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RELATIONSHIP BETWEEN STRUCTURE AND ANTI-COAGULANT ACTIVITY OF COUMARIN DERIVATIVES

BY

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Thirty-five coumarin derivatives have been examined for their anticoagulant activity in rabbits by determining the prothrombin time by a modification of Quick's one-stage method, in order to find out the structural features eliciting the activity. The compounds include methoxylated dicoumarols, substituted 4-hydroxycoumarins, coumarins devoid of a 4-hydroxyl group, such as 3- and 4-phenylcoumarins and 4-methylcoumarins, and some complex coumarin derivatives having additional rings. The results show the complexity of the problem and the involvement of various factors. Among these the importance of molecular geometry is emphasized by the high activity of calophyllolide (31)* and a new synthetic compound, 4-methyl-2,5-dioxo-3-phenyl-2*H*,5*H*-pyrano[3,2-*c*][1]-benzopyran (30). The importance for the anticoagulant activity of a substituent in position 8 of the coumarin moiety, and the role of ability to ionize with regard to the vitamin-K-like property of some hydroxylated phenylcoumarins, are also indicated.

Following the discovery of the anticoagulant action of dicoumarol by Stahmann, Huebner & Link (1941), several other 4-hydroxycoumarins have been found to be useful in therapy. The correlation of chemical structure with the anticoagulant activity among coumarin derivatives has been studied by Link (1943), Mentzer, Meunier, Lacocq, Billet & Xuong (1945) and Chmielewska & Cieślak (1958). In the present work coumarins, with or without a 4-hydroxyl group, have been examined and an attempt has been made to find out the essential structural features responsible for the anticoagulant activity.

METHODS

The anticoagulant activity of the coumarins was studied by determining the prothrombin time by the one-stage method of Quick, Stanley-Brown & Bancroft (1935) as modified by Montigel & Pulver (1952). Blood (0.80 ml.) was collected from the marginal vein of the rabbit ear into a syringe containing sodium citrate (0.20 ml. of a 3.13% solution). The blood was then centrifuged (1,700 revs/min) for 7 min. The separated plasma was transferred to a test-tube kept in a water-bath at 37±0.5° C. A suspension prepared from a tablet of "Thrombokinase with Calcium" (Geigy), containing 10 mg of active substance, thromboplastin from rabbit brain, 2.5 ml. of distilled water and $CaCl_2$ to yield a final concentration of 0.18%, were placed in a separate test-tube kept in the same water-bath. The time for the

* Numbers in parenthesis following names of compounds refer to compound numbers as set out in Table 1.

TABLE 1
CHEMICAL STRUCTURES

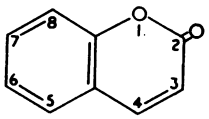
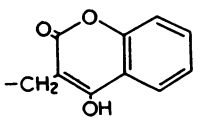
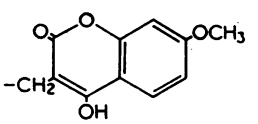
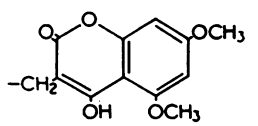
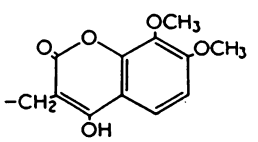
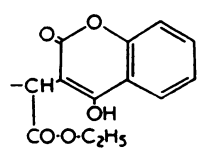
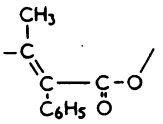
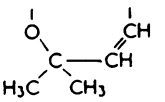
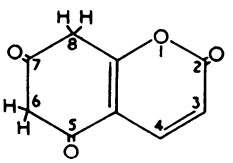
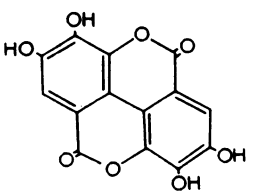
Compound no.	Positions of substituents in coumarin structure					
	3	4	5	6	7	8
(I) 3- and 4-substituted coumarins of the general formula: 						
1	—	—	—	—	—	—
2	–OH	—	—	—	—	—
3	$-OCH_3$	—	—	—	—	—
4	$-NH.CO.C_6H_5$	—	—	—	—	—
5	$-CO_2H$	—	—	—	—	$-OCH_3$
6	$-C_6H_5$	—	—	—	—	—
7	$-C_6H_5$	—	—	—	$-OCH_3$	—
8	$-C_6H_5$	–OH	—	—	$-OCH_3$	—
9	$-C_6H_4.NO_2$-*p*	—	—	—	—	—
10	—	$-CH_3$	—	–OH	—	—
11	—	$-CH_3$	—	—	–OH	—
12	—	$-CH_3$	—	—	–OH	$-CO.CH_3$
13	—	$-C_6H_5$	—	—	$-OCH_3$	—
14	—	$-C_6H_5$	—	–OH	$-OCH_3$	—
15	—	$-C_6H_5$	—	$-OCH_3$	$-OCH_3$	—
16	—	$-C_6H_5$	—	—	$-OCH_3$	$-OCH_3$
17	—	–OH	—	—	—	—
18	$-CO.CH_3$	–OH	—	—	—	—
19	–Br	–OH	—	—	—	—
20	—	–OH	—	—	–OH	—
21	—	–OH	$-OCH_3$	—	$-OCH_3$	—
22	—	–OH	—	—	$-OCH_3$	$-OCH_3$
23	$-CH(C_6H_5).CO.CH_2.CH_3$	–OH	$-OCH_3$	—	$-OCH_3$	—
24	$-CH(C_6H_4.NO_2$-*p*$).CO.CH_2.CH_3$	–OH	—	—	—	—
25		–OH	—	—	—	—
26		–OH	—	—	$-OCH_3$	—
27		–OH	$-OCH_3$	—	$-OCH_3$	—
28		–OH	—	—	$-OCH_3$	$-OCH_3$
29		–OH	—	—	—	—

TABLE 1—*continued*

Compound no.	Positions of substituents in coumarin structure					
	3	4	5	6	7	8
30			—	—	—	—
31	—	$-C_6H_5$			$-OCH_3$	$-CO.C(CH_3):CH.CH_3$
(II) Nuclear methylated tetrahydrocoumarins of the general formula:						
32	$-CH_3$	—	—	$-CH_3$, $-CH_3$	—	$-CH_3$
33	—	$-CH_3$	—	$-CH_3$, $-CH_3$	—	$-CH_3$
34	—	$-CH_3$	—	$-CH_3$, $-CH_3$	—	$-CH_3$, $-CH_3$
(III) 3,4-Benzocoumarin derivative:						
35	Ellagic acid	Formula:				

plasma (0.05 ml.) to solidify following the addition of the warmed thrombokinase suspension (0.1 ml.) was taken as the prothrombin time.

The coagulation valency was evaluated according to the procedure of Montigel & Pulver (1952), by plotting on two-way logarithmic graph paper a curve of the prothrombin times for the dilution series (100, 90, 80, 70, 60, 50, 40, 30, 20 and 10% prothrombin) of the normal plasma (100% prothrombin content) from thirty male albino rabbits of the Haffkine strain. The normal prothrombin times of these rabbits were between 6 and 8 sec (mean, 7 sec).

After determining the normal prothrombin time, groups of five male rabbits were given single doses (50 mg/kg by mouth through a small rubber catheter passing a mouth-gag) of each of the compounds, dissolved either in sodium hydroxide solution or in aqueous alcohol. The prothrombin time was again determined at 24 hr intervals until it returned to normal.

RESULTS

The chemical structures of the 35 coumarins investigated are shown in Table 1. The maximum hypoprothrombinaemic response obtained at varying periods after single equal doses of each compound was taken as the criterion for anticoagulant activity. The results giving the mean of the times of onset, the peak and duration of the anticoagulant activity and the coagulation valency at the peak hypopro-

TABLE 2

STRUCTURE AND ANTICOAGULANT ACTIVITY OF THIRTY-FIVE COUMARINS

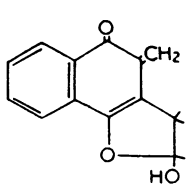
Anticoagulant activity was tested after single oral doses of 50 mg/kg of each coumarin in groups of five rabbits, and the activity was expressed relative to 7-methoxy-3-phenylcoumarin (7). Arabic numerals indicate the compound numbers, and roman numerals indicate the formula numbers of the compounds given in the discussion

No.	Name of compound	Molecular weight	Relative molar activity	Hypoprothrombinaemic activity: Action (hr) Onset	Peak	Duration	Coagulation valency (% prothrombin in plasma) at the maximum response
1	Coumarin	146	3·2	24	48	120	55
2	3-Hydroxycoumarin	162	0	Inactive			100
3	3-Methoxycoumarin	176	1·5	48	72	96	82
4	3-Benzamidocoumarin	265	0	Inactive			100
5	3-Carboxy-8-methoxycoumarin	220	2·7	24	24	72	75
6	3-Phenylcoumarin	222	3·1	24	24	120	72
7	7-Methoxy-3-phenylcoumarin	252	1	24	48	96	92
8	4-Hydroxy-7-methoxy-3-phenylcoumarin	268	4·9	24	72	120	63
9	3-*p*-Nitrophenylcoumarin	267	2·6	24	24	48	80
10	6-Hydroxy-4-methylcoumarin	176	1·3	24	24	72	85
11	7-Hydroxy-4-methylcoumarin	176	1·0	24	24	72	88
12	8-Acetyl-7-hydroxy-4-methylcoumarin	218	3·2	24	72	96	70
13	7-Methoxy-4-phenylcoumarin	252	1·8	24	72	120	85
14	6-Hydroxy-7-methoxy-4-phenylcoumarin	268	2·4	24	48	72	82
15	6,7-Dimethoxy-4-phenylcoumarin	282	2·8	24	48	72	80
16	7,8-Dimethoxy-4-phenylcoumarin	282	2·1	24	72	96	85
17	4-Hydroxycoumarin	162	4·8	24	48	120	40
18	3-Acetyl-4-hydroxycoumarin	204	8·1	24	24	96	20
19	3-Bromo-4-hydroxycoumarin	242	6·3	24	24	96	48
20	4,7-Dihydroxycoumarin	178	4·7	24	48	96	45
21	4-Hydroxy-5,7-dimethoxycoumarin	222	7·2	24	48	72	37
22	4-Hydroxy-7,8-dimethoxycoumarin	222	9·4	48	96	120	15
23	3-(2-Acetyl-1-*p*-nitrophenylethyl)-4-hydroxy-5,7-dimethoxycoumarin	308	5·4	24	24	72	65
24	3-(2-Acetyl-1-*p*-nitrophenylethyl)-4-hydroxycoumarin (nicoumalone; sintrom)	353	11·5	24	48	96	35
25	3,3′-Methylenebis(4-hydroxycoumarin) (dicoumarol) (V)	336	15·2	24	144	288	10
26	3,3′-Methylenebis(4-hydroxy-7-methoxycoumarin)	396	16·4	24	72	96	17
27	3,3′-Methylenebis-(4-hydroxy-5,7-dimethoxycoumarin)	456	13·9	24	48	120	38
28	3,3′-Methylenebis-(4-hydroxy-7,8-dimethoxycoumarin)	456	18·3	24	96	120	20
29	Ethyl di(4-hydroxycoumarin-3-yl)-acetate (ethyl biscoumacetate; tromexan)	408	10·2	24	24	72	50
30	4-Methyl-2,5-dioxo-3-phenyl-2*H*,5*H*-pyrano-[3,2-*c*][1]-benzopyran (VI)	304	10·6	24	48	72	30
31	5-Methoxy-2,2-dimethyl-8-oxo-10-phenyl-6-tigloyl-2*H*,8*H*-benzo-[1,2-*b*: 3,4-*b*′]dipyran (Calophyllolide) (VII)	416	16·6	24	48	168	20
32	5,6,7,8-Tetrahydro-3,6,6,8-tetramethyl-5,7-dioxocoumarin	234	2·3	24	24	48	80
33	5,6,7,8-Tetrahydro-4,6,6,8-tetramethyl-5,7-dioxocoumarin	234	4·1	24	48	120	64
34	5,6,7,8-Tetrahydro-4,6,6,8,8-pentamethyl-5,7-dioxocoumarin	248	6·3	24	48	144	49
35	Ellagic acid	302	0	Inactive			100

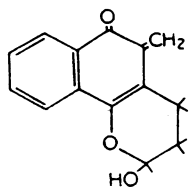
thrombinaemic response are shown in Table 2, along with the molar activity ratios of the compounds to compare anticoagulant activities. These values have been calculated as the ratio of the percentage decrease from normal of the prothrombin in the plasma produced by one molar dose of each compound, to the activity of a molar dose of 7-methoxy-3-phenylcoumarin (7).

DISCUSSION

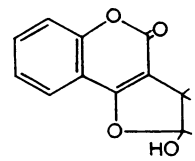
Link (1943) and co-workers examined some typical coumarins and concluded that the minimum requirement for anticoagulant activity was an intact 4-hydroxycoumarin with a substituent in position 3 and a keto-group in position 5. For maximal activity a bis arrangement was considered to be necessary. Such an arrangement was considered important also by Mentzer *et al.* (1945).



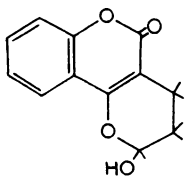
I



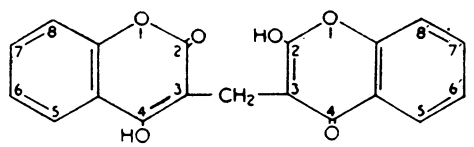
II



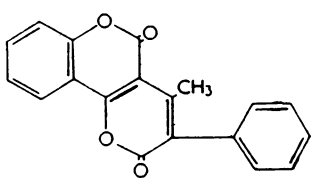
III



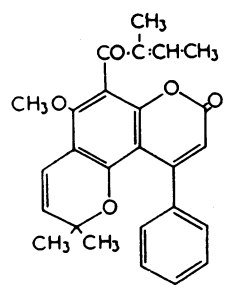
IV



V (25)



VI (30)



VII (31)

Chmielewska & Cieślak (1958) analysed the structural requirements for coumarin anticoagulants from the point of view of their vitamin K antagonism. They postulated that the active forms of vitamin K can be represented by the formulae I and II. On the other hand, an anticoagulant which is an antivitamin K should have the structure III or IV, which are cyclic hemiacetals obtained from the appropriate 3-substituted 4-hydroxycoumarins. For such acetal formation the carbon chain in position 3 should carry a carbonyl group or a potential carbonyl group in position 2′ or 3′. Working on these lines Chmielewska & Cieślak (1958) suggested

a revision of the structure of dicoumarol (25). In the new structure, V, one of the units is 2-hydroxychromone, and this structure alone explains the anticoagulant activity on the basis of the above hypothesis.

The present investigations were undertaken in an attempt to define in more exact terms the structural features responsible for the anticoagulant activity of coumarins. In most of the earlier studies only 4-hydroxycoumarins were examined. In the present study three major lines of approach to this problem have been made. (1) The influence of substituents, such as hydroxyl and methoxyl groups, on the activity of 4-hydroxycoumarin and dicoumarol has been studied. (2) The work of Chmielewska & Cieślak (1958) suggested the examination of compounds in which the oxygen atom in position 4 is involved in a preformed ring system simulating in shape the cyclic acetals. Link's (1943) coumapyran was one such early example. One compound of this type, namely 4-methyl-2,5-dioxo-3-phenyl-2*H*,5*H*-pyrano-[3,2-*c*][1]-benzopyran (VI, 30), has now been studied. (3) Some coumarin derivatives, devoid of an oxygen function in the 4-position substituent, have been examined. In coumarin (1), substituents in the pyrone ring modify considerably the chemical reactivity of the molecule. The most important among these compounds are 3- and 4-phenylcoumarins and 4-methylcoumarins, representative members of which have been studied (Table 1). These three different groups of coumarin derivatives can conveniently be considered separately.

(1) *Dicoumarol analogues and other 4-hydroxycoumarins.* The results show that the introduction of a methoxyl group into each nucleus of dicoumarol increases the molar anticoagulant activity. Loading the molecule with additional methoxyl groups either potentiates or inhibits activity depending on the positions of the substituents. Thus, there is considerable difference in activity between the isomeric dicoumarol derivatives, 3,3′-methylenebis(4-hydroxy-5,7-dimethoxycoumarin) (27) and 3,3′-methylenebis(4-hydroxy-7,8-dimethoxycoumarin) (28). The higher activity of the compound having substituents in position 8 shows the importance of their location. Similar findings exist for the 4-hydroxycoumarins. Further, in 4-hydroxycoumarin a bromine atom in position 3 (19) slightly increases the molar activity, while an acetyl group (18) in the same position confers even greater activity.

(2) *4-Methyl-2,5-dioxo-3-phenyl-2*H,5H-*pyrano-[3,2-*c*][1]-benzopyran.* The considerable activity of this compound (VI, 30), in which the 4-hydroxyl group is incorporated in an extra ring system, shows the importance of molecular shape and supports the hypothesis of Chmielewska & Cieślak (1958). However, the lack of other similar examples makes a delineation of this factor impossible at the present time.

(3) *Other coumarin derivatives without a 4-hydroxyl group.* The most interesting among these are the 3- and 4-phenylcoumarins, most of which are only very weak anticoagulants. Among these compounds there exists a significant difference between the activity of the various hydroxylated derivatives and that of their corresponding methyl ethers. Thus, invariably, the methylation of a free hydroxyl group increases the anticoagulant activity. This result indicates the probable importance of ionization as one of the factors ; a greater ability to ionize presumably aids the vitamin-K-like activity, and *vice versa.*

The 4-methylcoumarins show very weak anticoagulant action ; introduction of an 8-acetyl group into 7-hydroxy-4-methylcoumarin (12) increases the anticoagulant activity.

Introduction of a hydroxyl, methoxyl or benzamido group in position 3 of coumarin decreases the anticoagulant activity. The nuclear methylated coumarins derived from 5,7-dihydroxy-3- or -4-methylcoumarins (Gupta & Seshadri, 1957) seem to be less haemostatic than the 4-methylcoumarins.

In terms of molar activity, calophyllolide (VII, 31) ranks second among the compounds studied and is more active than dicoumarol (25). The inhibitory effect of the phenyl group in position 4 is obviously counteracted by an effective combination of several potentiating factors. These are presumably the methoxyl group in position 7, the acyl function in position 8 and the additional dimethylpyrano-group built on to the condensed benzene system. To these must be added the overall molecular geometry.

It may be concluded that the anticoagulant activity among coumarins is governed not by individual structural features but by a combination of several, none of which can be defined precisely at the present time. This is particularly due to ignorance regarding the biochemical mode of action. However, certain broad indications are available. Molecular shape is one of the factors, and six-membered heterocyclic rings (other than pyrone) such as a cyclic acetal, augment anticoagulant activity. Further, a substituent in position 8 is important, though its exact role is not clear. The relationship between the hydroxyl compounds and their methyl ethers indicates a probable role of the ability to ionize. A methoxyl group is relatively more conducive to anticoagulant activity, whereas a free hydroxyl group, which means greater ability to ionize, augments vitamin-K-like activity.

It must, however, be stressed that the above suggestions need confirmation.

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