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Short communication

Synthesis, structure, toxicological and pharmacological investigations of 4-hydroxycoumarin derivatives

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Abstract

Twenty 4-hydroxycoumarin derivatives were synthesized. Five of them are described for the first time. The X-ray crystal structure analysis of 3,3′-(2,3,4-trimethoxyphenylmethylene)bis-(4-hydroxy-2*H*-1-benzopyran-2-one) (**7**) and 3,3′-(3,5-dimethoxy-4-hydroxyphenylmethylene)bis-(4-hydroxy-2*H*-1-benzopyran-2-one) (**9**) confirmed the structure of these compounds. A comparative pharmacological study of the anticoagulant effect with respect to Warfarin showed that the synthesized compounds have different anticoagulant activities. The most prospective compound is 3,3′-(4-chlorophenylmethylene)bis-(4-hydroxy-2*H*-1-benzopyran-2-one) (**12**) with low toxicity, very good index of absorption and dose dependent anticoagulant activity.

Keywords: 4-Hydroxycoumarin derivatives; Aromatic aldehydes; X-ray crystal structure analysis; Coagulation

1. Introduction

4-Hydroxycoumarin derivatives are of interest because of their anticoagulant [1–3], spasmolytic [4,5], and rodenticidal [6–9] activities. Some coumarin derivatives are known for their antifungal and anti-HIV activities [10,11]. They are also extensively used as analytical reagents [12–14]. The most widely used antithrombotic in the USA and Canada is the racemic sodium Warfarin. Vitamin K is a cofactor of the microsomal enzyme 2,3-epoxide reductase which function is essential for the synthesis of active prothrombin, factors VII, IX, and X, proteins C and S [15]. The coumarin anticoagulants are antagonists of vitamin K. Their target is vitamin K 2,3-epoxide reductase in the liver microsomes. The latter is an enzyme that is inhibited by therapeutical doses of anticoagulants by reducing the synthesis of anticoagulant factors [16]. Recently, an 18-kDa protein has been identified in the endoplasmic reticulum, the quantity of which increases in the presence of coumarin anticoagulants. This protein inhibits the activity of 2,3-epoxide reductase in a dose-dependent manner [17]. However, the drugs of this group exhibit some side effects including the Warfarin-related purple toes syndrome. By synthesis of different 3,3′-arylidenebis-4-hydroxycoumarins it is possible to obtain compounds with biological activity comparable to that of Warfarin, but with lower toxicity and fewer side effects. We synthesized 20 4-hydroxycoumarin derivatives to study their toxicity and anticoagulant activity in vivo.

2. Results and discussion

2.1. Chemistry

Different substituted aromatic aldehydes were condensed with 4-hydroxycoumarin in ethanol, glacial acetic acid or acetic acid anhydride in a molar ratio 1:2. The products were 3,3′-arylidenebis-4-hydroxycoumarins, epoxydicoumarins and tetrakis-4-hydroxycoumarin derivatives. The structure of these compounds was confirmed by mass spectral, IR, ^{1}H NMR analyses.

The condensation process lasted for 5 h and the product was compound **1**—3,3′-phenylmethylenebis-(4-hydroxy-2*H*-1-benzopyran-2-one)—(without a substituent in the aromatic nucleus) (MS: 412). 3,3′-(2-Methoxyphenylmethylene)bis-(4-hydroxy-2*H*-1-benzopyran-2-one) (MS: 442) (**2**) and 3,3′-(4-methoxyphenylmethylene)bis-(4-hydroxy-2*H*-1-benzopyran-2-one) (MS: 442) (**3**) are the final products of the condensation process between 4-hydroxycoumarin and the aldehyde with

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one methoxy group in the aromatic nucleus. The condensation process lasted for 3–5 min. 3,3′-(2,3-Dimethoxyphenylmethylene)bis-(4-hydroxy-2*H*-1-benzopyran-2-one) (MS: 472) (**4**), 3,3′-(2,4-dimethoxyphenylmethylene)bis-(4-hydroxy-2*H*-1-benzopyran-2-one) (MS: 472) (**5**) and 3,3′-(3,4-dimethoxyphenylmethylene)bis-(4-hydroxy-2*H*-1-benzopyran-2-one) (MS: 472) (**6**) are the final products of the condensation process between 4-hydroxycoumarin and the aldehyde, containing two methoxy groups in the aromatic nucleus. The condensation process lasted for 3 h (2,3-dimethoxy-) (**4**), 15 min (2,4-dimethoxy-) (**5**), and 5 min (3,4-dimethoxy-) (**6**). When there were three methoxy groups in the aromatic nucleus of the aldehyde, two compounds, namely 3,3′-(2,3,4-thrimethoxyphenylmethylene)bis-(4-hydroxy-2*H*-1-benzopyran-2-one) (MS: 502) (**7**) and 3,3′-(3,4,5-trimethoxyphenylmethylene)bis-(4-hydroxy-2*H*-1-benzopyran-2-one) (MS: 502) (**8**) were synthesized. The condensation process lasted for 2 h (3,4,5-trimethoxy-) in glacial acetic acid medium at reflux, but in ethanol it lasted for 10 min. The condensation process to produce 2,3,4-trimethoxyphenylmethylene isomer lasted for 240 h in ethanol, assumingly because of a steric hindrance in this case. When the condensation process took place in glacial acetic acid medium there was no product. When there were three substituents—two methoxy groups and a hydroxyl group between them—3,3′-(3,5-dimethoxy-4-hydroxyphenylmethylene)bis-(4-hydroxy-2*H*-1-benzopyran-2-one) (MS: 488) (**9**) the condensation process lasted for 30 min in ethanol. When the three substituents in the aromatic nucleus were different (nitro group, methoxy group and hydroxyl group) 3,3′-(4-hydroxy-3-methoxy-5-nitrophenylmethylene)bis-(4-hydroxy-2*H*-1-benzopyran-2-ones) (MS: 503) (**10**) was synthesized. The condensation process lasted for 5.5 h in glacial acetic acid medium. The base peak was *m*/*z* 44 (100) and when FAB-technique was applied the base peak of the same substance was *m*/*z* 317 (100). The condensation process lasted for 2 h in glacial acetic acid medium when there was a methyl group in *p*-position of the aromatic aldehyde (**11**). The condensation process lasted for 6 h in glacial acetic acid medium when there was a carboxylic group in o-position of the aromatic aldehyde. The product is 2-carboxyphenylmethylenebis-(4-hydroxy-2*H*-1-benzopyran-2-one (MS: 456) (**15**). Four 4-hydroxycoumarin derivatives were synthesized using dialdehydes. The mass-spectral investigation proved that the molecular cation-radical was rather unstable and decomposed releasing a water molecule.

Various substituted aromatic aldehydes were condensed with 4-hydroxycoumarin in glacial acetic acid or ethanol in a molar ratio of 1:2. The products were 3,3′-arylidenebis-4-hydroxycoumarins. Using dialdehydes tetrakis-4-hydroxycoumarin derivatives were the final products. When the condensation process took place in acetic acid anhydride the final products were epoxydicoumarins. We synthesized 20 4-hydroxycoumarin derivatives, five of them being new, namely: 3,3′-(3,5-dimethoxy-4-hydroxyphenylmethylene)bis-(4-hydroxy-2*H*-1-benzopyran-2-one) (**9**), 3,3′-(4-hydroxy-3-methoxy-5-nitrophenylmethylene)bis-(4-hydroxy-2*H*-1-benzopyran-2-ones) (**10**), 3,3′-(4-chloro-3-nitrophenylmethylene)bis-(4-hydroxy-2*H*-1-benzopyran-2-ones) (**13**), 3,3′-(4-hydroxy-3-methoxy-5-nitrophenylmethylene)-4,4′-epoxydicoumarin (**18**), 3,3′,3″,3‴-(*m*-phenylenedimethylidine)-4,4′,-4″,-4‴-diepoxytetracoumarin (**20**). The structures of all compounds were confirmed by IR, ^{1}H NMR, and mass-spectral data.

2.2. X-ray crystal structure analysis of 3,3′-(2,3,4-thrimethoxyphenylmethylene)-bis-(4-hydroxy-2H-1-benzopyran-2-one) (7) and 3,3′-(3,5-dimethoxy-4-hydroxyphenylmethylene)bis-(4-hydroxy-2H-1-benzopyran-2-one) (9)

Crystallographic data for the compounds **7** and **9** are listed in Table 1. The solid state structures are shown in Figs. 1 and 2.

Fig. 1: DIAMOND drawing (50% probability level); selected bond length [A]: $O_{(35)}$–$C_{(36)}$ 1.361 (4) ; $O_{(36)}$–$C_{(36)}$ 1.226 (5) ; $C_{(31)}$–$C_{(36)}$ 1.436 (5) ; $O_{(25)}$–$C_{(26)}$ 1.357 (5) ; $O_{(26)}$–$C_{(26)}$ 1.234 (5) ; $C_{(21)}$–$C_{(26)}$ 1.447 (6). Selected bond angles [°]: $O_{(36)}$–$C_{(36)}$–$O_{(35)}$ 115.2 (3); $O_{(36)}$–$C_{(36)}$–$C_{(31)}$ 125.0 (3); $O_{(35)}$–$C_{(36)}$–$C_{(31)}$ 119.8 (3); $O_{(32)}$–$C_{(32)}$–$C_{(31)}$ 124.0 (3); $O_{(26)}$–$C_{(26)}$–$O_{(25)}$ 115.6 (3); $O_{(25)}$–$C_{(26)}$–$C_{(21)}$ 119.8 (3); $O_{(22)}$–$C_{(22)}$–$C_{(21)}$ 124.2 (3). Compound **7** crystallized from acetonitrile as colorless plates in the orthorhombic space group P212121 with $a = 11.5002$ (8) A, $b = 13.8707$ (15) A, $c = 14.2137$ (17) A, $\alpha = \beta = \gamma = 90°$, $V = 2267.3$ (4) A^3.

Fig. 2: DIAMOND drawing (50% probability level); selected bond length [A]: $O_{(22)}$–$C_{(23)}$ 1.357 (8) ; $O_{(4)}$–$C_{(23)}$ 1.218 (8) ; $C_{(18)}$–$C_{(23)}$ 1.456 (10) ; $C_{(9)}$–$O_{(10)}$ 1.335 (8) ; $O_{(5)}$–$C_{(9)}$ 1.225 (8) ; $C_{(8)}$–$C_{(9)}$ 1.470 (9). Selected bond angles [°]: $O_{(4)}$–$C_{(23)}$–$O_{(22)}$ 115.7 (6) ; $O_{(4)}$–$C_{(23)}$–$C_{(18)}$ 125.3 (6); $O_{(22)}$–$C_{(23)}$–$C_{(18)}$ 119.0 (6); $O_{(10)}$–$C_{(9)}$–$C_{(8)}$ 119.0 (7) ; $O_{(5)}$–$C_{(9)}$–$C_{(8)}$ 123.9 (7) ; $O_{(5)}$–$C_{(9)}$–$O_{(10)}$ 117.0 (6). Compound **9** crystallized from methanol as colorless plates in the triclinic space group P-1 with $a = 7.0383$ (7) A, $b = 12.327$ (2) A, $c = 16.567$ (3) A, $\alpha = 68.837°$ (18), $\beta = 88.569°$ (12), $\gamma = 73.878°$ (12), $V = 1283.1$ (4).10^6 pm^3.

2.3. Pharmacology—acute toxicity and blood anticoagulant activity

Acute per oral and intra peritoneal toxicity as well as the in vivo effects of the compounds on blood coagulation time were studied in mice. Warfarin was used as a reference compound. The studies were approved by the Institutional Animal Care Committee at the Faculty of Pharmacy, Medical University of Sofia, Bulgaria.

The most toxic compounds after intra peritoneal administration were **4**, **8**, **9** and **17**—with LD_{50} values ranging between 282 and 596 mg kg^{-1} b.w. Compound **9** showed higher toxicity than Warfarin. Compounds **1**, **10** and **17** showed similar acute toxicity values in comparison with Warfarin. All other compounds were less toxic than Warfarin.

After oral administration the only compound with similar toxicity to Warfarin was **4**. All other compounds were less toxic. Compounds **11**, **16**, **19** and **20** were practically nontoxic according to the scale of Hodge and Sterner [18] (p.o. LD_{50} > 5000 mg kg^{-1} b.w.).

Compounds **4**, **5** and **12** showed an index of absorption approximately twice higher than that of Warfarin. Compounds **6**,

Table 1
Crystal data, details for data collection and structure analysis of **7** and **9**

Data	Compound **7**	Compound **9**
Empirical formula	$C_{28}H_{22}O_9$	$C_{27}H_{20}O_9$
Formula weight	502.46	488.43
Crystal system/Space group	Orthorhombic, P212121	Triclinic, P-1
Unit cell dimensions	*a* = 11.5002 (8) A;	a = 7.0383 (7) A;
	α = 90°	α = 68.837 (18)
	b = 13.8707 (15) A;	b = 12.327 (2) A;
	β = 90°	β = 88.569 (12)
	c = 14.2137 (17) A;	c = 16.567 (3) A;
	γ = 90°	γ = 73.878 (12)
Volume	2267.3 (4) A^3	1283.1 (4) A^3
Z	4	2
Density (calculated)	1.472 Mg m^{-3}	1.264 Mg m^{-3}
Absorption coefficient	0.929 mm^{-1}	0.096 mm^{-1}
F (000)	1048	508
Crystal description	Colorless plates	colorless plates
Crystal size	0.3 0 × 0.2 5 × 0.20 mm	0.4 0 × 0.3 5 × 0.15 mm
Θ range for data collection [°]	5.00°–64.95°.	6.81°–23.25 .
Index ranges	$-\ell \le h \le 13$, $-\ell \le k \le 16$,	$-\ell \le h \le 7$, $-13 \le k \le 13$,
	$-16 \le \ell \le 16$	$-18 \le \ell \le 18$
Reflections collected	4770	4573
Independent reflections	3633 [R(int) = 0.0413]	3600 [R(int) = 0.0578]
Reflections observed	……	1754
$I \ge 2\sigma(I)$		
Absorption correction	semi empirical	DIFABS
Max. and min. transmission	Full-matrix least-squares of	0.708 and 0.251
And refinement method	F^2	
Scan method	ω scans	ω scans
Decay	1.0%	1.0%
Data/restraints/parameters	3633/0/423	3600/0/365
Final R indices [I > 2σ (I)]	R1 = 0.0475, wR2 = 0.1181	R1 = 0.0891, wR2 = 0.2365
Final R indices (all data) R1/ wR2	0.0688/0.1325	0.2076/0.2935
Extinction coefficient	0.0034 (4)	
Largest diff. peak and hóle	0.226 and–0.215 e A^{-3}	0.616 and–0.289 e A^{-3}

13, **16** and Warfarin had a comparable index of absorption. All other compounds had an index of absorption lower than that of Warfarin (Table 2).

After oral administration of 1/20 of LD_{50} of compounds **1**, **2**, **4**, **9**, **12** and **14**, a statistically significant prolongation of coagulation time compared to the controls was observed. These compounds were tested for a dose-dependent effect after 2 days of oral administration using 1/20, 1/50 and 1/100 of LD_{50} (Table 3). The other compounds did not affect significantly blood coagulation time.

When orally administered at a dose of 1/20 of LD_{50} the most pronounced effect exerted compounds **12** and **14** (about 14-fold increase in blood coagulation time) in comparison with the controls (vehicle treated group). Compound **14** caused 62% increase in the coagulation time at a dose of 1/100 of oral LD_{50} in comparison with the vehicle treated control group (Table 3).

3. Experimental procedures

3.1. Chemistry

Melting points were measured on Boetius hot plate microscope (Germany) and are 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 (δ) 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 °C min^{-1}. 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 of 0.25 mm and UV detection (254 nm). Yields of TLC-homogeneous isolated products are presented. Results of elemental analyses were within ± 0.4% of the theoretical values.

3.2. General synthesis

4-Hydroxycoumarin and the respective aromatic aldehyde at a molar ratio 2:1 in ethanol or in glacial acetic acid were mixed under stirring and heated at reflux until the appearance of an insoluble product. After cooling the product was filtered and was recrystallized. The following 17 3,3′-arylidenebis-(4-hydroxy-2*H*-1-benzopyran-2-ones) or tetrakis-4-hydroxycoumarin derivatives were synthesized according to this procedure (name of the compound, its number, aromatic aldehyde, reaction medium, duration of the condensation process, solvent for

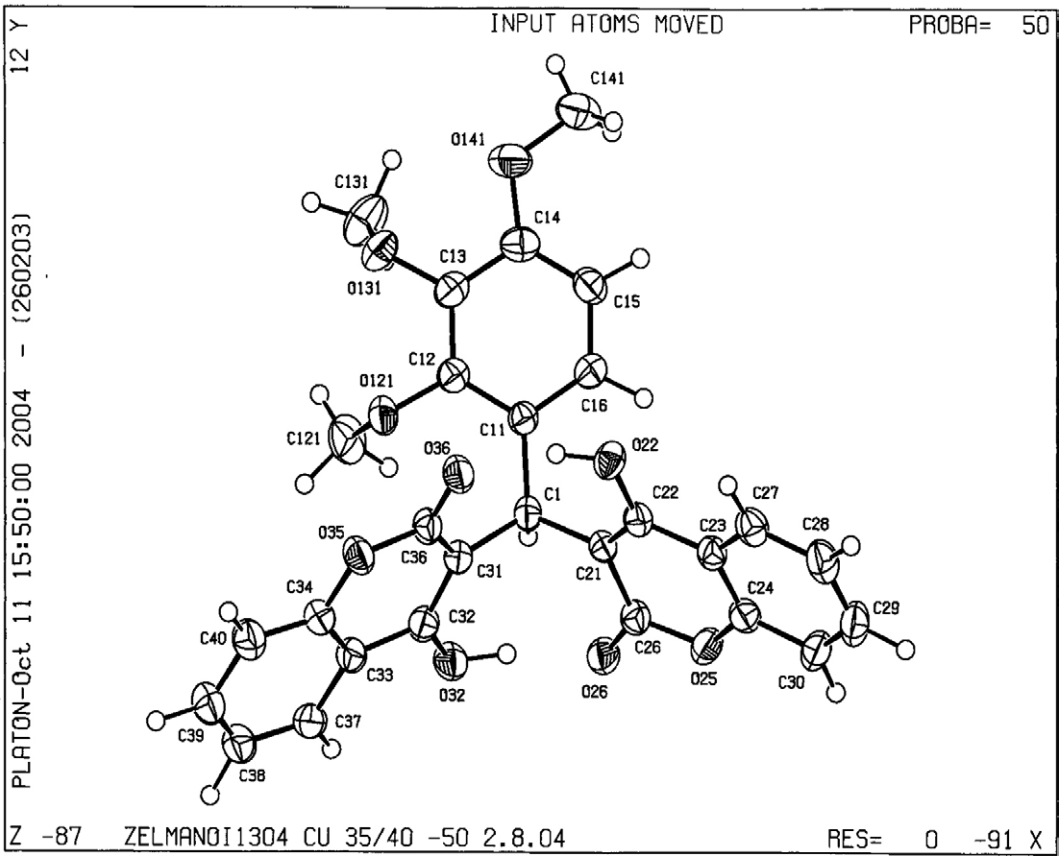


Fig. 1. A solid state structure of 3,3′-(2,3,4-trimethoxyphenylmethylene)bis-(4-hydroxy-2*H*-1-benzopyran-2-one) (**7**).

Table 2
Acute toxicity (LD_{50} values) in male mice after intra peritoneal (i.p.) and per oral (p.o.) administration and index of absorption (IA) in %

Compound	i.p. LD_{50} (mg kg^{-1})	p.o. LD_{50} (mg kg^{-1})	IA (%)
1	529.3 (337.7 ÷ 829.7)	1379.5 (1045.3 ÷ 1820.6)	38.3
2	664.4 (632.5 ÷ 1354.1)	1614.2 (1193.4 ÷ 2183.4)	41.1
3	983.9 (953.9 ÷ 1014.9)	2695.1 (2414.7 ÷ 3008.1)	36.5
4	578.5 (544.1 ÷ 606.5)	807.1 (596.7 ÷ 1091.7)	71.6
5	925.0 (893.1 ÷ 1091.7)	1290.2 (1229.4 ÷ 1354.1)	71.6
6	1206.3 (1103.3 ÷ 1318.9)	2260.0 (1609.5 ÷ 3176.1)	53.3
7	639.5 (578.2 ÷ 707.2)	4296.6 (4034.9 ÷ 4573.3)	14.8
8	586.6 (428.5 ÷ 802.9)	2752.8 (2404.1 ÷ 3152.0)	21.3
9	292.0 (239.8÷355.5)	1257.8 (1009.5 ÷ 1567.2)	23.2
10	489.1 (381.8 ÷ 626.7)	4716.8 (4382.4 ÷ 5076.8)	10.4
11	803.7 (714.9 ÷ 905.5)	4842.6 (3818.9 ÷ 6140.7)	16.5
12	2447.4 (2247.6 ÷ 2665.0)	2735.6 (2632.9 ÷ 2842.3)	89.4
13	2184.4 (1683.7 ÷ 2833.9)	4134.8 (3359.7 ÷ 5088.6)	52.8
14	1612.1 (1418.0 ÷ 1832.7)	> 5000	32.4
15	626.3 (577.9 ÷ 678.8)	2209.2 (1885.8 ÷ 2588.0)	28.3
16	1093.4 (958.1 ÷ 1247.9)	> 5000	21.9
17	485.9 (375.0÷629.6)	3724.9 (3405.3 ÷ 4074.5)	13.0
18	2661.4 (2197.2 ÷ 3223.7)	> 5000	53.2
19	1233.3 (1137.5 ÷ 1337.1)	> 5000	24.7
20	3946.7 (3574.4 ÷ 4357.7)	> 5000	78.9
Warfarin	363.7 (288.5 ÷ 442.4)	762.1 (612.1 ÷ 901.3)	47.0

LD_{50} values are expressed as mean and confidence interval is given in parenthesis. The index of absorption (IA) was calculated as the ratio of LD_{50} i.p. to LD_{50} p.o.

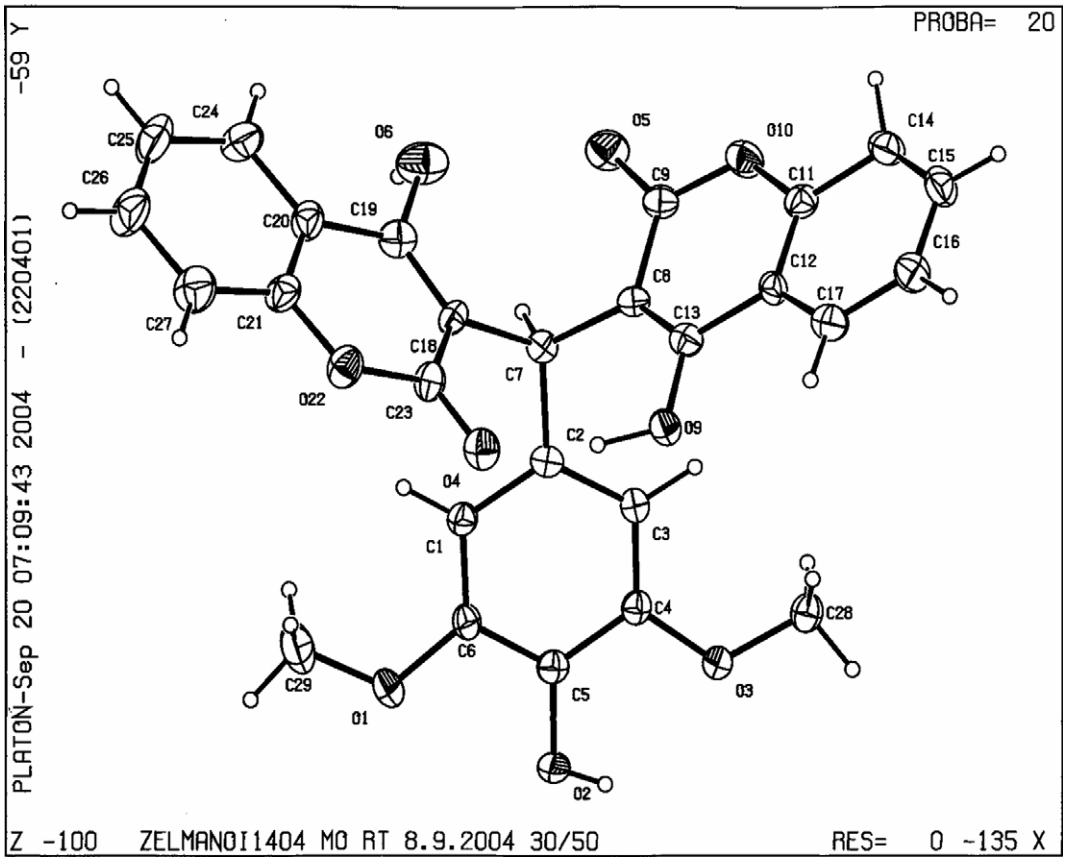


Fig. 2. A solid state structure of 3,3′-(3,5-dimethoxy-4-hydroxyphenylmethy-lene)bis-(4-hydroxy-2*H*-1-benzopyran-2-one) (**9**).

Table 3
The effects of selected compounds (**1**, **2**, **4**, **9**, **12** and **14**) on blood coagulation time after p.o. administration of 1/20, 1/50 and 1/100 of LD_{50}

Compound	Dose (parts of p.o. LD_{50})	Coagulation time (%) mean ± SD	Statistical significance
Control	–	100.0 ± 14.1	–
Warfarin	1/20	256.3 ± 25.2	$P < 0.05$[a]
	1/100	240.1 ± 19.8	$P < 0.05$[a]
1	1/20	147.6 ± 29.8	NS
	1/50	144.7 ± 31.2	NS
	1/100	122.8 ± 19.6	NS
2	1/20	225.9 ± 27.5	$P < 0.05$[a]
	1/50	156.6 ± 21.9	NS
	1/100	117.9 ± 14.9	NS
4	1/20	165.4 ± 21.2	$P < 0.05$[a]
	1/50	146.7 ± 27.5	NS
	1/100	145.1 ± 35.1	NS
9	1/20	190.4 ± 29.8	$P < 0.05$[a]
	1/50	143.0 ± 27.9	NS
	1/100	142.1 ± 25.5	NS
12	1/20	1136.1 ± 98.6	$P < 0.05$[a,b,c]
	1/50	343.5 ± 51.5	$P < 0.05$[a]
	1/100	137.0 ± 25.5	NS
14	1/20	1412.5 ± 148.6	$P < 0.05$[a,b,c]
	1/50	662.0 ± 69.8	$P < 0.05$[a]
	1/100	162.7 ± 21.8	$P < 0.05$[a]

Coagulation time 208.2 s = 100%.
[a] Statistically significant compared to control group.
[b] Statistically significant compared to Warfarin treated group with a dose 1/20 part of LD_{50}.
[c] Statistically significant compared to dose 1/50 p.o. LD_{50}.

recrystallization, yield, m.p., R_f). The last three compounds (epoxycoumarins) were synthesized in acetic acid anhydride medium (see synthetic scheme [Scheme 1]):

*3.2.1. 3,3′-Phenylmethylenebis-(4-hydroxy-2H-1-benzopyran-2-one) (**1**)*

Benzaldehyde, ethanol, 5 h, acetonitrile, 66%, 229–230 °C, [19–24], 0.43 (hexane/chloroform/-acetone 5:3:1).

*3.2.2. 3,3′-(2-Methoxyphenylmethylene)bis-(4-hydroxy-2H-1-benzopyran-2-one) **(2)***

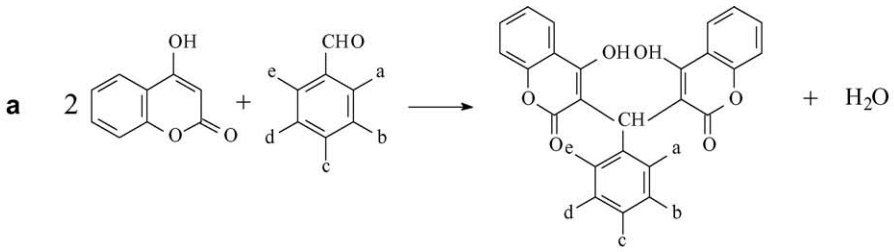
2-Methoxybenzaldehyde, ethanol, 5 min, acetonitrile, 81%, 214–215 °C (lit. 218 °C) [21,24–26], 0.33 (hexane/chloroform/acetone 5:3:1).

*3.2.3. 3,3′-(4-Methoxyphenylmethylene)bis-(4-hydroxy-2H-1-benzopyran-2-one) (**3**)*

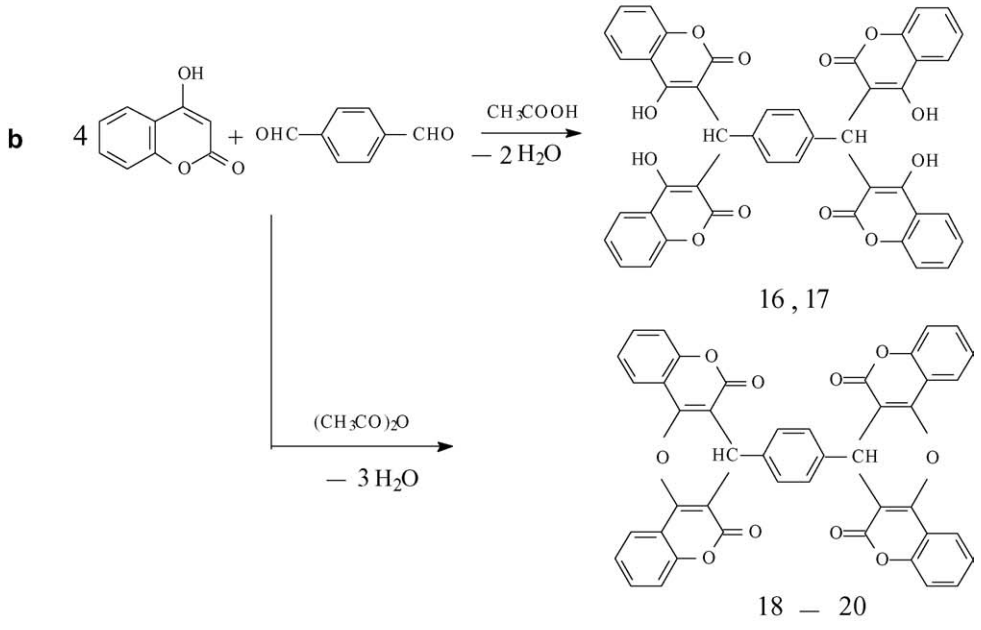
4-Methoxybenzaldehyde, ethanol, 3 min, acetonitrile, 79%, 250–251 °C (lit. 242 °C decomposition) [20,21,24,25,27–31], 0.42 (hexane/chloroform/acetone 5:3:1).

*3.2.4. 3,3′-(2,3-Dimethoxyphenylmethylene)bis-(4-hydroxy-2H-1-benzopyran-2-one) (**4**)*

2,3-Dimethoxybenzaldehyde, ethanol, 3 h, acetonitrile, 72%, 189.5–190.5 °C (lit. 282 °C) [26].



Comp. No	a	b	C	d	e
1	H	H	H	H	H
2	OCH_3	H	H	H	H
3	H	H	OCH_3	H	H
4	OCH_3	OCH_3	H	H	H
5	OCH_3	H	OCH_3	H	H
6	H	OCH_3	OCH_3	H	H
7	OCH_3	OCH_3	OCH_3	H	H
8	H	OCH_3	OCH_3	OCH_3	H
9	H	OCH_3	OH	OCH_3	H
10	H	OCH_3	OH	NO_2	H
11	H	H	CH_3	H	H
12	H	H	Cl	H	H
13	H	NO_2	Cl	H	H
14	H	H	$C_6H_5CH_2O$	H	H
15	COOH	H	H	H	H



Scheme 1. Synthetic scheme.

*3.2.5. 3,3′-(2,4-Dimethoxyphenylmethylene)bis-(4-hydroxy-2H-1-benzopyran-2-one) (**5**)*

2,4-Dimethoxybenzaldehyde, ethanol, 15 min, acetonitrile, 74%, 197–198 °C (lit. 197–199 °C) [21,32,33], 0.37 (hexane/chloroform/acetone 5:3:1).

*3.2.6. 3,3′-(3,4-Dimethoxyphenylmethylene)bis-(4-hydroxy-2H-1-benzopyran-2-one) (**6**)*

3,4-Dimethoxybenzaldehyde, ethanol, 5 min, 1,4-dioxane, 66%, 266.5–268 °C (lit. 264–266 °C) [24,30,32], 0.34 (hexane/chloroform/acetone 5:3:1).

3.2.7. 3,3′-(2,3,4-Thrimethoxyphenylmethylene)bis-(4-hydroxy-2H-1-benzopyran-2-one) (7)

2,3,4-Trimethoxybenzaldehyde, ethanol, 18 h and after cooling stayed for 7 days at room temperature, acetonitrile, 69%, 265–267 °C (lit. 265–266 °C) [32,33], 0.36 (hexane/chloroform/acetone 5:3:1).

3.2.8. 3,3′-(3,4,5-Trimethoxyphenylmethylene)bis-(4-hydroxy-2H-1-benzopyran-2-one) (8)

3,4,5-Trimethoxybenzaldehyde, ethanol, 10 min, acetonitrile, 54%, 241–243 °C (lit. 246–248 °C) [32,34–39], 0.26 (hexane/chloroform/acetone 5:3:1).

3.2.9. 3,3′-(3,5-Dimethoxy-4-hydroxyphenylmethylene)bis-(4-hydroxy-2H-1-benzopyran-2-one) (9)

3,5-Dimethoxy-4-hydroxybenzaldehyde, ethanol, 30 min, methanol, 64%, 188–189 °C, 0.14 (hexane/chloroform/acetone 5:3:1). Anal. $C_{27}H_{20}O_9$ (488) (C, H) (calcd/found): % C = 66.39/66.55; % H = 4.10/4.46. IR (nujol) cm^{-1}: 1665, 1605, 1269, 1215, 1100, 765. ^1H NMR (DMSO-d_6): δ = 3.6–3.7 s (6H), 4.4–4.6 s (1H), 5.3–5.5 s (1H), 6.2–6.3 s (1H), 6.4–6.5 s (1H), 7.2–8.1 m (10). MS: 488 (1), 326 (64), 295 (100), 279 (16), 162 (50), 120 (93), 92 (55), 63 (22).

3.2.10. 3,3′-(4-Hydroxy-3-methoxy-5-nitrophenylmethylene) bis-(4-hydroxy-2H-1-benzopyran-2-one) (10)

4-Hydroxy-3-methoxy-5-nitrobenzaldehyde, glacial acetic acid, 5.5 h, acetonitrile, 87%, 171–173 °C, 0.13 (hexane/chloroform/acetone 5:3:2). Anal. $C_{26}H_{17}NO_{10}$ (503) (C, H, N) (calcd/found): % C = 62.03/62.08; % H = 3.38/3.62; % N = 2.78/2.63. IR (nujol) cm^{-1}: 1651, 1607, 1456, 1377, 1098, 762. ^1H NMR (DMSO): δ = 3.6–3.8 s (3H), 5.1–5.2 s (1H), 5.4–6.1 s (2H), 6.1–6.3 s (1H), 7.0–8.0 m (10H). MS: 503 (13), 468 (4), 341 (15), 310 (5), 264 (2), 149 (3), 120 (100), 92 (64), 63 (46).

3.2.11. 3,3′-(4-Methylphenylmethylene)bis-(4-hydroxy-2H-1-benzopyran-2-one) (11)

4-Methylbenzaldehyde, glacial acetic acid, 2 h, acetonitrile, 67%, 265–267 °C (lit. 254–257 °C, decomposition) [24,27,40–43], 0.69 (hexane/chloroform/acetone 5:3:2).

3.2.12. 3,3′-(4-Chlorophenylmethylene)bis-(4-hydroxy-2H-1-benzopyran-2-one) (12)

4-Chlorobenzaldehyde, glacial acetic acid, 30 min, acetone, 91%, 260–262 °C (lit. 239–240 °C) [21,24,27,29,43].

4-Hydroxycoumarin (1.62 g, 10 mmol) and 4-chlorobenzylideneaniline (4.31 g, 20 mmol) in 15 ml glacial acetic acid were mixed. 3,3′-(4-Chlorobenzylidene)-bis-(4-hydroxy-2*H*-1-benzopyran-2-one) was the condensation product. The structure of the compound was confirmed by elemental analysis, IR, ^1H NMR, mass-spectrum. Anal. $C_{25}H_{15}ClO_6$ (445.5) (C, H, Cl) (calcd/found): % C = 67.34/67.51; % H = 3.14/3.39; % Cl = 7.97/7.73. IR (nujol) cm^{-1}: 1669, 1618, 1543, 1380, 763. ^1H NMR (DMSO-d_6): δ = 4.6–4.8 s (1H), 5.3–5.7 s (1H), 6.2–6.3 s (1H), 7.2–8.1 m (12 H). MS (FAB NEG): 446.5 (16), 444.8 (65), 331 (19), 199 (48), 167 (70), 161 (79) [28].

3.2.13. 3,3′-(4-Chloro-3-nitrophenylmethylene)bis-(4-hydroxy-2H-1-benzopyran-2-one) (13)

4-Chloro-3-nitrobenzaldehyde, glacial acetic acid, 3.5 h, acetonitrile, 68%, 264–267 °C, 0.18 (hexane/acetone 2:1). Anal. $C_{25}H_{14}ClNO_8$ (491) (C, H, Cl, N) (calcd/found): % C = 61.04/61.27; % H = 2.85/3.16; % Cl = 7.22/7.13; % N = 2.85/3.01. IR (nujol) cm^{-1}: 1667, 1615, 1538, 1377, 766. ^1H NMR (DMSO-d_6): δ = 4.7–4.9 s (1H), 5.5–5.9 s (1H), 6.3–6.4 s (1H), 7.2–8.0 m (11H). MS (FAB NEG): 491.5 (4), 490 (13), 331 (22), 306 (56), 199 (41), 168 (70).

3.2.14. 3,3′-(4-Benzyloxyphenylmethylene)bis-(4-hydroxy-2H-1-benzopyran-2-one) (14)

4-Benzyloxybenzaldehyde, glacial acetic acid, 5 h, acetonitrile, 50%, 224–226 °C, 0.60 (hexane/chloroform/acetone 5:3:2). Anal. $C_{32}H_{22}O_7$ (518) (C, H) (calcd/found): % C = 74.13/74.10; % H = 4.25/4.43. IR (nujol) cm^{-1}: 1669, 1607, 1377, 1182, 772. ^1H NMR (DMSO): δ = 5.0–5.1 s (1H), 5.2–5.6 s (2H), 6.2–6.3 s (2H), 6.8–8.0 m (17H). MS: 518 (38), 464 (5), 427 (40), 356 (20), 265 (6), 167 (3), 162 (39), 120 (20), 91 (100), 77 (12), 41 (19) [10,44–46].

3.2.15. 2-Carboxyphenylmethylenebis-(4-hydroxy-2H-1-benzopyran-2-one) (15)

2-Carboxybenzaldehyde, glacial acetic acid, 6 h, methanol, 54%, 228–231 °C (lit.228–230 °C) [35], 0.32 (cyclohexane/chloroform/acetic acid 10:10:4).

3.2.16. 3,3′,3″,3‴-(p-Phenylenedimethylidine)tetrakis-(4-hydroxy-2H-1-benzopyran-2-one) (16)

Terephthaldialdehyde, glacial acetic acid, 5 min, 1,4-dioxane, 51%, 313–315 °C (lit. 313–314 °C) [10,47–54], 0.79 (cyclohexane/chloroform/acetic acid 10:10:4).

3.2.17. 3,3′,3″,3‴-(m-Phenylenedimethylidine)tetrakis-(4-hydroxy-2H-1-benzopyran-2-one) (17)

Isophthaldialdehyde, glacial acetic acid, 3 h, ethylacetate, 72%, 233–235 °C (lit. 230–234 °C) [10,44], 0.77 (cyclohexane/chloroform/-acetic acid 10:10:4).

3.2.18. 3,3′-(4-Hydroxy-3-methoxy-5-nitrophenylmethylene)-4,4′-epoxydicoumarin (18)

4-Hydroxycoumarin, 4-hydroxy-3-methoxy-5-nitrobenzaldehyde, acetic acid anhydride, 3 h, acetonitrile, 43%, 278–281 °C, 0.37 (hexane/acetone 2:1). Anal. $C_{26}H_{15}NO_9$ (485) (C, H, N) (calcd/found): % C = 64.33/63.98; % H = 3.09/3.46; % N = 2.89/3.12. IR (nujol) cm^{-1}: 1779, 1732, 1672, 1613, 1456, 1179, 762. ^1H NMR (DMSO-d_6): δ = 3.1–3.4 s (3H), 5.0–5.1 s (1H), 6.8–7.0 s (1H), 7.5–8.5 m (10H). MS: 503 (13), 468 (4), 341 (15), 310 (5.5), 264 (1.5), 149 (3), 120 (100), 92 (64), 63 (46).

*3.2.19. 3,3′,3″,3‴-(p-Phenylenedimethylidine)-4,4′,4″,4‴-diepoxytetracoumarin (**19**)*

4-Hydroxycoumarin, terephthaldialdehyde, acetic acid anhydride, 90 min, ethanol, 66%, 346–348 °C (lit. 350 °C) [55], 0.78 (cyclohexane/chloroform/acetic acid 10:10:4).

*3.2.20. 3,3′,3″,3‴-(m-Phenylenedimethylidine)-4,4′,4″,4‴-diepoxytetracoumarin (**20**)*

4-Hydroxycoumarin, isophthaldialdehyde, acetic acid anhydride, 3 h, dimethylformamide, 8%, 360 °C (decomposition), 0.67 (cyclohexane/chloroform/acetic acid 10:10:4). Anal. $C_{44}H_{22}O_{10}$ (710) (C, H) (calcd/found): % C = 74.37/74.45; % H = 3.10/3.40. IR (nujol) cm^{-1}: 1728, 1667, 1611, 1456, 1369, 1175, 756. ^1H NMR ($C_6H_5NO_2$-d_5): δ = 2.3–2.5 s (1H), 5.3–5.5 s (1H), 7.1–8.3 m (20H). MS (FAB): 710 (2), 460 (4), 329 (20), 307 (68), 289 (43), 178 (16), 176 (100), 162 (35).

3.3. Structure determination

Crystal structures of compounds **7** and **9** were determined by the single crystal X-ray diffraction method. Data collection of these compounds was done at –60 °C using graphite monochromator Cu-$K_{\acute{\alpha}}$ (λ = 154.18 pm) and Mo-$K_{\acute{\alpha}}$ (λ = 71.07 pm) radiation on an ENRAF NONOIUS for circle diffractometer. Complete data collection parameters and details of the structure solution and refinement are given in Table 1. Further details on the crystal structure investigation are available free of charge at www.ccdc.ac.uk/conts/retrieving.html or deposit@ccdc.cam.ac.uk.

The unit cell was determined and refined using the Cad4-EXPRESS program. The space group was determined using the check-*hkl* and the semiempirical absorption correction was performed with the PLATON/ABS PSI program [56]. The structures were solved with direct methods by SHELXS97 and refined with SHELXL97 [57]. The structures were refined by least-square methods based on F^2. All non-hydrogen atoms were fully refined in the calculated position. The hydrogen atoms were taken from the electron density map and refined isotropically. The plots of the molecular structures were made using the DIAMOND program (CRYSTAL IMPACT GbR, Bonn, Germany).

3.4. Pharmacology

Animals: Male albino mice, line H, 25–30 g b.w. were used for acute intra peritoneal and oral toxicity and anticoagulation activity studies. The animals were housed individually, water and food being supplied ad libidum; animal room temperature 22 ± 3 °C; humidity 30%; lighting schedule 12 h light/dark cycle. Prior to administration the animals fasted for one day.

The compounds were suspended using Tween 80. Acute per oral and intra peritoneal toxicity studies were performed according to OECD Guideline 425 "Up and Down procedure" (FDA, 2001) [58]. The index of absorption (IA) in % was calculated (100 xi.p.LD_{50}/p.o.LD_{50}) using the data from acute toxicity studies. The compounds were administered for 3 days at a dose equivalent to 1/20–1/100 of LD_{50} and blood coagulation time was assessed 24 h thereafter according to the method of Moravitz [59,60].

Compounds **12** and **14** exerted dose dependent prolongation of blood coagulation time. The most prospective compound is **12** with low toxicity (p.o. $LD_{50} \approx 2735$ mg kg^{-1} b.w.), very good index of absorption (89%) and dose-dependent (1/20–1/50 p.o. LD_{50}) anticoagulant activity in vivo.

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