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Styrylheterocycles: A Novel Class of Inhibitors on Lipopolysaccharide-Induced Nitric Oxide Production

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Abstract—A series of styrylheterocycles was prepared and their inhibitory activities against nitric oxide (NO) production were evaluated in a cell culture system using lipopolysaccharide-stimulated RAW264.7 macrophage cells. Our studies have identified a new series of inhibitors on NO production, providing the basis for further development of potent inhibitors. The preliminary structure–activity relationship, to elucidate the essential structural requirements, has been described. Mechanistic studies suggest that the suppression of iNOS mRNA transcription is, at least in part, related to the inhibitory activity of styrylheterocycles.

Endogenous nitric oxide (NO) is an inorganic free radical that has been implicated in a variety of pathophysiological responses, including vasodilation, nonspecific host defense, ischemia reperfusion injury, chronic inflammation and carcinogenesis.[1–6] It is enzymatically synthesized from L-arginine in numerous tissue and cell types by a nitric oxide synthase (NOS).[7–10] There are three distinct isoforms of NOS: neural (nNOS), endothelial (eNOS) and inducible (iNOS). Among them, the iNOS is involved in the pathological responses mediated by the overproduction of NO, and is overexpressed by pro-inflammatory and/or carcinogenic stimuli, such as interleukin-1β, tumor necrosis factor-α and lipopolysaccharide (LPS) in macrophages, endothelial cells and smooth muscle cells.[11,12] Thus, inhibitors of iNOS enzyme activity or its induction have been postulated to be beneficial in the treatment of numerous disease mediated by the overproduction of NO.

Intensive efforts are currently under way to try and discover potent and selective inhibitors of NOS. Some of the earliest synthetic inhibitors are analogues of L-arginine, such as *N*-monomethyl arginine (L-NMMA),[13] *N*-nitroarginine (L-NA)[14] and its methyl ester (L-NAME), but the low potency and minimal selectivity have limited their utility. More recently, a diverse class of non-aminoacid synthetic NOS inhibitors has appeared in the literature, including guanidines,[15] isothioureas,[16] isoquinolinamines[17] and carboxamidines.[18] Even though some of these exhibited the adequate levels of selectivity and potency in vitro, their usefulness in vivo has generally been limited by their poor bioavailability or toxicity.[19]

Many natural products that inhibit the production of NO have also been found, such as flavonoids, coumarins and stilbenoids.[20–22] Of these, stilbenoid has attracted our attention as a starting point for a new class of inhibitors, due to its simplicity of structure and ease of synthesis. It was seen, by comparing the reported activities of the various natural stilbenoids,[22] that the inhibitory potency of stilbene compounds, against NO production, was sensitive to the substitution patterns on the stilbene template. On the basis of these studies, we began to investigate the biological evaluation of synthetic stilbene derivatives to find a potent inhibitor, which might serve as a new lead for chemical optimization.

We designed and prepared a series of compounds, with the general structure **1** (Fig. 1), where one of the phenyl

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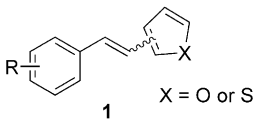
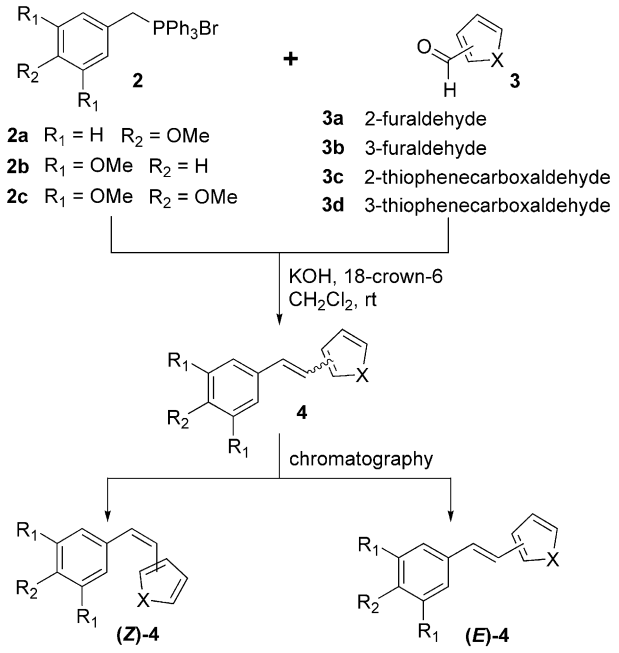


Figure 1. General structure **1**.

rings of the stilbene was replaced by a five-membered heteroaromatic ring. One of the rationales for this was the observation of Hamley et al.,[23] where the replacement of the phenyl ring in 3,4-dihydro-1-isoquinolinamines, with five-membered aromatic heterocycles, resulted in increased potency. In addition, furanocoumarins have shown greater inhibitory effects than simple coumarins.[20] Thus, we envisaged that the substitution by a five-membered heteroaromatic ring on the stilbene template could enhance the activity. Herein, we report the preparation and evaluation of this class of compounds, as well as the results of the preliminary structure–activity relationship studies.

The preparation of the styrylheterocycles was carried out according to Scheme 1. The stilbenoid skeletons of styrylfuran and styrylthiophene could be constructed by Wittig reactions, between a five-membered aromatic heterocyclic aldehyde and an aromatic phosphonium ylide. Modified Wittig reactions,[24] between phosphonium bromide **2a–c** and commercially available aldehydes **3a–d** (1.1 equiv) in the presence of freshly powdered KOH and a catalytic amount of 18-crown-6 in CH_2Cl_2 at room temperature yielded a mixture of olefins **4** (>85%) with a *Z*/*E* ratio of ca. 1:1 to 5:1 by TLC intensity. The *cis*/*trans* isomers were separated by silica gel column chromatography, and the structures were determined from the characteristic ^{1}H NMR coupling constants of the olefinic protons [16–17 Hz for (*E*)-**4** and 12 Hz for (*Z*)-**4**]. When the separation of the *Z*/*E* isomers was not possible by silica gel column chromatography, the *Z*/*E* mixtures **4** were converted to the *E*-isomers [**4a**, **4h**, **4o**, and **4r** (Table 1)] by heating with catalytic amounts of iodine in refluxing heptane.[25]

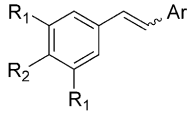


Scheme 1. Synthetic approach employed for the preparation of the styrylheterocycles described in Table 1.

The compounds were evaluated for their inhibitory activities on NO production in a cell culture system, using LPS-activated RAW264.7 macrophage cells, according to a previously documented procedure.[26,27] When the cells had been treated with 1 μg/mL LPS for 20 h, the NO production was markedly increased from the basal level of 2.5±0.5 to 42.5±0.7 μM. Under this experimental condition, the positive control L-NMMA exhibited an IC_{50} value of 44.0 μM. The inhibitory potencies, expressed as the IC_{50} values, of the synthesized styrylfurans and styrylthiophenes are shown in Table 1 and are compared with that of prototype natural stilbene resveratrol[22,28] (IC_{50} = 15.9 μM). The results in the present study demonstrated that nine styrylheterocycles (**4a**, **4h**, **4j** and **4o–t**) inhibited the LPS-induced NO production more effectively than resveratrol, whereas eight compounds (**4d–g** and **4k–n**) showed little activity (IC_{50} > 50 μM).

The styrylheterocycles having a 3,4,5-trimethoxy group on the phenyl ring, **4o–t**, generally exhibited greater inhibitory activities than the corresponding compounds with 3,5-dimethoxy (**4h–n**) or 4-methoxy groups (**4a–g**). As with the 3,4,5-trimethoxyphenyl series, all the furanyl (**4o–q**) and thiophenyl (**4r–t**) derivatives showed 2- to 3-fold greater inhibitory activities than resveratrol. However, for the 3,5-dimethoxyphenyl (**4h–n**) or

Table 1. Inhibitory effects of styrylfurans and styrylthiophenes on the NO production in LPS-activated mouse macrophage RAW264.7 cells



Compd	R_1	R_2	Ar	*E*/*Z*	IC_{50} (μM)
4a	H	OCH_3	2-Furanyl	*E*	13
4b	H	OCH_3	3-Furanyl	*Z*	34.6
4c	H	OCH_3	3-Furanyl	*E*	31.6[a]
4d	H	OCH_3	2-Thiophenyl	*Z*	>50
4e	H	OCH_3	2-Thiophenyl	*E*	>50
4f	H	OCH_3	3-Thiophenyl	*Z*	>50
4g	H	OCH_3	3-Thiophenyl	*E*	>50
4h	OCH_3	H	2-Furanyl	*E*	4.8[a]
4i	OCH_3	H	3-Furanyl	*Z*	18.7
4j	OCH_3	H	3-Furanyl	*E*	13.5
4k	OCH_3	H	2-Thiophenyl	*Z*	>50
4l	OCH_3	H	2-Thiophenyl	*E*	>50
4m	OCH_3	H	3-Thiophenyl	*Z*	>50
4n	OCH_3	H	3-Thiophenyl	*E*	>50
4o	OCH_3	OCH_3	2-Furanyl	*E*	5.9
4p	OCH_3	OCH_3	3-Furanyl	*Z*	6.6
4q	OCH_3	OCH_3	3-Furanyl	*E*	9.5
4r	OCH_3	OCH_3	2-Thiophenyl	*E*	5.3
4s	OCH_3	OCH_3	3-Thiophenyl	*Z*	5.6
4t	OCH_3	OCH_3	3-Thiophenyl	*E*	7.8[a]
Resveratrol	OH	H	4-Hydroxyphenyl	*E*	15.9

[a]These compounds exhibited cytotoxicity at 50 μM. At lower concentrations the cell viability was maintained over 90%.

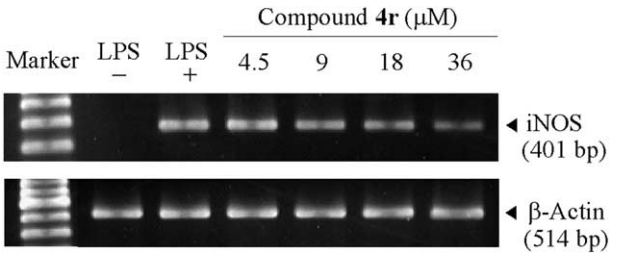


Figure 2. Effects of **4r** on the expression of iNOS mRNA in LPS-activated RAW264.7 cells.

4-methoxyphenyl (**4a**–**g**) series, only the furanyl derivatives exhibited comparable activities. These results indicate that the potency of these compounds is highly dependent on the substitution pattern of the phenyl ring. However, to our surprise, there was no significant correlation between the double-bond geometry and the inhibitory activity. Compounds with *Z*-double bonds (**4b**, **4i**, **4p** and **4s**) showed almost equipotent activities compared with their corresponding *E*-double bonds compounds (**4c**, **4j**, **4q** and **4t**). In the 2-furanyl derivatives (**4a**, **4h** and **4o**), the inhibitory activity on NO production by iNOS was enhanced with respect to their corresponding 3-furanyl analogues (**4c**, **4j** and **4q**).

To elucidate the possible mechanisms of active styrylheterocycles, the inhibition of the production of NO by compound **4r** was examined in relation to the suppression of the iNOS mRNA expression in the LPS-activated RAW264.7 cells, using RT-PCR analysis.[29] Treatment with LPS, for 6 h, dramatically increased the level of iNOS mRNA expression, and the induction mRNA was suppressed by treatment with **4r** in a dose-dependent manner, as shown in Figure 2. The RT-PCR analysis indicated that the inhibition of the NO production by active compounds was correlated with the suppression of the iNOS mRNA gene expression. This result indicates that active styrylheterocycles, at least in part, suppress the pathway of stimulation by LPS to iNOS gene expression, thereby alleviating the production of NO.

In conclusion, we have designed and evaluated a series of styrylheterocycles as inhibitors on NO production by iNOS. Several compounds have shown potent inhibitory activity towards the LPS-induced NO production. The present study suggests that the suppression of iNOS mRNA transcription is, at least in part, related to the inhibitory activity of styrylheterocycles. This new series of inhibitors are suggested as lead compounds for the development of potent and selective inhibitors for potential therapeutic use. Further studies toward more potent inhibitors, based on these findings, are currently in progress in our laboratory.

Acknowledgements

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27. **Measurements of Nitric oxide formation by iNOS in cultured LPS-induced RAW264.7 cells:** RAW264.7 macrophage cells, in 10% FBS-DMEM without phenol red media, were plated in 24-well plates (8×10^5 cells/mL), and then incubated for 24 h. The cells were replaced with new media, and then incubated in the medium, with 1 μg/mL of LPS, in the presence or absence of test samples. After an additional 20 h of incubation, the media were removed and analyzed for nitrite accumulation, as an indicator of NO production, using the Griess reaction.[26] Briefly, 150 μL of Griess reagent (0.1% naphthylethylenediamine and 1% sulfanilamide in 5% H_3PO_4 solution) was added to 100 μL of each LPS supernatant, or sample-treated cells, in triplicate. The absorbance was read at 540 nm, and compared against a standard curve of sodium

nitrite. The percentage inhibition was expressed as [1−(NO level of sample/NO level of vehicle-treated control)]×100. Dose-response curves were prepared, and the results typically expressed as the IC_{50} values.

28. For a good review with citations, see: Gusman, J.; Malonne, H.; Atassi, G. *Carcinogenesis* **2001**, *22*, 1111.

29. **Reverse transcription-polymerase chain reaction (RT-PCR) analysis of iNOS mRNA expression**: RAW264.7 cells (5×10^6 cells—10 cm dish) were incubated for 6 h with, and without, various concentrations of test samples and LPS (1 μg/mL). After washing with twice PBS, total RNA was isolated from the cell pellet, using an RNA isolation kit (Tri-reagent, Sigma Chemical Co., St. Louis, MO, USA). The total amount of RNA was determined by the absorbance at 260 nm. One microgram (1 μg) of RNA was reverse transcribed into cDNA using an avaian myeloblastosis virus (AMV) reverse transcriptase and oligo$(dT)_{15}$ primers (Promega Co., Madison, WI, USA). The PCR samples, contained in 50 μL of the reaction mixture, comprised of 50 mM KCl, 5 mM $MgCl_2$, 0.16 mM dNTP, 5.0 units of Taq DNA polymerase (Qiagen, Valencia, CA, USA) and 20 pmol of sense and antisense primers in 10 mM Tris–HCl (pH 8.3). The sense and antisense primers for iNOS were 5′-ATGTCCGAAGCAAACATCAC-3′ and 5′-TAATGTCCAGGAAGTAGGTG-3′, respectively. The sense and antisense primers for β-actin were 5′-TGTGATGGTGGGAATGGGTCAG-3′ and 5′-TTTGATGTCACGCACGATTTCC-3′, respectively. The PCR amplification was performed under the following conditions: 25 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 30 s, using a thermal cycler (GeneAmp PCR Systems 2400; PE Applied Biosystems, USA). The amplified PCR products were separated on a 2% agarose gel and visualized by SYBR Gold staining. The bands in the gel were photographed, and the fluorescence intensities analyzed using an Alpha Imager™ (Alpha Innotech Corp., USA).