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Original Scientific Article

Synthesis and Anti-inflammatory Activity of Novel Furochromenes

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Abstract. A series of variously substituted furochromenes, hemiacetals **2**, acetals **3**, and rearranged compounds **4**, were synthesized from variously substituted 4-hydroxycoumarins and evaluated in several *in vitro* assays, inhibition of mast cell degranulation induced by the activation of Fcε receptor type I or calcium ionophore and leukotriene B_4 (LTB_4) inhibition. The most active derivatives, **3p** and **4p** (8-*iso*-propyl substitution in coumarin ring) and **3r** (5-methyl-8-chloro substitution), showed significant inhibition of mast cell degranulation (Fcε–triggered) and LTB_4, and exhibited significant local anti-inflammatory activity in PMA induced ear edema in CD1 mice, with potency equal (compounds **3p** and **4p**) or better (compound **3r**) in comparison with zileuton, a reference drug used. It might be a promising direction for developing novel drugs as potential agents for the treatment of allergies and other inflammatory diseases.(doi: 10.5562/cca2240)

Keywords: furochromenes; 4-hydroxycoumarins; mast cell degranulation; leukotriene B_4; anti-inflammatory activity; ear edema

INTRODUCTION

Coumarins represent an important class of oxygenated heterocycles. Since many of them, both naturally occurring and synthetic, possess diverse array of biological activities, they are also of potential pharmaceutical interest. In nature they act as plant growth regulation factors, UV-filters and protectors from sun radiation, or as insecticides, antivirals or antibacterial agents.[1]

4-Hydroxycoumarin was determined as a building unit of natural product dicoumarol, isolated from spoiled sweet clover and identified as a causative agent of haemorrhagic cattle deaths.[2] The intensive investigations of many natural and synthetic coumarins resulted in discovery of warfarin and other 3-alkyl-4-hydroxycoumarins as potent, clinically used anticoagulants,[3–8] mainly used in postsurgical prevention of wound induced thrombus formation. Intensive investigation on the molecular level of action of these compounds showed that they prevent reduction of vitamin K epoxide to active vitamin K thus inhibiting the biosynthesis of protrombin and other clothing factors.[9]

Coumarins belonging to different chemical classes have been studied.[10] There are evidences of the anti-retroviral activity of coumarin derivatives and their ability to inhibit HIV integrase.[11–14] Some 3-aryl and 3-heteroaryl derivatives are inhibitors of macrophage migration inhibitory factor (MIF),[15] while natural coumarin product cnidicin[16] (Figure 1), isolated from the root extract of *Angelica koreana* (Umbelliferae), is an active component responsible for the inhibitory effect on the degranulation process of cultured mast cells. Cnidicin demonstrated a significant inhibition upon the release of β-hexosaminidase from the cultured RBL-2H3 cells in a dose dependent manner and the IC50 value was calculated as 25 ± 2.1 μM, which is comparable to that of azelastine (26 ± 3.2 μM) an anti-allergic drug known to inhibit the degranulation of mast cells.

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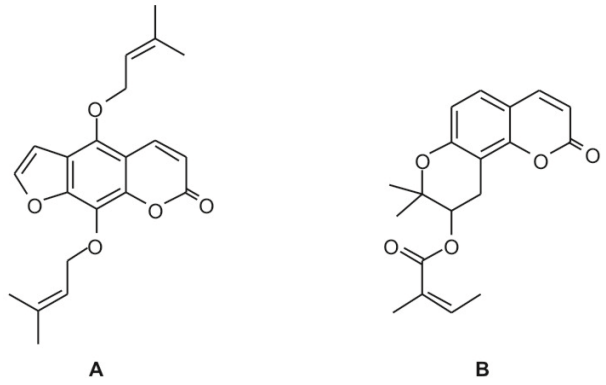


Figure 1. Cnidicin (**A**) and selinidin (**B**).

This result implied an anti-allergic action of cnidicin *in vitro*.

Another coumarin compound, selinidin (Figure 1),[17] isolated from the extract of *A. keiskei* is an antiallergic compound in screening employing rat basophilic leukemia RBL-2H3 cells. Selinidin had an inhibitory effect on degranulation of RBL-2H3 cells upon engagement of Fcε receptor type I with IgE and antigen. Furthermore, it was shown that selinidin modulates Fcε receptor type I mediated signaling although it does not prevent IgE-Fcε receptor type I binding and cell surface Fcε receptor type I expression. These results suggest that selinidin suppresses IgE-mediated mast cell activation by inhibiting signal transduction pathways through Fcε receptor type I.

Also, it was documented that some synthetic aryl substituted 3-tetrazolylcoumarins,[18,19] and 3-nitro-4-hydroxycoumarins[20,21] inhibit degranulation of mast cells thus preventing release of chemical mediators such as cysteinyl leukotrienes C_4, D_4 and E_4 (originally described as slow-reacting substances of anaphylaxis, SRS-A) and histamine. In addition, a broad array of other therapeutic applications of natural and synthetic coumarins has been reported in the literature, especially concerning their anti-inflammatory / antioxidant properties,[22–25] their application as neuroprotecting agents,[26] as well as in pharmacotherapy of breast cancer.[27]

Furocoumarins are a class of natural and synthetic compounds used as photoreactive, antiviral, anti-inflammatory agents as well as kinase inhibitors.[28] Furo[4,3-c]coumarins are generally prepared by condensation of aryl substituted 4-hydroxycoumarins as dinucleophiles and vicinal dielectrophiles.[26,29]

In a quest for new anti-inflammatory / antiallergic therapeutics we screened our compound collection in mast cell degranulation assays and leukotriene B_4 assay. Here, we wish to present the synthesis of several so far unreported classes of coumarin compounds, variously substituted furochromenes **2–4**, as well as to report the results on evaluation of their biological activity.

Anti-inflammatory activity of prepared compounds was examined on the basis of *in vitro* tests, inhibition of mast cell (RBL-2H3 cell line of rat basophilic leukaemia) degranulation induced by the activation of Fcε receptor type I. There is significant evidence that mast cells, basophils, and their released mediators are primary effectors of allergic inflammation. Mast cells may contribute to the pathophysiology of a wide variety of diseases including asthma, cardiomyopathy, schleroderma, arthritis, and fibrotic lung disorders. It is believed that many of the deleterious effects that mast cells mediate are due to a variety of proinflammatory molecules exocytosed with exposure to antigen and the subsequent crosslinking of cell-surface-bound immunoglobulin E (IgE), including histamine, serotonin, and the proteases tryptase, chymase, and carboxypeptidase. Furthermore, mast cell activation results in the release and increased production of cytokines including interleukins 4–6, tumor necrosis factor- alpha, prostaglandin D_2, and leukotrienes A_4 and C_4. Clearly, modulation of mast cell activation and degranulation could significantly affect a number of disease states.[30]

The inhibition of degranulation may prevent the symptoms and arrest the inflammation progress, which has been proven by the clinical use of degranulation inhibitors (sodium chromoglycate, nedochromyl sodium and ketotifen). Cysteinyl leukotriene antagonists have also proven efficacy in asthma therapy.[31] Therapeutically, the 5-lipoxygenase inhibitor, zileuton, is as effective as cysteinyl leukotriene antagonists, and its therapeutic effects are indistinguishable from those of the cysteinyl leukotriene antagonists. Number of LTB_4 antagonists were reported as well.[32]

Discovery of new generation of compounds which would inhibit mast cell degranulation as well as prevent synthesis of leukotrienes potentially provides a therapeutic approach for treatment of allergies and other inflammatory diseases.

Anti-inflammatory potential of selected compounds was also evaluated in the *in vivo* anti-inflammatory model of phorbol 12-myristate 13-acetate (PMA) induced ear oedema in CD1 mice. Mouse ear inflammation induced by different irritants, including PMA provides a range of skin inflammation models suitable for the evaluation of both topical and systemic anti-inflammatory agents.[33]

EXPERIMENTAL SECTION

General methods

Starting 4-hydroxycoumarins **1a, d, f–h** and **l** were commercially available. All other 4-hydroxycoumarins **1b–c, e, i–k, m–r** were synthesised according to protocols described in literature[34] and references cit-

ied therein. NMR spectra were recorded at 24 °C on a Bruker Avance DPX 300 spectrometer. Chemical shifts are reported as δ (ppm) relative to TMS as internal standard, and J is in Hz. High performance liquid chromatography-mass spectrometry (HPLC-MS) analysis were performed using a Waters XTerra RP18 analytical column (packing: 3.0 × 100 mm) on a Quattro micro™ (Micromass) mass spectrometer, using external calibration. Gradient was from 20 % MeCN / 0.1 % TFA in water to 80 % MeCN / 0.1 % TFA (flow rate 0.6 or 1 ml min^{-1}) over 15 min. The UV absorption was monitored at 310 nm. Reported values are monoisotopic masses. For all synthesized compounds determined purities were ≥ 95 % by HPLC-UV-MS.

Synthesis

*General synthetic methodology for Furochromenes **2–4***
General methodology to prepare hemiacetals **2**. Appropriate 4-hydroxycoumarin **1** (1 mmol) was mixed with a 40 % aqueous solution of glyoxal (2.5 mmol). Acetonitrile (5–10 ml) was added thereto and the reaction mixture was refluxed for 2–6 hours. After cooling the product was filtered and washed with acetonitrile, affording hydroxy furochromen derivatives **2a–p** as pure compounds.

General methodology to prepare acetals **3**. Into a solution of appropriate 4-hydroxycoumarin **1** (1 mmol) in MeOH (5 ml) was added aqueous dimethoxyacetaldehyde (60 % solution, 8 mmol). Reaction mixture was stirred at reflux temperature for 5 hours. After cooling to room temperature, product was collected by filtration and washed with MeOH, affording **3a–r** as pure compounds.

General methodology to prepare rearranged compounds **4**. Appropriate metoxy furochromen derivative **3** (0.3 mmol) was suspended in acetic acid (5 ml). The reaction mixture was refluxed for 1–24 hours, whereat the starting material was dissolved, and then a precipitate was formed. After cooling to the room temperature the product was collected by filtration and washed with acetic acid and diethyl ether, affording furochromen derivatives **4** as pure compounds.

Synthetic procedure for enol ether **5**. To a suspension of **2a** (0.412 mmol) in Me_2CO (10 ml) powdered anhydrous K_2CO_3 (2.89 mmol) was added, followed by Me_2SO_4 (2.12 mmol). Reaction mixture was heated to reflux and vigorously stirred for 24 hours, cooled to room temperature, followed by filtration. The filtrate was evaporated to dryness and the residue then triturated with ice-cold water for 10 minutes, filtered off and recrystallized from MeOH (140 mg / 25 ml) to obtain product **5**.

Synthetic procedure for acetylation of compound **3a**. A suspension of **3a** (1.05 mmol) in Ac_2O (12 ml) was stirred at reflux for 1 h. After cooling to room temperature, resulting precipitate was collected by filtration, washed with CH_3CN to afford product **6**, which was further recrystallized from Ac_2O.

*2-hydroxy-3-(4-hydroxy-2-oxo-2H-chromene-3-yl)-2,3-dihydro-4H-furo[3,2c]chromene-4-one (**2a**)*. (0.13 g, 74 %); white solid; ^{1}H-NMR (300 MHz, DMSO-d_6) δ / ppm: 4.84 (s, 1H), 6.29 (bs, 1H), 7.28–7.58 (m, 4H), 7.63–7.80 (m, 3H), 8.04 (d, J = 7.6 Hz, 1H), 12.02 (bs, 1H); ^{13}C-NMR (75.4 MHz, DMSO-d_6,) δ / ppm: 43.8, 101.6, 101.9, 109.6, 111.9, 115.8, 116.3, 116.6, 122.6, 123.3, 123.9, 124.3, 132.3, 132.7, 152, 154.1, 158.4, 161.4, 161.9, 164.9; ES-MS m / z: 363.2 $[M-H]^-$.

*3-(4-hydroxy-2-oxo-2H-benzo[g]chromene-3-yl)-2-methoxy-2,3-dihydro-4H-benzo[g]furo[3,2-c]chromene-4-one (**3a**)*. (0.15 g, 80 %); white solid; ^{1}H-NMR (300 MHz, DMSO-d_6) δ / ppm: 3.69 (s, 3H), 5.02 (d, J = 2 Hz, 1H), 6.24 (d, J = 3.5 Hz, 1H), 7.54-7.71 (m, 2H), 7.90 (s, 1H), 7.98 (s, 1H), 7.99-8.10 (m, 2H), 8.21 (d, J = 7.9 Hz, 1H), 8.51 (s, 1H), 8.67 (s, 1H); ^{13}C-NMR (75.4 MHz, DMSO-d_6,) δ / ppm: 42.5, 56.6, 100.8, 102.1, 111.9, 114.6, 115.8, 116.3, 116.6, 122.6, 123.5, 124, 124.4, 132.5, 132.9, 152.1, 154.1, 158.2, 161.4, 162, 165; ES-MS m / z : 379.2 $[MH]^+$.

*2-(4-hydroxy-2-oxo-2H-chromene-3-yl)-4H-furo[3,2-c]chromene-4-one (**4a**)*. (0.05 g, 45 %); yellow solid; ^{1}H-NMR (300 MHz, DMSO-d_6) δ / ppm: 7.28 (s, 1H), 7.39-7.49 (m, 3H), 7.52-7.79 (m, 3H), 7.96 (dd, J = 7.8 Hz, J = 1.4 Hz, 1H), 8.09 (dd, J = 8.2 Hz, J = 1.7 Hz, 1H); ^{13}C-NMR (75 MHz, DMSO-d_6) δ / ppm: 108.2, 110, 112, 115.9, 116.2, 116.6, 120.6, 123.7, 123.9, 124.4, 130.4, 132.8, 149.7, 151.7, 151.9, 152.3, 155.6, 156.7, 159.6, 163.6; ES-MS m / z : 345 $[M-H]^-$.

*1,1-bis(4-methoxy-2-oxo-2H-chromen-3-yl)-2-methoxy-ethene (**5**)*. (0.08 g, 48 %); colorless crystals; ^{1}H-NMR (300MHz, $CDCl_3$) δ / ppm : 3.85 (s, 3H), 4.05 (s, 3H), 4.13 (s, 3H), 6.89 (s, 1H), 7.22–7.32 (m, 4H), 7.45–7.52 (m, 2H), 7.73 (dd, J = 1.5, 7.9 Hz, 1H), 7.83 (dd, J = 1.5, 7.9 Hz, 1H); ^{13}C-NMR (75 MHz, $CDCl_3$) δ / ppm: 59.8, 60.9, 61, 101.8, 106.9, 113.5, 116.3, 116.5, 118.1, 118.2, 123.6, 123.7, 123.8, 124, 131.3, 152.2, 152.3, 154.3, 162.66, 162.71, 162.97, 163; ES-MS m /z: 407.24 $[M-H]^-$.

*3-(4-acetoxy-2-oxo-2H-chromen-3-yl)-2,3-dihydro-2-methoxy-4H-furo[3,2-c]chromen-4-one (**6**)*. (0.38 g, 86 %); white solid; recrystallization from Ac_2O afforded colorless crystals; ^{1}H-NMR (300 MHz, DMSO-d_6) δ / ppm : 2.26 (2, 3H), 3.69 (s, 3H), 5.02 (d, J = 2 Hz, 1H), 6.24 (d, J = 3.5 Hz, 1H), 7.61-7.75 (m, 2H), 7.90 (s, 1H), 7.98 (s, 1H), 7.89-8.13 (m, 2H), 8.21 (d, J = 7.9 Hz, 1H), 8.51 (s, 1H), 8.67 (s, 1H); ES-MS m / z: 421.3 $[M-H]^-$.

Table 1. Crystal data and structure refinement for compounds **5** and **6**

Compound	**5**	**6**
Empirical formula	$C_{23}H_{18}O_7$	$C_{23}H_{16}O_8$
Formula weight	406.37	420.36
Temperature (K)	100(2)	100
Wavelength (Å)	1.54178	1.54178
Crystal system	monoclinic	monoclinic
Space group	$P2_1/c$	$P2_1/n$
Unit cell dimensions	a = 14.0614(5) Å	a = 9.5110(3) Å
	b = 8.3013(4) Å	b = 16.5891(6) Å
	c = 17.9313(5) Å	c = 12.6032(6) Å
	$\alpha = \gamma = 90°$	$\alpha = \gamma = 90°$
	β = 112.060(5)°	β = 111.200(11)°
Volume (Å^3)	1939.85(15)	1853.9(2)
Z	4	4
Calculated density (Mg m^{-3})	1.391	1.506
Absorption coefficient (mm^{-1})	0.868	0.115
F(000)	848	872
Crystal size (mm)	0.4 × 0.2 × 0.2	0.3 × 0.3 × 0.2
Limiting indices	$-16 \le h \le 16$	$-11 \le h \le 11$
Limiting indices	$-9 \le k \le 9$	$-19 \le k \le 19$
Limiting indices	$-21 \le l \le 20$	$-14 \le l \le 14$
Reflections collected / unique	5638 / 3284 [R(int) = 0.1009]	5834 / 3098 [R(int) = 0.0474]
Refinement method	Full-matrix least-squares on F^2	Full-matrix least-squares on F^2
Data / restraints / parameters	3284 / 0 / 271	3098 / 0 / 281
Goodness-of-fit on F^2	1.027	0.981
Final R1 / wR2 [$I > 2\sigma(I)$]	0.0685 / 0.1921	0.0529 / 0.1325
R1 / wR2 (all data)	0.0818 / 0.2102	0.0833 / 0.1517
Largest diff. peak / hole (e Å^{-3})	0.310 / -0.233	0.459 / -0.503
Extinction coefficient	-	0.036(5)

X-Ray Crystallography

Crystal structures of compounds **5** and **6** were determined by the single crystal X-ray diffraction method. Data were collected at 100 K with a Kappa-CCD Nonoius diffractometer using graphite monochromated CuKα radiation. Data reduction was carried out using DENZO and SCALEPACK[35] software. The details of crystal data collection and refinement parameters for compounds **5** and **6** are listed in Table 1. Further details on crystal structure investigation are available free of charge on application to CCDC, 12 Union Road, Cambridge CB2 IEZ, UK, www.ccdc.ac.uk/conts/retrieving.html or deposit@ccdc.cam.ac.uk under following deposition numbers; CCDC 941948 for compound **5** and CCDC 941949 for compound **6**.

The structure was solved by direct methods implement in SHELXS97[36] and refined by a full-matrix least-squares method based on F^2 using SHELXL97.[35] Hydrogen atoms were positioned geometrically at calculated positions and allowed to ride on their parent atoms.

Computational Studies

Geometry optimization of examined compounds was performed using the density functional theory (DFT) at B3LYP[37,38] level with 6-31G(d,p) basis set. Zero-point vibration energy (ZPVE) correction with scaling factor

of 0.9608[39] was applied. Computations and visualizations were performed using Jaguar[40] and Maestro[41] programs, respectively, from the Schrödinger Suite 2011.

Inhibition of RBL-2H3 Mast Cell Degranulation

RBL-2H3 cell line of rat basophilic leukaemia (from ATCC) was used for the investigation of inhibition of degranulation induced by the activation of Fcε receptor type I or calcium ionophors. RBL-2H3 cell line was cultivated in DMEM medium (Invitrogen Corporation Cat. No. 31966-021) with 10 % of phoetal calf serum (Invitrogen Corporation) at 37 °C, 5 % CO_2, 90 % relative humidity. Cells were seeded in the same medium into 24-well plates, 50000 per well, and left to reach 80–90 % of confluence.

Dilutions of compounds were prepared in DMEM medium without phenol red (Invitrogen Corporation) in concentrations from 200 μM to 1 μM. The medium was removed from the cells and the diluted of compounds were added to the wells with the exception of the positive and the negative control where pure DMEM medium was added. Subsequently,

1. *for the IgE-induced degranulation by Fcε receptor type I*, a solution of SPE-1 (dinitrophenyl specific IgE) antibodies (Sigma) and dinitrophenylalbumin (Sigma), both in a final concentration of 0.5 μg ml^{-1},
2. *for Ca^{2+}-induced degranulation by means of a calcium ionophore*, the solution A23187 (Calbiochem) in a final concentration of 250 ng ml^{-1}, were added to the wells.

In the case of the negative control wells, pure DMEM medium was added. The cells were incubated for one hour at 37 °C, 5 % CO_2, and 90 % relative humidity. Each dilution as well as the positive and the negative controls were performed in triplicate.

The supernatant (50 μl) was transferred in duplicate to a 96-well plate. Thereto 100 μl of 50 mM sodium citrate buffer with 1 mg ml^{-1} para-nitrophenyl-N-acetyl-β-D-glucosaminide (Calbiochem) were added and it was incubated for 1 hour at 37 °C. The reaction was stopped with 100 μl of a saturated sodium carbonate solution. The absorbance was measured at 405 nm. The percentage of inhibition was expressed by the formula:

% inhibition = (1-(OD_{405}sample - OD_{405}negative control) / (OD_{405}positive control - OD_{405}negative control)) × 100.

Ketotifen, used as a standard, significantly inhibits degranulation in concentrations from 200-50 μM.

Leukotriene B_4 (LTB_4) Inhibition Assay

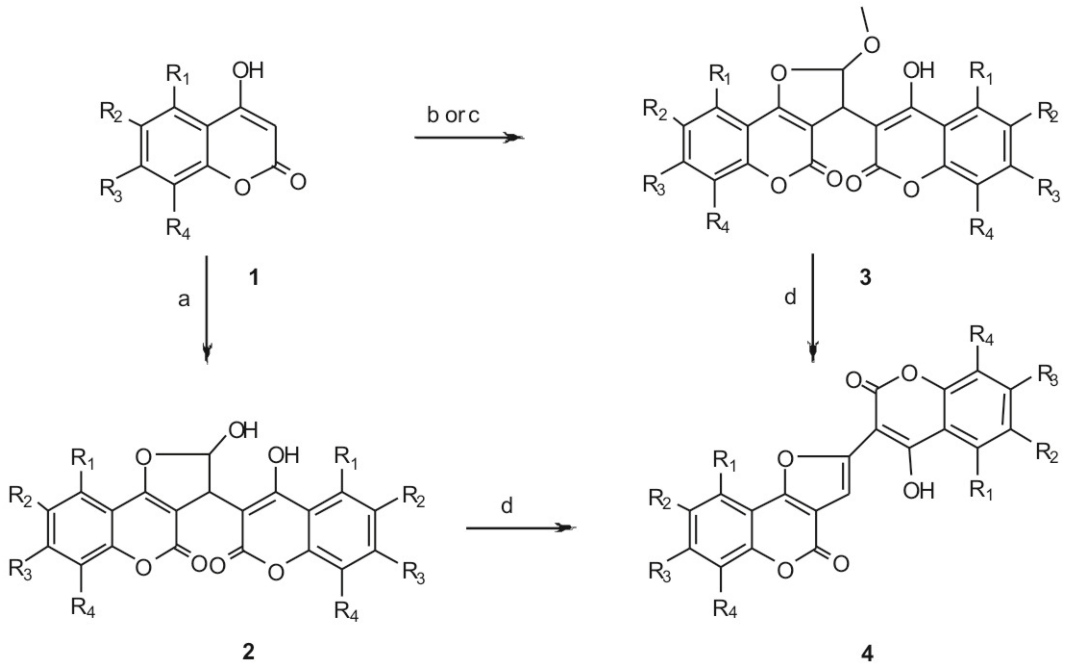
Compounds were assayed for their ability to inhibit the production of leukotriene B_4 in A23187 stimulated RBL-2H3 cells. RBL-2H3 cell line (ATCC 2256) is grown in DMEM medium (Invitrogen) supplemented with 10 % FBS (Invitrogen) in the atmosphere of 5 % CO_2, 90 % humidity, 37 °C. Cells are trypsinazed, washed with fresh DMEM medium and adjusted to 1 × 10^5 cells / ml. 500 μl / well of cell suspension is transferred into 24 well plate (Falcon) and grown overnight in culturing condition described herein. 10 mM solutions of tested compounds are prepared in DMSO (Sigma), and dissolved in working concentrations in DMEM medium without phenol red (Invitrogen). Dilutions of tested compounds are placed on cells, whereas for positive and negative controls only DMEM medium without phenol red are used and left in culturing conditions for 30 minutes. A23187 (Sigma) was added into all wells except negative controls in the final concentration of 250 nM and left for 45 minutes in culturing conditions. 10 μl of cellular supernatant was used to determine leukotriene B_4 levels using ELISA (R&D systems). Total concentrations of LTB_4 are calculated in samples and total inhibition was calculated using the formula:

% inhibition = (1 - LTB_4 sample concentration / LTB_4 positive control concentration) × 100.

Phorbol 12-myristate 13-acetate (PMA) Induced Ear Oedema in CD1 Mice

Male CD1 mice (Iffa Credo, France) weighing ≈ 35–40 g were randomly grouped (n = 8 in vehicle treated test group, dexamethasone treated control group as well as in groups treated with compounds to be assayed). Test compounds, dexamethasone as well as vehicle (Transphase Delivery System, containing benzyl alcohol 10 %, acetone 40 % and isopropanol 50 %) (all from Kemika, Croatia), were administered topically to the internal surface of the left ear thirty minutes prior to administration of phorbol 12-myristate 13-acetate (PMA) (Alexis biochemicals, USA). Test compounds were administered at a single dose of 250 or 100 μg / 15 μl / ear and dexamethasone at a single dose of 50 μg / 15 μl / ear. Thirty minutes later, 0.01 % PMA solution in acetone was applied topically to the same area of each animal in a volume of 12 μl / ear. During the treatment and challenge, animals were anaesthetized by using inhalation anaesthesia. Six hours after the challenge, animals were euthanized by asphyxiation in 100 % CO_2 atmosphere. For assessing the auricular oedema, 8 mm discs were cut out of left and right auricular pinna and weighed. The degree of oedema was calculated by subtracting the weight of 8 mm disc of the untreated ear from that of the treated contralateral ear.

The compound at appropriate dose is considered active if the supression of ear oedema in compound treated group is statistically significantly different in comparison to positive control group, as calculated by the non-parametric ANOVA statistical method (statistical analysis was done using the program GraphPad



Scheme 1. Synthesis of furochromen compounds **2-4**. Reagents and conditions: a) glyoxal, MeCN, reflux; b) glyoxal, MeOH, reflux; c) dimethoxyacetaldehyde, MeOH, reflux; d) AcOH or TFA, reflux.

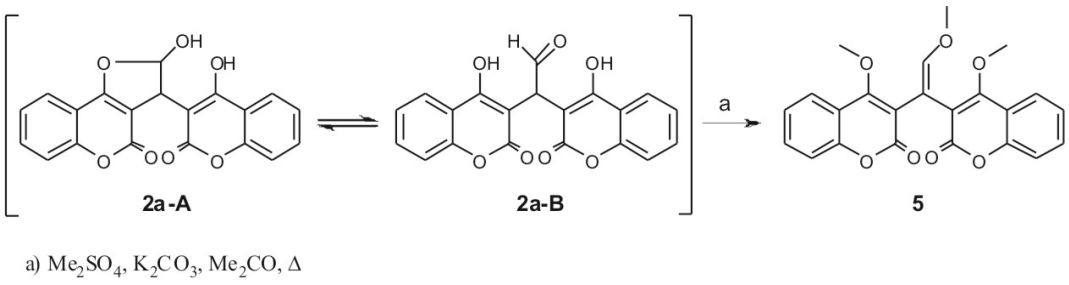
InStat v.3.06.

Statement. All procedures on animals were performed in accordance with (a) the EEC Council Directive 86 / 609 of 24th November 1986 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes; and (b) Statute of Republic Croatia, Animal Welfare Law, NN 135 of 13th December 2006.

RESULTS AND DISCUSSION

Chemistry

The compounds under study were prepared by condensation reaction of variously substituted hydroxycoumarins with glyoxal or dimethoxyacetaldehyde as dielectrophiles via tandem Knöevenagel / Michael addition reaction, followed by annulation and dihydrofuran ring formation (Scheme 1). When reaction was performed in nonnucleophilic solvent, such as acetonitrile, hemiacetals **2** were isolated as final products while acetals **3** were isolated after condensation in alcoholic solvent, *e.g.* methanol (as exemplified in this paper) or ethanol (not shown). Treatment of either **2** or **3** with dehydrating agents such as acetic acid or trifluoroacetic acid (TFA) resulted in the formation of new class of 2-substituted furo[3,2-*c*]chromens **4**. In this case elimination of C-2 substituent is accompanied by rearrangement and migration of the second coumarin moiety to C-2 position *via* neighbouring participation of 3'-4' double bond and formation of spiro[chroman-cycopropane]dione intermediate. 2-Methyl-4*H*-furo[3,2-c]chromen-4-one is already reported as a product of such rearrangement.[42]



Scheme 2. Cyclic (**2a-A**) and open chain (**2a-B**) isomeric forms of **2** and product of methylation (**5**).

Keto-enol tautomerism is the most common structural isomerism in 4-hydroxycoumarins. Due to dynamic equilibrium tautomers are rapidly interconvertible from one isomeric form to another and tautomer determination is quite challenging.[43] Other structural isomers are possible as well. In our studies we noticed that in compounds **2** ring-chain tautomerism[44] also exists (Scheme 2). Which of the isomeric form prevails in the mixture, cyclic (hemiacetal) or open chain (aldehyde), depends on the nature and number of the aromatic substituents, whether the compound is in a free acid form or as a salt. Type of the salt, type of the solvent in which the compound is dissolved, as well as the pH value of the solution also have an effect on the tautomer ratio.

For the purpose of structure elucidation NMR spectra of investigated compounds were run in DMSO-d_6. The cyclic form (i.e. **2a-A**) prevails in such solutions with anomeric proton (H-2) at ppm 6.2 and H-3 at 4.8. Existence of open chain form was confirmed by standard methylation reaction with dimethyl sulphate in the presence of potassium carbonate. Enol ether **5** was isolated as the only reaction product (Scheme 2). The molecular and crystal structures of **5** were determined by single-crystal X-ray diffraction (Figure 2, Table 1).

Both series of compounds **2** and **3** contain two stereocenters and therefore 4 possible stereoisomers could be expected. However, the pair of enantiomers with *trans*- configuration around C2-C3 bond in **3** is calculated to be more favorable (Figure 3), which was determined experimentally as well.

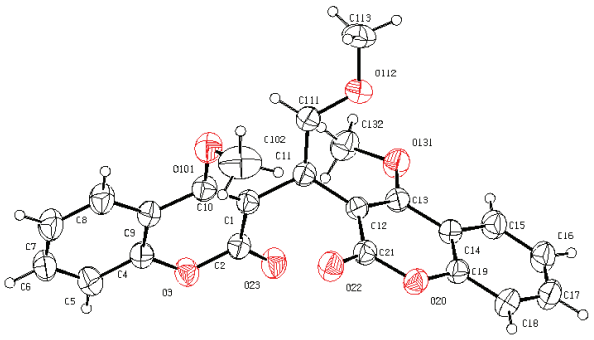
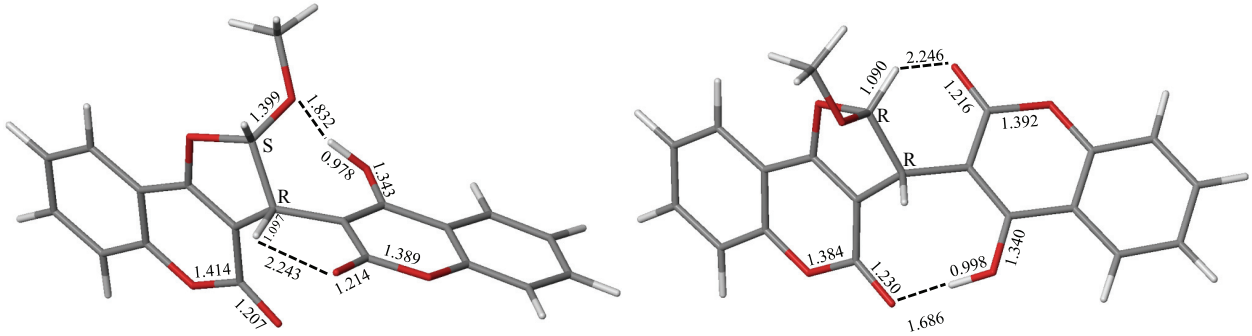


Figure 2. The molecular structure of compound **5**.

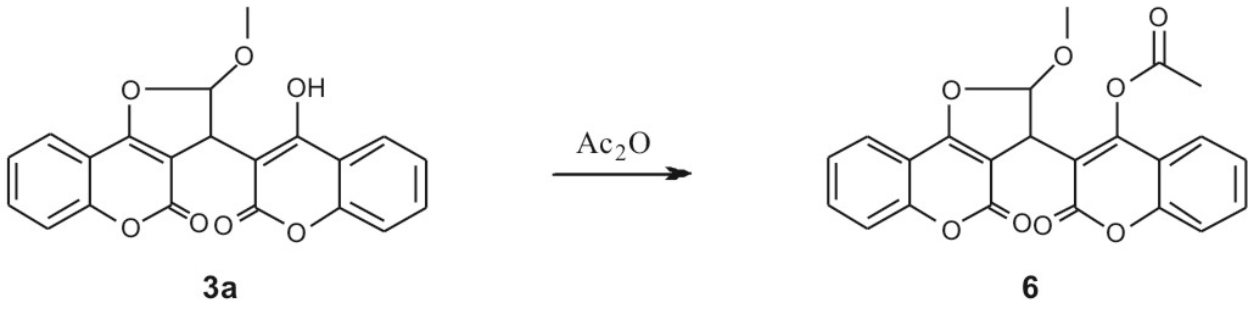
Acetylation of **3a** afforded compound **6** (Scheme 3), whose molecular and crystal structures (Figure 4) were determined by single-crystal X-ray diffraction (Table 1) and product was shown to be a racemic mixture with *trans* configuration around C2-C3 bond, suggesting (*trans*)-diastereoselectivity in the formation of acetal **3a**.



cis-(*R*,*S*)-**3a** [(*S*,*R*)-**3**] *trans*-(*R*,*R*)-**3a** [(*S*,*S*)-**3**]

ΔE_0(*cis-trans*) = 6.1 kcal mol^{-1}

Figure 3. *Trans*-**3a** recognized as favored product over *cis*-**3a**.



3a **6**

Scheme 3. Acetylation of **3a** affords compound **6.**

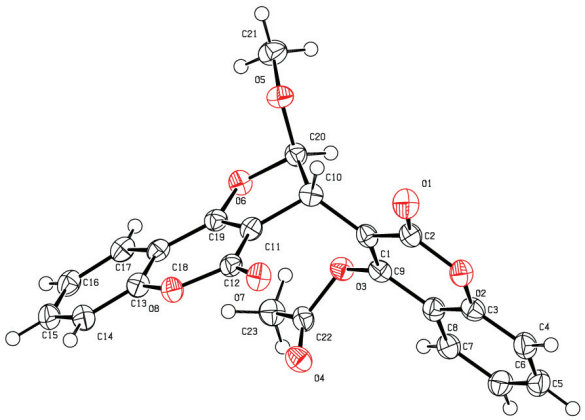


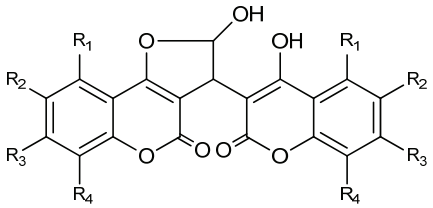
Figure 4. The molecular structure of compound **6**.

Pharmacology

The synthesized compounds were evaluated for their anti-inflammatory activity in the *in vitro* RBL-2H3 rat mast cells degranulation which was Fcε receptor or Ca^{2+}-triggered, and by inhibition of leukotriene B_4 (LTB_4), at inhibitor concentration of 10 μM.[45] For most of them Fcε receptor triggered degranulation were repeated with inhibitor concentration of 30 μM indicating dose-response behavior of analyzed compounds. Results are summarized in Tables 2–4. Compounds that significantly inhibited (*i.e.* 50 % or higher) degranulation of mast cells or LTB_4 are considered to be fully "active" and to possess anti-inflammatory activity.

The central role of the mast cell in the initiation of allergic responses makes it an ideal target for anti-allergic drug therapy. Mast cells are the primary initiating cells of immediate hypersensitivity reactions so their degranualtion is indicated as invoked in immediate or delayed type hypersensitivity reaction, allergy, anaphylaxis, inflammation, asthma and urticaria.[46] Immunological activation of mast cells results not only in degranulation but also in the liberation of arachidonic acid from phospholipids in the cell membrane. Their further

Table 2. Inhibition (%) of Fcε receptor triggered (Fcε) and Ca^{2+}-induced (Ca^{2+}) degranulations as well as leukotriene B_4 (LTB_4) production in RBL-2H3 rat mast cells for hemiacetales **2a**–**2p** at an inhibitor concentration of 10 μM

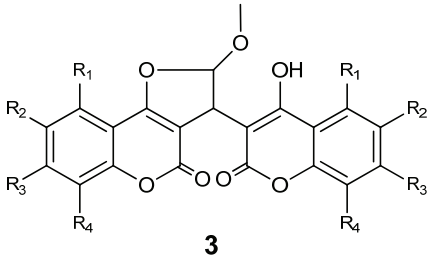


2

					Fcε		Ca^{2+}	LTB_4
Cpd	R_1	R_2	R_3	R_4	10 μM	30 μM[(a)]	10 μM	10 μM
2a	H	H	H	H	0	ND[(b)]	0	0
2b	H	OH	H	H	0	ND[(b)]	0	0
2c	H	OMe	H	H	0	ND[(b)]	2	23
2d	H	Cl	H	H	64	83	0	0
2e	H	H	Cl	H	75	87	13	13
2f	H	Me	H	H	0	ND[(b)]	0	18
2g	H	H	Me	H	15	35	0	34
2h	H	Me	Me	H	50	95	23	100
2i	Me	H	H	Me	90	95	22	100
2j	Me	H	Me	H	67	92	50	100
2k	H	Me	H	Me	50	95	12	39
2l	H	Cl	Me	H	50	90	18	33
2m	H	Et	H	H	30	90	11	100
2n	H	H	H	Et	64	93	10	68
2o	H	*i*-Pr	H	H	70	80	0	100
2p	H	H	H	*i*-Pr	80	91	5	100
Dicoumarol					45	45	13	0
Ketotifen					0	30	ND[(b)]	ND[(b)]
Zileuton					22	28	0	100

[(a)] Inhibitor concentration of 30 μM. [(b)] ND = not determined.

Table 3. Inhibition (%) of Fcε receptor triggered (Fcε) and Ca^{2+}-induced (Ca^{2+}) degranulations as well as leukotriene B_4 (LTB_4) production in RBL-2H3 rat mast cells for acetals **3a**–**3r** at an inhibitor concentration of 10 μM



3

					Fcε		Ca^{2+}	LTB_4
Cpd	R_1	R_2	R_3	R_4	10 μM	30 μM[(a)]	10 μM	10 μM
3a	H	H	H	H	0	ND[(b)]	0	0
3b	H	OH	H	H	0	ND[(b)]	0	0
3c	H	OMe	H	H	0	ND[(b)]	5	0
3d	H	Cl	H	H	30	50	14	0
3e	H	H	Cl	H	39	74	13	0
3f	H	Me	H	H	26	33	0	0
3g	H	H	Me	H	ND[(b)]	ND[(b)]	ND[(b)]	ND[(b)]
3h	H	Me	Me	H	46	51	18	31
3i	Me	H	H	Me	62	92	0	0
3j	Me	H	Me	H	65	96	10	0
3k	H	Me	H	Me	45	50	3	3
3l	H	Cl	Me	H	58	91	0	7
3m	H	Et	H	H	58	80	2	0
3n	H	H	H	Et	69	94	10	0
3o	H	*i*-Pr	H	H	ND[(b)]	ND[(b)]	10	67
3p	H	H	H	*i*-Pr	56	89	18	100
3r	Me	H	H	Cl	96	91	26	97

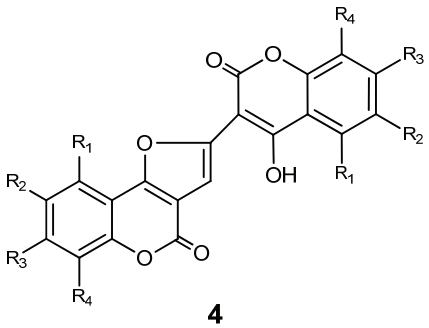
[(a)] Inhibitor concentration of 30 μM. [(b)] ND = not determined.

metabolism may proceed along either of two independent pathways, the cyclooxygenase pathway toward prostaglandins and the lypoxygenase pathway which has the capacity to generate a wide variety of leukotriene products. Leukotrienes are important mediators in host defense mechanisms and in inflammatory disease states due, for example, to their effects on cell migration, muscle contraction, vascular permeability, and the release of lysozomal enzymes.[47]

It was observed that majority of studied compounds inhibit mast cells degranulation triggered by Fcε receptor, and only few of them inhibit degranulation triggered by Ca^{2+}. Significant inhibition of mast cell degranulation induced by the activation of Fcε receptor type I showed certain derivatives with alkyl and halogen substituents as well as combinations of those substituents, whereas unsubstituted compounds and those with hydroxy and methoxy substituents gave the poorest inhibitions. Further, hemiacetals **2** proved to be the most active ones, regarding the *in vitro* activity, but during evaluation were proven to be chemically unstable and because of that were not fully investigated.[48] Acetals **3** and rearranged compounds **4** are equally potent inhibitors of mast cell (RBL-2H3) degranulation (Fcε–triggered).

The most potent inhibitors of Fcε–triggered mast cell degranulation bear liphophilic substituent at R_4 position. The potency of derivatives at R_4 follows the tendency of **2**–**4p** (isopropyl) > **2**–**4n** (ethyl). Methyl at R_4 is well tolerated in combination with additional methyl group at R_1 position as in **2**–**4i**. Introduction of lipophilic substituent at R_3 position, like chlorine in **2**–**4e** and methyl in **2g** and **4g** has positive effect on Fcε–triggered inhibition of mast cell degranulation, especially in combination with additional substitution with methyl at R_1 as in compounds **2**–**4j**. However, R_1 / R_3 combination in **2**–**4j** was somewhat less potent than R_1 / R_4 in **2**–**4i**. Finally, R_2 position tolerates only lipophilic substituents, and the inhibitory potency of derivatives followed the tendency of **2**–**4o** (isopropyl) > **2**–**4d** (chlorine) > **2**–**4m** (ethyl). Less lipophilic or polar groups like methyl (**2**–**4f**), hydroxy (**2**–**4b**), methoxy (**2**–

Table 4. Inhibition (%) of Fcε receptor triggered (Fcε) and Ca^{2+}-induced (Ca^{2+}) degranulations as well as leukotriene B_4 (LTB_4) production in RBL-2H3 rat mast cells for 2-substituted furo[3,2]coumarins **4a**–**4p** at an inhibitor concentration of 10 μM



4

					Fcε		Ca^{2+}	LTB_4
Cpd	R_1	R_2	R_3	R_4	10 μM	30 μM[(a)]	10 μM	10 μM
4a	H	H	H	H	0	ND[(b)]	0	0
4b	H	OH	H	H	0	ND[(b)]	15	0
4c	H	OMe	H	H	0	ND[(b)]	0	0
4d	H	Cl	H	H	0	ND[(b)]	2	24
4e	H	H	Cl	H	78	87	18	32
4f	H	Me	H	H	0	ND[(b)]	6	0
4g	H	H	Me	H	40	40	19	40
4h	H	Me	Me	H	32	ND[(b)]	0	0
4i	Me	H	H	Me	55	78	6	41
4j	Me	H	Me	H	35	65	0	29
4k	H	Me	H	Me	26	30	24	6
4l	H	Cl	Me	H	68	80	15	31
4m	H	Et	H	H	ND[(b)]	ND[(b)]	1	46
4n	H	H	H	Et	68	71	59	32
4o	H	*i*-Pr	H	H	59	64	14	100
4p	H	H	H	*i*-Pr	79	90	37	97

[(a)] Inhibitor concentration of 30 μM. [(b)] ND = not determined.

4c), were not tolerated and led to almost complete loss of activity. Introduction of additional methyl group at R_3 position increase activity, and generally it was determined that disubstituted R_2 / R_3 (**2–4h** and **2–4l**) derivatives were more potent that monosubstituted R_2 analogues (**2–4f** and **2–4d**, respectively). On the other hand, influence of substitutions on LTB_4 inhibition among three classes is not equivocal. Mostly, substitutions with positive effect on Fcε–triggered inhibition of mast cell degranulation also produce significant LTB_4 inhibition. This trend correlates in series **2** and **4**, but regarding series **3**, most of the substitutions at R_1, R_2 and R_3 led to complete loss in LTB_4 inhibition. Exceptions are most lipophilic substituents like isopropyl at R_2 in **3o** and at R_4 in **3p**. Also combination of methyl at R_1 and chlorine at R_4 in series **3**, led to very potent compound **3r**.

The most promising compounds were then selected for further evaluation in the *in vivo* anti-inflammatory model of phorbol 12-myristate 13-acetate (PMA) induced ear oedema in CD1 mice (Table 5). PMA induction of acute skin inflammation was conducted according to Napoletano *et al.*[49] with some modifications. PMA is tumor-promoting agent, and protein kinase C activator widely used to induce cutaneous inflammation in experimental animal models.

Table 5. *In vivo* anti-inflammatory activity of selected compounds in the model of phorbol 12-myristate 13-acetate (PMA) induced ear oedema in CD1 mice

		Ear oedema
Cpd	μg / ear	% inhibition*
3p	250	71
3p	100	39
3r	100	68
4p	100	33
Zileuton	100	38
Dexamethasone	50	89

*$P < 0.05$, Non-parametric ANOVA for all compounds

Epicutaneous application of PMA results in vascular leakage, leukocyte infiltration, epidermal hyperplasia, activation of protein kinase C and increased release of arachidonic acid (AA) and its metabolites.[50] The initiation of inflammatory responses by metabolites of AA and suppression of acute inflammatory responses by inhibitors of cyclooxygenase (COX) and lypoxygenase (LOX) establish an important role for metabolites of AA in acute inflammation induced by AA and PMA.[51]

Compounds **3r**, **3p** and **4p** showed statistically significant suppression of ear eodema in comparison to positive control group, with comparable or even better activity to 5-LO and LTB_4 inhibitor zileuton.

These findings attracted us to extend our research to this class of compounds in the quest for anti-inflammatory – antiallergic medication.

CONCLUSION

Several new series of coumarin compounds, hemiacetals **2**, acetals **3**, and rearranged compounds **4**, have been synthesized and their inhibitory potential to prevent mast cell degranulation as well as LTB_4 production was evaluated. Significant inhibition of mast cell degranulation induced by the activation of Fcε receptor type I showed certain derivatives with alkyl and halogen substituents as well as combinations of those substituents, whereas unsubstituted compounds and those with hydroxy and methoxy substituents gave the poorest inhibitions.

Position R_1 is tolerated for Me substituent like in **2i** and **3r**. Majority of the active compounds are unsubstituted on position R_2, however, some compounds with R_2 = iPr or Cl shown inhibition larger than 50 % (**2d**, **2o**, **4o**, **3l**, **4l**). On the other hand, compounds with oxygen in R_2 (R_2 = OH and OMe) haven't shown any activity. Compounds **2e** and **4e** where R_3 = Cl shown inhibitor activity larger than 75 %. Position R_4 is tolerated for larger lipophilic substituents like Et (**2n**, **3n**, **4n**) and iPr (**2p**, **4p**). Additionally, in combination with R_2 = Me, compounds where R_4 = Cl (**3r**) and Me (**2i**, **3i**, **4i**) shown the best activities in this set.

Significant inhibition of LTB_4 exhibited mono- and bi-alkyl substituted compounds **2h–j**, **2m–p**, **3o–p**, and **4o–p** as well as 5-methyl-8-chloro substituted derivative **3r**.

Selected compounds, **3p**, **4p** and **3r**, exhibited significant anti-inflammatory activity in PMA induced ear edema in CD1 mice, with potency equal (compounds **3p** and **4p**) or better (compound **3r**) in comparison with zileuton as the reference compound. This is very significant finding since it opens a new possibility of developing new class of non-steroidal compounds for the treatment of inflammatory diseases.

Further structural modifications are in progress with a view to shedding light on the better understanding of anti-inflammatory activity of 4-hydroxycoumarin compounds and critical structural parameters that affect biological activity.

Abbreviations

RBL-2H3 – Cell line of rat basophilic leukaemia
LTB_4 – Leukotriene B_4
IgE – Immunoglobulin E
Fcε receptors – Fc receptors that bind IgE
PMA – Phorbol 12-myristate 13-acetate
DMEM – Dulbecco's modification of Eagle's medium
FBS – Fetal bovine serum
ELISA – Enzyme-linked immunosorbent assay
ANOVA – Analysis-of-variance statistical test

Supplementary Materials. – Supporting informations to the paper are enclosed to the electronic version of the article. These data can be found on the website of *Croatica Chemica Acta* (http://public.carnet.hr/ccacaa).

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