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The Synthesis and Effect of Fluorinated Chalcone Derivatives on Nitric Oxide Production

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Abstract—Dimethoxy- and trimethoxychalcone derivatives, with various patterns of fluorination, were synthesized and evaluated for their influence on nitric oxide production. Some of them, chalcones **1**, **5**, **7**, **10**, **11** and **17**, inhibited NO production with an IC_{50} in the *submicromolar* range; **17** is especially noteworthy because of its potency (IC_{50} 30 nM). These effects were not the consequence of a direct inhibitory action on enzyme activity but the inhibition of enzyme expression.

Introduction

Activated macrophages play a key role in inflammatory responses and release a variety of mediators, including nitric oxide (NO).[1] NO is a potent vasodilator that facilitates leukocyte migration[2] and formation of edema, as well as leukocyte activity and cytokine production.[3] In addition, NO can also react with superoxide anion to form peroxynitrite, a potent oxidizing molecule that contributes to tissue injury during inflammatory responses.[4] Nitric oxide is generated from L-arginine by nitric oxide synthase (NOS).[5] Neuronal and endothelial NOS are constitutive, calcium-dependent isoforms, whereas the inducible, calcium-independent enzyme, inducible nitric oxide synthase (iNOS), is expressed in many cell types in response to a diverse range of inflammatory cytokines and bacterial metabolites such as lipopolysaccharide (LPS).[6,7] The inherent activity exhibited by iNOS results in the production of NO. In this regard, NO can enhance the release of tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β). Thus, in addition to acting as a powerful effector molecule mediating the cytotoxic activities of mouse macrophages, it can play a role in enhancing the production of a variety of other inflammatory mediators, and thus can contribute both directly and indirectly to the immunopathology of macrophage-dependent inflammation.[3,8]

We have previously studied several chalcone derivatives as potential anti-inflammatory agents[9–11] and reported that some of them are able to control NO, superoxide and PG production in vitro as well as in vivo, having a potential role in modulating the inflammatory process. In the present paper, we describe the synthesis and effects of seventeen dimethoxy- and trimethoxychalcone derivatives, with various patterns of fluorination, on NO production in LPS-stimulated murine RAW 264.7.

Chemistry

The general synthetic plan employed to prepare the chalcone derivatives used the Claisen–Schmidt condensation, which has been previously reported.[12] As shown in Tables 1 and 2, a series of six dimethoxychalcone[13] (**1**–**6**) and eleven trimethoxychalcone[14] (**7**–**17**) derivatives were prepared by condensing aromatic aldehydes and methyl ketones, using solid sodium hydroxide in methanol at room temperature. In most cases, the starting materials were commercially available and the products were always obtained as the *trans*-alkene (*E*-form) as determined by NMR spectroscopy.[12,15]

Results and Discussion

Lipopolysaccharide stimulation of RAW 264.7 macrophages for 20 h induced iNOS with the consequent generation of large quantities of NO. As shown in Tables 1

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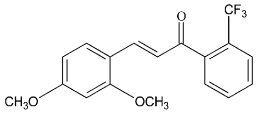
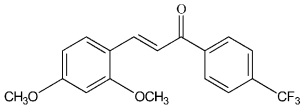
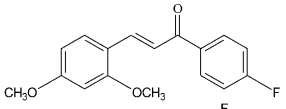
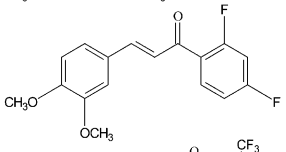
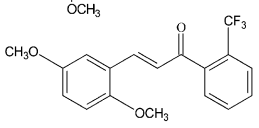
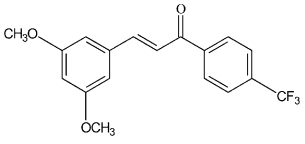
Table 1. Dimethoxychalcone derivatives

Compd	Structure	% Yield	Mp (°C)[a]	Nitrite[b] (% inhibition)	IC_{50} (μM)[c]
1		86	93–95	77.5±3.4**	0.84 (0.46–1.81)
2		73	82–84	46.8±3.5**	ND
3		56	115–116	55.7±5.1**	ND
4		86	103–105	52.4±2.5**	ND
5		84	92–94	99.0±0.5**	0.91 (0.43–1.85)
6		64	88–90	60.1±4.0**	ND

Details of the assay procedures are provided.[16]
[a]Mps are uncorrected.
[b]Results show mean ± SEM of percentages of inhibition at the concentration of 10 μM ($n=6$). *$p<0.05$; **$p<0.01$, Dunnett's test.
[c]Values represent the concentration required to produce 50% inhibition of the response, along with the 95% confidence limits. ND, not determined.

and 2, under these conditions some of the chalcones tested inhibited the generation of this mediator at 10 μM. The IC_{50} values for the inhibition of nitrite accumulation of the most active (more than 65% inhibition at 10 μM) chalcone derivatives were determined. At the highest concentration tested (10 μM), these compounds did not exert cytotoxic effects (<5%) during the 20 h incubation period as indicated by MTT reduction (data not shown).

To determine if the inhibition of nitrite production was either due to interference with the enzyme induction by LPS or due to direct action of this compound on NOS activity, chalcone derivatives were incubated for 2 h with cells after the induction of the enzyme by LPS. No significant reduction of nitrite production during these 2 h was observed (data not shown). This suggests that the inhibition of nitric oxide production by some chalcone derivatives in macrophages may occur at the level of enzyme expression.

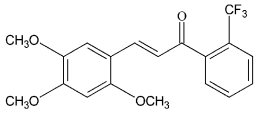
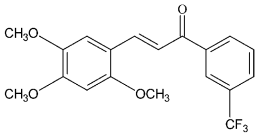
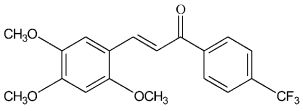
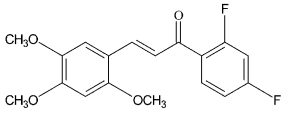
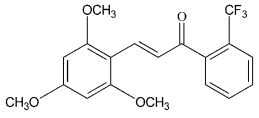
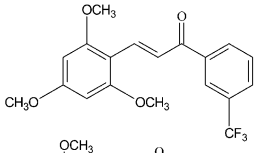
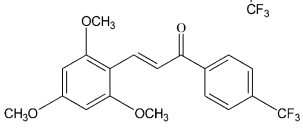
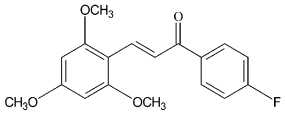
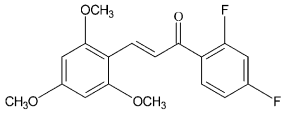
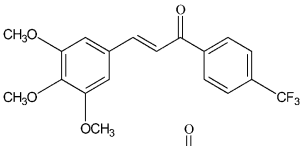
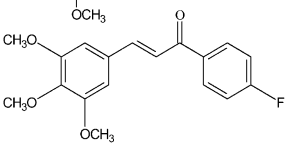
Western blot analysis was carried out on lysates of macrophages obtained as described. LPS induced iNOS expression which correlated with an increase in nitrite accumulation in the medium (Fig. 1). The addition of selected chalcone derivatives (**1**, **5**, **7**, **10**, **11**, **12**, **17**) and the reference compound, dexamethasone, reduced iNOS expression as well as nitrite levels.

From the structure–activity study, the following trends can be deduced: the trimethoxychalcone derivatives with a fluoro substituent at position C-4′ (**14**, **17**) were found to be better inhibitors of nitrite production than with a trifluoromethyl substituent at the same position (**13**, **16**). The presence of a trifluoromethyl group at C-2′, in dimethoxychalcone as well as in trimethoxychalcone derivatives, is associated with a very potent inhibition of nitrite accumulation (**1**, **5**, **7**, **11**). In contrast, this substituent is less interesting at position C-3′ (**8**, **12**) or C-4′ (**2**, **6**, **9**, **13**, **16**). The influence of the trimethoxy moiety on the activity depends strongly on the substitution pattern of the fluorine/CF_3 on the benzoyl portion of the compound and the best two compounds are **7** and **17**.

Compounds that inhibit excess production of NO by macrophages might be of benefit for the prevention and treatment of autoimmune diseases, septic shock and different inflammatory pathologies.

Figure 1. Effect of fluorinated chalcone derivatives (10 μM) on iNOS expression in RAW 264.7 cells. B, normal cells (without LPS); C, control (with LPS); Dex, dexamethasone.

Table 2. Trimethoxychalcone derivatives

Compd	Structure	% Yield	Mp (°C)[a]	Nitrite[b] (% inhibition)	IC_{50} (μM)[c]
7		62	153–155	73.7±3.1**	0.28 (0.15–0.47)
8		62	130–131	26.7±1.5	ND
9		56	110–112	49.3±3.5**	ND
10		82	108–110	81.7±6.5**	0.64 (0.43–1.12)
11		78	129–130	76.3±5.1**	1.02 (0.81–1.70)
12		72	123–125	75.0±2.6**	1.84 (0.54–1.16)
13		60	156–158	43.6±4.6*	ND
14		83	140–142	63.9±1.9**	ND
15		61	138–140	59.0±8.7*	ND
16		58	228–230	55.8±8.9*	ND
17		64	108–109	94.1±1.4**	0.03 (0.02–0.47)

Details of the assay procedures are provided.[16]

[a]Mps are uncorrected.

[b]Results show mean±SEM of percentages of inhibition at the concentration of 10 μM ($n=6$). *$p<0.05$; **$p<0.01$, Dunnett's test.

[c]Values represent the concentration required to produce 50% inhibition of the response, along with the 95% confidence limits. ND, not determined.

Acknowledgements

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References and Notes

1. Laskin, D. L.; Pendino, K. J. *Annu. Rev. Pharmacol. Toxicol.* **1995**, *35*, 655.
2. Wright, C. D.; Mülsch, A.; Busse, R.; Osswald, H. *Biochem. Biophys. Res. Commun.* **1989**, *160*, 813.
3. Marcinkiewicz, J.; Grabowska, A.; Chain, B. *Eur. J. Immunol.* **1995**, *25*, 947.
4. Kaur, H.; Halliwell, B. *FEBS Lett.* **1994**, *350*, 9.
5. Knowles, R. G.; Moncada, S. *Biochem. J.* **1994**, *298*, 249.
6. MacMicking, J.; Xie, Q. W.; Nathan, C. *Annu. Rev. Immunol.* **1997**, *15*, 323.
7. Xie, Q. W.; Nathan, C. *J. Leucocyte Biol.* **1994**, *56*, 576.
8. Alcaraz, M. J.; Guillen, I. *Curr. Pharm. Des.* **2002**, *8*, 125.
9. Herencia, F.; Ferrándiz, M. L.; Úbeda, A.; Dominguez, J. N.; Charris, J. E.; Lobo, G. M.; Alcaraz, M. J. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 1169.
10. Herencia, F.; Ferrándiz, M. L.; Úbeda, A.; Guillén, I.; Dominguez, J. N.; Charris, J. E.; Lobo, G. M.; Alcaraz, M. J. *FEBS Lett.* **1999**, *453*, 129.
11. Herencia, F.; Ferrándiz, M. L.; Úbeda, A.; Guillén, I.; Dominguez, J. N.; Charris, J. E.; Lobo, G. M.; Alcaraz, M. J. *Free Radic. Biol. Med.* **2001**, *30*, 43.
12. Li, R.; Kenyon, G. L.; Cohen, F. E.; Chen, X.; Gong, B.; Dominguez, J. N.; Davidson, E.; Kurzban, G.; Miller, R. E.; Rosenthal, P. J.; McKerrow, J. H. *J. Med. Chem.* **1995**, *38*, 5031.
13. Spectral data for dimethoxychalcone derivatives: (**1**): ^{1}H NMR ($CDCl_3$) δ 3.84 (s, 3H, OCH_3), 3.86 (s, 3H, OCH_3), 6.46 (d, J = 2.1 Hz, 1H, 3-H), 6.52 (dd, J = 2.1 and 8.6 Hz, 1H, 5-H), 7.12 (d, J = 16.1 Hz, 1H, α-H), 7.32–7.51 (m, 5H), 7.70 (d, J = 16.1 Hz, 1H, β-H). (**2**): ^{1}H NMR ($CDCl_3$) δ 3.82 (s, 3H, OCH_3), 3.84 (s, 3H, OCH_3), 6.47 (d, J = 2.1 Hz, 1H, 3-H), 6.58 (dd, J = 1.9 and 8.5 Hz, 1H, 5-H), 7.14 (d, J = 15.8 Hz, 1H, α-H), 7.30–7.47 (m, 4H), 7.65 (d, J = 15.9 Hz, 1H, β-H), 7.82 (d, J = 8.7 Hz, 1H, 6′-H). (**3**): ^{1}H NMR ($CDCl_3$) δ 3.80 (s, 3H, OCH_3), 3.86 (s, 3H, OCH_3), 6.45 (d, J = 2.0 Hz, 1H, 3-H), 6.56 (dd, J = 2.0 and 8.6 Hz, 1H, 5-H), 7.10 (d, J = 16.0 Hz, 1H, α-H), 7.32–7.52 (m, 4H), 7.68 (d, J = 15.9 Hz, 1H, β-H), 7.80 (d, J = 8.6 Hz, 1H, 6′-H). (**4**): ^{1}H NMR ($CDCl_3$) δ 3.88 (s, 3H, OCH_3), 3.90 (s, 3H, OCH_3), 6.99 (d, J = 8.4 Hz, 1H, 5-H),7.63–8.19 (m, 5H), 8.20 (d, J = 15.7 Hz, 1H, β-H). (**5**): ^{1}H NMR ($CDCl_3$) δ 3.78 (s, 3H, OCH_3), 3.84 (s, 3H, OCH_3), 6.91 (d, J = 9.0 Hz 1H), 7.00–7.42 (m, 5H), 7.76 (d, J = 8.5 Hz 1H), 7.93 (d, J = 15.9 Hz, 1H, β-H). (**6**): ^{1}H NMR ($CDCl_3$) δ 3.86 (s, 3H, OCH_3), 4.02 (s, 3H, OCH_3), 6.80–7.40 (m, 6H), 7.46 (d, J = 14.8 Hz, 1H, α-H), 7.70 (d, J = 15.5 Hz, 1H, β-H), 8.05 (d, J = 8.0 Hz, 1H, 6′-H). In addition, all compounds had IR, LSIMS and elemental analysis in complete agreement with the assigned structures.
14. Spectral data for trimethoxychalcone derivatives: (**7**): ^{1}H NMR ($CDCl_3$) δ 3.99 (s, 3H, OCH_3), 4.04 (s, 3H, OCH_3), 4.11 (s, 3H, OCH_3), 6.68–8.02 (m, 8H). (**8**): ^{1}H NMR ($CDCl_3$) δ 4.03 (s, 3H, OCH_3), 4.09 (s, 3H, OCH_3), 4.14 (s, 3H, OCH_3), 7.30 (d, J = 14.8 Hz, 1H, α-H), 7.38–7.47 (m, 4H), 7.88 (d, J = 15.9 Hz, 1H, β-H), 7.98 (d, J = 8.6 Hz, 1H, 6′-H). (**9**): ^{1}H NMR ($CDCl_3$) δ 4.67 (s, 3H, OCH_3), 4.78 (s, 3H, OCH_3), 4.84 (s, 3H, OCH_3), 6.35–7.70 (m, 5H), 7.80 (d, J = 15.5 Hz, 1H, β-H), 7.98 (d, J = 8.1 Hz, 1H, 6′-H). (**10**): ^{1}H NMR ($CDCl_3$) δ 3.92 (s, 3H, OCH_3), 4.15 (s, 3H, OCH_3), 4.20 (s, 3H, OCH_3), 6.89 (d, J = 16.1 Hz, 1H, α-H), 7.28–7.85 (m, 6H). (**11**): ^{1}H NMR ($CDCl_3$) δ 4.65 (s, 3H, OCH_3), 4.76 (s, 3H, OCH_3), 4.78 (s, 3H, OCH_3), 6.68–7.80 (m, 7H), 7.90 (d, J = 15.5 Hz, 1H, β-H). (**12**): ^{1}H NMR ($CDCl_3$) δ 4.53 (s, 3H, OCH_3), 4.62 (s, 3H, OCH_3), 4.65 (s, 3H, OCH_3), 6.32 (d, J = 2.1 Hz, 1H), 6.50 (d, J = 2.0 Hz, 1H), 7.15 (d, J = 15.8 Hz, 1H, α-H), 7.25–7.50 (m, 4H), 7.88 (d, J = 8.2 Hz, 1H, 6′-H). (**13**): ^{1}H NMR ($CDCl_3$) δ 4.53 (s, 3H, OCH_3), 4.68 (s, 3H, OCH_3), 4.72 (s, 3H, OCH_3), 6.38 (d, J = 2.1 Hz, 1H), 6.58 (d, J = 1.8 Hz, 1H), 7.22 (d, J = 16.0 Hz, 1H, α-H), 7.28–7.65 (m, 3H), 7.70 (d, J = 15.6 Hz, 1H, β-H), 7.85 (d, J = 8.0 Hz, 1H, 6′-H). (**14**): ^{1}H NMR ($CDCl_3$) δ 3.59 (s, 3H, OCH_3), 3.75 (s, 3H, OCH_3), 3.91 (s, 3H, OCH_3), 6.48 (d, J = 2.1 Hz, 1H), 6.66 (d, J = 2.0 Hz, 1H), 7.20 (d, J = 15.8 Hz, 1H, α-H), 7.38–7.47 (m, 3H), 7.67 (d, J = 15.9 Hz, 1H, β-H), 7.78 (d, J = 8.6 Hz, 1H, 6′-H). (**15**): ^{1}H NMR ($CDCl_3$) δ 4.48 (s, 3H, OCH_3), 4.56 (s, 3H, OCH_3), 4.61 (s, 3H, OCH_3), 6.85–7.90 (m, 6H), 8.02 (d, J = 15.8 Hz, 1H, β-H). (**16**): ^{1}H NMR ($CDCl_3$) δ 4.61 (s, 3H, OCH_3), 4.86 (s, 3H, OCH_3), 4.92 (s, 3H, OCH_3), 7.54–7.75 (m, 6H), 7.80 (d, J = 15.9 Hz, 1H, β-H), 7.92 (d, J = 8.0 Hz, 1H, 6′-H). (**17**): ^{1}H NMR ($CDCl_3$) δ 3.76 (s, 3H, OCH_3), 3.90 (s, 3H, OCH_3), 3.95 (s, 3H, OCH_3), 7.62 (d, J = 15.6 Hz, 1H, α-H), 7.64–7.75 (m, 5H), 7.78 (d, J = 15.8 Hz, 1H, β-H), 7.96 (d, J = 8.5 Hz, 1H, 6′-H). In addition, all compounds had IR, LSIMS and elemental analysis in complete agreement with the assigned structures.
15. The substituted methyl ketone (1 mmol) and the aldehyde (1 mmol) were dissolved in a minimum amount of methanol (normally 2–4 mL) and then a single NaOH pellet was added (about 100 mg). The reaction mixture was stirred at room temperature until an off-white to bright yellow solid was formed (within a few min to 24 h). The solid was collected by filtration and washed with cold methanol. The product was recrystallized from appropriate solvent(s) whenever necessary.
16. **Cell culture**: The mouse macrophage cell line RAW 264.7 was cultured in DMEM medium containing 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin and 10% fetal bovine serum. Cells were resuspended at a concentration of 2×10^6/mL and co-incubated with *Escherichia coli* LPS (1 μg/mL) at 37 °C for 20 h in the presence of test compounds or vehicle. The nitrite concentration as a reflection of NO release was assayed fluorometrically. The amount of nitrite was obtained by extrapolation from a standard curve with sodium nitrite as a standard. The mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan was used to assess the possible cytotoxic effects of compounds. **iNOS activity in intact cells**: RAW 264.7 macrophages stimulated for 20 h with LPS were washed and Hank's buffer supplemented with L-arginine (0.5 mM) was added for 2 h incubation with test compounds to determine their effects on iNOS activity. Supernatants were collected for the measurement of nitrite accumulation for the last 2 h fluorometrically as above. **Western blot assay**: Cellular lysates from cell line RAW 264.7 (10^6/well) incubated for 18 h with LPS were obtained with lysis buffer (1% Triton X-100, 1% deoxycholic acid, 20 mM NaCl, and 25 mM Tris, pH 7.4). Following centrifugation (10,000× *g*, 15 min), supernatant protein was determined and 25 μg protein were loaded on 12% SDS–PAGE and transferred onto nitrocellulose membranes for 90 min at 125 mA. Membranes were blocked in PBS–Tween 20 containing 3% w/v unfatted milk and incubated with anti-iNOS polyclonal antibody (1/1000 dilution). Blots were washed and incubated with peroxidase-conjugated goat anti-rabbit IgG (1/20,000 dilution; Dako; Glostrup, Denmark). The immunoreactive bands were visualized using an enhanced chemiluminescence system (ECL; Amersham Iberica, Spain). **Statistical analysis**: Statistical evaluation included one-way analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons. p Values of $p < 0.05$ (*) or $p < 0.01$ (**) were taken as significant. Results are shown as mean ± SEM for n experiments. Inhibitory concentration 50% (IC_{50}) values were calculated from at least four significant concentrations.