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Original article

# Synthesis and anti-inflammatory effects of a series of novel 7-hydroxycoumarin derivatives

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ABSTRACT

A number of 7-hydroxycoumarins have been synthesised by Pechmann cyclisation using differently substituted resorcinols employing perchloric acid as the condensing agent. All the compounds have been characterised by analytical and spectroscopic methods. The anti-inflammatory properties were tested with LPS-induced inflammation in J774 macrophages. Expression of iNOS and COX-2 was determined by Western blot, NO by nitrite assay and IL-6 by ELISA analyses. Fifteen of the tested 7-hydroxycoumarins also inhibited IL-6 production but none of them had any major inhibitory effect on COX-2 expression.


## 1. Introduction

Coumarins, also known as 2*H*-benzo-1-pyran-2-ones, are naturally occurring heterocyclic compounds that contain oxygen [1]. In the literature, some coumarin derivatives have been reported to inhibit NO production [2,3]. Further studies have shown some plant derived coumarins to inhibit the activation of NF-κB [4]. This transcriptional factor is known to bind to the promoter region of iNOS gene and to play a pivotal role in LPS mediated iNOS expression in macrophages and other cells.

A few studies on the synthesis of 7-hydroxycoumarins have been reported [5,6], and these served as a starting point for our more extensive approach. Some 7-methoxycoumarins have been reported to inhibit the iNOS – NO pathway [7]. Coumarins are metabolised to 7-hydroxycoumarins in man [8] and reported activities of different coumarins may be partially related to their metabolites.

In this study, we focused on 7-hydroxycoumarins because it can help to design new pro-drugs based on coumarin skeleton. We synthesised twenty-two 7-hydroxycoumarin derivatives and investigated systematically their anti-inflammatory effects on the expression of inflammatory enzymes iNOS and COX-2, and on the production of inflammatory factors NO and IL-6 in activated macrophages.

## 2. Chemistry

A sulphuric acid or perchloric acid catalysed von Pechmann reaction is a common method to prepare 4-substituted coumarins in a laboratory scale [5]. Knoevenagel, Wittig and Perkin reactions have also been used [6,9]. The differences in reactions are obvious if we examine the starting materials for them. In Knoevenagel, Wittig and Perkin reactions, salicylaldehyde with β-ketoester, triphenylphosphine derivative or anhydride, respectively, have been used as starting materials, while in the von Pechmann reaction, substituted phenols and β-ketoesters serve as starting materials. In commercial use, the Knoevenagel and Perkin reactions are used more often than the von Pechmann reaction.

In the literature, reactions in perchloric acid and at a temperature above 80 °C have been left to proceed for 3–5 h [5]. Experiments have also been carried out in sulphuric acid, in which the addition of β-ketoester has been done at 0 °C [10]. However, we

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used a perchloric acid catalysed von Pechmann reaction (Scheme 1) at room temperature. This was expected to diminish side-reactions but may also have an effect on reaction time or yield. We conducted every step, including the dissolving resorcinol to acid, the addition of β-ketoester and reaction, at room temperature. The perchloric acid is practical to use because it does not give rise to warming in contrast to sulphuric acid, which heats up in pouring the reaction mixture into the water. The desired coumarins were prepared from properly substituted β-ketoesters and resorcinols. The studied coumarin derivatives are presented in Scheme 1.

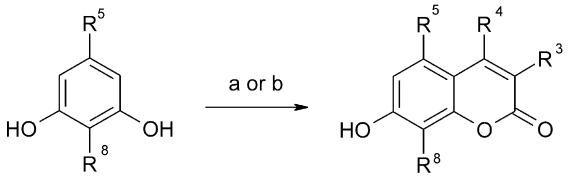
Five of the twenty-two coumarins (**14**, **17**, **18**, **21** and **22**) reported here are new compounds. All the compounds were synthesised by a modified method which has not previously been reported in the literature. The differences are in principal used catalytic acid and the conduction of the reaction at room temperature. The mechanism of the von Pechmann reaction has been presented in the literature. The mechanism points out the importance of an acidic catalyst by showing the ketoester activation step in the beginning of the reaction [11].

Compounds **1** and **3** were prepared using a different method for producing 4-unsubstituted compounds from resorcinol and ethylpropiolate. Dioxane was used as the solvent and zinc chloride as the catalyst [12]. The reaction conditions had to be kept dry because of the catalyst. This method gave lower yields than the acidic von Pechmann reaction.

Crude products were recrystallised from ethanol and to analyse the purity and structure of the compounds we measured standard $^{1}H$ NMR, $^{13}C$ NMR and IR spectra. The purity of all compounds was confirmed by combustion analysis. Also, the exact masses of the $[M - H]^-$ ions of the studied compounds were measured. The skeletal structure was studied by X-ray crystallographic determination of three compounds **10**, **21** and **22**.

## 3. Pharmacology

In the present study, we aimed to investigate the possible anti-inflammatory effects of 7-hydroxycoumarins because they are important metabolites of coumarins in man [8]. Since some 7-oxycoumarins have been reported as potential anti-inflammatory compounds the study of 7-hydroxycoumarins may reveal new possibilities to development of coumarin based drugs to treat diseases caused by inflammation.




**1.** $R^3 = R^4 = R^5 = R^8 = H$
**2.** $R^3 = R^5 = R^8 = H$, $R^4 = Me$
**3.** $R^3 = R^4 = R^8 = H$, $R^5 = CH_3$
**4.** $R^3 = R^8 = H$, $R^4 = R^5 = Me$
**5.** $R^3 = R^5 = H$, $R^4 = R^8 = Me$
**6.** $R^3 = R^4 = Me$, $R^5 = R^8 = H$
**7.** $R^3 = F$, $R^4 = Me$, $R^5 = R^8 = H$
**8.** $R^3 = R^4 = R^5 = Me$, $R^8 = H$
**9.** $R^3 = Et$, $R^4 = Me$, $R^5 = R^8 = H$
**10.** $R^3 = R^5 = R^8 = H$, $R^4 = Pr$
**11.** $R^3 = R^5 = H$, $R^4 = Me$, $R^8 = OMe$
**12.** $R^3 = Et$, $R^4 = R^5 = Me$, $R^8 = H$
**13.** $R^3 = Et$, $R^4 = R^8 = Me$, $R^5 = H$
**14.** $R^3 = R^8 = H$, $R^4 = Pr$, $R^5 = Me$, $R^8 = H$
**15.** $R^3 = R^8 = H$, $R^4 = iPr$, $R^5 = Me$
**16.** $R^3 = R^4 = Me$, $R^5 = H$, $R^8 = OMe$
**17.** $R^3 = F$, $R^4 = Me$, $R^5 = H$, $R^8 = OMe$
**18.** $R^3 = R^5 = H$, $R^4 = Pr$, $R^8 = OMe$
**19.** $R^3 = R^5 = R^8 = H$, $R^4 = Ph$
**20.** $R^3 = R^5 = H$, $R^4 = Ph$, $R^8 = Me$
**21.** $R^3 = R^5 = R^8 = H$, $R^4 = 2\text{-}FC_6H_4$
**22.** $R^3 = R^5 = H$, $R^4 = Ph$, $R^8 = OMe$

Reaction conditions: a: i) ethylpropiolate. dioxane. ii) dry $ZnCl_2$, reflux, 24 h, iii) 5% HCl. b: i) $HClO_4$, ii) $R^4COCH(R^3)CO_2Et$, RT, iii) $H_2O$

**Scheme 1.** Syntheses of 7-hydroxycoumarins via methods a and b.

The effects of the 7-hydroxycoumarins on NO production and iNOS expression were investigated in macrophages activated by exposing them to an inflammatory stimulus, lipopolysaccharide (LPS), which activates the cells through a toll-like-receptor 4 (TLR4) pathway. To evaluate the mechanism of action, three of the most effective compounds were selected for studies, in which their effects on iNOS mRNA expression and on the activation of NF-κB, an important transcription factor for iNOS gene, were measured. In addition, effects of the compounds on the expression of an inflammatory enzyme COX-2 (which produces prostaglandins) and on a proinflammatory cytokine IL-6 were also investigated. Measurements were carried out with two concentrations of the tested 7-hydroxycoumarins, 10 μM and 100 μM.

The pharmacological studies on the newly synthesised compounds were started by cytotoxicity testing. Cytotoxicity as a contributing factor was ruled out by using a XTT test. The test measures cells' mitochondrial dehydrogenase activity that only occurs in viable cells. Triton-X (0.1%) was used as a positive control of cell death.

## 4. Results and discussion

### 4.1. Synthesis

Investigation of the crude products indicated good purity and in consequence of room temperature the diminution of the side-reactions was more significant than the elongated reaction time. The monitoring of reactions by TLC indicated the reaction time to be mostly from 4 to 6 h. Longer period did not increase the yield remarkably.

The compound **15** differed from the others by both the needed reaction time and the isolation method. The best result was achieved within a period of one and a half day. The precipitated product started immediately to decompose after the reaction mixture was poured into water. The extraction of diluted reaction mixture with ethyl acetate, followed directly by neutralisation of the organic layers with sodium bicarbonate solution was used to protect the product. The combined organic phases were washed several times with water before drying and evaporation.

The zinc chloride catalysed reaction used is very sensitive to moisture and both dioxane and zinc chloride should be dried carefully before the use. The perchloric acid catalysed von Pechmann reaction at elevated temperature to produce 4-unsubstituted 7-hydroxycoumarins has been reported in literature [5].

In the perchloric acid catalysed von Pechmann reaction used starting materials were DL-malic acid and resorcinol. The study indicated the acid being capable to activate malic acid partially but at room temperature the reaction did not produce any detectable coumarin structure.

### 4.2. Inhibition of NO production

All of the compounds significantly inhibited LPS-induced NO production when used at 100 μM concentrations, and nine compounds inhibited it by more than 80% as compared to cells treated with LPS alone. These nine compounds were 5-methyl (**3**), 3,4-dimethyl (**6**), 3,4,5-trimethyl (**8**), 3-ethyl-4-methyl (**9**), 3-ethyl-4,8-dimethyl (**13**), 8-methoxy-4-propyl (**18**), 4-phenyl (**19**), 4-(-2-$FC_6H_4$) (**21**) and 8-methoxy-4-phenyl (**22**) derivatives. Five of the compounds (**8**, **18**, **19**, **21** and **22**) also inhibited LPS-induced NO production by more than 50% at the lower concentration tested (10 μM) (Table 1). A highly selective iNOS inhibitor 1400W [13] was used as a positive control compound. 1400W (1000 μM) inhibited NO production in LPS-stimulated cells by 99%.

**Table 1**
The effect of 7-hydroxycoumarins on LPS-induced NO production and iNOS protein expression in J774 macrophages.

| Compound | NO production (inhibition %) | | iNOS protein expression (inhibition %) | |
|---|---|---|---|---|
| | 10 μM | 100 μM | 10 μM | 100 μM |
| **1** | 5.5 ± 1.2 | 64.4 ± 2.3 | 10.0 ± 12.1 | 47.2 ± 7.3 |
| **2** | −14.2 ± 2.5 | 42.9 ± 5.4 | 22.1 ± 12.4 | 61.8 ± 8.7 |
| **3** | 9.2 ± 2.7 | 80.0 ± 1.9 | 2.7 ± 5.4 | 86.2 ± 3.9 |
| **4** | −8.6 ± 2.8 | 71.0 ± 1.6 | −19.7 ± 8.6 | 74.3 ± 3.5 |
| **5** | 17.3 ± 3.2 | 65.7 ± 1.9 | 20.1 ± 7.0 | 61.4 ± 1.2 |
| **6** | 37.6 ± 2.4 | 88.9 ± 0.6 | 28.6 ± 6.7 | 85.5 ± 0.4 |
| **7** | 7.4 ± 4.5 | 62.8 ± 3.0 | 46.5 ± 7.5 | 53.0 ± 1.3 |
| **8** | 51.8 ± 3.1 | 92.4 ± 0.8 | 49.6 ± 1.6 | 94.7 ± 1.5 |
| **9** | 43.3 ± 1.4 | 92.3 ± 0.1 | 7.5 ± 6.4 | 91.0 ± 1.1 |
| **10** | 25.3 ± 1.3 | 67.6 ± 2.7 | 25.3 ± 4.2 | 89.7 ± 1.3 |
| **13** | 46.5 ± 1.9 | 87.0 ± 0.9 | 18.9 ± 3.2 | 65.2 ± 1.1 |
| **14** | 38.5 ± 2.0 | 62.5 ± 2.3 | 7.8 ± 4.3 | 87.8 ± 1.8 |
| **16** | 21.7 ± 6.2 | 50.5 ± 1.5 | 18.0 ± 8.3 | 74.2 ± 5.9 |
| **17** | 15.8 ± 1.5 | 50.2 ± 2.4 | 2.7 ± 5.4 | 86.2 ± 3.9 |
| **18** | 57.0 ± 2.0 | 83.7 ± 0.8 | 13.4 ± 6.7 | 74.6 ± 2.2 |
| **19** | 54.8 ± 2.9 | 85.0 ± 0.2 | 46.5 ± 5.6 | 93.0 ± 1.3 |
| **21** | 50.4 ± 3.3 | 87.5 ± 0.9 | 48.8 ± 6.0 | 95.4 ± 2.1 |
| **22** | 71.5 ± 3.6 | 81.8 ± 1.8 | 55.9 ± 2.3 | 84.6 ± 3.2 |

Mean ± SEM, $n = 4$.

In inflammation, NO is principally formed in a reaction catalysed by iNOS which is an inducible enzyme. Therefore, we investigated if the 7-hydroxycoumarins also effect on iNOS expression, which was studied by Western blot analysis. Unstimulated cells did not express detectable amounts of iNOS protein, but LPS enhanced iNOS expression considerably. All tested 7-hydroxycoumarins suppressed iNOS expression significantly when studied at a 100 μM concentration, and ten of them (**3**, **6**, **8**, **9**, **10**, **14**, **17**, **19**, **21** and **22**) inhibited iNOS protein expression by more than 80%. With the lower concentration (10 μM), most compounds had a minor inhibitory effect on iNOS expression, and five of them (**7**, **8**, **19**, **21** and **22**) inhibited iNOS expression by more than 45% (Table 1). Recent study on 3-(1′,1′-dimethyl-allyl)-6-hydroxy-7-methoxycoumarin isolated from *Ruta graveolens* L. indicated it to inhibit NO production and iNOS expression through suppression of NF-κB activation in a macrophage cell line. *In vivo* experiments on endotoxin-induced inflammation in mice the effect was 75% (i.p. injection, 1 mg/25 g body weight) [14]. Also, oxycoumarin derivatives have been shown to inhibit iNOS expression and NO production, most effective compounds were the 5,7-dimethoxycoumarins, which inhibited NO production up to 95% with 50 μM in RAW245.7 cells stimulated with LPS [7]. Sesquiterpene coumarins extracted from roots of Ferula Fukanensis have also been shown to inhibit NO production and iNOS expression (with IC50-values in range of 9–25 μM) [2].

Since NF-κB is an important transcription factor for iNOS expression we used pyrrolidinedithiocarbamate (PDTC), an inhibitor of NF-κB activation [15] as a positive control for inhibition of iNOS expression. PDTC (100 μM) inhibited iNOS expression by 97%.

### 4.3. Anti-inflammatory mechanism of coumarins

To investigate the mechanisms of action in more detail, we measured the effects of three of the most potent 7-hydroxycoumarins (**6**, **8** and **9**) on iNOS mRNA expression, and on the activation of NF-κB. iNOS mRNA was measured by quantitative RT-PCR after 3 h incubation with a combination of LPS and the tested compounds. The iNOS mRNA levels were very low in resting cells, but they increased significantly when the cells were exposed to LPS. The three compounds inhibited LPS-induced iNOS mRNA expression by more than 50%. Activation of the transcription factor NF-κB was investigated by measuring the nuclear translocation of the p65 subunit of NF-κB by Western blotting. LPS induced NF-κB translocation, and it peaked at 30 min after the addition of LPS. All three tested 7-hydroxycoumarins inhibited LPS-stimulated NF-κB p65 translocation by more than 65%. A previously known NF-κB inhibitor PDTC [15] was used as a control compound, and when used at 100 μM concentrations it inhibited NF-κB activation by more than 95%.

### 4.4. Inhibition of IL-6 production

We also tested the effects of 7-hydroxycoumarins on the LPS-induced production of proinflammatory cytokine IL-6, by measuring its concentration in the cell culture medium by immunoassay. IL-6 production in resting cells was very low, but it was significantly enhanced when cells were activated by adding LPS. With the higher concentration used (100 μM), eight of the studied compounds (**5**, **6**, **8**, **9**, **10**, **13**, **19** and **21**) inhibited IL-6 production by more than 80%, and only two compounds (**3** and **17**) had an inhibitory effect smaller than 30%. With the lower concentration used (10 μM), ten compounds inhibited IL-6 production slightly but for none of them was the inhibition over 30% (Table 2). Dexamethasone (10 μM) was used as a control compound and inhibited IL-6 production by 74%.

### 4.5. Effect on COX-2 production

The effect of 7-hydroxycoumarins on LPS-stimulated COX-2 expression in J774 macrophages was also tested. None of the studied eighteen compounds inhibited COX-2 expression in a significant manner (Table 3). Dexamethasone (10 μM) was used as a control compound and inhibited COX-2 expression by 87%.

### 4.6. Cytotoxicity

None of the compounds at concentrations of 100 μM showed cytotoxic effects. XTT testing was carried out in similar conditions where the experiments for anti-inflammatory activities were carried out.

**Table 2**
The effect of 7-hydroxycoumarins on LPS-induced IL-6 production in J774 macrophages.

| Compound | IL-6 production (inhibition %) | |
|---|---|---|
| | 10 μM | 100 μM |
| **1** | 3.2 ± 3.5 | 34.1 ± 1.1 |
| **2** | 4.6 ± 5.2 | 44.1 ± 2.5 |
| **3** | −22.5 ± 2.6 | 4.2 ± 4.9 |
| **4** | 14.5 ± 4.8 | 66.4 ± 0.9 |
| **5** | 17.5 ± 1.9 | 82.8 ± 0.45 |
| **6** | 16.7 ± 1.5 | 85.7 ± 0.8 |
| **7** | 21.2 ± 4.4 | 38.1 ± 3.7 |
| **8** | −34.2 ± 6.3 | 92.2 ± 1.4 |
| **9** | 27.9 ± 2.4 | 90.3 ± 0.3 |
| **10** | 25.4 ± 4.2 | 88.9 ± 0.7 |
| **13** | 26.1 ± 3.3 | 94.9 ± 0.1 |
| **14** | 12.2 ± 2.7 | 54.3 ± 5.8 |
| **16** | −0.9 ± 7.0 | 43.4 ± 2.9 |
| **17** | 10.7 ± 5.2 | 14.8 ± 4.5 |
| **18** | 11.0 ± 4.1 | 73.4 ± 1.0 |
| **19** | −13.8 ± 8.8 | 95.7 ± 0.9 |
| **21** | 0.3 ± 4.4 | 92.7 ± 0.5 |
| **22** | −21.8 ± 3.3 | 46.9 ± 3.7 |

Mean ± SEM, $n = 4$.

**Table 3**
The effect of 7-hydroxycoumarins on LPS-induced COX-2 expression in J774 macrophages.

| Compound | COX-2 protein expression (inhibition %) | |
|---|---|---|
| | 10 μM | 100 μM |
| **1** | −17.2 ± 9.6 | −20.4 ± 8.9 |
| **2** | 3.2 ± 3.2 | 22.7 ± 6.7 |
| **3** | −44.1 ± 9.2 | −60.4 ± 8.3 |
| **4** | −14.8 ± 7.2 | −17.5 ± 8.6 |
| **5** | 2.3 ± 2.1 | 7.8 ± 3.2 |
| **6** | −57.7 ± 9.3 | 3.7 ± 6.8 |
| **7** | −2.2 ± 3.8 | −25.1 ± 4.6 |
| **8** | −31.7 ± 6.5 | 11.1 ± 10.3 |
| **9** | −29.9 ± 8.9 | −73.2 ± 13.0 |
| **10** | −11.1 ± 8.3 | 25.0 ± 2.7 |
| **13** | −36.8 ± 3.2 | 21.0 ± 1.5 |
| **14** | −6.8 ± 11.6 | −45.6 ± 9.3 |
| **16** | −12.5 ± 11.4 | −16.3 ± 11.9 |
| **17** | −15.4 ± 4.2 | −36.8 ± 5.7 |
| **18** | −38.9 ± 10.0 | 8.6 ± 4.5 |
| **19** | −47.1 ± 8.3 | −0.4 ± 6.6 |
| **21** | −34.7 ± 9.3 | −16.6 ± 9.2 |
| **22** | −59.1 ± 8.4 | −34.7 ± 5.3 |

Mean ± SEM, $n = 4$.

### 4.7. Structure–activity relationship

When investigating the structure–activity relationship, some observations can be made. All three compounds containing phenyl ring at position 4 were highly active in inhibiting NO production and iNOS expression. From the five tested 3-alkyl substituted compounds only 7-hydroxy-8-methoxy-3,4-dimethylcoumarin (**16**) did not show high iNOS, NO and IL-6 inhibition activity. High activities indicate the activation effect of 3-alkyl substitution to be significant. The reason for lower activity of compound **16** is supposed the methoxy group being capable to hinder the membrane solubility. The effect is not clear, because in comparison the 4-phenyl and the 4-phenyl-8-methoxy compounds (**19** and **22**) the activity was only slightly lowered by methoxy group. The reason for this difference could be based on the big electrostatic effect of the phenyl which diminish the effect of methoxy group. However, a lipophilic substituent at position 3 increases the activity whereas the 3-fluorine does not activate the compounds.

## 5. Conclusions

Twenty 7-hydroxycoumarins were synthesised by a new modification of the perchloric acid catalysed von Pechmann reaction and two by a zinc chloride catalysed reaction. The procedure differed from earlier in the reaction temperature. Instead of classical heating the reaction was kept at room temperature. The method is practical because it showed only a low appearance of side-reactions and required only a little longer reaction time.

Also, 7-hydroxycoumarins inhibit iNOS expression and NO and IL-6 production in a dose-dependent manner at non-cytotoxic concentrations. Based on the experiments, the 7-hydroxycoumarins also inhibit LPS-induced iNOS mRNA expression and nuclear translocation (i.e. activation) of NF-κB. The inhibition of COX-2 expression is only slightly affected which increase their value as selective inhibitors. Phenyl ring at position 4 or a lipophilic substituent at position 3 increases the inhibition. However, the 3-fluorine does not activate the compounds.

In conclusion, these findings suggest that 7-hydroxycoumarins inhibit the early signalling mechanisms needed to induce transcription of iNOS gene in inflammation and introduce them as compounds with promising anti-inflammatory effects in conditions complicated by increased iNOS expression and NO production.

## 6. Experimental section

### 6.1. Materials and methods

The X-ray diffraction data were collected on a Nonius KappaCCD diffractometer using Mo Kα radiation ($\lambda = 0.710\ 73$ Å). The crystals were immersed in cryo-oil, mounted in a Nylon loop, and measured at a temperature of 100 K. Chemicals were used as received if not elsewise mentioned. Reactions were monitored by TLC using Merck Kieselgel 60 $F_{254}$ plates and ethyl acetate–toluene (1:1) as an eluent. Melting points were measured with GWB Gallenkamp Melting Point Apparatus and are uncorrected. NMR spectra were measured either with a Bruker Avance 250 (250 MHz for $^1$H, 62.9 MHz for $^{13}$C) or with a Bruker Avance 400 FT NMR (400 MHz for $^1$H, 100.6 MHz for $^{13}$C). IR spectra were measured with an Avatar 320 FT-IR. Combustion analyses were done using an Elementar Analysensystem GmBH varioMICRO.

Reagents were obtained as following: goat polyclonal mouse COX-2, rabbit polyclonal mouse iNOS, donkey anti-goat polyclonal, and goat anti-rabbit polyclonal antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

#### 6.1.1. Cell culture

J774 murine macrophages (American Type Culture Collection, Rockville, MD) were cultured in Dulbecco's modified Eagle's medium with glutamax-I. The culture media contained 10% heat-inactivated foetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 250 ng/mL amphotericin B (all from Gibco, Paisley, Scotland). Cells were seeded on 24-well plates, and the cell monolayers were grown for 72 h to confluence before the experiments were started and the compounds of interest were added in fresh culture medium.

#### 6.1.2. XTT test

Cell viability was tested by using Cell Proliferation Kit II, which measures cells' ability to metabolise sodium 30-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis-(4-methoxy-6-nitro) benzene sulphonic acid hydrate (XTT) to formazan by mitochondrial dehydrogenases that only occur in viable cells (Boehringer Mannheim, Indianapolis, IN, USA).

#### 6.1.3. Nitrite assays

At indicated time points, the culture medium was collected for nitrite measurement, which was used as a measure of NO production. Culture medium (100 μl) was incubated with 100 μl of Griess reagent (0.1% napthalethylenediamine dihydrochloride, 1% sulfanilamine, and 2.5% $H_3PO_4$), and the absorbance was measured at 540 nm. The concentration of nitrite was calculated with sodium nitrite as the standard.

#### 6.1.4. Preparation of cell lysates for Western blotting

At indicated time points, cells were rapidly washed with ice-cold phosphate-buffered saline (PBS) that contained 2 mM sodiumorthovanadate. The cells were resuspended in a lysis buffer containing 1% Triton-X, 50 mM NaCl, 10 mM Tris-base pH 7.4, 5 mM EDTA, 0.5 mM phenylmethylsulfonylfluoride, 1 mM sodiumorthovanadate, 40 μM leupeptin, 50 μg/mL aprotinin, 5 mM NaF, 2 mM sodiumpyrophosphate, and 10 μM N-octyl-β-D-glucopyranoside. After incubation for 15 min on ice, the lysates were centrifuged (13 500 g, 5 min). The protein content of the supernatants was measured by the Coomassie blue method.

#### 6.1.5. Preparation of nuclear extracts for Western blotting

At indicated time points, the cells were rapidly washed with ice-cold PBS and solubilised in hypotonic buffer A (10 mM HEPES-KOH

pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiotreitol, 0.2 mM phenylmethylsulfonylfluoride, 10 μg/mL leupeptin, 25 μg/mL aprotinin, 0.1 mM EGTA, 1 mM sodiumorthovanadate, and 1 mM NaF). After incubation for 10 min on ice, the cells were vortexed for 30 s and the nuclei were separated by centrifugation at 4 °C, 21 000 g for 10 s. The pellet was resuspended in buffer C (20 mM HEPES-KOH pH 7.9, 420 mM NaCl, 25% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiotreitol, 0.2 mM phenylmethylsulfonylfluoride, 10 μg/mL leupeptin, 25 μg/mL aprotinin, 0.1 mM EGTA, 1 mM sodiumorthovanadate, and 1 mM NaF) and incubated on ice for 20 min. Nuclei were vortexed for 30 s and nuclear extracts were obtained by centrifugation at 4 °C, 21 000 g for 2 min. The protein content of the supernatant was measured by the Coomassie blue method. The samples were boiled in a SDS sample buffer and stored at −20 °C.

*6.1.6. Western blot analysis*

After boiling for 5 min, equal aliquots of protein (20 μg) were loaded onto a 10% SDS-polyacrylamide electrophoresis gel and electrophoresed for 4 h at 100 V in a buffer containing 95 mM Tris–HCl, 960 mM glycine, and 0.5% SDS. After electrophoresis, the proteins were transferred to a Hybond enhanced chemiluminescence nitrocellulose membrane (Amersham, Buckinghamshire, U.K.) with a semi-dry blotter at 2.5 mA/$cm^2$ for 60 min. After transfer, the membrane was blocked in TBS/T (20 mM Tris-base pH 7.6, 150 mM NaCl, 0.1% Tween-20) containing 5% nonfat milk for 1 h at room temperature and incubated overnight at 4 °C with NF-κB p65, COX-2 or iNOS antibodies in TBS/T containing 5% nonfat milk. Thereafter, the membrane was washed 4 times in TBS/T for 5 min, incubated with a secondary antibody coupled to horseradish peroxidase in the blocking solution for 0.5 h at room temperature, and washed four times with TBS/T for 5 min. A bound antibody was detected using a SuperSignal West Pico chemiluminescent substrate (Pierce, Cheshire, U.K.) and a FluorChem 8800 imaging system (Alpha Innotech Corp., San Leandro, CA). The chemiluminescent signal was quantified by using FluorChem software version 3.1.

*6.1.7. RNA extraction and real-time RT-PCR*

At the indicated time points, cell monolayers were rapidly washed with ice-cold PBS, and cells were homogenised using a QIAshredder (QIAGEN, Valencia, CA). RNA extraction was carried out with the use of an RNeasy kit for isolation of total RNA (QIAGEN). Total RNA (25 ng) was reverse-transcribed to cDNA using TaqMan Reverse Transcription reagents and random hexamers (Applied Biosystems, Foster City, CA). cDNA obtained from the RT reaction (amount corresponding to approximately 1 ng of the total RNA) was subjected to PCR using a TaqMan Universal PCR Master Mix and an ABI PRISM 7000 Sequence detection system (Applied Biosystems). The primer and probe sequences and concentrations were optimised according to the manufacturer's guidelines in the TaqMan Universal PCR Master Mix Protocol part number 4304449 revision C, and were: 5′-CCTGGTACGGGCATTGCT-3′, 5′-GCTCATGC GGCCTCCTT-3′ (forward and reverse mouse iNOS primers, respectively, both 300 nM), 5′-CAGCAGCGGCTCCATGACTCCC-3′ (mouse iNOS probe of 150 nM, containing 6-FAM as 5′-reporter dye and TAMRA as 3′-quencher), 5′-GCATGGCCTTCCGTGTTC-3′ (mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward primer, 300 nM), 5′-GATGTCATCATACTTGGCAGGTTT-3′ (mouse GAPDH reverse primer, 300 nM), and 5′-TCGTGGATCT-GACGTGCCGCC-3′ (mouse GAPDH probe, 150 nM, containing 6-FAM as 5′-reporter dye and TAMRA as 3′-quencher). The PCR reaction parameters were as follows: incubation at 50 °C for 2 min, incubation at 95 °C for 10 min, and, thereafter, 40 cycles ofdenaturation at 95 °C for 15 s and annealing and extension at 60 °C for 1 min. Each sample was determinated in duplicate.

A standard curve method was used to determine the relative mRNA levels, as described in the Applied Biosystems User Bulletin: a standard curve for each gene was created using RNA isolated from LPS-stimulated J774 macrophages. Isolated RNA was reverse-transcribed and the dilution series of cDNA ranging from 1 pg to 10 ng were subjected to real-time PCR. The obtained threshold cycle values were plotted against the dilution factor to create a standard curve. Relative mRNA levels in the test samples were then calculated from the standard curve. When calculating the results the iNOS mRNA levels were first normalised against GAPDH.

*6.1.8. Enzyme-linked immunosorbent assay (ELISA)*

Culture medium samples were kept at −20 °C until assayed. The concentration of mouse IL-6 (DuoSet® ELISA, R&D Systems Europe Ltd, Abindgon, U.K.) was determined by ELISA according to the manufacturer's instructions.

*6.1.9. Statistics*

The results are expressed as the mean ± S.E.M. Statistical significances of the differences were calculated by analyses of variance, supported by the Dunnett's multiple comparisons test. Differences were considered significant at $P < 0.05$.

*6.2. Preparation of studied compounds*

*6.2.1. General method for 4-unsubstituted 7-hydroxycoumarins (**1**, **3**)*

Properly substituted resorcinol (100 mmol) was dissolved in dioxane (100 ml) and ethylpropiolate (100 mmol), and dried zinc chloride (100 mmol) was added. The mixture was refluxed for 24 h. After cooling to room temperature, 100 ml of 5% HCl was added slowly. After addition, the mixture was concentrated to half volume and kept at +4 °C. The precipitate was filtered and dried at room temperature. The product was recrystallised from ethanol. Analytical data of compounds **1** and **3** is presented in Supplementary data.

*6.2.2. General method for 4-substituted 7-hydroxycoumarins (**2**, **4**–**22**)*

30 mmol of properly substituted resorcinol was dissolved in 30 ml of perchloric acid. 30 mmol of β-ketoester was added and the reaction mixture was stirred at room temperature until TLC showed that resorcinol had reacted. The mixture was poured into 250 ml of cold water and the precipitate was filtered and dried at room temperature and recrystallised from ethanol. Analytical data of compounds **2**–**13**, **15**, **16**, **19** and **20** is presented in Supplementary data.

*6.2.3. 7-Hydroxy-5-methyl-4-propyl-2H-benzo-1-pyran-2-one (**14**)*

Yield 86%. mp 165–167 °C. TLC $R_f = 0.685$ (ethyl acetate/toluene, 1:1, v/v). $^1$H NMR ($d_6$-DMSO) $\delta$ (ppm): 0.96 (t, 3H, $J = 7.5$ Hz, 3′-$CH_3$), 1.61 (sxt, 2H, $J = 7.4$ Hz, 2′-$CH_2$), 2.28 (s, 3H, 5- $CH_3$), 2.90 (t, 2-H, $J = 7.9$ Hz, 1′-$CH_2$), 6.02 (s, 1H, H-3), 6.59 (d, 1H, $J = 1.47$ Hz, H-6), 6.63 (d, 1H, $J = 1.47$ Hz, H-8), 10.61 (s, 1H, OH). $^{13}$C NMR ($d_6$-DMSO) $\delta$ (ppm): 13.71, 20.96, 22.38, 37.17, 105.74, 107.84, 111.25, 112.06, 142.52, 155.10, 155.86, 158.00, 159.83. IR (KBr): 3231, 2962, 2875, 1681, 1628, 1601, 1512, 1457, 1398, 1334, 1283, 1250, 1213, 1153, 1095, 1047, 1015, 996, 926, 887, 825, 705. Anal. calcd for $C_{13}H_{14}O_3$: C 71.54, H 6.47; found C 71.37, H 6.59. ESI-MS ($m/z$) 217.0876 ([M − H]$^-$).

*6.2.4. 3-Fluoro-7-hydroxy-8-methoxy-4-methyl-2H-benzo-1-pyran-2-one (**17**)*

Yield 96%. mp 168–170 °C. TLC $R_f = 0.625$ (ethyl acetate/toluene, 1:1, v/v). $^1$H NMR ($d_6$-DMSO) $\delta$ (ppm): 2.32 (d, 3H, $J = 2.9$ Hz, 4-$CH_3$), 3.83 (s, 3H, $OCH_3$), 6.95 (d, 1H, $J = 8.9$ Hz, H-6), 7.34 (d, 1H, $J = 8.9$ Hz, H-5), 10.37 (br, 1H, OH). $^{13}$C NMR ($d_6$-DMSO) $\delta$ (ppm): 10.19, 61.77, 113.94, 115.21, 121.27, 133.95, 134.16, 136.11, 146.04,

154.29, 157.07. IR (KBr): 3259, 3006, 2943, 2842, 1716, 1641, 1600, 1514, 1464, 1437, 1384, 1361, 1329, 1291, 1242, 1198, 1160, 1115, 1021, 1000, 936, 822, 749. Anal. calcd for $C_{11}H_9O_4F$: C 58.93, H 4.05; found C 58.73, H 4.40. ESI-MS ($m/z$) 223.0411 ([M − H]$^-$).

*6.2.5. 7-Hydroxy-8-methoxy-4-propyl-2H-benzo-1-pyran-2-one (**18**)*

Yield 89%. mp 129–131 °C. TLC $R_f = 0.603$ (ethyl acetate/toluene, 1:1, v/v). $^1$H NMR ($d_6$-DMSO) $\delta$ (ppm): 0.92 (t, 3H, $J$ = 7.4 Hz, 3′-$CH_3$), 1.56 (sxt, 2H, $J$ = 7.4 Hz, 2′-$CH_2$), 2.66 (t, 2H $J$ = 7.4 Hz, 1′-$CH_2$), 3.77 (s, 3H, $OCH_3$), 6.07 (s, 1H, H-3), 6.84 (d, 1H, $J$ = 8.8 Hz, H-6), 7.36 (d, 1H, $J$ = 8.8 Hz, H-5), 10.48 (br, 1H, OH). $^{13}$C NMR ($d_6$-DMSO) $\delta$ (ppm): 13.58, 21.37, 32.90, 60.54, 109.28, 112.06, 113.04, 120.19, 134.28, 147.85, 153.42, 157.41, 160.15. IR (KBr): 3268, 2963, 2938, 2873, 1726, 1680, 1601, 1512, 1457, 1434, 1387, 1352, 1264, 1201, 1166, 1056, 1006, 933, 843, 809, 721. Anal. calcd for $C_{13}H_{14}O_4$: C 66.66, H 6.02; found C 66.54, H 6.15. ESI-MS ($m/z$) 233.0819 ([M − H]$^-$).

*6.2.6. 7-Hydroxy-4-(2-fluoro-phenyl)-2H-benzo-1-pyran-2-one (**21**)*

Yield 98%. mp 224–225 °C. TLC $R_f$ = 0.607 (ethyl acetate/toluene, 1:1, v/v). $^1$H NMR ($d_6$-DMSO) $\delta$ (ppm): 6.26 (s, 1H, H-3), 6.76 (d, 1H, $J$ = 8.7 Hz, H-6), 6.80 (s, 1H, H-5), 7.05 (d, 1H, $J$ = 8.7 Hz, H-8), 7.50 (m, 4H, $C_6H_4F$), 10.76 (s, 1H, OH). $^{13}$C NMR ($d_6$-DMSO) $\delta$ (ppm): 104.43, 112.46, 113.91, 115.27, 117.77, 124.42, 126.98, 129.77, 132.61, 133.73, 152.10, 157.01, 158.40, 161.81, 163.47. IR (KBr): 3344, 3155, 1693, 1597, 1563, 1490, 1446, 1377, 1308, 1263, 1237, 1152, 1119, 1090, 1001, 943, 849, 817, 765. Anal. calcd for $C_{15}H_9O_3F$: C 70.31, H 3.54; found C 70.13, H 3.85. ESI-MS ($m/z$) 255.0463 ([M − H]$^-$).

*6.2.7. 7-Hydroxy-8-methoxy-4-phenyl-2H-benzo-1-pyran-2-one (**22**)*

Yield 94%. mp 181 °C. TLC $R_f = 0.622$ (ethyl acetate/toluene, 1:1, v/v). $^1$H NMR ($d_6$-DMSO) $\delta$ (ppm): 3.86 (s, 3H, $OCH_3$), 6.18 (s, 1H, H-3), 6.86 (d, 1H, $J$ = 8.7 Hz, H-6), 7.02 (d, 1H, $J$ = 8.7 Hz, H-5), 7.55 (m, 5H, $C_6H_5$), 10.54 (br, 1H, OH). $^{13}$C NMR ($d_6$-DMSO) $\delta$ (ppm): 60.53, 110.37, 111.50, 113.20, 121.99, 128.35, 128.72, 129.50, 134.54, 135.12, 148.28, 153.82, 155.66, 159.70. IR (KBr): 3344, 1695, 1602, 1564, 1474, 1429, 1362, 1308, 1287, 1201, 1119, 1078, 849, 823. Anal. calcd for $C_{16}H_{12}O_4$: C 71.64, H 4.51; found C 71.27, H 4.76. ESI-MS ($m/z$) 267.0660 ([M − H]$^-$).

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## Appendix. Supplementary information

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejmech.2011.05.052.

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