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Simple synthesis and biological evaluation of flocoumafen and its structural isomers

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Abstract. Simple synthesis and biological properties of flocoumafen **1** and its structural isomers are described. The key synthetic strategies involve Knoevenagel condensation, Grignard reaction, intramolecular ring cyclization and coupling reaction. Flocoumafen **1** was easily separated into *cis* and *trans* forms using flash column chromatography. They were then evaluated for suppression of LPS-induced NO generation and anti-excitotoxicity *in vitro*. It was found that the *trans*-flocoumafen was potent suppressor of NO generation with the concentration of 10 μM *in vitro*, while no significant effect for neurotoxicity in cultured cortical neurons.

Keywords. Flocoumafen; intramolecular ring cyclization; coupling reaction, NO-generation; neurotoxicity.

1. Introduction

Many 4-hydroxycoumarins have been naturally obtained from several plants such as tonka bean, lavender, sweet clover grass, strawberries, and cinnamon. The 4-hydroxycoumarin and its derivatives have been effectively used as anticoagulants such as warfarin, brodifacoum, difethialone, bromadiolone, coumatetralone, and flocoumafen for the treatment of disorders in which there is excessive or undesirable clotting, such as thrombophlebitis, pulmonary embolism, and certain cardiac conditions.[1] Recently, the 4-hydroxycoumarins have evoked a great deal of interest due to their characteristic structural features and biological activities. The coumarin have been studied as cell growth stimulation, bacteriostatic activity as well as various pharmacological effectiveness such as analgesic,[2] arthritis,[3] anti-inflammatory,[4] anti-pyretic,[5] anti-bacterial,[6] anti-viral,[7] and anti-cancer effects.[8] Especially, the flocoumafen **1** and thioflocoumafen **2** are very effective anticoagulant agents among the 4-hydroxycoumarin derivatives.[9] The coumarin nucleus is derived from cinnamic acid as phenylacrylic skeleton in the biosynthesis. Especially, the hydroxy group attached to coumarin nucleus at 4-position is important in biosynthesis pathway. Currently, 4-hydroxycoumarins are registered for the control of rats and mice in and around farm structures, households, and domestic dwellings, inside transport vehicles, commercial transportation facilities, and industrial areas. Recent report[10] described a simple synthesis of 4-hydroxycoumarin derivatives and their screening assay for their biological properties. Actually, most of the work to date, regarding the synthetic approaches of warfarin type anticoagulants were reported[11] by the Ferreira group through the formation of the carbon backbone using organocopper methodology, the ring cyclization, and the coupling reaction with the 4-hydroxycoumarin moiety.

The scheme 1 reveals representative retrosynthetic approach for syntheses of **1** and **2**. The major synthetic strategies involve intramolecular ring cyclization, *O*-alkylation for preparation of key fragments tetrahydronaphthalen-1-ol **3** or tetrahydronaphthalen-1-bromide **4** and coupling reaction of **3**, **4** with 4-hydroxycoumarin **5** or 4-hydroxythiocoumarin **6** to generate the target flocoumafen **1** or thioflocoumafen **2**. The skeletons of 2*H*-1-benzopyran-2-ones and tetrahydronaphthalens are

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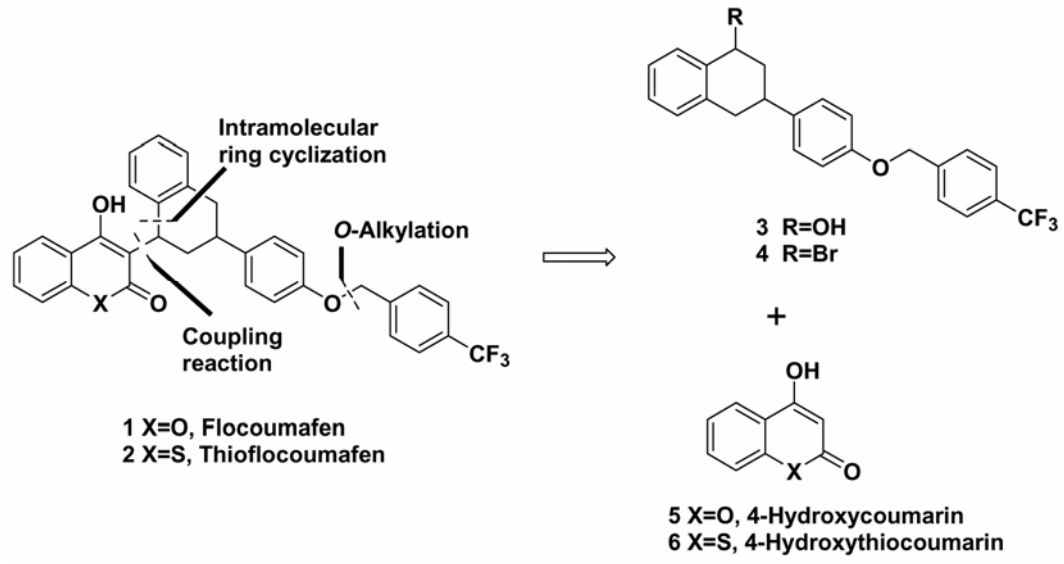


Scheme 1. Retrosynthetic analysis of flocoumafen **1** and thioflocoumafen **2**.

essential structural features for the 4-hydroxycoumarin derivatives of second generation rodenticides **1**–**2**. They have traditionally been coupled with acidic media. Even though improved condensation reactions of 4-hydroxycoumarins **5–6** with compounds **3** or **4** using Bronsted–Lowry acids (HCl, H_2SO_4) have been reported,[11–12] these reactions led to preferential dehyrdohalogenation to give low yield. Thus, an efficient coupling condition was required to obtain high yield.

In the context of our medicinal research program dealing with the synthesis of biologically active 4-hydroxycoumarin derivatives, we hope to report a simple synthesis of flocoumafen from intramolecular ring cyclization, *O*-alkylation, and coupling reaction. Flocoumafen **1** was firstly separated into *cis* form and *trans* form using flash silica-column chromatography. Biological activities of flocoumafen and its structural isomers for suppression of LPS-induced NO generation *in vitro* suggests that they can be possible lead compounds for anti-inflammatory agents.

2. Experimental

2.1 *General*

Reactions requiring anhydrous conditions were performed with the usual precautions for rigorous exclusion of air and moisture. Tetrahydrofuran was distilled from sodium benzophenone ketyl prior to use. Thin layer chromatography (TLC) was performed on precoated silica gel G and GP uniplates from Analtech and visualized with 254 nm UV light. Flash chromatography was carried out on silica gel 60 [Scientific Adsorbents Incorporated (SAI), particle size 32–63 μm, pore size 60 Å]. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker DPX 500 at 500 MHz and 125 MHz, respectively. The chemical shifts are reported in parts per million (ppm) downfield from tetramethylsilane, and *J* values are in Hz. Infrared (IR) spectra were obtained on an ATI Mattson FT/IR spectrometer. Mass spectra were recorded with a Waters Micromass ZQ LC–Mass system and high resolution mass spectra (HRMS) were measured with a Bruker BioApex FTMS system by direct injection using an electrospray interface (ESI). When necessary, chemicals were purified according to the reported procedures.[13]

2.1a *Synthesis of 3-[1,2,3,4-Tetrahydro-3-[4-(4-trifluoromethylbenzyloxy)phenyl]-1-naphthalen-1-ol* (**3**): To a stirred solution of ketone (**13**, 0·8 g, 2·0 mmol) in EtOH (6 mL) was added portion-wise sodium borohydride (91 mg, 2·4 mmol) and then the mixture was stirred at room temperature for 2 h. The reaction mixture was diluted with water (5 mL) and acidified with 1 N HCl aqueous solution. The resulting mixture was extracted with dichloromethane (10 mL × 3). The combined organic layer was washed with saturated aqueous NH_4Cl solution (15 mL) and organic phase was separated, dried over anhydrous

$MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography (silica gel, ethyl acetate/hexanes = 1 : 3, *v*/*v*) to afford alcohol **3** (0·56 g, 70%) as a white solid. R_f = 0·3 (30% ethyl acetate/hexanes); m.p. 111·6°C. IR (neat, NaCl) ν 3388 (OH), 2922 (C–H), 1611 (C=C), 1584, 1512, 1454, 1244, 1066 (C–O), 824 cm^{-1}. ^1H NMR ($CDCl_3$, 500.14 MHz) δ 7·63 (*dd*, 3H, *J* = 8·5, 8·5 Hz, aromatic-H), 7·56 (*d*, 2H, *J* = 8·0 Hz, aromatic-H), 7·29–7·20 (*m*, 4H, aromatic-H), 7·10 (*d*, 1H, *J* = 7·5 Hz, aromatic-H), 6·95 (*d*, 2H, *J* = 8·0 Hz, aromatic-H), 5·13 (*s*, 2H, benzyl-H), 5·02–4·96 (*m*, 1H, CH), 3·09–2·88 (*m*, 2H, CH_2), 2·51–2·45 (*m*, 1H, CH), 1·92 (*q*, 1H, *J* = 12·5 Hz, CH_2), 1·78 (*d*, 1H, *J* = 8·0 Hz, CH_2). ^{13}C NMR ($CDCl_3$, 125·76 MHz) δ 157·2, 141·4, 139·3, 138·4, 136·4, 128·7, 127·9 (2C), 127·6, 127·5 (2C), 126·9, 126·7, 125·7 (2C), 125·6 (2C), 115·1 (2C), 70·3, 69·4, 41·0, 38·7, 38·6. HRMS: *m*/*z* = 399·1585 (calcd. 399·1572 for $C_{24}H_{22}F_3O_2$: $[M + H]^+$).

2.1b *Synthesis of flocoumafen; 4-hydroxy-3-[1,2,3,4-tetrahydro-3-[4-(4-trifluoromethylbenzyloxy)phenyl]-1-naphthyl]coumarin* (**1**): To a stirred solution of secondary alcohol (**3**, 0·2 g, 0·5 mmol) in dichloromethane (4 mL) was added *p*-toluenesulfonic acid (5 mg, cat) and 4-hydroxycoumarin (0·1 g, 0·6 mmol) and then the mixture was refluxed for 6 h. The reaction mixture was cooled to room temperature and washed with water (5 mL). The organic layer was separated and the aqueous layer was extracted with dichloromethane (10 mL × 3). The combined organic layer was washed with saturated aqueous NH_4Cl solution (15 mL) and organic phase was separated, dried over anhydrous $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography (silica gel, ethyl acetate/hexanes = 1 : 4, *v*/*v*) to give flocoumafen **1** (0·18 g, 64%) as a white solid. R_f = 0·2 (25% ethyl acetate/hexanes); m.p. 137·5°C. IR (neat, NaCl) ν 3396 (OH), 1668 (C=O), 1610 (C=C), 1570, 1510, 1452, 1419, 1326, 1240, 1066 (C–O), 825 cm^{-1}. ^1H NMR ($CDCl_3$, 500·14 MHz) δ 7·72 (*d*, 1/2H, *J* = 8·0 Hz, aromatic-H), 7·68–7·61 (*m*, 5/2H, aromatic-H), 7·57–7·50 (*m*, 3H, aromatic-H), 7·38–7·29 (*m*, 4H, aromatic-H), 7·28–7·23 (*m*, 2H, aromatic-H), 7·19 (*dd*, 2H, *J* = 8·5, 8·5 Hz, aromatic-H), 6·91 (*dd*, 2H, *J* = 8·5, 8·5 Hz, aromatic-H), 5·12 (*s*, 1H, benzyl-H), 5·10 (*s*, 1H, benzyl-H), 4·86 (*q*, 1/2H, *J* = 6·0 Hz, CH), 4·72 (*t*, 1/2H, *J* = 4·5 Hz, CH), 3·23 (*d*, 1/2H, *J* = 12·5 Hz, CH_2), 3·13–2·98 (*m*, 5/2H, CH, CH_2), 2·51–2·40 (*m*, 1/2H, CH_2), 2·38–2·24 (*m*, 1/2H, CH_2), 1·96–1·82 (*m*, 1/2H, CH_2). ^{13}C NMR ($CDCl_3$, 125·76 MHz) δ 163·5 (C=O), 160·8 (C), 157·2 (C), 157·1 (C), 152·7 (C), 152·6 (C), 141·3 (C), 138·1 (C), 137·9 (C), 137·7 (C), 134·3 (C), 132·1 (CH), 132·0 (CH), 130·8 (C), 130·7 (C), 130·6 (CH), 129·3 (CH), 128·7 (CH), 128·1 (CH), 128·0 (C), 127·9 (C), 127·8 (C), 127·5 (C), 127·4 (C), 125·7 (C), 125·6 (C), 124·1 (CH), 124·0 (CH), 123·2 (CH), 123·1 (CH), 116·6 (CH), 116·5 (CH), 116·3 (C), 115·1 (C), 115·0 (C), 109·4 (C), 108·8 (C), 69·3 (benzyl-C), 39·8 (CH), 38·6 (CH_2), 38·1 (CH_2), 37·5 (CH), 37·0 (CH), 36·5 (CH_2), 35·9 (CH_2). HRMS: *m*/*z* = 543·1772 (calcd. 543·1783 for $C_{33}H_{26}F_3O_4$: $[M + H]^+$); *cis*-Flocoumafen: m.p. 180·1°C. ^1H NMR ($CDCl_3$, 500·14 MHz) δ 7·72 (*d*, 1H, *J* = 7·5 Hz, aromatic-H), 7·62 (*d*, 2H, *J* = 8·0 Hz, aromatic-H), 7·53 (*dd*, 3H, *J* = 7·5, 2·0 Hz, aromatic-H), 7·34 (*d*, 1H, *J* = 8·0 Hz, aromatic-H), 7·32–7·23 (*m*, 5H, aromatic-H), 7·21 (*d*, 2H, *J* = 9·0 Hz, aromatic-H), 6·92 (*d*, 2H, *J* = 9·0, 8·5 Hz, aromatic-H), 5·64 (*brs*, 1H, OH), 5·12 (*s*, 2H, benzyl-H), 4·87 (*q*, 1H, *J*=5·5 Hz, CH), 3·13–3·02 (*m*, 3H, CH, CH_2), 2·52–2·42 (*m*, 1H, CH_2), 1·95–1·80 (*m*, 1H, CH_2); LC–MS (ESI^+) *m*/*z* 565·1 – [M + Na]; *trans*-Flocoumafen: m.p. 107·3°C. ^1H NMR ($CDCl_3$, 500·14 MHz): δ = 7·66 (*dd*, 1H, *J* = 1·5, 1·5 Hz, aromatic-H), 7·63 (*d*, 2H, *J* = 8·0 Hz, aromatic-H), 7·57–7·52 (*m*, 3H, aromatic-H), 7·39–7·34 (*m*, 2H, aromatic-H), 7·33–7·29 (*m*, 2H, aromatic-H), 7·27–7·20 (*m*, 2H, aromatic-H), 7·16 (*d*, 2H, *J* = 8·5 Hz, aromatic-H), 6·90 (*d*, 2H, *J* = 8·5 Hz, aromatic-H), 5·12 (*s*, 2H, benzyl-H), 4·72 (*t*, 1H, *J*=4·0 Hz, CH), 3·23 (*d*, 1H, *J* = 12·0 Hz, CH_2), 3·12–2·99 (*m*, 2H, CH, CH_2), 2·36–2·32 (*m*, 2H, CH_2); LC–MS (ESI^+) *m*/*z* 565·3 – [M + Na].

2.2 *Biological activity test*

2.2a *Cell culture*: Cerebral cortices were removed from the brains of 15·5-day-old fetal mice. The neocortices were triturated and plated on 24-well plates (with approximately 10^6 cells/mL) precoated with 100 μg/mL poly-D-lysine and 4 μg/mL laminine, in Eagle's minimal essential media (Earle's salts, supplied glutamine-free), and supplemented with horse serum (5%), fetal bovine serum (5%), 2 mM glutamine, and 20 mM glucose. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2. After 6 days *in vitro* (DIV), the cultures were shifted

to the plating media containing 10 μM cytosine arabinoside without fetal serum. Cultures were then fed twice per week. Mixed cortical cell cultures containing neurons and glia (DIV 16-14) were exposed to excitatory amino acid, glutamate, in Eagle's minimal essential media without serum. The morphology of the degenerating neurons was observed under a phase contrast microscope over the next 24 h. The murine BV2 cell line (a generous gift from Dr. W. Kim, Korea Research Institute of Bioscience and Biotechnology, Korea), which is immortalized after infection with a v-raf/v-myc recombinant retrovirus, exhibits the phenotypic and functional properties of reactive microglial cells[14] BV2 cells were maintained at 37°C at 5% CO_2 in DMEM supplemented with 10% FBS, 100 μg/mL streptomycin, and 100 U/mL penicillin. BV2 cells were grown in 24-well plates at a concentration of 1×10^5 cells/well followed by proper treatment.

2.2b *Nitrite assay:* NO production from activated microglial cells was determined by measuring the amount of nitrite, a relatively stable oxidation product of NO, as described previously.[15] Cells were incubated with or without LPS (1 μg/mL) in the presence or absence of various concentrations of compounds for 24 h. The nitrite accumulation in the supernatant was assessed by Griess reaction. In brief, an aliquot of the conditioned medium was mixed with an equal volume of 1% sulfanilamide in water and 0·1% *N*-1-naphthylethylenediamine dihydrochloride in 5% phosphoric acid. The absorbance was determined at 540 nm in an automated microplate reader.

2.2c *Cell viability:* Cortical neuronal cell number and viability were assessed by using the reagent WST-1 (Roche, Indianapolis, IN). This colorimetric assay measures the metabolic activity of viable cells based on cleavage of the tetrazolium salt WST-1 substrate 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio-1,3-benzene disulphonate into formazan by mitochondrial dehydrogenase in live cells. This was followed by incubation with WST-1 reagent at a dilution of 1 : 10 in the original conditioned media at 37°C for 2 h. After thorough shaking, the formazan produced by the metabolically active cells in each sample was measured at a wavelength of 450 nm and a reference wavelength 650 nm. Absorbance readings were normalized against control wells with untreated cells. Neuronal death was analysed 24 h later, and the percentage of neurons undergoing actual neuronal death was normalized to the mean value that is found after a 24 h exposure to 300 μM NMDA (defined as 0) or a sham control (defined as 100).

3. Results and discussion

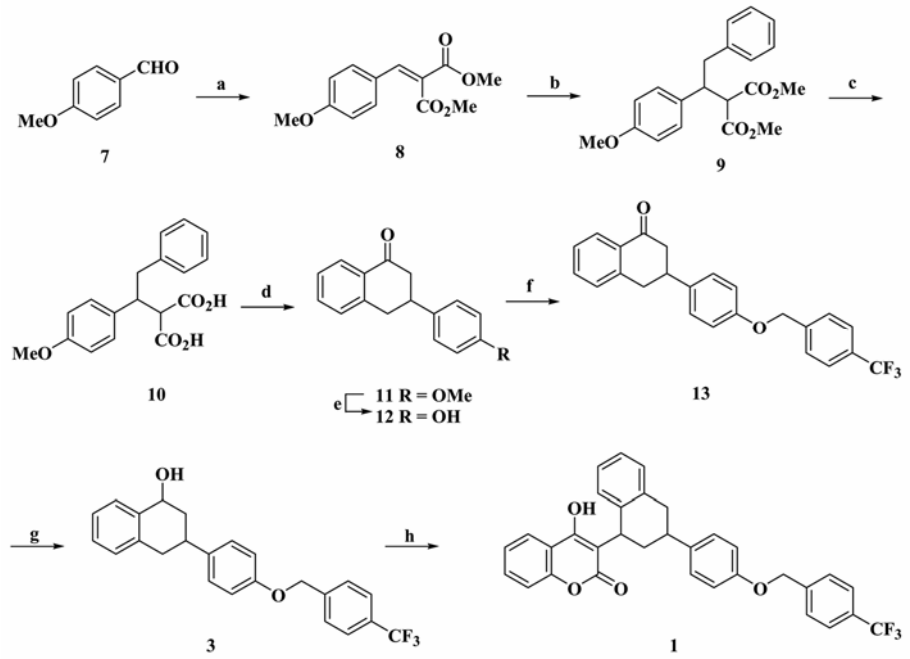
3.1 *Chemistry*

The linear synthesis of flocoumafen **1** is summarized in scheme 2. Commercially available 4-methoxybezaldehyde **7** was condensed with dimethyl malonate as a Knoevenagel condensation in the presence of AcOH and pyrrolidine to afford diketo ester **8**, which was treated with freshly prepared benzylmagnesium bromide in the presence of copper (I) chloride to give compound **9**. Subsequent hydrolysis of **9** was accomplished with aqueous potassium hydroxide. The resulting diacid **10** was decarboxylated under acidic condition and then cyclized intramolecularly using polyphosphoric acid to yield tetralone **11** in two steps.[16] Demethylation of tetralone **11** was performed by hydrobromic acid in acetic acid to give phenol **12**. It was *O*-alkylated with freshly prepared 3-(trifluoromethyl)benzyl bromide in sodium hydride/THF to generate ether **13**.[17] Reduction of ketone group of compound **13** with sodium borohydride in methanol gave secondary alcohol **3**, which was treated with *p*-toluenesulfonic acid to give flocoumafen **1**[18] in 64% yield as 1 : 1 structural isomeric mixture.

On the other hand, the coupling reaction of bromide **4** with 4-hydroxycoumarin **5** was not effective for preparation of flocoumafen **1** due to generation of undesired dehydroxyhalogenated product and almost recovered starting materials. The obtained flocoumarin **1** was a 1 : 1 mixture of *cis*-flocoumafen (*cis*-FCF) and *trans*-flocoumafen (*trans*-FCF). For the biological activity test, we separated it into *cis*-FCF and *trans*-FCF using flash silica-column chromatography. Also, we could recrystallize flocoumarin **1** using the co-solvent (ethyl acetate/hexanes) system in order to separately yield *cis* and *trans* forms.

3.2 *Suppression of NO-generation and anti-neurotoxicity*

NO production from activated microglial cells was determined by measuring the amount of nitrite after incubation with or without LPS (1 μg/mL) in the presence or absence of various concentrations of



Scheme 2. Reagents and conditions: (**a**) Dimethyl malonate, AcOH, pyrrolidine, toluene, reflux, 3 h; (**b**) benzylmagnesium bromide, Cu_2Cl_2, THF, 0°C, 1 h; (**c**) KOH/H_2O, reflux, 3 h; (**d**) H_2SO_4/H_2O, reflux, 8 h; and then PPA, 80°C, 1 h; (**e**) HBr, AcOH, reflux, 6 h; (**f**) 3-(trifluoromethyl)benzyl bromide, sodium hydride, THF, 0°C, 1 h; (**g**) $NaBH_4$, MeOH, r.t., 2 h; (**h**) 4-hydroxycoumarin, *p*-TsOH, dichloromethane, 80°C, 3 h.

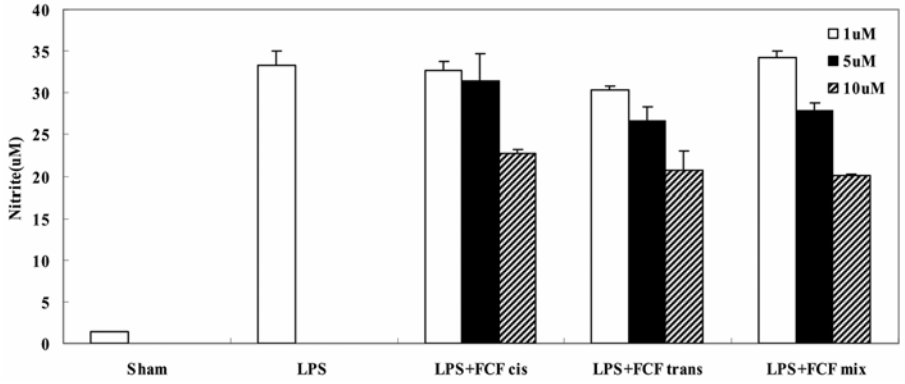


Figure 1. Suppression of NO production in LPS-treated microglia. The cells were treated with 1 μg/mL of LPS only or LPS plus different concentrations (1, 5, and 10 μM) of compounds at 37°C for 24 h. At the end of incubation, 50 μL of the medium was removed to measure nitrite production. All values represent mean ± SE of three independent experiments performed in triplicate.

compounds for 24 h. All compounds (10 μM) showed considerable suppression of LPS-induced NO generation (figure 1). The *trans* form of FCF has more potent activity than that of *cis*-FCF in anti-inflammatory action. However, *cis*-FCF is slightly more active than *trans*-FCF in the inhibition of glutamate-induced neurotoxicity in cultured cortical neurons. Exposure of cortical cell cultures to 300 μM NMDA (prototype of glutamate receptor agonist) resulted in a rapid swelling of the neuronal cell body within 2 h, and caused 90 to 100% neuronal death over the next day. The 60 μM of glutamate induced the 60% of neurotoxicity after 24 h exposure in cultured neurons. These excitotoxic neuronal deaths were weakly prevented by all of compounds (1 μM) (figure 2). The results revealed that suppression of NO production in LPS-treated microglia cell of the *trans*-flocoumafen is increased

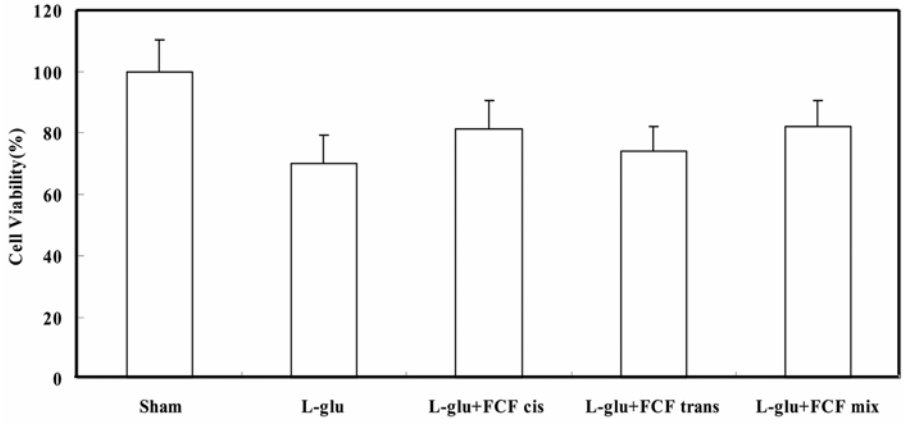


Figure 2. Inhibition of glutamate-induced neurotoxicity in cultured cortical neurons. Glutamate (60 μM) and compounds (1 μM) were applied for 24 h at 37°C. After incubation of neurons with water soluble tetrazolium salts WST-1 for 2 h, the compounds were quantified spectrophotometrically. All values represent the mean ± S.E. of three independent experiments performed in triplicate.

much more than that of the *cis*-flocoumafen due to the favourable electronic conjugation system. We have found that the *trans*-FCF exhibited significantly high efficacy comparable to mix-FCF or *cis*-FCF *in vitro* LPS-induced NO generation, while all of them did not show significant neurotoxicity in cultured cortical neurons.

4. Conclusion

We synthesized flocoumafen **1** from inexpensive and readily available materials and then separated it into *cis*-flocoumafen (*cis*-FCF) and *trans*-flocoumafen (*trans*-FCF). Their biological activities have been evaluated for the NO generation and anti-excitotoxicity *in vitro*. We expect that this simple synthesis of flocoumafen **1** and key fragments are useful for the synthesis of flocoumafen analogues.

Acknowledgements

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