyrazole was generated by condensation of 4-chromone and alkylhydrazine. Among the analogs, **13b** and **14a** showed stronger inhibitory activities with IC_{50} values of 8.9 and 1.2 μM, respectively, than honokiol.

Chronic neuroinflammation induced by activated microglia is the major cause of neurodegenerative disorders such as Parkinson's disease and Alzheimer's disease, as well as ischemic brain diseases.[1,2] Microglia are the primary immune cells that play a crucial role in the innate immune response in the normal brain and central nervous system (CNS).[3] Although activated microglia scavenge dead cells from the CNS and secrete different neurotrophic factors for neuronal survival, severe activation causes various autoimmune responses leading to neuronal cell death and brain injury. Activated microglia promote neuronal injury by releasing proinflammatory and cytotoxic factors, including tumor necrosis factor-α, nitric oxide (NO), inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2). Thus, inhibition of NO production in activated microglia may be effective for treating neurodegenerative disorders.

The natural products biphenyl-neolignans honokiol (**1**) and 4′-*O*-methylhonokiol (**2**) were isolated from *Magnolia officinalis* and have been reported to exert a wide range of biological and pharmacological effects, including anti-inflammatory, anti-allergic, anti-cancer, anti-osteoclastogenic, anti-bacterial, anti-thrombotic, anti-depressant, and anti-neurodegenerative activities[4] (Figure 1). Among these biological activities, the neuroprotective and neurotropic effects of honokiol and its related analogs are regarded as a major characteristic.[5] For instance, honokiol was shown to have neurotrophic activity in cultures of rat cortical neurons at concentrations of 0.1 ~ 10 μM. Honokiol promoted neurite outgrowth in serum-free medium supplemented with B27 and significantly enhanced neuron survival and growth in serum-free medium supplemented with N2. We previously reported the efficient synthesis of biphenyl-neolignans and novel anti-inflammatory effects of their analogs.[6,7] Recently, Kim et al. demonstrated that the natural neolignan balanophonin delayed the progression of neuronal cell death by inhibiting NO production in lipopolysaccharide (LPS)-mediated activated BV2 cells by suppressing iNOS expression.[8] Structurally, honokiol (**1**) and 4′-*O*-methylhonokiol (**2**) consist of a 5,3′-diallyl-biphenyl skeleton bearing a combination of hydroxy or methoxy groups on C2 of the A ring and C4′ of the B ring. Although numerous studies have examined biphenyl-neolignan derivatives, few studies have evaluated heterocyclic substituents in the A or B ring of a biphenyl-neolignan.[9] Neolignans were reported to have poor absorption, high systemic clearance, and low oral bioavailability, although they were potentially effective for treating CNS disorders.[10] For therapeutic use, chemical modifications can be used to increase neuroprotective activity and optimize the physicochemical properties of CNS drugs. Our focuses on the synthesis and medicinal chemistry of biphenyl-neolignan; here, we describe the synthesis and biological evaluation of two heterocyclic analogs with isoxazole and pyrazole scaffolds as novel neuroprotective agents. In this study, we investigated the inhibitory activities of isoxazole and pyrazole derivatives on NO production and iNOS and COX-2 expression

* Corresponding author. Tel.: +82-32-820-4949; fax: +82-32-820-4829. E-mail: syseo@gachon.ac.kr (S.-Y.S.) and sunnykim@gachon.ac.kr (S.Y.K.). † These authors have contributed equally to the preparation of this manuscript

in LPS-activated BV-2 cells.



Honokiol (**1**) 4-*O*'-Methylhonokiol (**2**)

Figure 1. Structure of natural biphenyl-neolignans honokiol and 4-O'-methylhonokiol.

The goal of this work was to improve the neuroprotective activity and physicochemical properties, such as metabolic stability and membrane permeability of the blood-brain barrier, of honokiol.[11] Therefore, we introduced an intramolecular hydrogen bond between the phenol group on the A ring and a neighboring moiety to reduce metabolic changes. Honokiol analogs with an *N*-heterocycle as a surrogate for the B ring of biphenyl-neolignans have been reported previously. To identify, anti-neuroinflammatory agents based on biphenyl-neolignan, both isoxazole and pyrazole were used as *N*-heterocyclic scaffolds to replace the B ring. (Figure 2) To decrease lipophilicity (*c*logP and *t*-PSA values), an alkoxymethyl group and a piperazinylmethyl group were incorporated at the C5 position of isoxazole. Using pyrazole analogs, *N*-allyl, benzyl, and phenyl were introduced to replace the allyl group in the B ring. The effect of these changes on the methoxy group in the A ring was examined to determine their structure-activity relationship.

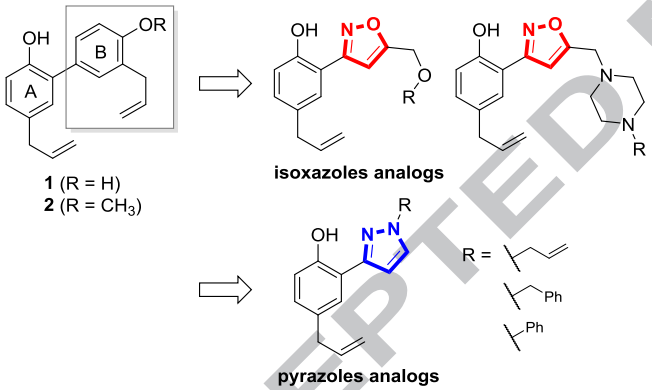
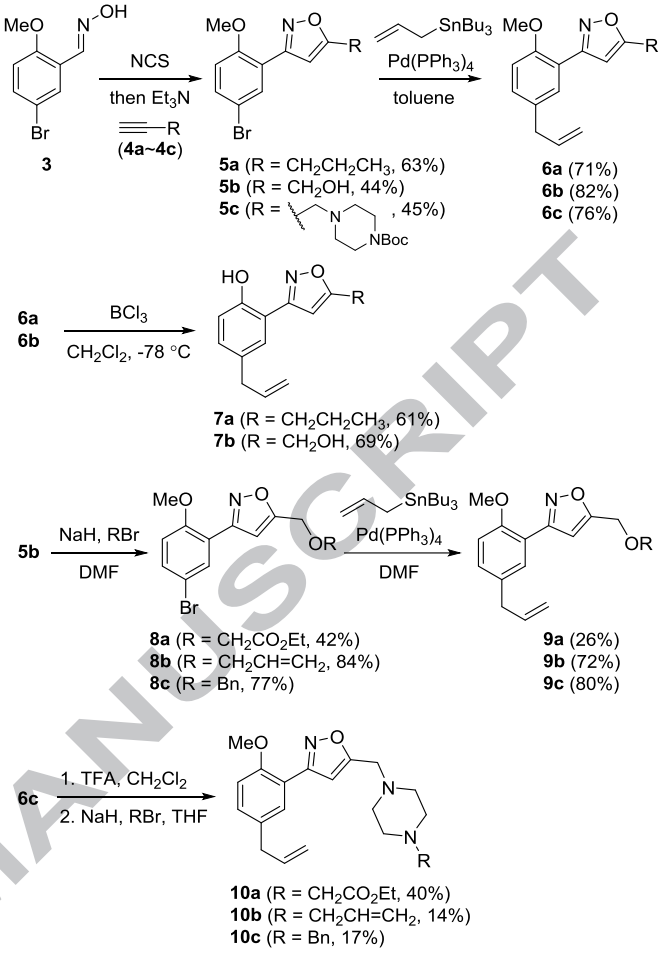


Figure 2. Design of biphenyl-neolignan heterocyclic analogs based on honokiol and 4′-O-methylhonokiol.

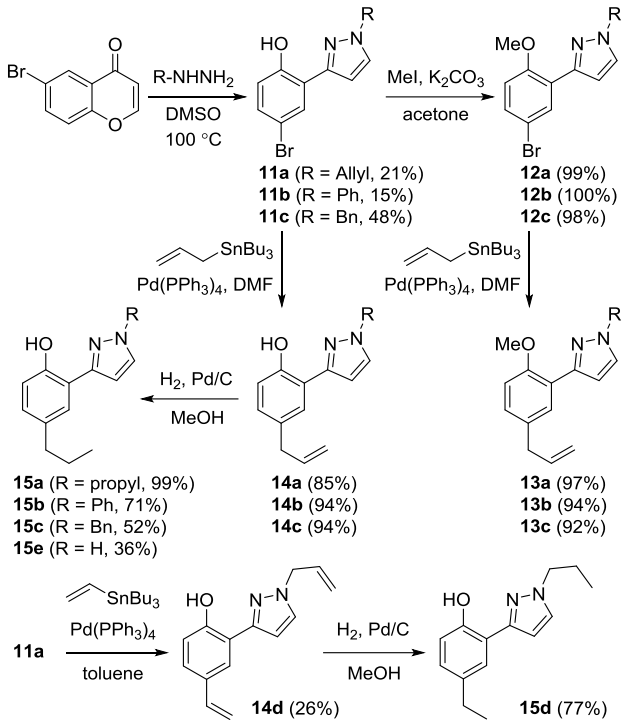
We previously reported the synthesis of honokiol analogs starting with introduction of isoxazole as a unique surrogate of the B ring in honokiol[7a] (Scheme 1). To prepared isoxazole analogs, oxime (**3**), generated by treatment of commercially available 5-bromo-2-methylbenzaldehyde with NH_2OH was converted to three isoxazoles via nitrile oxide cyclcoaddition using three alkyne reagents; 1-pentyne, propargyl alcohol, and 1-Boc-4-propargylpiperidine. Stille cross-coupling allylation of isoxazoles (**5a-5c**) with allyltributyltin and $Pd(PPh_3)_4$ afforded allylated compounds (**6a-6c**), followed by demethylation to provide the desired phenols (**7a-7b**) in moderate yield. **5b** was treated with NaH and alkyl halide to afford *O*-alkylated products (**8a-8c**), followed by the Stille cross-coupling allylation to afford allylated products (**9a-9c**) containing 2-ethoxy-2-oxoethyl, allyl, and benzyl groups, respectively. The Boc group was removed from 6c in trifluoroacetic acid-mediated condition and subsequently three alkyl groups were introduced onto the nitrogen at the opposite site by *N*-alkylation using ethyl bromoacetate, allyl bromide, and benzyl bromide, respectively, to afford *N*-alkylated products (**10a-10c**).



Scheme 1. Synthesis of isoxazole analogs

To synthesize pyrazole analogs, condensation of mono-substituted hydrazine and 6-bromo-4-chromone was conducted as shown in Scheme 2.[6b,12] Allylhydrazine was prepared using our previously described method and benzylhydrazine and phenylhydrazine were commercially available. 6-Bromo-4-chromone was reacted with three hydrazines at 100 °C to afford the pyrazoles (**11a-11c**). *O*-methylation of **11a-11c** using MeI and K_2CO_3 generated the aryl bromides (**12a-12c**), after which Stille cross-coupling of **12a-12c** with allyltributyltin and $Pd(PPh_3)_4$ afforded allylated products (**13a-13c**), respectively, in good yield. Despite the slightly lower yield than that observed in the synthesis from **12a-12c**, **11a-11c** containing a phenolic group were directly converted to the desired pyrazole analogs (**14a-14c**). Catalytic hydrogenation of **14a-14c** afforded the saturated compounds (**15a-15c**) along with the benzylated compound **15e**. In the same manner, **11a** was treated with vinyltributyltin and $Pd(PPh_3)_4$ to afford **14d**, which was reduced to **15d** under catalytic hydrogenation.

The inhibitory activities of isoxazole and pyrazole analogs on iNOS-mediated NO production were evaluated in LPS-activated BV-2 cells stimulated with 100 ng/mL LPS in the presence or absence of samples for 24 h. The nitrite level was measured in the culture media using Griess reagent (1% sulfanilamide and 0.1% *N*-1-napthylethylenediamine dihydrochloride in 5% phosphoric acid). For this reaction, 50 μL of the supernatant was mixed with an equal volume of the Griess reagent and the OD was measured at 570 nm. NG-Mono-methyl-L-arginine (L-NMMA), a well-known NO synthase (NOS) inhibitor, was used as a positive control. Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay.[8]

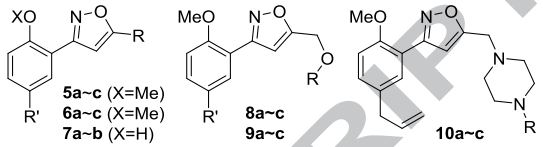


Scheme 2. Synthesis of pyrazole analogs.

The inhibitory effect of the synthesized isoxazole analogs on NO production is shown in Table 1. The intermediates **5c**-**5c** with a C5-bromide in the A ring showed no inhibitory activity. The inhibitory activity of C5-allylated compounds (**6a**-**6c** and **7a**-**7b**) was not improved, although **6c** showed weak activity. The inhibitory activity of isoxazole analogs with an *O*-alkylated and *N*-alkylated on C5 position of the isoxazole ring was evaluated. Among the alkoxymethyl derivatives, bromide compounds (**8a**-**8c**) showed no inhibitory activity, while allylated compounds (**9a** and **9b**) showed moderate activity with IC_{50} = 25.9 and 28.7 μM, respectively. Compared to 2-ethoxy-2-oxoethyl and the allyl group, the benzyl group at the C5 position of isoxazole was not favorable for activity. The piperazine derivatives (**10a**-**10c**) showed no inhibitory activity. Most derivatives did not show any cytotoxicity. The inhibitory activity of **9a** and **9b** was not as strong as that of honokiol, but was similar to that of L-NMMA, the positive control. These compounds exhibited better drug-like properties as new agents for treating CNS-related diseases compared to honokiol. The NO production inhibition by isoxazole analogs was dependent on the C5-allyl substituent in the phenyl ring and the *O*-alkylated analogs have potential activity compared to the *N*-alkylated analogs at the C5 position of isoxazole.

The inhibitory activities of pyrazole analogs against iNOS-mediated NO production from LPS-stimulated BV-2 cells are listed in Table 2. We prepared three pyrazole derivatives with *N*-allyl, benzyl, and phenyl groups. Based on the functional groups on honokiol, the allyl and vinyl group were introduced at the C5-position of the A ring. The C5-bromide intermediates (**11a**-**11d** and **12a**-**12c**) did not inhibit NO production regardless of the presence or absence of the C2-methyl group in the A ring. However, the allyl group at the C5 position in the A ring affected the inhibitory activity. The C2-methoxy derivatives **13b** and **13c** significantly inhibited NO production. Particularly, **13b** strongly inhibited NO production with an IC_{50} = 8.92 μM, which is nearly 2-fold as strong as that of honokiol. Unexpectedly, **14a**, which has two allyl groups at the C5 position of the phenyl ring and N1 position of the pyrazole ring, strongly inhibited NO production with an IC_{50} = 1.2 μM, which is 14-fold stronger than that of honokiol and much stronger than those of the known inhibitors, L-NMMA (IC_{50} = 26.6 μM) and shogaol (IC_{50} = 5.59 μM).[13] Two derivatives, **14b** and **14c** which have a benzyl and phenyl group as *N*-substituents, showed decreased activity compared to **14a**. Replacement of allyl with vinyl decreased the NO inhibitory activity with an IC_{50} = 30.5 μM. The compounds saturated by reducing the allyl or vinyl group (**15a**-**15e**) showed no inhibitory activity. **13b** and **14a** showed slight cytotoxicity at 20 μM, but no cytotoxicity at less than 10 μM (Figure 3(b)).

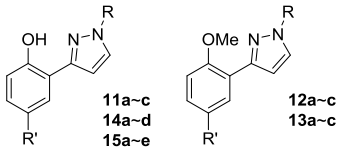
Table 1. Inhibitory effect of isoxazole compounds on NO production and viability of LPS-activated BV-2 cells.



Compound	R	R'	IC_{50} [a] (μM)	Cell viability (%) [b]	cLogP
L-NMMA[c]	-	-	26.6	94.4±4.2	-
1	-	-	17.0	118.4±5.0	4.49
2	-	-	52.3	115.1±2.7	5.16
5a		Br	>200	97.9±2.7	3.95
5b	OH	Br	>200	94.1±4.5	1.59
5c	N NBoc	Br	>200	100.2±4.0	4.34
6a			>200	98.2±3.7	4.08
6b	OH		>200	120.4±5.9	1.72
6c	N NBoc		76.9	117.3±8.7	4.48
7a			>200	93.3±1.9	3.84
7b	OH		>200	126.2±5.8	1.47
8a	O O	Br	>200	121.6±1.8	2.68
8b		Br	90.4	94.6±5.2	3.06
8c		Br	>200	103.8±4.8	3.78
9a	O O		25.9	125.8±8.7	2.82
9b			28.7	141.0±9.3	3.19
9c			>200	144.7±5.9	3.91
10a	O O	-	>200	93.2±3.0	3.72
10b		-	111	100.6±8.5	3.72
10c		-	>200	98.6±7.2	4.79

[a] The IC_{50} value was defined as the concentration (μM) that caused 50% inhibition of NO production in LPS-activated BV-2 cells; [b] Cell viability after treatment with 20 μM of each compound was determined by the MTT assay. The results are averages of three independent experiments, and the data are expressed as mean ± SD; [C] L-NMMA was used as a positive control.

Table 2. Inhibitory effect of pyrazole compounds on NO prod uction and viability of LPS-activated BV-2 cells.



Compound	R	R'	IC_{50} [a] (μM)	Cell viability (%) [b]	cLogP
11a		Br	111.73	112.8±5.2	3.36
11b		Br	>200	109.3±8.0	4.53
11c		Br	>200	107.4±5.1	4.54
12a		Br	>200	92.2±2.4	3.51
12b		Br	>200	124.0±3.9	4.68
12c		Br	>200	121.8±1.7	4.70
13a			>200	109.0±2.9	3.62
13b			**8.92**	68.6±14.5	4.78
13c			35.96	112.2±9.4	4.80
14a			**1.2**	59.9±5.4	3.33
14b			73	125.0±9.3	4.49
14c			92.45	99.5±8.0	4.51
14d			30.47	104.7±2.5	2.98
15a			91.33	129.4±4.1	4.10
15b			109.4	125.4±2.9	4.98
15c			116.06	126.0±6.5	5.00
15d			>200	108.8±0.5	3.57
15e	H		60.22	94.7±2.6	3.11
L-NMMA[c]	-	-	26.58	94.4±4.2	-

[a] The IC_{50} value was defined as the concentration (μM) that caused 50% inhibition of NO production in LPS-activated BV-2 cells; [b] Cell viability after treatment with 20 μM of each compound was determined by the MTT assay. The results are averages of three independent experiments, and the data are expressed as mean ± SD; [C] L-NMMA was used as a positive control.

Compounds **13b** and **14a** inhibited NO production in a dose-dependent manner (Figure 3(a)). To further investigate the mechanism of action of **13b** and **14a**, we evaluated the effect of **13b** and **14a** on iNOS protein and COX-2 enzyme expression in LPS-activated BV-2 cells. As shown in Figure 3(c) and Figure 4, compounds **13b** and **14a** strongly inhibited iNOS expression in a dose-dependent manner. Particularly, **14a** shows more potent inhibition of iNOS. As shown in Figure 3(d) and Figure 4, compounds **13b** and **14a** also showed strong dose-dependent inhibition of COX-2. Similarly, **14a** shows more potent inhibition of COX-2. These results suggest that inhibition of LPS-induced NO production in BV-2 cells by compound **13b** and **14a** occurred mainly through inhibition of iNOS and COX-2 expression.

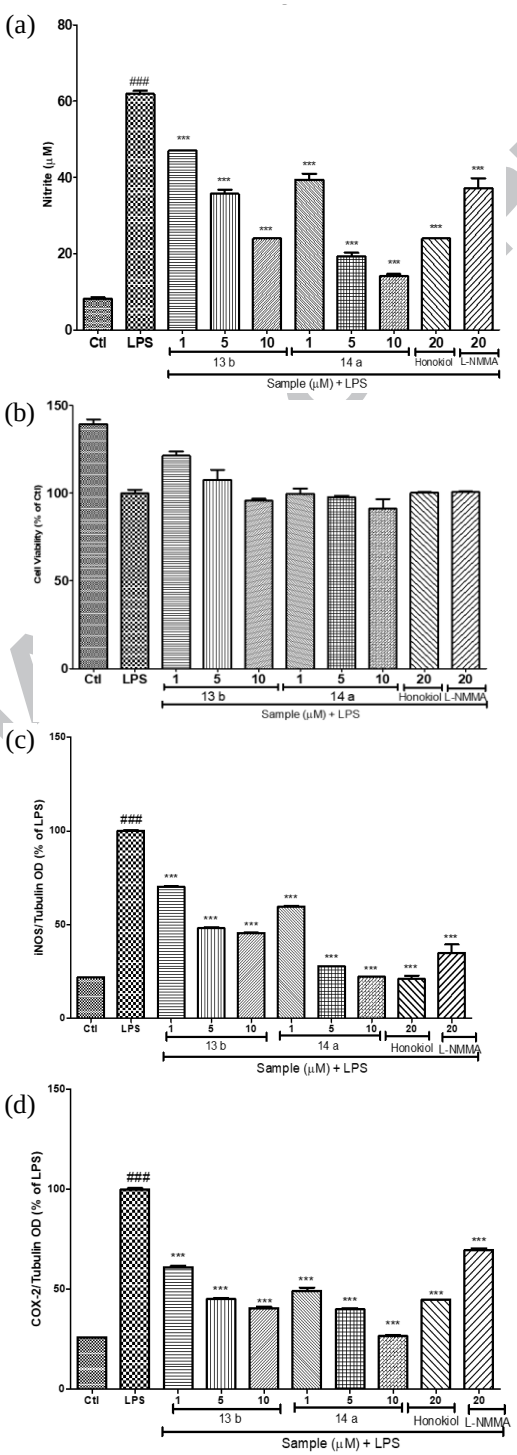


Figure 3. Role of **13b** and **14a** to inhibit nitrite production, expression of inducible nitric oxide synthase (*i*NOS) and cyclooxygenase (COX-2) against LPS-activated BV-2 cells. (a) Inhibitory effect of **13b** and **14a** on NO production in LPS-activated BV-2 cells. (b) Effect of **13b** and **14a** on cell viability. (c) Inhibitory effect of **13b** and **14a** on iNOS expression. (d) Inhibitory effect of **13b** and **14a** on COX-2 expression. All data are presented as the mean ± standard error of the mean of three independent experiments. ***P < 0.001 indicate significant differences compared with treatment with LPS alone, while ###P < 0.001 indicate significant differences compared with an untreated control group. Here Ctl is control, LPS is lipopolysaccharide. L-NMMA was used as positive control.

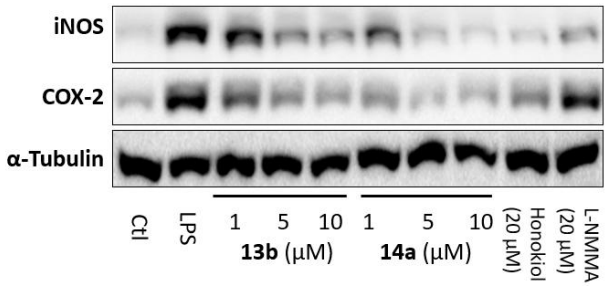


Figure 4. Effect of compounds **13b** and **14a** on *i*NOS and COX-2 protein expression in LPS-stimulated BV-2 cells.

In summary, we synthesized a series of 17 isoxazole and 18 pyrazole derivatives and evaluated their inhibitory effects on NO production in LPS-activated BV-2 cells. Among the prepared compounds, **13b** and **14a** inhibited NO production in LPS-activated BV-2 cells by suppressing iNOS and COX-2 expression. The pyrazole analog **14a** was the most potent inhibitor. These findings provide insight into the structural features influencing biological activities of this class of compounds and provide a foundation for further studies of analog design. Recently, retinoid X receptor was studied as the only binding protein of honokiol derivatives, although the various biological activities of honokiol were well known.[14] Our synthetic and medicinal chemistry work would be exploited to design photoaffinity probes for target identification using a forward chemical genetic approach.[15] Further analysis of the mode of action and biological activity *in vivo* of these pyrazole analogs as well as their potential as novel drug candidates is underway.

Acknowledgement

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Supplementary Material

Supplementary data associated with this article can be found, in the online version, at doi:

Highlights

- Isoxazole and pyrazole analogs in B ring of honokiol
- Isoxazole and pyrazole were constructed by nitrile oxide cycloaddition and condensation of 4-chromone and alkylhydrazine
- Two pyrazole analogs strongly inhibit NO production in LPS-activated BV-2 cells by suppressing iNOS and COX-2 expression