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Laboratory note

Synthesis, toxicological and pharmacological assessment of some 4-hydroxycoumarin derivatives

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Summary — The synthesis of seven coumarin derivatives from 4-hydroxycoumarin and different unsaturated ketones is described. The structures of the synthesized compounds were proved by IR, ^{1}H-NMR and mass-spectral data. Acute toxicity studies of the compounds were performed on mice by oral and intraperitoneal administration. A comparative pharmacological study of the *in vivo* anticoagulant effects of the derivatives with respect to Warfarin showed that the new compounds have different anticoagulant activities. 4-Acetoxy-3-[1-(4-nitrophenyl)-3-oxobutyl]-2*H*-1-benzopyran-2-one **2** showed slight acute toxicity and a higher anticoagulant effect than Warfarin.

Warfarin / Acenocoumarol / 4-hydroxycoumarin derivative / blood coagulation time

Introduction

Warfarin [1, 2], Acenocoumarol [3, 4], Coumachlor [5–8] and the 4-hydroxycoumarin derivatives possess anticoagulant and rodenticidal activities. The most widely used of these drugs in USA and Canada is Warfarin. However, the drugs of this group exhibit some side effects including the Warfarin-related purple toes syndrome [9]. By chemical modification of the Warfarin structure, it is possible to obtain new compounds with biological activity and toxicity comparable to that of Warfarin, but with lower toxicity and fewer side effects.

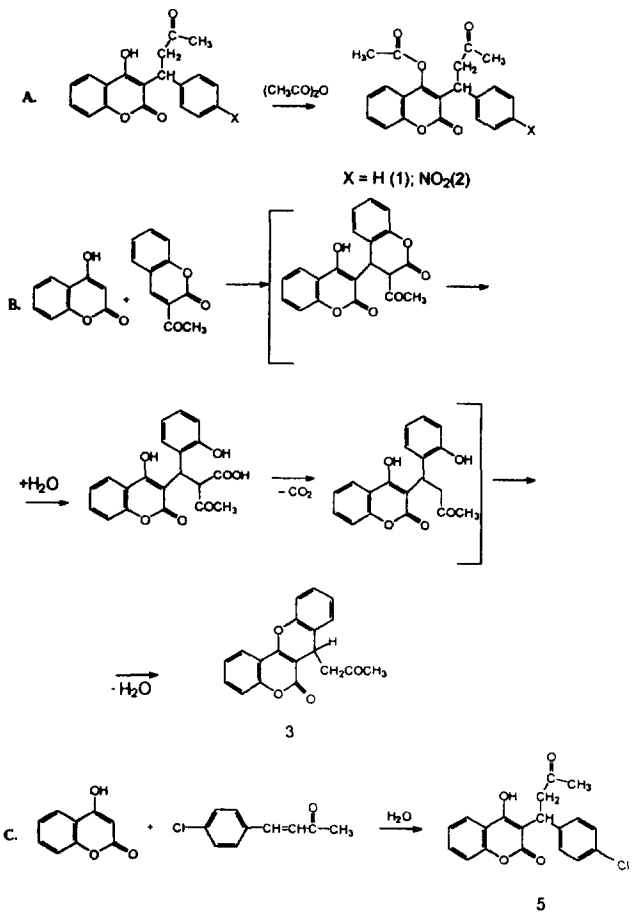
Chemistry

We synthesized Acenocoumarol [10–12] and Warfarin [12, 13] and treated them with acetic anhydride at a molar ratio 1:16 to produce 4-acetoxy derivatives [14] (scheme 1, A). The structures of these compounds were confirmed by mass-spectral analysis. The possible fragmentation pathways are proposed in the schemes 2 and 3. The initial fragmentation step of 4-acetoxy-3-(3-oxo-1-phenylbutyl)-2*H*-1-benzopyran-2-one **1** involves an elimination of ketene thus obtaining the cation-radical with *m/z* 353. The latter decomposed by two pathways through an elimination of $CH_3C^{\bullet}O$ to produce the ion with *m/z* 310, and through an elimination of ketene to produce the ion with *m/z* 311. The base peak of the compound is the resonance-stabilized acetyl-cation with *m/z* 43 (scheme 2).

The mass-spectral fragmentation of 4-acetoxy-3-[1-(4-nitrophenyl)-3-oxobutyl]-2*H*-1-benzopyran-2-one **2** is analogous to compound **1**. The highest intensity peak of the ion with *m/z* 265 was obtained by consecutive elimination of a ketene and acetyl radical from M^+ (scheme 3).

4-Hydroxycoumarin was treated with 3-acetylcoumarin at a molar ratio 1:1 with solution of 0.4 g NaOH in 10 ml water. The reaction mixture was refluxed and stirred for 20 h. The reaction time for the appearance of **3** [1] and the exhaustion of 4-hydroxycoumarin and 3-acetylcoumarin was monitored by TLC (scheme 1, B). The structure of 7-acetonyl-6-oxo-6*H*,7*H*-(1)-benzopyrano[4,3-*b*]-1-benzopyran **3** was confirmed by mass-spectral analysis. The possible fragmentation pathways are proposed in the scheme 4.

Treatment of 4-hydroxycoumarin with an ammonium acetate in a molar ratio 1:10 afforded 4-aminocoumarin **4** [15, 16]. Treatment of 4-hydroxycoumarin with 4-chlorophenyl-3-butene-2-one in a molar ratio 1:1 afforded Coumachlor **5**. Coumachlor was treated with an aqueous solution of sodium hydroxide in a molar ratio 1:0.95 to produce the coumachlor sodium salt **6**.



Scheme 1.

Pharmacology

The freshly prepared compounds were investigated for acute toxicity in mice by oral and intraperitoneal administration. An assessment of the coumpounds' effect on blood coagulation time after single and 3 day treatment was made. Warfarin and Acenocoumarol were used as reference compounds.

Results and discussion

Analysis of the experimental data for the acute toxicity (LD_{50}) for the freshly prepared compounds and comparison with Warfarin and Acenocoumarol (tables I and II) show that the compound **3** has a low toxicity (LD_{50} = 2099 mg/kg body weight (bw)) by intraperitoneal administration (table II). Compound **5** exhibited higher toxicity upon oral application. Compounds **1–4** were practically nontoxic (LD_{50} > 5000 mg/kg bw).

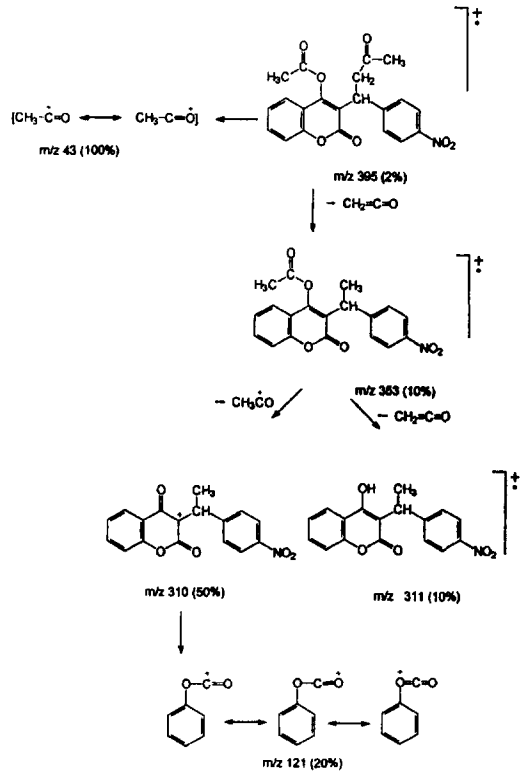
Table I. Acute toxicity (LD_{50}) of the compounds after oral application on male mice.

Compound	*LD_{50} (mg/kg) range of values*	*Statistically significant differences*[a]
1	> 5000	$p \leq 0.05$ (**5,6,W**)
2	> 5000	$p \leq 0.05$ (**5,6,W**)
3	> 5000	$p \leq 0.05$ (**5,6,W**)
4	> 5000	$p \leq 0.05$ (**5,6,W**)
5	391.8 (339.3–452.7)	$p \leq 0.05$ (**1,2,3,4,6,W**)
6	1315.4 (1079.9–1601.1)	$p \leq 0.05$ (**1,2,3,4,5**)
Warfarin (W)	1468.6 (1212.7–1778.6)	$p \leq 0.05$ (**1,2,3,4,5**)

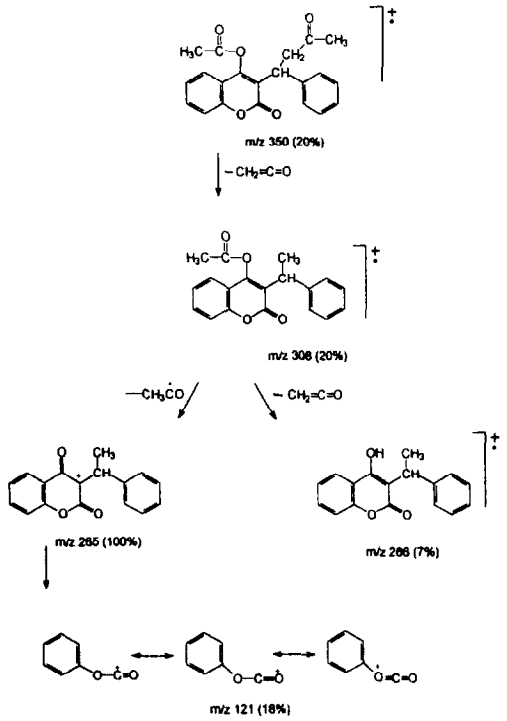
[a]Compared with the compounds indicated in parentheses.

After intraperitoneal application, the observed toxicities of the compounds were as follows. Compounds **5** and **6** have similar high toxicities ($LD_{50} \approx 220$ mg/kg bw). Warfarin was more toxic than compounds **1–4** ($p \leq 0.05$) and less toxic ($p \leq 0.05$) than the compounds **5** and **6** (table II).

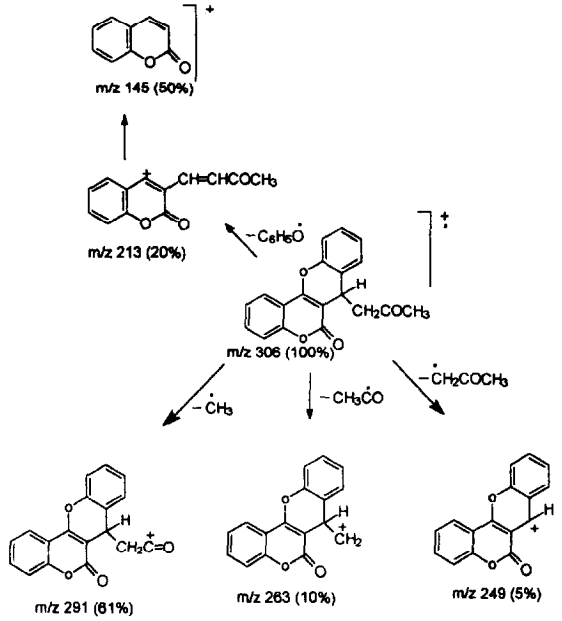
As shown by *in vivo* experiments after a single oral administration of the compounds in doses of 10% of



Scheme 2.



Scheme 3.



Scheme 4.

the LD_{50}, a high increase in the blood coagulation time (measured 24 h after administration) was in the groups treated with compounds **5** and **6**. The effect was statistically significant ($p \leq 0.05$) in comparison to the control group and Warfarin (table III). The data for the blood coagulation time after a 3 day treatment in doses of 10% and 5% of the LD_{50} are presented in table IV. All the tested compounds increased the blood coagulation time in comparison with the control saline-treated group. The highest effect was observed in the group treated with compound **2**, followed by the groups treated with Warfarin, and compounds **5** and **1** (table IV). It is known that the anticoagulant effect of Warfarin is related to the change of the synthesis of inactive group of the vitamin-K-requiring clotting factors II, VII, IX and X. The effect of Warfarin proteins C and S explains the thrombolytic effect of the drug [17].

Table II. Acute toxicity (LD_{50}) of the compounds after intraperitoneal application on male mice.

Compound	*LD_{50} (mg/kg) range of values*	*Statistically significant differences*[a]
1	1479.9 (1058.9–2068.5)	$p \leq 0.05$ (**5,6,W**)
2	1330.9 (967.3–1830.9)	$p \leq 0.05$ (**5,6,W**)
3	2099.8 (1849.4–2384.5)	$p \leq 0.05$ (**2,5,6,W**)
4	1780.4 (1302.6–2434.4)	$p \leq 0.05$ (**5,6,W**)
5	220.9 (190.2–256.6)	$p \leq 0.05$ (**1,2,3,4,W**)
6	224.7 (197.4–256.3)	$p \leq 0.05$ (**1,2,3,4,W**)
Warfarin (**W**)	750.0 (581.9–966.6)	$p \leq 0.05$ (**1,2,3,4,5,6**)

[a]Compared with the compounds indicated in parentheses.

Table III. Effect of the compounds on blood coagulation time of the mice after single oral administration.

Compound	*Dose (mg/kg)*	*Coagulation time (s)*	*Statistically significant differences*[a]
Control (**C**)		155.6 ± 29.5	–
1	500	209.5 ± 40.0	$p \leq 0.05$ (**C,2,4**)
2	500	391.4 ± 32.2	$p \leq 0.05$ (**C,1,3,4,W**)
3	500	229.3 ± 47.9	$p \leq 0.05$ (**C**)
4	500	168.0 ± 42.1	$p \leq 0.05$ (**1,2,5,6,W**)
5	40	343.3 ± 95.1	$p \leq 0.05$ (**C,4**)
6	130	358.8 ± 105.8	$p \leq 0.05$ (**C,4**)
Warfarin (**W**)	150	290.0 ± 35.9	$p \leq 0.05$ (**C,2,4**)

[a]Compared with the compounds indicated in parentheses.

Table IV. Effect of the compounds on blood coagulation time of the mice after 3-day per oral administration.

Compound	*Dose (mg/kg)*	*Coagulation time (s)*
Control		254.0 ± 65.9
1	250	321.2 ± 47.8
	500	1080 ± 242.1[ab]
2	250	870.4 ± 68.3[a]
	500	1200 ± 0[ab]
3	250	368.7 ± 54.1
	500	930 ± 258.6[ab]
4	250	262 ± 34.5
	500	798.7 ± 70.2[ab]
5	20	548.6 ± 79.4[a]
	40	1045 ± 49.1[ab]
6	65	611.8 ± 78.5[ab]
	130	1080 ± 268.3[ab]
Warfarin	75	561.7 ± 69.8[a]
	150	1182.8 ± 45.4[ab]

[a]$p \leq 0.05$ statistically significant difference in comparison with the control group; [b]$p \leq 0.05$ statistically significant difference in comparison with dose A.

Conclusion

Comparative pharmacological and toxicological experiments of the compounds indicated that compound **2** has a slow toxicity upon oral (LD_{50} > 5000 mg/kg bw) and intraperitoneal administration (LD_{50} = 1330.9 mg/kg bw). The toxicity was significantly lower than the reference compound Warfarin. On the other hand, the effect of the compound **2** on blood coagulation time, after single and 3 day administration in equitoxic doses was more remarkable than Warfarin. These results illustrate that the compound **2** has prospects for further pharmacological and toxicological investigation.

Experimental protocols

Chemistry

Melting points were measured on Boetius hot plate microscope (Germany) and were corrected. IR spectra (KBr) were recorded on a UR 20 (Karl Zeiss, Jena apparatus). ^{1}H-NMR spectra were recorded at ambient temperature on Brucker 250 WM (250 MHz) spectrometer in DMSO-d_6, $CDCl_3$ and D_2O. Chemicals shifts are given in ppm (σ) relative to TMS used as an internal standard. Mass spectra were recorded on a Jeol JMS D300 double focusing mass spectrometer coupled to a JMA 2000 data system. The compounds were introduced by direct inlet probe, heated from 50°C to 400°C at a rate of 100°C/min. The ionization current was 300 mA, the accelerating voltage 3 kV and the chamber temperature 150°C. TLC was performed on precoated plates Kieselgel 60 F_{254} Merck (Germany) with layer thickness 0.25 mm and detection by UV (254 nm). Yields of TLC-homogeneous isolated products are given. The elemental analysis was performed at the Faculty of Chemistry, University of Sofia. Analyses indicated by the symbols of the elements were within ± 0.4% of theoretical values.

We synthesized Warfarin and Acenocoumarol and treated them with an acetic anhydride at a molar ratio to produce 4-acetoxy derivatives. 4-Hydroxycoumarin was treated with 3-acetylcoumarin at a molar ratio 1:1 with a solution of 0.4 g NaOH in 10 ml water. The reaction mixture was refluxed and stirred for 20 h. The reaction time for the appearance of **3** and exhaustion of 4-hydroxycoumarin and 3-acetylcoumarin was monitored by TLC.

Treatment of 4-hydroxycoumarin with 4-chlorophenyl-3-buten-2-one at a molar ratio 1:1 afforded Coumachlor **5**. Coumachlor was treated with an aqueous solution of NaOH at a molar ratio 1:0.95 to produce the Coumachlor sodium salt **6**.

4-Acetoxy-3-(3-oxo-1-phenylbutyl)-2H-1-benzopyran-2-one 1
Warfarin (20.0 mmol, 6.16 g) was added to 30 ml acetic anhydride and refluxed for 1 h. After cooling, the reaction mixture was poured into water to eliminate excess acetic anhydride. The organic phase was crystallized and the product was filtered. It was washed with water until removal of the odor of acetic acid. The crude product was recrystallized by heating once with 2-propanol. Yield 5 g (71.4%), mp 116–118°C, TLC (toluene/chloroform/acetone, 8:8:1) R_f 0.52. Anal $C_{21}H_{18}O_5$ (350) (C, H). IR, cm^{-1}: 1760, 1712, 1628, 1608, 1490, 1455, 1420, 1185. ^{1}H-NMR: 2.8 s (3H), 2.5 s (3H, ester), 3.5 d (2H), 4.9 t (1H), 7–7.8 m (9H, arom).

4-Acetoxy-3-[1-(4-nitrophenyl)-3-oxobutyl]-2H-1-benzopyran-2-one 2
Acenocoumarol (10 mmol, 3.53 g) was added to 15 ml acetic anhydride and refluxed for 1 h. After cooling, the reaction mixture was poured into water to eliminate excess acetic anhydride. The organic phase was crystallized and the product filtered. The precipitate was washed with water until the disappearance of odor of acetic acid. The crude product was recrystallized from methanol. Yield 49.4%, mp 149–150.5°C, R_f 0.42 (as above in **1**). Anal $C_{21}H_{17}NO_7$ (395) (C, H, N). IR (KBr) cm^{-1}: 1790, 1720, 1630, 1610, 1495, 1465, 1410, 1165.

7-Acetonyl-6-oxo-6H,7H-1-benzopyrano[4,3-b]-1-benzopyran 3
4-Hydroxycoumarin (10 mmol, 1.62 g) and 3-acetylcoumarin (10 mmol, 1.88 g) were added to NaOH (10 mmol, 0.4 g) in 30 ml water. The reaction mixture was refluxed and stirred for 20 h. After cooling the separated product was recrystallized from ethylacetate. Yield 13%, mp 263°C TLC (toluene/chloroform/acetone 8:8:1). R_f 0.74. Anal $C_{19}H_{14}O_4$ (306) (C, H). IR (KBr) cm^{-1}: 1709, 1638, 1615, 1578, 1150. ^{1}H-NMR: 2.00 s (3H, side chain), 2.28 d (2H), 4.31 t (CH), 6.8–7.9 m (8H).

4-Amino-2H-1-benzopyran-2-one (4-aminocoumarin) 4
A mixture of 4-hydroxycoumarin (50.0 mmol, 8.10 g) and dry ammonium acetate (500.0 mmol, 38.7 g) was refluxed in 50 ml glacial acetic acid for 2 h. After cooling the mixture was poured into 300 ml water and allowed to stand several hours at 5–10°C. An almost colorless solid formed, which was filtered, washed with water and recrystallized from ethyl acetate. Yield 4.67 (58%), mp 232–234°C. Anal $C_9H_7NO_2$ (161) (C, H, N) [18].

4-Hydroxy-3-[1-(4-chlorophenyl)-3-oxobutyl]-2H-1-benzopyran-2-one 5
4-Hydroxycoumarin (50 mmol, 8.1 g) was added to 4-(4-chlorophenyl)-3-buten-2-one in 150 ml distilled water. The reaction

mixture was refluxed under vigorous stirring for 14 h. The product was filtered and washed successively with hot water (3 x 100 ml) and, after cooling, with ether (2 x 50 ml). The product was recrystallized from methanol. Yield 12.6 g (75%) mp 162–163°C, TLC (hexane/acetone; 2:1) R_f 0.29. Anal $C_{19}H_{15}ClO_4$ (342.5) (C, H, Cl). IR (KBr) cm^{-1}: 3600, 1715, 1630, 1575, 1490, 900. ^{1}H-NMR (80 MHz, $CDCl_3$): 1.77 s (3H, side chain), 2.25–2.6 m (2H), 3.6–3.9 s (1H, OH) (in D_2O the signal at C-4 disappeared), 4.0–4.3 t (1H, CH); 6.6–8.0 m (8H).

For the preparation of Coumachlor phase-transfer catalyst benzyltriethylammonium chloride in optimum concentration of 5 mol% is more suitable. The use of this catalyst reduces the reaction time to about 30 min.

4-Hydroxy-3-[1-(4-chlorophenyl)-3-oxo-butyl]-2H-1-benzopyran-2-one, sodium salt 6

Coumachlor (10 mmol, 3.43 g) was suspended in 40 ml distilled water containing 10 ml solution of NaOH (9.5 mmol, 0.38 g). The suspension was stirred and left at room temperature until clarity was obtained. The solution was filtered and the filtrate evaporated *in vacuo*. The pale-yellow residue was recrystallized from 2-propanol and carbo. After cooling the product was separated. Yield 3.24 g (89%) mp 208–211°C. Anal $C_{19}H_{14}NaClO_4$ (C, H, Cl). IR (KBr) cm^{-1}: 3600–3200, 1725, 1630, 1575, 1490, 910. ^{1}H-NMR (60 MHz, D_2O): 2.3 s (3H, side chain), 3.5 d (2H), 4.8–5.05 t (1H, CH), 7.05–8.15 m (8H).

4-Hydroxy-3-(3-oxo-1-phenylbutyl)-2H-1-benzopyran-2-one, Warfarin

Yield 83%, mp 159–161°C [12].

Biological evaluation

The experiments were conducted on 340 white male mice weighing 23–25 g. Acute toxicity (LD_{50}) of water soluble compounds was assessed by dissolving in saline (0.9% NaCl). The water-insoluble compounds were dissolved in saline with 1–2 drops of Tween 80. After dissolution they were administered to mice *via* oral and intraperitoneal routes. The LD_{50} was evaluated for 4 or 5 different doses each on 6 animals and calculated by the method of Litchfield–Wilcoxon [19], using a personal Pravetz-8M computer. The anticoagulation effect of the compounds was evaluated likewise for *in vivo* experiments according to the method of Moravitz [20]. The compounds were administered orally in doses of 10% and 5% of the LD_{50} and blood coagulation times were measured in seconds 24 h after single and 3 day applications.

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