7-(2-Oxoalkoxy)coumarins: Synthesis and Anti-Inflammatory Activity of a Series of Substituted Coumarins

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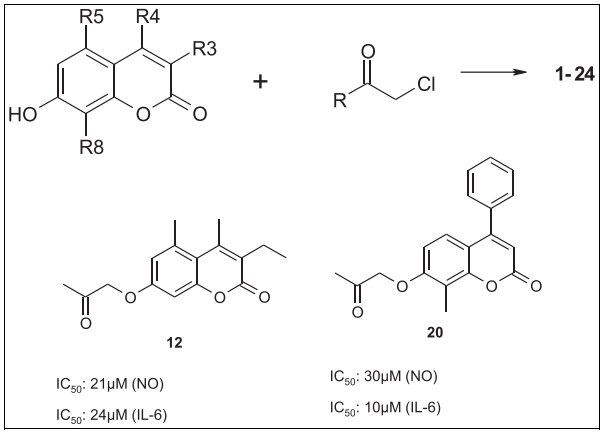
Additional Supporting Information may be found in the online version of this article.
Received April 28, 2013
DOI 10.1002/jhet.2173
Published online 00 Month 2014 in Wiley Online Library (wileyonlinelibrary.com).



A series of 7-(2-oxoalkoxy)coumarins have been synthesized by conjugating substituted 7-hydroxycoumarins with different chloroketones. The anti-inflammatory properties of 7-(2-oxoalkoxy)coumarins were studied in LPS-induced inflammatory response in J774 macrophages. Western blot was used to determine the expression of iNOS and COX-2, NO was determined by measuring its metabolite nitrite by Griess reaction and IL-6 was measured by ELISA. Seventeen of the studied compounds inhibited NO and IL-6 production over 50% at 100 μM concentrations. IC_{50} values of the best inhibitors were 21 μM/24 μM (NO/IL-6) for compound **12** and 30 μM/10 μM (NO/IL-6) for compound **20**. The main result was that the substitution with 7-(2-oxoalkoxy) group improved the anti-inflammatory properties of most of the investigated 7-hydroxycoumarins.

J. Heterocyclic Chem., **00,** 00 (2014).

INTRODUCTION

Coumarins are widely presented in plants, including Orchidaceae, Rutaceae, and Umbelliferae, and both synthetic and plant-derived coumarins are utilized in a wide range of applications in chemical, pharmaceutical, and agricultural industry [1–3]. Coumarins are also important ingredients of plants used as anti-inflammatory remedies in traditional medicine [4–6]. There are, however, limited data on the anti-inflammatory effects of plant-derived coumarins [7] and their synthetic derivatives [8].

7-Acetonyloxycoumarins or 7-(2-oxopropoxy)coumarins (Fig. 1) are widely used as intermediates for synthetic furocoumarins namely psoralens and angelicins [9–11]. Some applications for other 7-(2-oxoalkoxy)coumarins have been developed such as the use of cyclic and acyclic coumaryloxy-ketones as fluorogenic substrates in an assay for monooxygenase enzyme activity [10]. Also, compounds of this structure other than 7-acetonyloxycoumarins are used as intermediates to furocoumarins [12] and coumarin-based structures, geiparvarins [13].

Nitric oxide (NO) is a gaseous signaling molecule that mediates various physiological and pathophysiological responses in the human body, e.g., in the immune response and inflammation, in cardiovascular system, and in neurotransmission. *In vivo*, NO is synthesized from amino acid L-arginine and molecular oxygen (O_2) in a reaction catalyzed by enzyme nitric oxide synthase (NOS). Three different forms of NOS have been identified, namely endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS) [14–18]. eNOS and nNOS are calcium-dependent constitutive enzymes that are responsible, in general, for the low physiological NO production. In inflammation, pro-inflammatory cytokines and bacterial products trigger iNOS expression in inflammatory and tissue cells, and iNOS is responsible for prolonged production of increased amounts of NO in the inflammatory focus [16,17,19]. At high concentrations, NO has pro-inflammatory and destructive properties, and selective iNOS inhibitors have been proven anti-inflammatory in experimentally induced arthritis and several other inflammatory models [20–22].

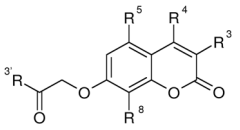


Figure 1. Structure of studied 7-(2-oxoalkoxy)coumarins 1–24.

Our previous study considering 7-hydroxycoumarins [23] showed potency of this scaffold as an inhibitor of several inflammatory processes. A few studies have been carried out using 7-oxycoumarins [24] but other oxygen-linked substituents at 7-position have not yet been published. Plant derived coumarins, i.e., 3-(1′-1′-dimethyl-allyl)-6-hydroxy-7-methoxy-coumarin isolated from *Ruta graveolens L.* and sesquiterpene coumarins extracted from roots of *Ferula Fukanensis* and oxycoumarin derivatives, have been shown to inhibit NO production and iNOS expression [7,24–26]. Because these 7-oxygen-substituted coumarins (7-methoxycoumarins) have been found to inhibit NO production, we were interested to investigate effects of 2-oxoalkoxygroup at the same position. These earlier studies encouraged us to investigate the effects of replacing 7-OH by 7-(2-oxoalkoxy). In this study, we synthesized twenty-four 7-(2-oxoalkoxy)coumarin derivatives and investigated their effects on the expression of inflammatory enzymes iNOS and COX-2, and on the production of inflammatory mediators NO and IL-6 in activated macrophages.

RESULTS AND DISCUSSION

Syntheses. To alter the structure of previously studied 7-hydroxycoumarins, we combined the coumarin scaffold with 2-oxoalkoxy group (Scheme 1). Twenty-two of synthesized compounds were substituted with the aid of chloroacetone and two compounds with phenacylchloride or 1-chloropinacolone. The method we used is a known substitution reaction of phenolic hydroxyl group. It was reported first time in the literature with phenol [27] and β-naphtol [28] and more recently with dihydroxynaphtalen [29].

Compounds **1–22** were synthesized by reaction of suitable 7-hydroxycoumarin and chloroacetone in the presence of K_2CO_3 and acetone as solvent. Usually, the mixture turned slightly brownish-yellow within reaction. The potassium carbonate bound the hydrogen chloride formed in conjugation by forming potassium chloride, which is presented as a very fine powder. To prevent the dissolution of inorganic salts during isolation, the acetone used was dried with drying agent before using.

Compound **23** containing phenacyl instead of acetyl group was prepared in the same manner as compounds **1–22**. The only exception was the use of phenacyl chloride in molar ratio 1:1 to coumarin rather than excess of chloride. The reason was the difficulties on the removal

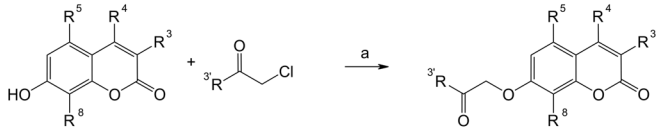
Table 1

Inhibition of NO production and iNOS expression by tested coumarins in J774 macrophages stimulated with LPS 100 ng/mL for 24 h.

	Inhibition of NO production (%)		Inhibition of iNOS protein expression (%)	
Compound	10 μM	100 μM	10 μM	100 μM
1	12±6.4	50±3.3	11±7.3	16±7.0
2	−1±6.7	22±5.1	11±3.3	42±2.4
3	0±4.7	19±0.4	−40±1.4	−5±5.1
4	20±7.1	58±1.3	24±5.0	68±1.2
6	−5±5.6	41±1.9	25±3.1	64±7.3
7	16±5.4	51±2.6	−15±7.4	34±7.0
8	38±3.5	94±0.3	−6±12.8	92±1.1
9	42±4.1	98±0.4	−9±11.1	97±0.8
10	−1±1.8	74±0.5	24±2.4	42±4.9
11	2±1.5	−3±2.8	18±4.6	36±1.6
12	27±3.6	89±0.4	47±7.2	97±0.6
13	49±2.6	94±0.3	32±4.4	97±0.4
14	28±2.0	71±1.2	15±7.2	69±1.5
15	23±3.4	74±1.4	17±8.5	66±4.7
16	17±3.3	66±1.5	−5±4.7	40±2.0
17	2±2.0	43±1.6	−5±3.3	25±2.7
18	14±3.2	73±1.4	16±6.5	70±1.2
19	31±6.5	95±1.2	33±4.2	93±0.7
20	49±2.0	90±1.6	58±5.5	99±0.1
21	47±5.2	76±2.8	38±7.2	72±3.9
22	49±3.2	88±0.7	36±5.1	85±2.2
23	13±4.2	48±3.4	46±1.9	63±4.8
24	25±4.7	65±0.5	46±4.5	88±0.7

Mean ± SEM, $n = 4$.

Scheme 1. Preparation of 7-(2-oxoalkoxy) coumarins **1–24**. Reagents and conditions: (a) Me_2CO, K_2CO_3, reflux 14–18 h.



1. R3 = R4 = R5 = R8 = H, R3'= Me
2. R3 = R5 = R8 = H, R4 = R3' = Me
3. R3 = R4 = R8 = H, R5 = R3' = Me
4. R3 = R8 = H, R4 = R5 = R3' = Me
5. R3 = R5 = H, R4 = R8 = R3' = Me
6. R3 = R4 = R3' = Me, R5 = R8 = H
7. R3 = F, R4 = R3' = Me, R5 = R8 = H
8. R3= R4 = R5 = R3' = Me, R8 = H
9. R3 = Et, R4 = R3' = Me, R5= R8 = H
10. R3 = R5 = R8 = H, R4 = Pr, R3' = Me
11. R3 = R5 = H, R4 = R3' = Me, R8 = OMe
12. R3 = Et, R4 = R5 = R3' = Me, R8 = H
13. R3 = Et, R4 = R8 = R3' = Me, R5 = H
14. R3 = R8 = H, R4 = Pr, R5 = R3' = Me, R8 = H
15. R3 = R8= H, R4 = i-Pr, R5 = R3' = Me
16. R3 = R4 = R3' = Me, R5 = H, R8 = OMe
17. R3 = F, R4 = R3' = Me, R5 = H, R8 = OMe
18. R3 = R5 = H, R4 = Pr, R8 = OMe, R3' = Me
19. R3 = R5 = R8 = H, R4 = Ph, R3' = Me
20. R3 = R5 = H, R4 = Ph, R8 = R3' = Me
21. R3 = R5 = R8 = H, R4 = 2-FC6H4, R3' = Me
22. R3 = R5 = H, R4 = Ph, R8 = OMe, R3' = Me
23. R3 = R5 = R8 = H, R4 = Me, R3' = Ph
24. R3 = R5 = R8 = H, R4 = Me, R3' = t-Bu

of the solid starting chloride traces from crude product. The formed crude product was almost pale powder. To provide *tert*-butyl group to oxoalkoxy tail in the compound **24**, 1-chloropinacolone was used. Equimolar amounts of starting materials were used to prevent the formation of scarcely separable liquid mixture of product and high boiling chloropinacolone. In addition to compounds **23** and **24**, compounds **15** and **18** were also prepared with equimolar amounts of starting materials.

Most of the compounds could be dried successfully by evaporating the solvent in rotary evaporator, but compound **18** did contain impurities unable to be removed by this method. Crude products were slightly brownish moist powder except compound **18**, which was dark brown sticky oil. After recrystallization from ethanol all products were pale crystalline powders. To get a solid product from compound **18** the reaction mixture was evaporated and kept at open air until mostly precipitated. After several careful recrystallizations from ethanol the product formed crystals. Yields of recrystallized products vary largely depending on the substitution but were generally good when compared with the yields reported in the literature [30–32].

Pharmacology. To study the anti-inflammatory effects of 7-(2-oxoalkoxy)coumarins, we measured their effects on the expression of inflammatory enzymes iNOS and COX-2 (which produce nitric oxide and prostaglandins, respectively) and on the production of inflammatory mediators nitric oxide (NO) and interleukin-6 (IL-6) in macrophages. As an inflammatory stimulus lipopolysaccharide (LPS) was used to activate the macrophages through a toll-like-receptor 4 (TLR4) pathway. In the primary testing, two concentrations of the 7-(2-oxoalkoxy)coumarins, i.e., 10 and 100 μM, were used.

The compounds were dissolved as a 100 mM stock solution in DMSO for the cell culture experiments. Compound **5** was excluded from the studies because it was not soluble in DMSO at desired concentrations. In all experiments (including controls), the final DMSO concentration was adjusted to be 0.1%, which was shown not to affect the measured parameters. The pharmacological studies on the newly synthesized compounds were started by cytotoxicity testing. For these purposes, XTT test was used. 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.

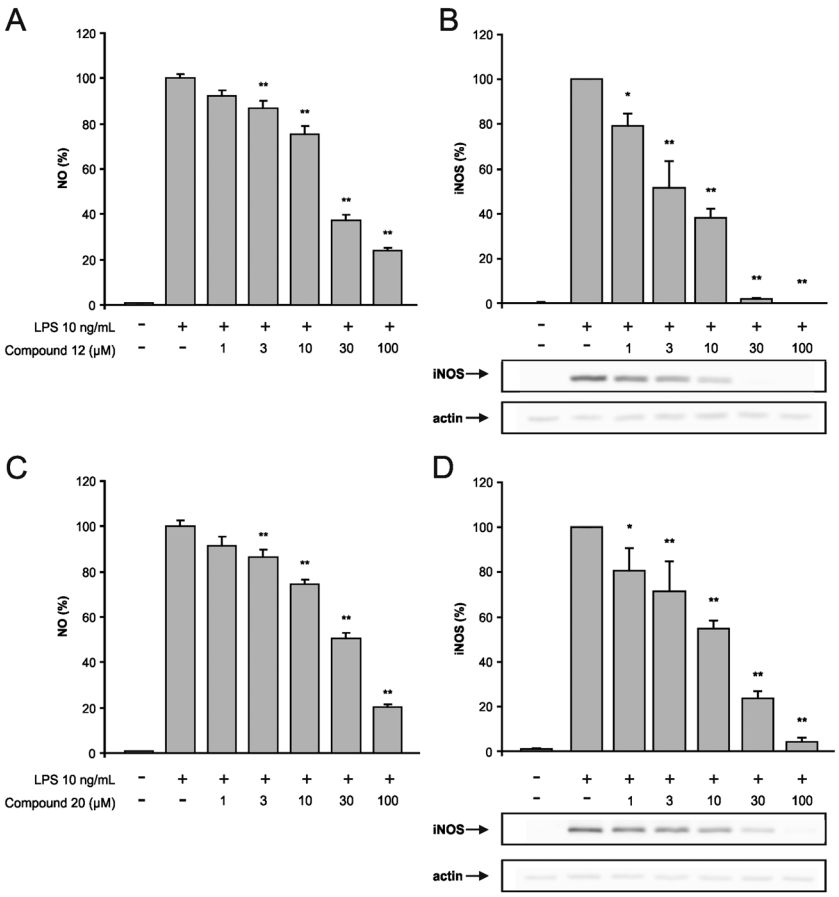


Figure 2. Dose-dependent effects of compounds **12** and **20** on iNOS and NO. Effects of compounds **12** (A and B) and **20** (C and D) on NO production (A and C) and iNOS expression (B and D) in J774 macrophages stimulated with LPS 100 ng/mL for 24 h. Results are expressed as mean ± SEM, $n = 4$.

None of the compounds (100 μM) showed cytotoxic effects when tested in similar conditions where the experiments for anti-inflammatory activities were carried out.

Inhibition of inducible nitric oxide synthase expression and nitric oxide production. In inflammation, NO is principally formed in a reaction catalyzed by iNOS, which is an inducible enzyme. Unstimulated cells did not produce NO or express iNOS protein at detectable levels, whereas LPS enhanced both considerably. Most of the compounds significantly inhibited LPS-induced NO production and iNOS expression when used at 100 μM concentrations.

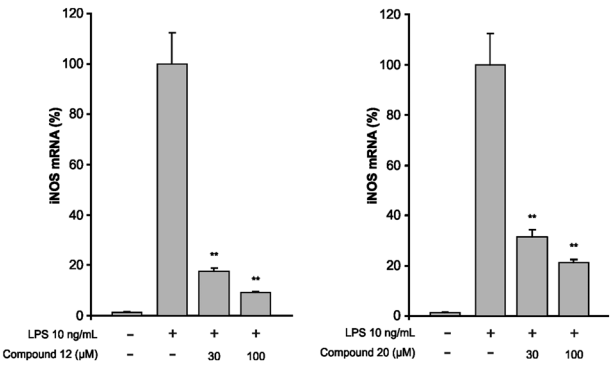


Figure 3. The effects of compounds **12** and **20** on iNOS mRNA expression. Effects measured with J774 macrophages stimulated with LPS 100 ng/mL for 8 h. Results are expressed as mean ± SEM, $n = 4$.

Seven compounds (**8**, **9**, **12**, **13**, **19**, **20**, and **22**) inhibited NO production and iNOS expression by more than 85% when used at 100 μM concentrations as compared with the cells treated with LPS alone. The compounds **12** and **20** appeared to be the most potent inhibitors of iNOS protein expression with more than 97% and 47% inhibition at 100 and 10 μM concentrations, respectively (Table 1). A highly selective iNOS inhibitor 1400 W [33] was used as a positive control compound. 1400 W (1000 μM) inhibited NO production in LPS-stimulated cells by more than 95%, but as an iNOS inhibitor, it did not affect iNOS protein expression. Because NF-κB is an important transcription factor for iNOS expression, we used pyrrolidinedithiocarbamate (PDTC), an inhibitor of NF-κB activation [34] as a positive control for inhibition of iNOS expression. PDTC (100 μM) inhibited iNOS expression by 97%.

The two most potent compounds (**12** and **20**) were selected for the further studies. To investigate dose-dependency, the compounds were added into to cell culture in 1–100 μM concentrations. Both of the compounds inhibited NO production in a dose-dependent manner with IC_{50} values of 21 μM (compound **12**) and 30 μM (compound **20**), respectively, and a similar effect was seen on iNOS expression (Fig. 2). iNOS mRNA was measured by quantitative RT-PCR after 8 h incubation with a combination of LPS and the compound **12** or **20**. The

Table 2

Inhibition of IL-6 production and COX-2 expression by tested coumarins in J774 macrophages stimulated with LPS (100 ng/mL) for 24 h.

	Inhibition of IL-6 production (%)		Inhibition of COX-2 protein expression (%)	
Compound	10 μM	100 μM	10 μM	100 μM
1	4±3.6	22±2.8	−19±7.6	−37±12
2	8±6.0	38±2.1	4±5.6	12±5.7
3	8±3.7	36±1.7	29±5.9	57±3.2
4	21±3.1	68±0.9	−11±9.3	41±6.2
6	28±1.3	71±0.7	35±6.9	65±1.9
7	7±5.0	40±2.1	−7±8.6	3±9.7
8	33±4.3	93±0.1	3±8.7	67±5.2
9	34±1.3	82±0.5	7±11.5	65±5.9
10	17±1.5	71±2.0	18±1.4	33±4.4
11	13±3.8	39±4.1	4±4.9	33±1.9
12	47±4.5	90±0.4	29±7.8	57±1.6
13	62±1.2	73±0.7	55±2.2	26±4.8
14	43±2.5	80±1.2	29±6.7	49±4.3
15	43±1.3	78±0.7	23±7.9	59±2.7
16	15±3.9	60±0.9	7±3.5	70±3.0
17	−1±4.8	46±2.4	−9±5.3	30±6.3
18	27±1.7	74±0.6	20±5.8	63±2.7
19	27±2.8	90±1.0	−2±10.8	58±4.7
20	33±4.6	89±0.7	−8±17.4	37±15.2
21	46±4.1	76±2.0	−4±13.5	12±5.6
22	51±4.8	82±1.8	13±3.0	19±3.6
23	21±13.3	56±3.0	17±3.6	−1±8.8
24	18±0.6	70±0.4	20±6.3	44±5.2

Mean ± SEM, $n = 4$.

iNOS mRNA levels were very low in resting cells, but they increased significantly when the cells were exposed to LPS. Compounds **12** and **20** had a clear inhibitory effect on iNOS mRNA levels (Fig. 3) which is consistent with their inhibitory effects of iNOS expression and NO production.

We also tested the effects of 7-(2-oxoalkoxy)coumarins on the LPS-induced production of pro-inflammatory 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 the cells were activated by adding LPS. With the higher concentration used (100 μM), seven of the studied compounds (**8**, **9**, **12**, **14**, **19**, **20**, and **22**) inhibited IL-6 production by more than 80%, and only one compound (**1**) had an inhibitory effect smaller than 30%. With the lower concentration used (10 μM), nine compounds inhibited IL-6 production by more than 30% (Table 2). Dexamethasone (10 μM) was used as a control compound [23,35], and it inhibited IL-6 production by 74%. Dose–response studies showed IC_{50} values of 24 μM for compound **12** and 10 μM for compound **20** on IL-6 production (Fig. 4).

The effects of 7-(2-oxoalkoxy)coumarins were also tested on LPS-stimulated COX-2 expression in activated J774 macrophages. Nine of the tested compounds (**3**, **6**, **8**, **9**, **12**, **15**, **16**, **18**, and **19**) inhibited the COX-2 expression by more than 50% when used at a concentration of 100 μM (Table 2). Dexamethasone (10 μM) was used as a control compound and inhibited COX-2 expression by 87%.

Structure-activity study. Our earlier study on 7-hydroxycoumarins contained 17 similar structures as the present study, namely **1–4**, **6–10**, **13**, **14**, **16–19**, **21**, and **22**. Comparison of the results showed over 90% inhibition of iNOS expression with compounds **8**, **9**, and **19** from both series, while derivative **13** showed over 90% activity only as 7-(2-oxoalkoxy)coumarin and derivative **21** only as 7-hydroxycoumarin. Results on NO production were similar: compounds **8** and **9** from both series inhibited NO production by more than 90% while compounds **13** and **19** were effective only as 7-(2-oxoalkoxy)coumarins. Generally, acetonyloxylation of 7-hydroxycoumarins affected more on NO production than on iNOS expression.

Instead of acetonyloxy group the substitution with 2-oxo-2-phenylethoxy or 3,3-dimethyl-2-oxobutoxy improved inhibition

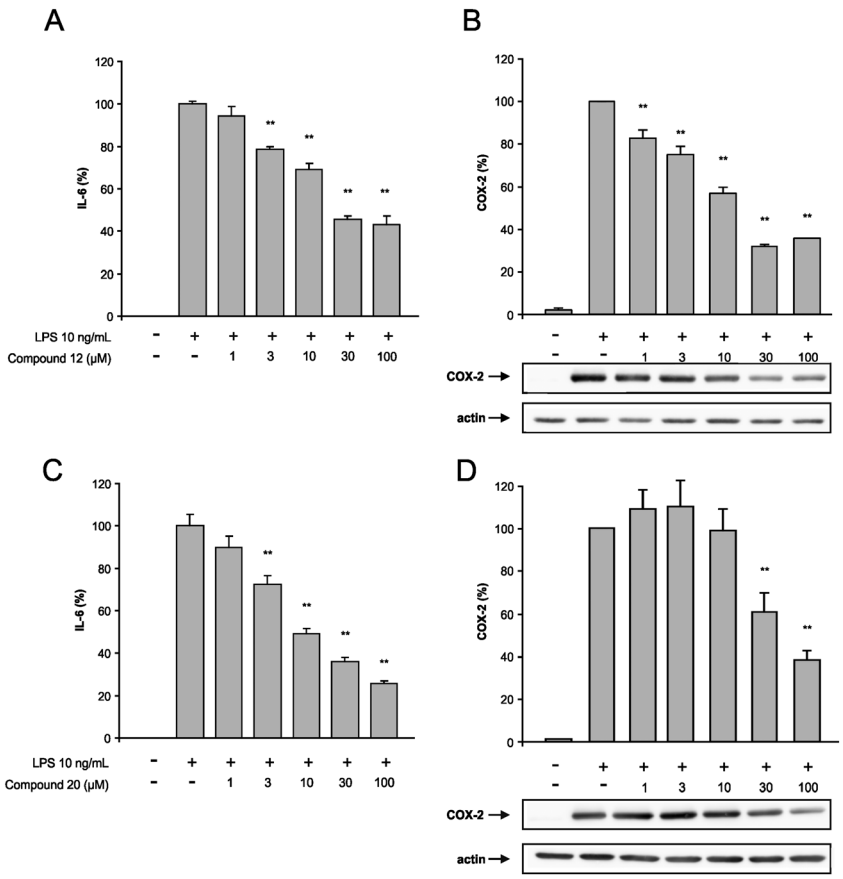


Figure 4. Dose-dependent effects of compounds **12** and **20** on IL-6 and COX-2. Effects of compounds **12** (A and B) and **20** (C and D) on IL-6 production (A and C) and COX-2 expression (B and D) in J774 macrophages stimulated with LPS 100 ng/mL for 24 h. Results are expressed as mean ± SEM, $n = 4$.

Table 3

The differences in the inhibition activities between 7-(2-oxoalkoxy)coumarins that improved activity in either iNOS/NO or IL-6 inhibition over 7-hydroxycoumarins[a] at 100 μM concentration. Positive value means improved activity of 7-(2-oxoalkoxy)coumarin over 7-hydroxycoumarin.

Compound	Difference in inhibition of NO production (percent unit)	Difference in inhibition of iNOS expression (percent unit)	Difference in inhibition of IL-6 production (percent unit)
4	−13	−6	2
8	2	−2	1
9	6	6	−8
10	6	−48	−18
13	7	32	−22
14	8	−19	26
16	15	−34	17
18	−11	0	1
19	10	0	−6
22	7	0	35
23	5	1	12
24	22	26	26

[a]Values of 7-hydroxycoumarins are presented in the supporting information.

activities remarkably. Compounds **23** and **24** were compared to 7-hydroxy-4-methylcoumarin. Substitution with 3,3-dimethyl-2-oxobutoxy increased activities more: the iNOS inhibition was almost 90% compared to 62% inhibition activity of 7-hydroxy-4-methylcoumarin.

Inhibition of IL-6 production was also improved by substitution with acetonyloxy group. Compounds **14** and **22** gained the major effect as the IL-6 inhibition of hydroxycoumarins were approximately 50% but acetonyloxylation rose them above 80%. The efficiency of the 7-(2-oxoalkoxy)coumarins to inhibit COX-2 expression is enhanced in the case of 13 out of the 17 comparable structures, with an average between 40% and 60% inhibition of LPS-induced COX-2 expression, while none of the 7-hydroxycoumarins were able to show over 30% inhibition.

All of the six most active compounds (**8**, **9**, **12**, **19**, **20**, and **22**) contain either 4-phenyl or 3-alkyl substituent. In addition to these compounds, the compound **13**, namely 7-acetonyloxy-3-ethyl-4,8-dimethylcoumarin inhibited iNOS expression over 95% and NO production by almost 95%, and the IL-6 production was reduced by 73%, which is good but below the 80% level. This was observed also by experiments with 7-hydroxycoumarins. Further, two out of three 3-alkylated compounds (**6** and **16**) lacking excellent anti-inflammatory activity contain substituent at position 8.

From the four tested 4-phenyl compounds, three (**19**, **20**, and **22**) are regarded as very effective anti-inflammatory compounds. There is also minor decreasing effect of the 8-substituent on the activity: the 8-methoxy substituted compound (**22**) is less active than the other two promising compounds (**19** and **20**). Within 4-phenyl compounds, the fluorine at the position 2 of the phenyl ring (compound **21**) as well as the 3-fluorine in the compounds **7** and **17** decreases the activity remarkably.

Generally, the substitution of hydroxyl by 2-oxoalkoxy group improved the anti-inflammatory activity of some of the previously studied 7-hydroxycoumarins. In Table 3, there are twelve 7-(2-oxoalkoxy)coumarins that have improved activity over respective 7-hydroxycoumarin in either iNOS/NO or IL-6 inhibition at 100 μM concentration and nine of them showed meaningful increase in the inhibition activity (difference over 5 percentage units compared with the respective 7-hydroxycoumarin). Results of the relevant 7-hydroxycoumarins are presented in supporting information and all values can be found from our previous paper [23].

CONCLUSIONS

Twenty-four substituted 7-(2-oxoalkoxy)coumarins were synthesized according to commonly known procedure. Ten of the coumarins (**3**, **4**, **7**, **8**, **12**, **15**, **17**, **18**, **21**, and **22**) synthesized in the present study are novel compounds.

In the study, 7-(2-oxoalkoxy)coumarins inhibited iNOS expression and NO production as well as IL-6 production and COX-2 expression in a dose-dependent manner. The most promising compounds were **8**, **9**, **12**, **19**, **20**, and **22**, i.e., 3,4,5-trimethyl, 3-ethyl-4-methyl, 3-ethyl-4,5-dimethyl, 4-phenyl, 8-methyl-4-phenyl, and 8-methoxy-4-phenyl derivatives. These compounds inhibited iNOS expression, and the production of IL-6 and NO by more than 80% at the concentration of 100 μM, without cytotoxicity.

The structure-activity elucidation points out two major properties of the most active coumarins. Lipophilic substituents, namely methyl or ethyl (compounds **8**, **9**, **12**, and **13**), at position 3 increased the activity whereas the 3-fluorine hindered the activity rather than activated the compounds **7** and **17**. The 4-phenyl substitution was also a clear enhancer of the inhibition activity. Within 4-phenyl compounds, the fluorine at the position 2 of the

phenyl ring (compound **21**) decreased the activity remarkably. The activity order of different 2-oxoalkoxy substituents seems to be 3,3-dimethyl-2-oxobutoxy > 2-oxo-2-phenylethoxy 2-oxopropoxy and substitution of 7-hydroxycoumarins to 7-(2-oxoalkoxy)coumarins seemed to improve the inhibitory activities.

EXPERIMENTAL

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

Cell culture. J774 murine macrophages (American Type Culture Collection, Rockville, MD) were cultured in Dulbecco's modified Eagle's medium with Ultraglutamine 1. The culture medium was supplemented with 10% heat-inactivated fetal 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 confluency before the experiments were started, and the compounds of interest were added in fresh culture medium.

XTT-test. Cell viability was tested by using Cell Proliferation Kit II, which measures cells' ability to metabolize 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).

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 [36] (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.

Preparation of cell lysates for western blotting. At indicated time points, cells were rapidly washed with ice-cold phosphate-buffered saline. 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, 20 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, 10 min). The protein content of the supernatants was measured by the Coomassie blue method.

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 1 h at 120 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, UK) 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°Cwith COX-2, iNOS or actin (loading control) antibodies in TBS/T containing 5% nonfat milk. Thereafter, the membrane was washed four 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. The bound antibody was detected and quantitated by using a SuperSignal West Pico chemiluminescent substrate (Pierce, Cheshire, UK) and an ImageQuant LAS 4000 mini imaging system (GE Healthcare Bio-Sciences, AB, Uppsala, Sweden). The chemiluminescent signal was quantified with ImageQuant TL 7.0 Image Analysis Software [37].

RNA extraction and quantitative reverse transcription polymerase chain reaction. After 8 h incubation, cell monolayers were rapidly washed with ice-cold phosphate-buffered saline, and cells were homogenized using a QIAshredder (QIAGEN, Valencia, CA, USA). RNA extraction was carried out with the use of an RNeasy kit for isolation of total RNA (QIAGEN). Total RNA (100 ng) was reverse-transcribed to cDNA using TaqMan Reverse Transcription reagents and random hexamers (Applied Biosystems, Foster City, CA, USA). cDNA obtained from the RT reaction (amount corresponding to approximately 2.5 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 optimized according to the manufacturer's guidelines in the TaqMan Universal PCR Master Mix Protocol part number 4304449 revision C, and were: 5′-CCTGGTACGGGCATTGCT-3′, and 5′-GCTCATGCGGCCTCCTT-3′ (forward and reverse mouse iNOS primers, respectively, both 300 nM); 5′-CAGCAGCGGCT CCATGACTCCC-3′ (mouse iNOS probe, 150 nM, containing 6-FAM as 5′-reporter dye and TAMRA as 3′-quencher); 5′-GCAT GGCCTTCCGTGTTC-3′ and 5′-GATGTCATCATACTTGG CAGGTTT-3′ [forward and reverse mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers, respectively, both 300 nM]; and 5′-TCGTGGATCTGACGTGCCGCC-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 of denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 min. Each sample was determined 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 normalized against GAPDH [37].

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

Statistics. The results are expressed as the mean ± SEM. Statistical significances of the differences were calculated by analyses of variance, supported by the Dunnett's multiple comparisons test. Differences were considered significant at $p < 0.05$ and shown as * = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$.

Syntheses. Chemicals were used as received if else has not been mentioned. 7-Hydroxycoumarins were synthesized according to the literature [23]. Melting points were measured with Gallenkamp Melting Point Apparatus (Gallenkamp, Loughborough, UK) and are uncorrected. NMR spectra were measured either with Bruker Avance 250 (250 MHz for ^{1}H, 62.9 MHz for ^{13}C; Bruker BioSpin AG, Fällanden, Swizerland) or with Bruker Avance 400 FT NMR (400 MHz for ^{1}H, 100.6 MHz for ^{13}C; Bruker BioSpin AG, Fällanden, Swizerland). IR spectra were measured with Nicolet Avatar 320 FTIR (Thermo Electron Scientific Instruments, LLC, Madison, WI, USA). Combustion analysis was done using vario MICRO cube (Elementar Analysensysteme GmbH, Hanau, Germany) Mass spectra were measured with 12-T APEX-Qe FT-ICR instrument (Bruker Daltonik GmbH, Bremen, Germany).

General method for synthesis of 7-acetonyloxycoumarins 1–24. 15 mmol of substituted 7-hydroxycoumarin was dissolved in 400 mL of dry acetone and 21 g (138 mmol) of potassium carbonate was added. The chloroacetone (6.66 g, 72 mmol) was added, and the mixture was refluxed for 24 h. After refluxing, the solution was filtered, and acetone was removed with rotary evaporator. The products were purified by recrystallization from ethanol.

7-Acetonyloxy-5-methylcoumarin (3). White crystals. Yield 68%; mp 127–128°C; IR (KBr, cm^{-1}): 3070 (ν C–H, aromatic), 1717 (ν C═O, lactone), 1711 (ν C═O, R–C(O)–R), 1606 (ν C═C, lactone), 1440 (d_{as} C–H, Ar–*CH_3*), 1387 (d_s C–H, Ar–*CH_3*), 1358 (d_s C–H, C(O)–*CH_3*), 1204 (ν O–C, lactone), 1123 (ν O–C, O–CH_2), 1079 (r C–C–C, CH_2–CO–CH_3), 855 (γ C–H, aromatic), 829 (γ C–H, RCH═*CH*R′); ^{1}H-NMR (DMSO-d_6) δ: 2.17 (s, 3H, OCH_2CO*CH_3*), 2.46 (s, 3H, 5-Me), 4.96 (s, 2H, O*CH_2*$COCH_3$), 6.28 (d, 1H, J = 9.75 Hz, 3-H), 6.81 (m, 2H, 6-H and 8-H), 8.08 (d, J = 9.5 Hz, 4-H); ^{13}C-NMR (DMSO-d_6) δ: 17.91, 26.06, 72.05, 99.09, 111.41, 111.83, 113.69, 137.94, 141.46, 155.75, 160.16, 160.42, 203.08; HRMS (ESI-FTICR) Calcd for $C_{13}H_{13}O_4$ (M+H$^+$): 233.0808; found, 233.0807; Anal. Calcd for $C_{13}H_{12}O_4$: C, 66.71; H, 6.37; found: C, 66.26; H, 5.15.

7-Acetonyloxy-4,5-dimethylcoumarin (4). White crystals. Yield 77%; mp 179–180°C; IR (KBr, cm^{-1}): 3066 (ν C–H, aromatic), 1713 (ν C═O, lactone), 1694 (ν C═O, R–C(O)–R), 1612 (ν C═C, lactone), 1454 (d_{as} C–H, CH═CR*CH_3*), 1440 (d_{as} C–H, CH═CR*CH_3*), 1369 (d_s C–H, CH═CR*CH_3*), 1358 (d_s C–H, C(O)–*CH_3*), 1206 (ν O–C, lactone), 1113 (ν O–C, O–CH_2), 1070 (r C–C–C, CH_2–CO–CH_3), 860 (γ C–H, aromatic), 844 (γ C–H, RR′C═*CH*R″); ^{1}H-NMR (DMSO-d_6) δ: 2.18 (s, 3H, OCH_2CO*CH_3*), 2.35 (s, 3H, 4-Me), 2.60 (s, 3H, 5-Me), 4.99 (s, 2H, O*CH_2*$COCH_3$), 6.15 (s, 1H, 3-H), 6.72 (s, 1H, 8-H), 6.81 (s, 1H, 6-H); ^{13}C-NMR (DMSO-d_6) δ: 21.24, 23.82, 26.24, 72.90, 107.56, 108.85, 109.76, 112.91, 143.01, 153.93, 154.55, 159.41, 202.65; HRMS (ESI-FTICR) Calcd for $C_{14}H_{15}O_4$ (M+H$^+$): 247.0965; found, 247.0963; Anal. Calcd for $C_{14}H_{14}O_4$: C, 68.28; H, 5.73; found: C, 68.51; H, 5.86.

7-Acetonyloxy-3-fluoro-4-methylcoumarin (7). White crystals. Yield 36%; mp 162–163°C; IR (KBr, cm^{-1}): 3095 (ν C–H, aromatic), 1721 (ν C═O, R–C(O)–R), 1618 (ν C═C, lactone), 1365 (d_s C–H, C(O)–*CH_3*), 1121 (ν O–C, O–CH_2), 1076 (r C–C–C, CH_2–CO–CH_3), 857 (γ C–H, aromatic), 834 (γ C–H, aromatic); ^{1}H-NMR (DMSO-d_6) δ: 2.17 (s, 3H, OCH_2CO*CH_3*), 2.34 (d, J = 2.8 Hz, 3H, 4-Me), 4.98 (s, 2H, O*CH_2*$COCH_3$), 7.02 (m, 2H, 6-H and 8-H), 7.67 (d, J = 9.2 Hz, 5-H); ^{13}C-NMR (DMSO-d_6) δ: 11.58, 27.75, 73.88, 103.10, 114.16, 114.21, 114.72, 128.11, 133.33, 141.48, 152.81, 161.62, 204.74; HRMS (ESI-FTICR) Calcd for $C_{13}H_{12}FO_4$ (M+H$^+$): 251.0714; found, 251.0712; Anal. Calcd for $C_{13}H_{11}FO_4$: C, 62.40; H, 4.43; found: C, 61.96; H, 4.67.

7-Acetonyloxy-3,4,5-trimethylcoumarin (8). White crystals. Yield 67%; mp 184–185°C; IR (KBr, cm^{-1}): 3073 (ν C–H, aromatic), 1744 (ν C═O, lactone), 1689 (ν C═O, R–C(O)–R), 1605 (ν C═C, lactone), 1476 (d_{as} C–H, CH═CR*CH_3*), 1385 (d_s C–H, CH═CR*CH_3*), 1358 (d_s C–H, C(O)–*CH_3*), 1348 (d_s C–H, C(O)–*CH_3*), 1186 (ν O–C, lactone), 1107 (ν O–C, O–CH_2), 1087 (r C–C–C, CH_2–CO–CH_3), 860 (γ C–H, aromatic); ^{1}H-NMR (DMSO-d_6) δ: 2.07 (s, 3H, 3-Me), 2.18 (s, 3H, OCH_2CO*CH_3*), 2.60 (s, 3H, 4-Me), 4.98 (s, 2H, O*CH_2*$COCH_3$), 6.68 (s, 1H, 8-H), 7.77 (s, 1H, 6-H); ^{13}C-NMR (DMSO-d_6) δ: 14.53, 21.24, 22.75, 27.93, 74.58, 109.73, 110.62, 111.19, 120.84, 143.05, 149.34, 154.3, 156.98, 162.22, 204.47; HRMS (ESI-FTICR) Calcd for $C_{15}H_{17}O_4$ (M+H$^+$): 261.1121; found, 261.1121; Anal. Calcd for $C_{15}H_{16}O_4$: C, 69.22; H, 6.20; found: C, 68.75; H, 6.20.

7-Acetonyloxy-3-ethyl-4-methylcoumarin (9). Light yellow crystals. Yield 72%; mp 140°C; IR (KBr, cm^{-1}): 3070 (ν C–H, aromatic), 1728 (ν C═O, lactone), 1697 (ν C═O, R–C(O)–R), 1612 (ν C═C, lactone), 1462 (d C–H, R′C–*CH_2*CH_3), 1393 (d_s C–H, R–CH_2–*CH_3*), 1366 (d_s C–H, C(O)–*CH_3*), 1231 (ν O–C, lactone), 1146 (ν O–C, O–CH_2), 1072 (r C–C–C, CH_2–CO–CH_3), 856 (γ C–H, aromatic), 814 (γ C–H, aromatic), 779 (r C–$(CH_2)_n$–C, R–CH_2–CH_3); ^{1}H-NMR (DMSO-d_6) δ: 1.03 (t, 3H, J = 7 Hz, CH_2*CH_3*), 2.17 (s, 3H, OCH_2CO*CH_3*), 2.37 (s, 3H, 4-Me), 2.50 (q, 2H, J = 7.5 Hz, *CH_2*CH_3), 4.96 (s, 2H, O*CH_2*$COCH_3$), 6.92 (m, 2H, 6-H and 8-H), 7.67 (d, 1H, J = 8.25 Hz, 5-H); ^{13}C-NMR (DMSO-d_6) δ: 14.55, 15.93, 27.76, 51.78, 73.83, 102.61, 113.84, 115.5, 125.45, 127.89, 148.04, 154.55, 161.45, 162.35, 204.88; HRMS (ESI-FTICR) Calcd for $C_{15}H_{17}O_4$ (M+H$^+$): 261.1121; found, 261.1121; Anal. Calcd for $C_{15}H_{16}O_4$: C, 69.22; H, 6.20; found: C, 68.81; H, 6.13.

7-Acetonyloxy-4-propylcoumarin (10). Light yellow crystals. Yield 70%; mp 89–90°C; IR (KBr, cm^{-1}): 3075 (ν C–H, aromatic), 1728 (ν C═O, lactone), 1713 (ν C═O, R–C(O)–R), 1612 (ν C═C, lactone), 1373 (d_s C–H, R–$(CH_2)_2$–*CH_3*), 1350 (d_s C–H, C(O)–*CH_3*), 1207 (ν O–C, lactone), 1146 (ν O–C, O–CH_2), 1069 (r C–C–C, CH_2–CO–CH_3), 852 (γ C–H, aromatic), 845 (γ C–H, RR′C═*CH*R″), 791 (γ C–H, aromatic), 756 (r C–$(CH_2)_n$–C, R–$(CH_2)_2$–CH_3); ^{1}H-NMR (DMSO-d_6) δ: 0.98 (t, 3H, J = 7.5 Hz, CH_2CH_2*CH_3*), 1.64 (sxt, 2H, J = 7.5 Hz, CH_2*CH_2*CH_3), 2.18 (s, 3H, OCH_2CO*CH_3*), 2.74 (t, 2H, *CH_2*CH_2CH_3), 5.00 (s, 2H, O*CH_2*$COCH_3$), 6.17 (s, 1H, 3-H), 6.96 (m, 2H, 6-H and 8-H), 7.73 (d, 1H, J = 9.75 Hz, 6-H); ^{13}C-NMR (DMSO-d_6) δ: 13.57, 21.23, 26.07, 28.37, 72.18, 101.44, 110.31, 112.40, 112.51, 126.11, 154.81, 156.65, 160.17, 160.65, 203.11; HRMS (ESI-FTICR) Calcd for $C_{15}H_{17}O_4$ (M+H$^+$): 261.1121; found, 261.1120; Anal. Calcd for $C_{15}H_{16}O_4$: C, 69.22; H, 6.20; found: C, 69.11; H, 6.16.

7-Acetonyloxy-3-ethyl-4,5-dimethylcoumarin (12). White crystals. Yield 47%; mp 166–167°C; IR (KBr, cm^{-1}): 3070 (ν C–H, aromatic), 1719 (ν C═O, lactone), 1693 (ν C═O, R–C(O)–R), 1619 (ν C═C, lactone), 1452 (d C–H, R–CH_2–*CH_3*), 1389 (d_s C–H, RR′C═CR″–*CH_3*), 1351 (d_s C–H, C(O)–*CH_3*), 1190 (ν O–C, lactone), 1114 (ν O–C, O–*CH_2*), 1067 (r C–C–C, CH_2–CO–CH_3), 856 (γ C–H, aromatic), 783 (r C–C–C, C–CH_2–CH_3); ^{1}H-NMR ($CDCl_3$-d_3) δ: 1.13 (t, 3H, J = 7.5 Hz, CH_2*CH_3*), 2.29 (s, 3H, OCH_2CO*CH_3*), 2.36 (s, 3H, 5-Me), 2.67 (m, 5H, *CH_2*CH_3 & 5-Me), 4,67 (s, 2H, O*CH_2*$COCH_3$), 6.35 (s, 1H, 6-H), 6.78 (s, 1H, 8-H); ^{13}C-NMR ($CDCl_3$-d_3) δ: 12.87,

19.42, 20.64, 21.76, 26.73, 73.56, 108.17, 109.37, 111.19, 126.47, 141.34, 147.13, 153.74, 155.44, 161.37, 203.24; HRMS (ESI-FTICR) Calcd for $C_{16}H_{19}O_4$ ($M+H^+$): 275.1278; found, 275.1278; Anal. Calcd for $C_{16}H_{18}O_4$: C, 70.06, H 6.61: found: C, 69.88; H, 6.56.

7-Acetonyloxy-5-methyl-4-propylcoumarin (14). White crystals. Yield 78%; mp 150–152°C; IR (KBr, cm^{-1}): 3062 (ν C–H, aromatic), 1724 (ν C═O, lactone), 1709 (ν C═O, R–C(O)–R), 1605 (ν C═C, lactone), 1385 (d_s C–H, R–$(CH_2)_2$–*CH_3*), 1373 (d_s C–H, C(O)–*CH_3*), 1204 (ν O–C, lactone), 1123 (ν O–C, O–CH_2), 1061 (r C–C–C, CH_2–CO–CH_3), 870 (γ C–H, aromatic), 829 (γ C–H, RR′C═*CH*R″), 752 (r C–C–C, R–$(CH_2)_2$–CH_3); ^{1}H-NMR (DMSO-d_6) δ: 0.95 (t, 3H, *J* = 7.5 Hz, CH_2CH_2*CH_3*), 1.55 (sxt, 2H, *J* = 7.5 Hz, CH_2*CH_2*CH_3), 2.19 (s, 3H, OCH_2CO*CH_3*), 2.34 (s, 3H, 5-Me), 2.98 (t, 2H, *J* = 7.5 Hz, *CH_2*CH_2CH_3), 5.02 (s, 2H, O*CH_2*$COCH_3$), 6.11 (s, 1H, 3-H), 6.70 (s, 1H, 8-H), 6.81 (s, 1H, 6-H); ^{13}C-NMR (DMSO-d_6) δ: 13.60, 21.19, 22.43, 26.13, 37.57, 72.77, 106.76, 108.81, 109.91, 112.35, 142.87, 154.90, 155.41, 157.58, 159.57, 202.28; HRMS (ESI-FTICR) Calcd for $C_{16}H_{19}O_4$ ($M+H^+$): 275.1278; found, 275.1278; Anal. Calcd for $C_{16}H_{18}O_4$: C, 70.06; H, 6.61; found: C, 69.65; H, 6.54.

7-Acetonyloxy-5-methyl-4-isopropylcoumarin (15). White crystals. Yield 52%; Decomposed over 250°C; IR (KBr, cm^{-1}): 3075 (ν C–H, aromatic), 1724 (ν C═O, lactone), 1609 (ν C═C, lactone), 1443 (d_{as} C–H, R–CH–(*CH_3*)$_2$), 1396 (d_s C–H, Ar–*CH_3*), 1362 (d_s C–H, C(O)–*CH_3*), 1331 (d C–H, R–*CH*–$(CH_3)_2$), 1200 (ν O–C, lactone), 1173 (r C–C–C, R–CH–$(CH_3)_2$), 1142 (r C–C–C, R–CH–$(CH_3)_2$), 1115 (ν O–C, O–CH_2), 1076 (r C–C–C, CH_2–CO–CH_3), 872 (γ C–H, aromatic), 818 (γ C–H, RR′C═*CH*R″); ^{1}H-NMR (DMSO-d_6) δ: 1.30 (d, 6H, *J* = 6.8 Hz, CH(*CH_3*)$_2$), 2.31 (s, 3H, O*C*H_2CO*CH_3*), 2.78 (m, 1H, C*H*$(CH_3)_2$) 4.62 (s, 2H O*CH_2*$COCH_3$), 6.03 (s, 1H, 3-H), 6.61 (s, 1H, 8-H), 6.71 (s, 1H, 6-H); ^{13}C-NMR(DMSO-d_6) δ: 20.04, 23.00,26.64, 32.61, 72.81, 99.18, 108.80,115.96, 143.18, 160.16, 171.78, 204.32; HRMS (ESI-FTICR) Calcd for $C_{16}H_{19}O_4$ ($M+H^+$): 275.1278; found, 275.1277; Anal. Calcd for $C_{16}H_{18}O_4$: C, 70.06; H, 6.61; found: C, 70.32; H, 6.91.

7-Acetonyloxy-3-fluoro-8-methoxy-4-methylcoumarin (17). Pale brown powder. Yield 57%; mp 155–157°C; IR (KBr, cm^{-1}): 1723 (ν C═O, lactone), 1697 (ν C═O, R–C(O)–R), 1606 (ν C═C, lactone), 1459 ($d_{as,s}$ C–H, Ar–O–*CH_3*), 1431 (d_{as} C–H, RR′C═CR″–*CH_3*), 1383 (d_s C–H, RR′C═CR″–*CH_3*), 1364 (d_s C–H, C(O)–*CH_3*), 1282 (ν C–O, Ar–*O*–CH_3), 1224 (ν O–C, lactone), 1181 (ν C–F, RR′C═CR″F), 1125 (ν O–C, O–CH_2), 1060 (r C–C–C, CH_2–CO–CH_3), 1014 (ν O–C, Ar–*O*–*C*H_3), 813 (γ C–H, aromatic); ^{1}H-NMR (DMSO-d_6) δ: 2.19 (s, 3H, OCH_2CO*CH_3*), 2.34 (d, 3H, *J* = 2.75 Hz 4-Me), 5.05 (s, 2H, O*CH_2*$COCH_3$), 7.05 (d, 1H, *J* = 9 Hz, 6-H), 7.42 (d, 1H, *J* = 9 Hz, 5-H); ^{13}C-NMR (DMSO-d_6) δ: 11.63, 27.76, 62.40, 74.17, 112.20, 115.33, 121.65, 136.93, 141.57, 145.52, 154.44, 155.59, 156.07, 204.92; HRMS (ESI-FTICR) Calcd for $C_{14}H_{14}O_5F$ ($M+H^+$): 281.0820; found, 281.0819; Anal. Calcd for $C_{14}H_{13}O_5F$: C, 60.00; H, 4.68; found: C, 59.88; H, 4.48.

7-Acetonyloxy-8-methoxy-4-propylcoumarin (18). White crystals. Yield 40%; mp 102–103°C; IR (KBr, cm^{-1}): 3067 (ν C–H, aromatic), 1730 (ν C═O, lactone), 1713 (ν C═O, R–C(O)–R), 1447 (d_{as} C–H, R–$(CH_2)_2$–*CH_3*), 1371 (d_s C–H, R–$(CH_2)_2$–*CH_3*), 1348 (d_s C–H, C(O)–*CH_3*), 1281 (ν C–O, *Ar–O*–CH_3), 1207 (ν O–C, lactone), 1115 (ν O–C, O–CH_2), 1018 (ν C–O, Ar–*O*–*CH_3*), 845 (γ C–H, RR′C═*CH*R″), 806 (γ C–H, aromatic), 750 (r C–C–C, R–$(CH_2)_2$–CH_3); ^{1}H-NMR (DMSO-d_6) δ: 0.99 (t, 3H, *J* = 7.5 Hz, CH_2CH_2*CH_3*), 1.55 (sxt, 2H, *J* = 7.5 Hz, CH_2*CH_2*CH_3), 2.21 (s, 3H, OCH_2CO*CH_3*), 2.75 (t, 2H, *J* = 7.5 Hz, *CH_2*CH_2CH_3), 3.89 (s, 3H, 8-OMe), 5.06 (s, 2H, O*CH_2*$COCH_3$), 6.21 (s, 1H, 3-H), 6.98 (d, 1H, *J* = 9.0 Hz, 6-H), 7.48 (d, 1H, *J* = 9.0 Hz, 5-H); ^{13}C-NMR (DMSO-d_6) δ: 13.56, 21.24, 26.08, 32.75, 60.61, 72.47, 109.58, 110.62, 113.56, 119.81, 135.30, 147.33, 153.39, 156.77, 159.76, 203.32; HRMS (ESI-FTICR) Calcd for $C_{16}H_{19}O_5$ ($M+H^+$): 291.1227; found, 291.1226; Anal. Calcd for $C_{16}H_{18}O_5$: C, 66.20; H, 6.25; found: C, 65.86; H, 6.24.

7-Acetonyloxy-4-(2-fluorophenyl)coumarin (21). White crystals. Yield 62%; Mp 174–175°C; IR (KBr, cm^{-1}): 3072 (ν C–H, aromatic), 1730 (ν C═O, lactone), 1713 (ν C═O, R–C(O)–R), 1617 (ν C═C, lactone), 1384 (d_s C–H, C(O)–*CH_3*), 1209 (ν O–C, lactone), 1159 (ν C–F, Ph–F), 1126 (ν O–C, O–CH_2), 1074 (r C–C–C, CH_2–CO–CH_3), 834 (γ C–H, RR′C═*CH*R″), 821 (γ C–H, aromatic), 754 (γ C–H, Ph); ^{1}H-NMR (DMSO-d_6) δ: 2.28 (s, 3H, OCH_2CO*CH_3*), 5.02 (s, 2H, O*CH_2*$COCH_3$), 6.36 (s, 1H, 3-H), 6.90 (dd, 1H, J_1 = 8.75 Hz, J_2 = 2.5 Hz, 5-H), 7.11 (m, 2H, 6-H and 8-H), 7.41 (m, 4H, 4-(2-FPh)); ^{13}C-NMR (DMSO-d_6) δ: 26.06, 72.25, 101.55, 111.84, 112.91, 115.82, 122.23, 122.47, 125.08, 127.48, 130.73, 132.02, 149.81, 154.81, 159.63, 161.10, 202.93; HRMS (ESI-FTICR) Calcd for $C_{18}H_{14}O_4F$ ($M+H^+$): 313.0871; found, 313.0871; Anal. Calcd for $C_{18}H_{13}O_4F$: C, 69.23; H, 4.20; found: C, 68.91; H, 4.39.

7-Acetonyloxy-8-methoxy-4-phenylcoumarin (22). White needles. Yield 64%; Mp 155–156°C; IR (KBr, cm^{-1}): 3098 (ν C–H, Ph), 3059 (ν C–H, aromatic), 1721 (ν C═O, lactone), 1454 ($d_{as,s}$ C–H, Ar–O–*CH_3*). 1373 (d_s C–H, C(O)–*CH_3*), 1111 (ν O–C, O–*CH_2*), 1065 (r C–C–C, CH_2–CO–CH_3), 1015 (ν C–O, Ar–*O*–*C*H_3), 841 (γ C–H, RR′C═*CH*R″), 818 (γ C–H, aromatic), 760 (γ C–H, Ph), 698 (γ C–H, Ph); ^{1}H-NMR (DMSO-d_6) δ: 2.18 (s, 3H, OCH_2CO*CH_3*), 3.82 (s, 3H, 8-OMe), 5.04 (s, 2H, O*CH_2*$COCH_3$), 6.21 (s, 1H, 3-H), 6.95 (d, 1H, *J* = 9.25 Hz, 5-H), 7.07 (d, 1H, *J* = 9 Hz, 6-H), 7.55 (m, 5H, 4-Ph); ^{13}C-NMR (DMSO-d_6) δ: 26.06, 60.65, 72.46, 109.71, 111.77, 113.11, 121.55, 128.35, 128.74, 129.59, 134.83, 135.54, 147.70, 153.64, 155.18, 159.49, 203.11; HRMS (ESI-FTICR) Calcd for $C_{19}H_{17}O_5$ ($M+H^+$): 325.1071; found, 325.1072; Anal. Calcd for $C_{19}H_{16}O_5$: C, 70.36; H, 4.97; found: C, 69.89; H, 5.11.

Acknowledgments. Mrs. Ritva Romppanen, Mrs. Taina Nivajärvi, Ms. Sonja Niskanen, Mrs. Salla Hietakangas, Ms. Meiju Kukkonen, and Ms. Petra Miikkulainen are warmly thanked for excellent technical assistance and Mrs. Heli Määttä for skillful secretarial help. Ms. Sanna Hiltunen is warmly thanked for personal assistance. We are thankful for Dr. Ewen Macdonald for language editing. The study was supported by grants from Medical Research Fund of Tampere University Hospital, Finland, The Academy of Finland, The National Graduate School of Organic Chemistry and Chemical Biology, University of Joensuu, and University of Eastern Finland.

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