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Synthesis and iNOS Inhibitory Activities of Thioflavones

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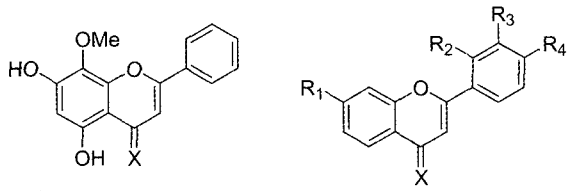
A number of thioflavones has been synthesized and evaluated for their iNOS inhibitory activities. Thiowogonin (**6**) was obtained from naturally occurring chrysin in 5 steps. Other thioflavones were prepared from the corresponding flavones in a single step by the reaction with Lawesson's reagent. The biological activities of thioflavones were not enhanced by the functional group conversion from carbonyl to thiocarbonyl. Compounds **11** and **13** showed potent NO inhibitory activity at high concentration (40 uM), leading to the possible development of novel neuroprotective agents based on wogonin.

Key words: Thiowogonin, Thioflavones, iNOS, Nitric oxide (NO), Anti-inflammatory activity

INTRODUCTION

Flavonoids are polyphenolic compounds of plant origin, exhibit various biological activities including anti-inflammatory, anti-oxidant, and anti-tumor activities (Read, 1995; Harborne *et al.*, 2000). Wogonin (5,7-dihydroxy-8-methoxyflavone), a flavonoid isolated from the root of a medicinal herb *Scutellaria baicalensis* Georgi, has been shown to possess various anti-inflammatory activities including inhibition of nitric oxide (NO) production (Lim *et al.*, 2004; Chi *et al.*, 2003; Park *et al.*, 2001; Chen *et al.*, 2001). Several studies have demonstrated that induction of iNOS produces a large amount of NO during endotoxemia and under inflammatory conditions. Therefore, drugs that inhibit iNOS expression and/or activity resulting in decreased NO generation may have beneficial therapeutic effects on the treatment of diseases due to overproduction of NO (Avontuur *et al.*, 1999). It has previously been reported that wogonin inhibit lipopolysaccharide (LPS)-induced NO production by suppressing inducible NO synthase (iNOS) in microglia (Lee *et al.*, 2003).

Recently we have synthesized flavone analogs modified at the A and B ring systems of chrysin and evaluated their inhibitory activities of prostaglandin production (Dao, Chi *et al.*, 2004; Dao, Kim *et al.*, 2004; Kim, S. J. *et al.*, 2004). Among synthetic flavones tested, several compounds showed strong inhibitory activities (IC_{50} = 0.1-0.5 μM) against COX-2 catalyzed PGE_2 production from LPS induced RAW 264.7 cells. In this study, our interest to yield potent iNOS inhibitors for the development of neuroprotective drugs has led to the structural modifications of wogonin and flavones. Herein, we described the chemical conversion of wogonin and flavones to thiowogonin and the corresponding thioflavones (Fig. 1) and their inhibitory activities against production of NO, a key mediator for inflammation.



X=O wogonin
X=S thiowogonin

thioflavones

Fig. 1. Structures of wogonin, thiowogonin, and thioflavones

MATERIALS AND METHODS

All chemicals were obtained from commercial suppliers, and used without further purification. All solvents used for reaction were freshly distilled from proper dehydrating agent under nitrogen gas. All solvents used for chromatography were purchased and directly applied without further purification. ^{1}H-NMR spectra were recorded on a Varian Gemini 2000 instrument (200 MHz) spectrometer. Chemical shifts are reported in parts per million (ppm) downfield relative to tetramethylsilane as an internal

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standard. Peak splitting patterns are abbreviated as m (multiplet), s (singlet), bs (broad singlet), d (doublet), bd (broad doublet), t (triplet) and dd (doublet of doublets). Analytical thin-layer chromatography (TLC) was performed using commercial glass plate with silica gel $60F_{254}$ purchased from Merck. Chromatographic purification was carried out by flash chromatography using Kieselgel 60 (230~400 mesh, Merck).

Chemistry

General Procedure for synthesis of thiowogonin (6)

It was not successful to prepare thiowogonin from wogonin following the general procedure as described in the section B. Therefore, thiowogonin (**6**) was prepared from chrysin (**1**) in 5 steps.

Chrysin (**1**) was converted to 7-benzyloxy-5-hydroxyflavone (**2**) following the known procedure (Dao, Kim *et al.,* 2004). To a around flask containing 250 mL of pyridine introduced with stirring 10 g of 7-benzyloxy-5-hydroxyflavone (**2**), then added partially solution of 9 g of potassium hydroxide in 200 mL of water. To the above mixture, solution of 15 g of potassium persulfate in 450 mL of water was added slowly in 2 h. The mixture was stirred at room temperature for 24 h. The deep brown solution was acidified slightly (pH 6) by slowly adding approximately 300 mL of concentrated hydrochloric acid. The pale solid was filtered and washed with water, yielding 5.5 g of starting material. The filtrate was extracted twice with 200 mL of diethyl ether. The ethereal extract was evaporated to yield more 0.5 g of starting material. The clear aqueous layer was treated with 30 g. of sodium sulfite and added slowly with 250 mL of c-HCl. The mixture was heated in water bath for 30 minutes then cooled in ice bath. The resulting yellow solid was filtered and washed with water the filtrate was extracted with ether and evaporated yielding more solid. The solid was collected and crystallized from methanol to yield 1.9 g of 7-benzyloxy-5, 8-dihydroxyflavone (**3**). 7-Benzyloxy-5, 8-dihydroxyflavone (1.0 g) in 60 mL of acetone was refluxed for 6 h with 0.25 mL of methyl sulfate and 3 g of potassium carbonate, monitoring by TLC with solvent system hexane : acetone (5:1). The solvent was distilled off and the residue was cooled to room temperature. To the remaining 150 mL of water was added. The bright yellow solid was filtered and washed with water and crystallized from acetone to yield 7-benzyloxy-5-hydroxy-8-methoxyflavone (**4**). The title compound (**6**) was obtained from the compound **4** in two steps. First, the compound **4** (0.94 g, 2.5 mmol) was converted 7-benzyloxy-5-hydroxy-8-methoxythiolflavone (**5**) by the reaction with Lawesson's reagent in the standard conditions (Clausen *et al.,* 1990). To the solution of 0.3 g of 7-benzyloxy-5-hydroxy-8-methoxythioflavone in 20 mL of glacial acetic acid, 6 mL of concentrated hydrochloric acid was added and refluxed in boiling water bath for 1 h. The reaction mixture was cooled to room temperature and 250 mL of water was added. The yellow solid was precipitated and filtered and washed with water and crystallized from methanol to yield thiowogonin (**6**).

5,7-Dihydroxy-8-methoxythioflavone (thiowogonin, 6)
^{1}H-NMR (200 MHz, DMSO-d_6), δ 13.50 (s, 1H, 5-OH); 11.36 (s, 1H, 7-OH); 8.22-8.28 (d, 2H, H 2' and H6'); 7.72-7.75 (m, 4H, H3', H4', H5' and H6); 6.52 (s, 1H, H3); 3.96 (s, 3H, OCH_3).

General Procedure for synthesis of thioflavones (11, 12, and 13)

A 200-mL, three-necked, round-bottomed flask is fitted with a rubber septum, thermometer, magnetic stirring bar, and reflux condenser equipped with a nitrogen bubbler. The flask is charged with a flavone (5 mmol) and Lawesson's reagent (2.5 mmol), whereupon the temperature of the reaction mixture increases to 75-80°C. After 5 min, 35 mL of benzene is added by syringe and the mixture is stirred while being brought to reflux. The mixture is heated at reflux for 2 h and then cooled to room temperature, whereupon it again becomes heterogeneous. The benzene is removed with the aid of a rotary evaporator and the resulting solid was crystallized from methanol-dichloromethane to produce the title products as solid.

3',4'-Dichloro-7-methoxythioflavone (11)
^{1}H-NMR (200 MHz, $CDCl_3$), δ 8.50-8.54 (d, 1H, J = 8.8 Hz, H5); 8.08 (ds, 1H, J = 1.2 Hz, H3'); 7.76-7.80 (d, 1H, J = 8.2 Hz, H6'); 7.62 (s, 1H, H8), 7.58-7.62 (d, 1H, J = 8.2 Hz, H 5'); 7.01-7.05 (d, 1H, J = 8.2 Hz, H6); 6.97 (s, 1H, H3); 3.97 (s, 3H, OCH_3).

4-Chloro-7-methoxythioflavone (12)
^{1}H-NMR (200 MHz, DMSO-d_6), δ 8.51-8.55 (d, 1H, J = 8.8 Hz, H5); 7.90-7.94 (d, 2H, J = 8.8 Hz, 7.65 (s, 1H, H8); 7.49-7.53 (d, 2H, J = 9.0 Hz, H3' and H5'); 6.96-7.06 (dd, 1H, J = 9.2 Hz, 2.6 Hz, H6); 3.96 (s, 3H, OCH_3).

4'-Methanesulfonylthioflavone (13)
^{1}H-NMR (200 MHz, DMSO-d_6), δ 8.54-8.58 (d, 1H, J = 8.6 Hz, H5); 8.50-8.54 (d, 2H, J = 8.2 Hz, H2' and H6'); 8.20-8.24 (d, 2H, J = 8.6 Hz, H3' and H5'); 8.15 (s, 1H, H3); 7.98-8.04 (t, 2H, H6 and H7); 7.65-7.70 (t, 1H, H8); 3.41 (s, 3 H, SO_2CH_3).

Biological evaluation

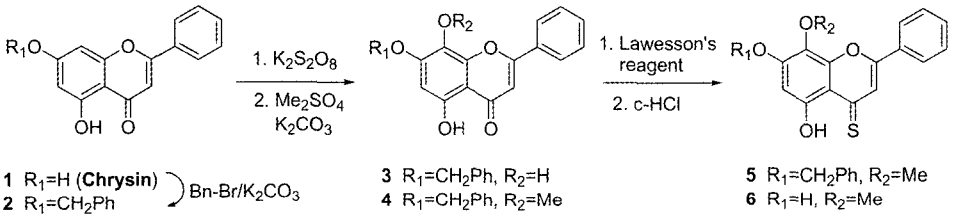
The nitrite concentration in the medium was measured as an indicator of NO production according to the Griess reaction (Kim *et al.,* 1995). Immortalized murine BV2 microglial cells, cultured in DMEM supplemented with

10% fetal bovine serum and antibiotics (100 U/mL of penicillin and 100 U/mL of streptomycin), were treated with a different concentrations of wogonin derivatives 1 h prior to LPS treatment for 18 h. At the end of treatment, one hundred microliters of each supernatant was mixed with the same volume of Griess reagent (0.1% naphthylethylenediamine dihydrochloride and 1% sulfanilamide in 5% phosphoric acid) in a 96-well microtiter plate. Absorbance of the mixture at 540 nm was measured on an ELISA plate reader. Sodium nitrite was used as standard to calculate nitrite concentrations.

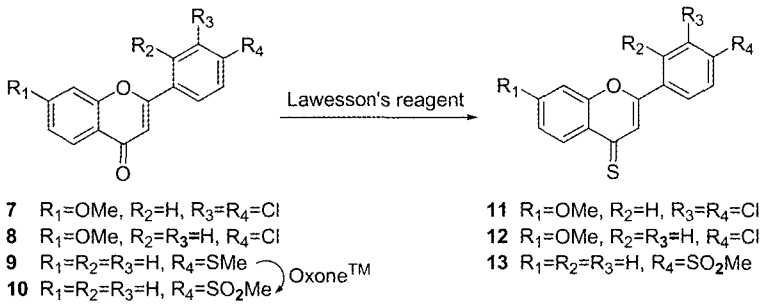
RESULTS AND DISCUSSION

Preparation of thiowogonin (**6**) from wogonin in general conditions was not successful. Thiowogonin was prepared from commercially available chrysin (**1**) following the procedure as shown in Scheme 1. Chrysin (**1**), benzyl bromide and anhydrous K_2CO_3 in acetone were refluxed at 60°C for 6 h. Solvent was distilled off, the residue was cooled to room temperature then poured into a cold water. Filtration of precipitated solid and washing with petroleum ether and water gave 7-*O*-benzylchrysin (**2**) as pale yellow solid. To the above solid in pyridine and aqueous KOH was added slowly a solution of potassium persulfate water during 2 h. The mixture was stirred at room temperature for 24 h and was acidified slightly. Filtration of the pale yellow solid and washing with water yielded 7-benzyloxy-8-hydroxyflavone (**3**). 7-Benzyloxy-8-hydroxyflavone (**3**) in acetone was refluxed for 6 h with Me_2SO_4 and K_2CO_3. The solvent was distilled off, and the residue was cooled to room temperature. To the reaction mixture was added water and filtered the bright yellow solid. The solid was washed with water and crystallized from acetone to yield 7-benzyloxy-8-methoxyflavone (**4**). A solution of the compound **4** and Lawesson's reagent in toluene was refluxed for 1 h. The resulting reaction mixture was concentrated under reduced pressure and subjected to flash chromatography using a mixture of hexane: ethylacetate: dichloromethane (1:1:1) as an eluant to yield 7-benzyloxy-8-methoxythioflavone (**5**) as deep green or solid. The solid was refluxed with c-HCl acid in glacial acetic acid for 1 h. The reaction mixture was cooled to room temperature and was added water. The precipitated yellow solid was filtered and washed with water. Recrystallization of the crude solid from methanol yielded thiowogonin (**6**).

Thioflavones (**11**, **12**, and **13**) were synthesized from the corresponding flavones by the reaction with Lawesson's reagent as shown in Scheme 2. We previously reported that these flavones (**7**, **8**, **9**, and **10**) exhibited strong inhibitory activities against COX-2 catalyzed PGE_2 production from LPS induced RAW 264.7 cells. The flavones (**7**, **8**, **9**, and **10**) were prepared following the previously reported procedure (Dao, Chi *et al.*, 2004). To a solution of 4'-methylthioflavone (**9**) in MeOH and THF was added slowly a solution of Oxone™ in H_2O at -10°C and the reaction mixture was stirred at 0°C for 3 h (Joo *et al.*, 2002). The solution was extracted two times with CH_2Cl_2 and the organic layer was washed with brine and dried over anhydrous $MgSO_4$. Filtration and followed by evaporation of the filtrated solution under reduced pressure gave the crude product as pale yellow solid. Recrystallization of the residue with methanol gave the 4'-methanesulfonylflavone (**10**) as white solid. Reaction of flavones (**7**, **8**, and **10**) with



Scheme 1. Synthetic procedure for thiowogonin from chrysin



Scheme 2. Synthetic procedure for thioflavones from the corresponding flavones

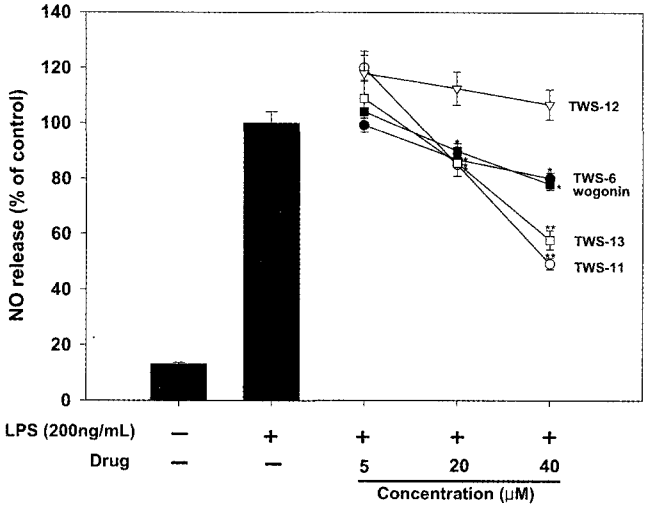


Fig. 2. Inhibitory effects of thiowogonin and thioflavones on LPS-induced nitrite production in BV2 microglial cells

Lawesson's reagent gave the corresponding thioflavones **11**, **12**, and **13**, respectively.

When cells were incubated with the indicated compounds alone, the concentration of nitrite in the medium was maintained at a background level similar to that of the unstimulated cells (data not shown). After treatment with LPS (200 ng/mL), nitrite concentrations in the medium were increased remarkably by about 10-fold (LPS alone in Fig. 2).

When BV2 microglial cells were treated with different concentrations of the indicated compounds together with LPS, a significant concentration-dependent inhibition of nitrite production was observed at all compounds except the compound **12**. The biological activities of thioflavones were not enhanced by the functional group conversion from carbonyl to thiocarbonyl, however, **11** and **13** showed potent NO inhibitory activity, leading to the possible development of novel neuroprotective agents. Although the compound **11** showed a significant inhibitory activity, it caused cell death at higher concentrations above 20 μM. Thiowogonin (**6**), the thiocarbonyl derivative of wogonin, showed approximately the same activity as wogonin, suggesting that the conversion of carbonyl functional group itself could not enhance inhibitory effect of wogonin.

In summary, we prepared thiowogonin and 5 thioflavones. Inhibitory activities of thiowogonin and thioflavones on LPS-induced NO production were observed in BV2 microglial cells. Functional group conversion of wogonin could not enhance NO inhibitory activity of wogonin as we observed. Compounds **11** and **13** showed potent NO inhibitory activity at high concentration (40 uM), leading to the possible development of novel neuroprotective agents based on wogonin based on wogonin.

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