Synthesis, Toxicological, and Pharmacological Assessment of Some Oximes and Aldehyde Condensation Products of 4-Hydroxycoumarin

Ilia Manolov[a)]* and Nicolay D. Danchev[b)]

[a)]Department of Industrial Pharmacy, Faculty of Pharmacy, 2 Dunav St, Sofia 1000, Bulgaria

[b)]Department of Pharmacology and Toxicology, Faculty of Pharmacy, 2 Dunav St., Sofia 1000, Bulgaria

Key Words: *Warfarin and Warfarin derivatives; oximes and aldehyde condensation products; blood coagulation time*

Summary

The synthesis of condensation products of 4-hydroxycoumarin and nitro-substituted aromatic aldehydes as well as oximes of drugs with anticoagulant activity is described. The acute toxicity of the compounds was studied in mice by oral and intraperitoneal administration. A comparative pharmacological study of the in vivo anticoagulant effects of Warfarin derivatives showed that the new compounds have different anticoagulant activity. 4-Hydroxy-3-[1-(4-chlorophenyl)-3-oxobutyl]-2*H*-1-benzopyran-2-one, oxime **3** showed anticoagulant effect similar to Warfarin and Coumachlor, but its acute toxicity was higher than that of the reference drugs.

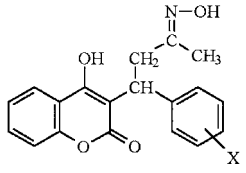
Introduction

4-Hydroxycoumarin derivatives possess anticoagulant and rodenticidal activities. The drugs of this group exhibit some side effects including the Warfarin-related purple toes syndrome [1]. By chemical modification of the structure of Warfarin [2, 3], Acenocoumarol [4, 5], and Coumachlor [6–9] it is possible to obtain compounds with biological activity and toxicity comparable to that of Warfarin, but with lower toxicity and fewer side effects.

Results and Discussion

Chemistry

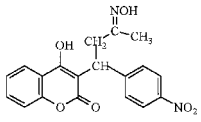
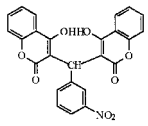
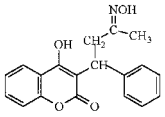
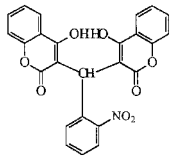
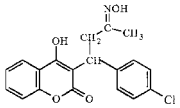
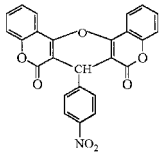
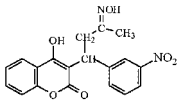
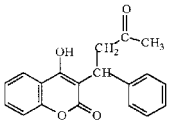
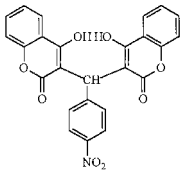
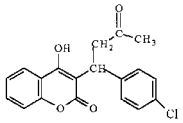
We synthesized Acenocoumarol [10–12], Coumachlor, and Warfarin [12, 13] and treated them with hydroxylamine hydrochloride at a molar ratio 1: 1 in ethanol to produce their oximes [14] with general formula (Table 1)



where X is: H, NO_2, Cl

We propose two probable mass spectral fragmentation pathways concerning compound **1**. The possible fragmentation pathways are presented in Scheme 1a,b [15]. The first phase of the fragmentation process of 4-hydroxy-3-[1-(4-nitrophenyl)-3-oxobutyl]-2*H*-1-benzopyran-2-one, oxime **1** involves a rather unstable molecular ion-radical. This undergoes further fragmentation resulting in a cascade of unstable ions, finally yielding ions with *m/z* 205 (100%), 162 (52%), 120 (77%), and 92 (46%). The base peak of the compound is the resonance-stabilized cation of 4-(4-nitrophenyl)-3-buten-2-one, oxime with *m/z* 205 (Scheme 1a).

Table 1

n°	Formula	n°	Formula
1		6	
2		7	
3		8	
4		9	
5		10	

The mass spectral fragmentation of 4-hydroxy-3-[1-(4-chlorophenyl)-3-oxobutyl]-2*H*-1-benzopyran-2-one, oxime **3** is analogous to that of compound **1**. The highest intensity peak of the ion with *m/z* 194 was obtained by elimination of a 4-hydroxycoumarin radical from M.

The mass spectral fragmentation of 4-hydroxy-3-(3-oxo-1-phenylbutyl)-2*H*-1-benzopyran-2-one, oxime **2** is analogous to the one of 4-acetoxy-3-(3-oxo-1-phenylbutyl)- 2*H*-1-benzopyran-2-one [16]. The base peak of the compound is the resonance-stabilized cation-radical with *m/z* 120.

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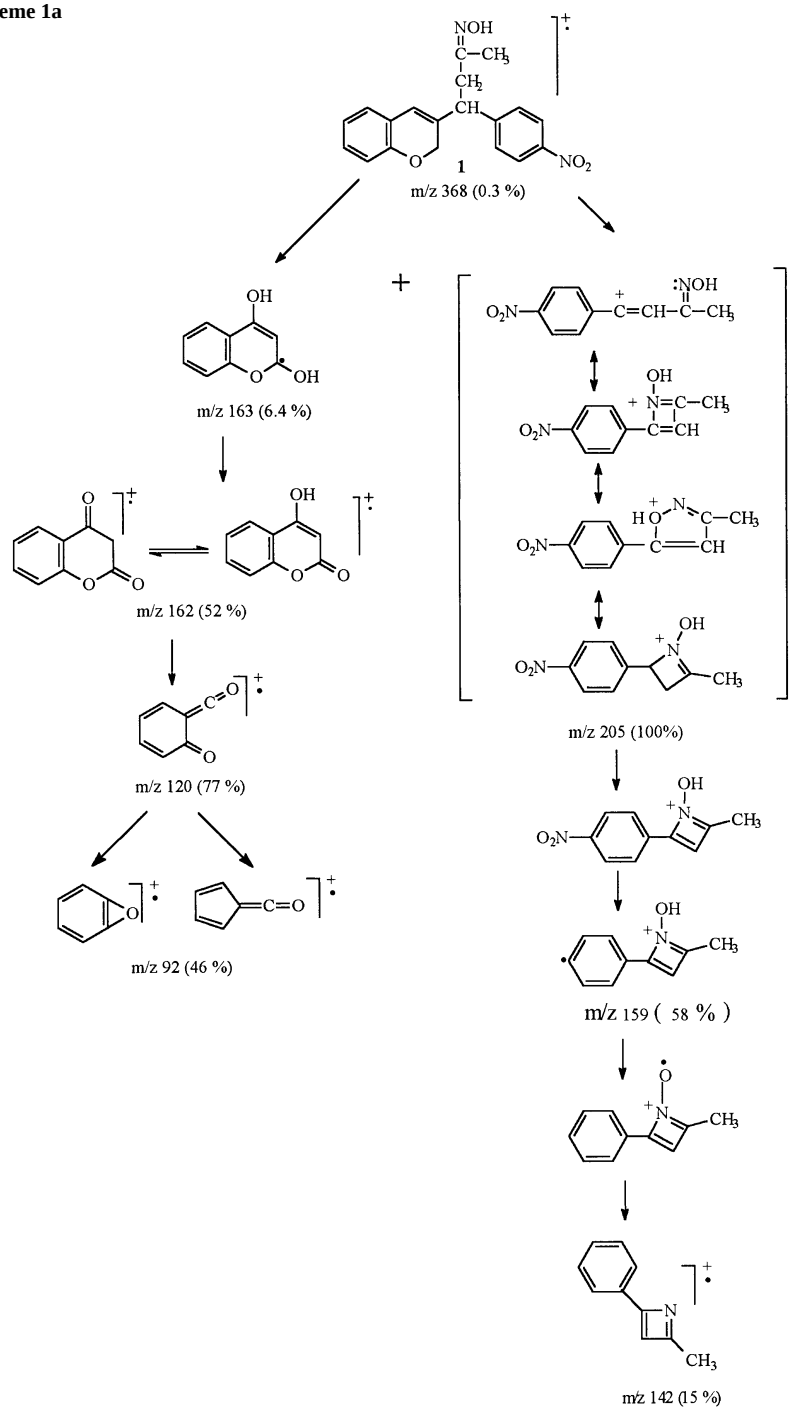
Scheme 1a



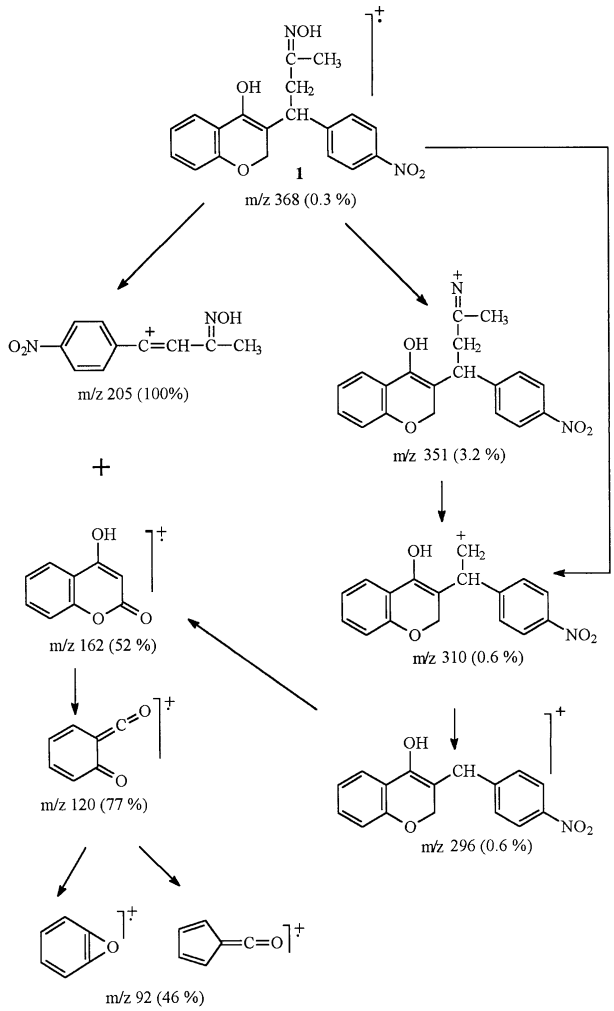
p-Nitrobenzaldehyde was added to 4-hydroxycoumarin in glacial acetic acid in a molar ratio of 1:2 [17]. During boiling a crystalline substance separated out. It was 3,3′-(*p*-nitrobenzylidene)-bis-4-hydroxycoumarin **5**. Substances **6** and **7** are obtained in a similar way. The structure of these compounds was confirmed by mass spectral analysis. As a result of this process an unstable molecular ion-radical is obtained. The base peak of all these three aldehyde condensation products is the resonance-stabilized cation-radical with *m/z* 120.

3,3′-(*p*-Nitrobenzylidene)-bis-4-hydroxycoumarin was added to acetic anhydride and the solution was boiled [18, 19]. After cooling 3,3′-(*p*-nitrobenzylidene)-4,4′-epoxydicoumarin **8** separated out. The mass spectral fragmentation of *p*-nitrobenzylidene-[4*H*]-pyrano-bis-4-hydroxycoumarin **8** is shown in Scheme 2. The base peak of the compound is the resonance-stabilized cation with *m/z* 317.

Pharmacology

The compounds investigated were tested for acute toxicity on mice by oral and intraperitoneal administration. An assessment of the compounds' effect on blood coagulation time

Scheme 1b



after a single day and a 3-day treatment was made. Warfarin and Coumachlor were used as references compounds.

Experimental data concerning the acute toxicity (LD_{50}) of the derivatives showed that compounds **5** and **8** had low toxicity (LD_{50} = 1115.4 and 1211.5 mg/kg body weight (b.w.) after intraperitoneal administration (Table 2), less than references Warfarin and Coumachlor. Compounds **1**, **2**, **3**, **4**, **6**, and **7** exhibited higher toxicity than Warfarin, but **1** and **3** exhibited similar toxicity to Coumachlor after i.p. application. After oral application compounds **5**, **6**, **7**, and **8** were practically nontoxic (LD_{50} value > 2500 mg/kg b.w.). Compounds **1**, **2**, and **3** were more toxic than Warfarin and Coumachlor after p.o. administration. The highest value of absorption (IR) showed compounds **2**, **1**, **3** and Warfarin – approximately 50% (Table 2). The index of absorption of Coumachlor was 17.1% (3 times less than Warfarin).

As shown by in vivo experiments after a single oral administration of the compounds at a dose of 10% of the LD_{50}, a large increase in blood coagulation time was measured 24 h after administration in the groups treated with compound **3**, Coumachlor, and Warfarin.

The data for the blood coagulation time after 3-day treatment (once a day) with the same doses are shown in Table 4. Compounds **1**, **3**, Coumachlor, and Warfarin increased the blood coagulation time in comparison with the control vehicle-treated group. The highest effect was observed in the group treated with compound **3**, Coumachlor and Warfarin.

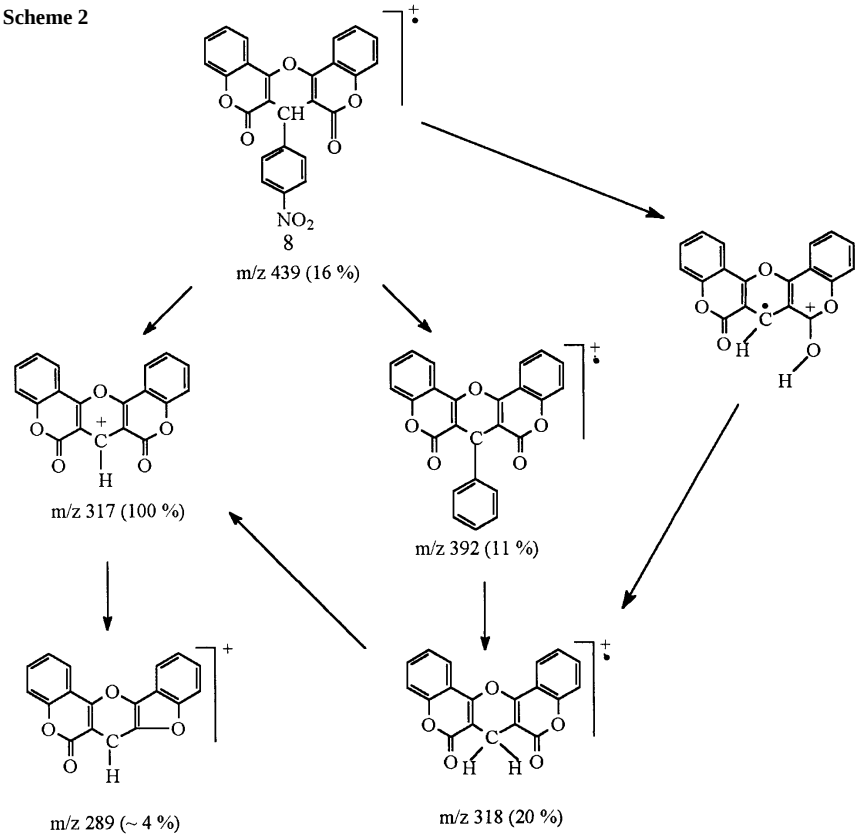
Table 2. Acute toxicity of the compounds after intraperitoneal and per oral administration on male mice.

Cmpd	LD_{50} (mg/kg); i.p.	LD_{50} (mg/kg); p.o.	Index of absorption (%)
1	265.7 (228.0 – 309.7)[a]	523.4 (456.7 – 599.8)[a]	50.7
2	349.1 (230.6 – 419.2)[a]	588.4 (546.4 – 633.7)[a]	59.3
3	265.7 (228.0 – 309.7)[a]	546.7 (505.3 – 591.1)[a]	48.6
4	403.6 (371.2 – 431.3)[a]	1172.6 (682.1 –1634.2)	34.4
5	1115.4 (985.3 –1262.6)[a]	> 2500[a]	<44.6
6	418.0 (359.7 – 485.8)[a]	> 2500[a]	<16.7
7	640.5 (541.9 – 756.9)	> 2500[a]	<25.6
8	1211.5 (991.7 – 1480.2)[a]	> 2500[a]	<48.4
9	750.1 (581.9 – 966.6)	1468.6 (1212.7 – 1778.6)	51
10	224.7 (197.4 – 256.3)	1315.4 (1079.9 – 1601.1)	17.1

Index of absorption was calculated as a LD_{50} i.p./LD_{50} p.o.

[a]Statistically significant differences in comparison with Warfarin

Scheme 2



Compound **5** decreased blood coagulation time statistically significantly. The anticoagulant effect of Warfarin is connected 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 anticoagulant effect of the drug [20].

Our pharmacological and toxicological experiments on the compounds show that compound **3** has a similar toxicity compared to Coumachlor but a higher (3 times) toxicity than Warfarin after i.p. administration. On the other hand the effect of compound **3** on blood coagulation time after a single and a 3-day administration in equitoxic doses is similar to that of Warfarin. We suggest that 4-hydroxy-3-[1-(4-chlorophenyl)-3-oxobutyl]-2*H*-1-benzopyran-2-one, oxime **3** acts as a whole molecule, because the value of LD_{50} after p.o. administration is three times less (more toxic) than Coumachlor and Warfarin. These results indicate that compound **3** has prospects for further pharmacological and toxicological experiments. The investigations made with the substances described in our work showed the following preliminary structure-activity relationships:

Compounds **5** and **8** had equal toxicity, i.e. elimination of a water molecule and formation of a pyran ring had no effect upon toxicity of compound **8.** The change of the position of nitro group in the aromatic nucleus (*o*- and *m*-isomers) increased the toxicity of the newly synthesized substances (**6** and **7**).

Substance **2** had no anticoagulant activity but introduction of a nitro group and especially a chlorine atom into the aromatic nucleus significantly increased the anticoagulant activity with no substantial effect upon acute toxicity. More compounds need to be synthesized in order to elucidate the structure-activity relationship.

Acknowledgments

We thank Mrs. S. Samurova from the Department of Organic Chemistry, Faculty of Chemistry, Sofia University, for performing the elemental analyses, as well as Dr. Achim Blocher from the Laboratory of Radiopharmacy, Eberhard-Karls University of Tübingen, Germany for mass spectra. We also thank Ms L. Alexandrova, Mr. A. Belchev, and Mrs. R. Christova for their technical assistance.

Experimental

Chemistry

Melting points were measured on Boetius hot plate microscope (Germany) and were uncorrected. IR spectra (nujol) were recorded on an IR-spectrometer FTIR-8101M Shimadzu. ^{1}H-NMR spectra were recorded at ambient temperature on a Bruker 250 WM (250 MHz) spectrometer in [D_6]-acetone, $CDCl_3$. Chemical shifts are given in ppm (σ) relative to TMS used as an internal standard. Mass spectra were recorded on a Jeol JMS D 300 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 °/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 and UV detection (254 nm). Yields of TLC-homogeneous isolated products are given. 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 the theoretical values.

We synthesized Warfarin, Acenocoumarol, Coumachlor and treated them with hydroxylamine hydrochloride at a molar ratio 1:1 to produce oxime derivatives.

Table 3. Effect of the compounds on the blood coagulation time in s after single administration.

Compound	Doses (mg/kg) p.o.	Blood coagulation time (s)
Control	–	203.5 ± 58.7
1	50	138.8 ± 45.7
2	50	322.5 ± 56.9[a]
3	50	243.8 ± 46.6
4	120	332.1 ± 42.8[a]
5	250	156.4 ± 22.3
6	250	56.4 ± 11.4[a]
7	250	221.6 ± 54.6
8	250	109.1 ± 36.2[a]
9	147	280.3 ± 31.9[a]
10	130	358.8 ± 65.8[a]

[a]Statistically significant differences in comparison with control vehicle treated group.

Table 4. Effect of the compounds on the blood coagulation time after 3 days' administration.

Compound	Doses	Blood coagulation time (s)
Control	–	156.3 ± 41.0
1	50	422.5 ± 61.2[a, b]
2	50	205.0 ± 41.9
3	50	980.1 ± 98.0[a]
4	120	242.1 ± 49.6[a, b]
5	250	73.5 ± 20.1[a, b]
6	250	149.9 ± 39.4[b]
7	250	146.6 ± 25.6[b]
8	250	251.1 ± 52.3
9	147	1082.8 ± 55.4[a]
10	130	950.2 ± 91.5[a]

[a] Statistically significant differences in comparison with control treated group; [b] Statistically significant differences in comparison with Warfarin treated group.

General Procedure

Coumarin derivative (10 mmol) was added to 150 ml ethanol, containing hydroxylamine hydrochloride (10 mmol). Three ml pyridine and 0.4 g sodium hydroxide were added to the reaction mixture. The solution was allowed to stand for 12 h and then refluxed for 6 h. After cooling the oxime crystallized out and was filtered off. The crude product was recrystallized from methanol. TLC (toluene/chloroform/acetone, 8:8:1). [The yield, mp, R_f, elemental analyses data, IR, ^{1}H-NMR and MS: *m/z* (% of intensity) are given under the respective heading.]

4-Hydroxy-3-[1-(4-nitrophenyl)-3-oxobutyl]-2H-1-benzopyran-2-one Oxime **1**

2.58 g (70%), 191–193 °C, 0.15, $C_{19}H_{16}N_2O_6$ (368) (C, H, N). IR, cm^{-1}: 2924, 1673, 1620, 1568, 1497, 1452, 1399, 1215, 766. ^{1}H-NMR ([D$_6$]-acetone): 1.84 s (3H), 3.1–3.3 d (2H – side chain), 4.9–5.1 t (1H), 7.2–8.1 m (8H – arom), 10.9 s (2H – two hydroxyl groups). MS: 368 (0.3); 351 (3.2); 310 (0.6); 296 (0.6); 205 (100); 163 (6.4); 162 (52); 159 (58); 143 (20); 142 (15); 120 (77); 92 (46).

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

2.75 g (77%), 194–195 °C, 0.30, $C_{19}H_{17}NO_4$ (323) (C, H, N), IR, cm^{-1}: 2855, 1672, 1620, 1568, 1499, 1453, 1399, 1215, 766, 700. ^{1}H-NMR ([D$_6$]-acetone): 1.84 s (3H), 2.4–2.6 d (2H – side chain), 3.1–3.5 t (1H), 7.1–8.1 m (9H – arom), 11.0 s (2H – two hydroxyl groups). MS: 323 (1.0); 306 (10.3); 289 (25); 275 (12); 162 (72); 121 (94); 120 (100); 92 (100).

4-Hydroxy-3-[1-(4-chlorophenyl)-3-oxobutyl]-2H-1-benzopyran-2-one Oxime **3**

2.65 g (74%), 192–194 °C, 0.28, $C_{19}H_{16}ClNO_4$ (357.5) (C, H, N, Cl). IR cm^{-1}: 2855, 1672, 1620, 1568, 1497, 1451, 1398, 1215, 766, 700. ^{1}H-NMR ([D$_6$]-acetone): 1.84 s (3H), 3.1-3.2 d (2H), 4.6–4.7 t (1H), 7.1–8.1 m (8H – arom), 11.0 s (2H – two hydroxyl groups). MS: 357 (0.3); 340 (3.2); 324 (1.2); 299 (2.5); 285 (4.7); 259 (1.5); 248 (2.1); 246 (6.0); 194 (100); 162 (31); 120 (31).

4-Hydroxy-3-[1-(3-nitrophenyl)-3-oxobutyl]-2H-1-benzopyran-2-one Oxime **4**

3.1 g (84%), 188–190 °C, 0.17, $C_{19}H_{16}N_2O_6$ (368) (C, H, N), IR cm^{-1}: 3358, 3195, 2953, 2924, 2855, 1674, 1616, 1570, 1524, 1458, 1377, 760, 740. ^{1}H-NMR ([D$_6$]-acetone): 1.9 s (3H), 3.3–3.4 d (2H), 4.8–4.9 t (1H), 7.2–8.2 m (8H – arom), 10.9 s (2H – two hydroxyl groups). MS: 368 (0.3); 279 (4.0); 249 (4.0); 212 (12); 205 (42); 201 (36); 162 (67); 120 (100); 92 (94).

4-Hydroxycoumarin was treated with *o*-, *m*-, *p*-nitrobenzaldehyde in a molar ratio 2:1 in glacial acetic acid for 30 min to 1 h at reflux. After cooling the product was filtered and the precipitate was washed until the disappearance of acetic acid odor. The crude product was recrystallized from acetone. [The name, number, yield, mp, R_f, elemental analyses data, IR, ^{1}H-NMR, and MS: *m/z* (% of intensity) are given]:

3,3′-(4-Nitrobenzylidene)-bis-4-hydroxy-2H-1-benzopyran-2-one **5**

3.5 g (76%), 237–239 °C, 0.41, $C_{25}H_{15}NO_8$ (457) (C, H, N), IR (nujol) cm^{-1}: 2953, 2924, 2855, 1653, 1616, 1597, 1564, 1491, 1377, 793. ^{1}H-NMR($CDCl_3$): 5.9 s (1H), 7.1–8.0 m (12H – arom), 8.5–9.0 s (1H), 11.0–11.2 s (1H). MS: 457 (1.2); 294 (12); 278 (36); 248 (48); 220 (7); 162 (34); 121 (27); 120 (100); 92 (80).

3,3′-(3-Nitrobenzylidene)-bis-4-hydroxy-2H-1-benzopyran-2-one **6**

3.7 g (81%), 228–230 °C, 0.37, $C_{25}H_{15}NO_8$ (457) (C, H, N), IR (nujol) cm^{-1}: 2951, 2924, 2855, 1654, 1614, 1598, 1564, 1490, 1377, 740. ^{1}H-NMR ($CDCl_3$): 5.8 s (1H), 7.1–8.1 m (12H – arom), 8.5–9.0 s (1H), 11.0 – 11.2 s (1H). MS: 457 (1.1); 439 (0.4); 294 (13); 278 (21); 248 (18); 162 (60); 120 (100); 92 (61); 63 (10).

3,3′-(2-Nitrobenzylidene)-bis-4-hydroxy-2H-1-benzopyran-2-one **7**

3.40 g (75%), 207–209 °C, 0.34, $C_{25}H_{15}NO_8$ (457) (C, H, N), IR (nujol) cm^{-1}: 2924, 2855, 1653, 1616, 1568, 1524, 1377, 1354, 1099, 762. ^{1}H-NMR ($CDCl_3$): 6.0 s (1H), 7.1–8.2 m (12H – arom), 8.6–9.0 s (1H), 11.0–11.2 s (1H). MS: 457 (0.3); 410 (13); 322 (8); 304 (53); 264 (86); 263 (70); 235 (51); 208 (6); 178 (14); 162 (41); 120 (100); 92 (68); 63 (31).

3,3′-(4-Nitrobenzylidene)-4,4′-epoxydicoumarin **8**

3,3′-(4-Nitrobenzylidene)-bis-4-hydroxy-2*H*-1-benzopyran-2-one (10 mmol, 4.57 g) was added to 25 ml acetic acid anhydride and the reaction mixture was refluxed for 30 min. The hot reaction mixture was poured into 300 ml distilled water to destroy the excess of acetic acid anhydride. The product was precipitated, filtered and washed until the disappearance of acetic acid odor. The crude product was recrystallized from acetone. Yield 3.88 g (85%), m.p. 338–342 °C, TLC (toluene/chloroform/acetone, 8:8:1),

R_f 0.28. Anal. $C_{25}H_{13}NO_7$(439) (C, H, N). IR (nujol) cm^{-1}: 2953, 2924, 2855, 1728, 1713, 1669, 1610, 1510, 1456, 1368, 1348, 1180, 764. ^{1}H-NMR ($CDCl_3$): 4.0 s (1H), 7.0–8.1 m (12H – arom). MS: *m/z* (% of intensity): 439 (16); 392 (11); 318 (20); 317 (100); 289 (4); 189 (4.0); 132 (4.0); 92 (4.0).

4-Hydroxy-3-(3-oxo-1-phenylbutyl)-2H-1-benzopyran-2-one, Warfarin **9**

Yield 83%, m.p. 159–161 °C [12].

4-Hydroxy-3-[1-(4-chlorophenyl)-3-oxobutyl]-2H-1-benzopyran-2-one, Coumachlor **10**

Yield 75%, m.p. 162–163 °C [16].

Pharmacology

The experiments were conducted on 340 white male mice weighing 23–25 g. Acute toxicity (LD_{50}) of water insoluble compounds was assessed by dissolving in saline (0.9% NaCl) 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 [21], using a Pravetz-8M personal computer. The anticoagulant effect of the compounds was evaluated likewise for *in vivo* experiments according to the method of Moravitz [22]. The compounds were administered orally in doses 10% and 5% of the LD_{50} and blood coagulation times were measured in seconds 24 h after a single and 3-day applications.

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