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Styrylheterocycles as a novel class inhibitor of cyclooxygenase-2-mediated prostaglandin E_2 production

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Abstract—The inhibitory effects of a series of styrylheterocycles on the production of cyclooxygenase-2-mediated prostaglandin E_2 (PGE_2) were evaluated in lipopolysaccharide-stimulated RAW264.7 murine macrophages. A new series of potential inhibitors, including 3-[2-(4-methoxy-phenyl)-vinyl]-thiophene, have been identified, thus providing novel chemical leads for the further development of potential inhibitors in this capacity. The suppression of COX-2 mRNA expression by the active styrylheterocycles, in part, was involved in the inhibitory activity against the overproduction of PGE_2.

Several lines of evidence suggest that the overproduction of prostaglandins (PGs) mediates a variety of pathophysiological processes, including inflammation, Alzheimer's disease, hypertension, heart failure and carcinogenesis, and inducible isoform of cyclooxygenase (COX)-2 is mainly responsible for the production of large amounts of the mediator.[1–3] Two isoforms of COX, designated COX-1 and COX-2, at least, have been identified, and catalyze the biosynthesis of PGs from arachidonic acid. Especially, COX-2 is an inducible enzyme, and is expressed in response to growth factors, tumor promoters, or various physiological and inflammatory stimuli.[4,5] With exposure to lipopolysaccharide (LPS), macrophages express the COX-2 gene and protein, and COX-2-mediated metabolites, including PGE_2, are also involved in the induction of inflammation, pain, and carcinogenesis.[1–3] Since COX-2-mediated PGE_2 modulates inflammation and tumor growth, the inhibitors of COX-2 activity, or suppressors of COX-2 expression, have recently received much attention as effective anti-inflammatory or cancer chemopreventive agents, with anticipated lower side effects. In our continuous efforts to develop COX-2 inhibitors, or suppressors, for anti-inflammatory or cancer chemopreventive agents from natural products, or synthetic chemicals, our study was extended to styrylheterocycles, based on the effects of stilbenoids in these capacities.

A number of naturally occurring *trans*-stilbenoids have been reported as inhibitors of COX, although these compounds were somewhat less active than the representative COX inhibitors, such as celecoxib and indomethacin. For examples, resveratrol (**1**, Fig. 1) exhibited in vitro inhibitory activity against both COX-1 and COX-2.[6] Aiphanol (**4**), a natural stilbenolignan, showed significant inhibitory activity against COX-1 and COX-2.[7] Isorhapontigenin (**2**), a natural hydroxystilbene, showed a COX inhibition, whereas piceatannol (**3**), the demethyl derivative of isorhapontigenin, was inactive.[7] Recently, some prenylated stilbenoid natural products (**5** and **6**) have been found to exhibit moderate COX-1-selective COX inhibiting activity.[8]

It was seen by comparing the reported activities of the various natural *trans*-stilbenoids[7–9] that the selectivity and inhibitory potency of stilbene compounds against COX were sensitive to the substitution patterns on the *trans*-stilbene template. On the basis of these studies, we investigated the biological evaluation of various synthetic *trans*-stilbene derivatives in order to establish the preliminary structure–activity relationships of stilbenes,

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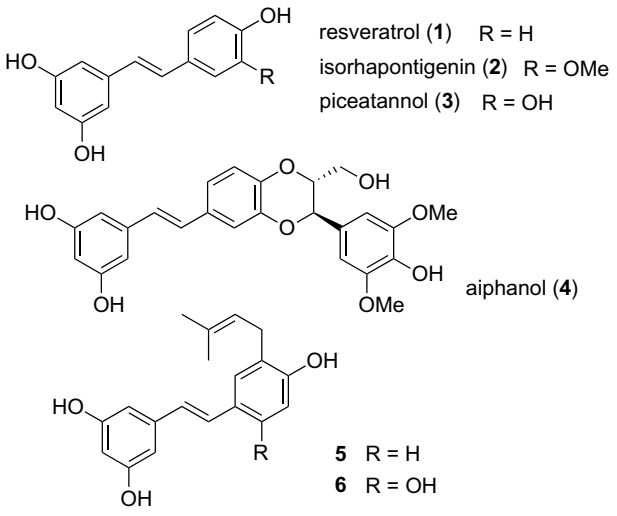
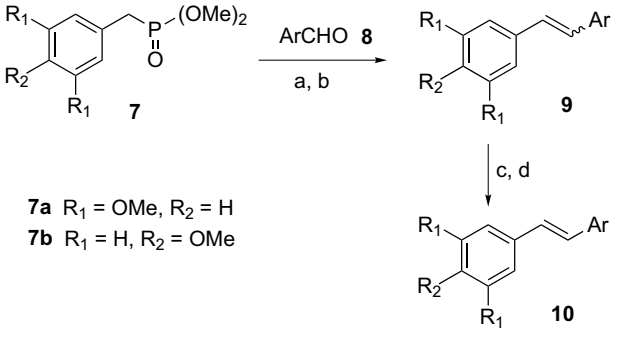


Figure 1.

and find a potent COX inhibitor that could serve as a new lead for chemical optimization.

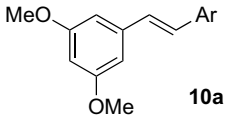
We designed and prepared several *trans*-stilbenoids in which one phenyl ring contains dimethoxy groups on the 3 and 5 positions instead of the corresponding dihydroxyl groups of the natural products **1**–**6**. We envisaged that the substitution by dimethoxy groups could enhance the activity since previously reported potent COX inhibitors or suppressors generally have at least one lipophilic aromatic ring.[10] We report here the inhibition of COX-2-mediated PGE_2 production of synthesized *trans*-stilbene analogues and our discovery of 3-[2-(4-methoxy-phenyl)-vinyl]-thiophene as a new lead compound.

Compounds were prepared through our previously reported solution-phase synthetic pathway (Scheme 1).[11] Briefly, Horner–Wadsworth–Emmons reactions between phosphonates **7** and aromatic aldehydes **8**



Scheme 1. Solution-phase synthetic approach employed for the preparation of the *trans*-stilbenoids described in Tables 1 and 2. Reagents and conditions: (a) **8**, 18-crown-6, KOH, CH_2Cl_2, rt; aqueous workup; (b) Girard's reagent T, AcOH, CH_2Cl_2, rt; aqueous workup; (c) I_2, heptane, reflux; (d) $NaHSO_3$, aqueous workup. Chemical yields of **10a** (94%); **10b** (93%); **10c** (94%); **10d** (90%); **10e** (92%); **10f** (90%); **10g** (90%); **10h** (92%); **10i** (91%); **10j** (88%); **10k** (89%); **10l** (87%); **10m** (89%).

Table 1. Inhibitory effects of the 3,5-dimethoxystilbene analogues on the PGE_2 production in LPS-stimulated RAW264.7 cells[a]



10a - 10j

Compounds[b]	Ar	IC_{50} (μM)[c]
10a	3,5-Dimethoxyphenyl	>33.3
10b	2,4,5-Trimethoxyphenyl	7.4
10c	2,5-Dimethoxyphenyl	5.8
10d	2-Hydroxy-4-methoxyphenyl	4.1
10e	2,3-Dimethoxyphenyl	1.5
10f	4-Bromophenyl	3.1
10g	4-Pyridyl	1.2
10h	2-Furyl	1.0
10i	3-Furyl	1.3
10j	3-Thienyl	1.5
1	Resveratrol	4.0

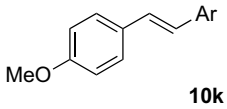
[a] The activities were measured as described previously.[12,13]
[b] Compounds **10a**–**e** and **10h** were previously known.
[c] The IC_{50} values were determined from triplicate tests.

(1.1 equiv) in the presence of KOH and a catalytic amount of 18-crown-6 in CH_2Cl_2 yielded a mixture of *Z* and *E* olefins **9**. After standard aqueous workup, the excess aldehydes **8** was removed from the mixture by treatment with Girard's reagent T and acetic acid. The *Z*/*E* mixture **9** was converted to the *E*-isomers **10** by heating with catalytic amounts of iodine in refluxing heptane. The reaction mixtures were diluted with diethyl ether and washed with saturated aqueous sodium bisulfite and water to remove iodine from the *trans*-stilbene derivatives. The NMR spectra of the synthesized *trans*-stilbenes showed that these were very clean and contained no notable impurities.

Initially, the compounds, in which one phenyl ring contains 3,5-dimethoxy groups, were prepared and evaluated for their inhibitory activities on the PGE_2 production in LPS-stimulated RAW264.7 cells.[12,13] As shown in Table 1, the effects of substituents introduced into the other phenyl ring were revealed to be highly dependent, as expected, on the position of substitution (compounds **10a**–**f**). For example, compound **10a**, which contains dimethoxy groups on the 3 and 5 positions, did not show any inhibitory activities up to 33 μM. However, the inhibitory activities of **10e** ($IC_{50} = 1.5\,\mu M$) and **10f** ($IC_{50} = 3.1\,\mu M$) were increased compared to resveratrol (**1**) ($IC_{50} = 4.0\,\mu M$). In another variation, we replaced the phenyl ring with 4-pyridyl, 2-furyl, 3-furyl, and 3-thienyl rings (compounds **10g**, **10h**, **10i**, and **10j**, respectively). Comparison of the styrylheterocycles **10g**–**j** with resveratrol (**1**) showed that the inhibitory activities were improved more than twofold by the introduction of the heteroaromatic rings.

Taking compounds **10h**–**j** as leads, we explored the modification of 3,5-dimethoxyphenyl moiety to 4-methoxyphenyl, in view of the fact that many potent COX inhibitors have characteristic *para*-substituted aryl groups.[11] As seen in Table 2, this replacement led to

Table 2. Inhibitory effects of the 4-methoxystilbene analogues on the PGE_2 production in LPS-stimulated RAW264.7 cells[a]



10k - 10m

Compounds	Ar	IC_{50} (μM)[b]
10k	2-Furyl	0.4
10l	3-Furyl	0.5
10m	3-Thienyl	0.1

[a] The activities were measured as described previously.[12,13]
[b] The IC_{50} values were determined from triplicate tests.

significant improvements in the inhibitory activity of the COX-2-mediated PGE_2 production. Compounds **10k–m** were found to be almost three to fifteen times more potent than their corresponding parent compounds (**10h–j**). Among these styrylheterocycles, 3-[2-(4-methoxy-phenyl)-vinyl]-thiophene **10m** exhibited a most appreciable inhibitory activity in the sub-micromolar range ($IC_{50} = 0.1$ μM). This result led us to consider it as a lead compound, with the view of developing a new series of potent inhibitors of the COX-2-mediated PGE_2 production.

To obtain a clearer knowledge of the inhibitory mechanism of PGE_2 production, the suppressive effect of one representative active styrylheterocycle **10h** on the COX-2 mRNA expression was investigated using RT-PCR analysis as described previously with the minor modifications.[12,14] Treatment of LPS for 4 h dramatically increased the expression of the COX-2 mRNA levels, and the induction was suppressed by the treatment of **10h** in a concentration-dependent manner, as depicted in Figure 2. This result suggests that the inhibitory effects of active styrylheterocycles, against LPS-stimulated PGE_2 production, might possibly be, at least, in part correlated with the suppression of COX-2 mRNA expression. However, since the suppressive effect of COX-2 mRNA expression was not reached completely at the IC_{50} value of PGE_2 production by the representative compound, other mechanisms such as direct COX-2 inhibitory activity could not be excluded. Therefore, the inhibition of PGE_2 production in LPS-activated RAW264.7 cells by styrylheterocyles might be due to either suppression of COX-2 expression or direct inhibition of COX-2 enzyme activity.

In conclusion, a series of lipophilic *trans*-stilbenoids have been prepared and evaluated as to their effects on the activity of COX with the goal of identifying a potent inhibitor. We found that 3-[2-(4-methoxy-phenyl)-vinyl]-thiophene **10m** had an appreciable inhibitory activity against the overproduction of the inflammatory mediator PGE_2, and could serve as a new lead for further chemical optimization. We have also gained an insight into the preliminary structure–activity relationships of stilbenoids, which is valuable in the design and development of a new class of COX inhibitors. Further studies for more potent inhibitors, based on the above findings, are in progress in our laboratory.

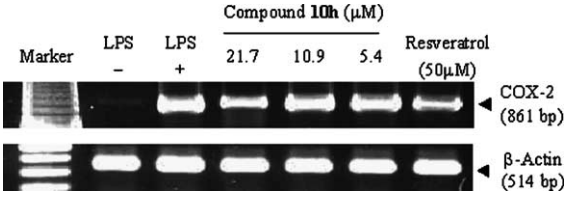


Figure 2. Effects of **10h** on the expression of COX-2 mRNA in LPS-stimulated RAW264.7 cells, using RT-PCR analysis.

Acknowledgements

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13. Measurements of PGE_2 accumulation by COX-2 in cultured LPS-induced RAW264.7 cells: The level of prostaglandin E_2 (PGE_2) production was measured by an enzyme-immunometric assay, as described previously.[12] RAW264.7 macrophage cells were maintained in DMEM, supplemented with penicillin–streptomycin and 10% FBS, at 37 °C in 5% CO_2 humidified air. To evaluate the inhibitory activity of the test materials on COX-2, the cells were allowed to adhere, for 2 h in the presence of aspirin (250 μM) in a 96-well culture plate, changed with fresh

media, and further incubated for 22 h. The attached cells were washed twice with PBS, and then incubated in the fresh medium, with 1 μg/mL of LPS. The test materials were simultaneously added to each well. After an additional 20 h incubation, the media were removed and analyzed by the PGE_2 enzyme immunometric assay (EIA). In these assays, 100% activity is defined as the difference between the PGE_2 accumulation in the absence and presence of LPS for 20 h from triplicate determinations. The percentage inhibition was expressed as $[1-(PGE_2$ level of sample/PGE_2 level of vehicle treated-control)$]\times 100$.

14. Reverse transcription-polymerase chain reaction (RT-PCR) analysis of COX-2 mRNA in RAW264.7 cells: RAW264.7 cells (5×10^6 cells—10 cm dish) were incubated for 4 h with or 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 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 COX-2 were 5′-GGAGAGACTATCAAGATAGTGATC-3′ and 5′-ATGGTCAGTAGACTTTTACAGCTA-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: 38 cycles of denaturation at 94 °C for 15 s, annealing at 55 °C for 1 min and extension at 72 °C for 1 min, using a thermal cycler (GeneAmp PCR Systems 2400; PE Applied Biosystems, USA). The amplified PCR products were run on a 2% agarose gel and visualized by SYBR Gold staining.