CHAPTER-I

CHEMISTRY OF ANTICOAGULANTS DERIVED FROM 4-HYDROXYCOUMARIN
PART A

CHEMISTRY AND BIOLOGICAL ACTIVITY OF ANTICOAGULANTS OF 4-HYDROXYCOUMARIN SERIES.
INTRODUCTION

Anticoagulants are some of the most important group of drugs, and are recommended in certain pathological conditions, as venous or arterial thrombosis, pulmonary embolism due to an intravascular clot, coronary occlusion with myocardial infarction, rheumatic heart disease, excessive traumatic injuries, burns or frostbite. The other major usage of oral anticoagulants is as rodenticides.

These drugs prevent blood clotting or prolong the coagulation time of blood by interfering with one or more steps in the chain of enzymatic reactions which lead to clot formation. They are generally divided as direct acting and indirect acting. The direct acting anticoagulants are administered parenterally, and interact with or inhibit directly one or more factors, involved in the coagulation process, eg. Heparin which activates release of inhibitors of clotting and, oxalates or citrates which bind to calcium, which is very essential for clotting. The indirect acting anticoagulants are given orally and generally bring about the release of anticoagulants present in the normal blood as antithrombin or heparin cofactor.

To understand the mechanism of anticoagulant action it will be appropriate to discuss briefly the blood clotting mechanism which is also known as the coagulation phenomenon or hemostasis, or fibrin cascade.
Blood clotting: It is a defence mechanism of the body to overcome the defects in the blood vessels in case of an injury. The site of injury is plugged with blood clots which prevent the further blood loss. A defective clotting mechanism could lead to a potential loss of blood through minor injuries to the small blood vessels, which leads to haemorrhages or haematomas. The injury to the blood vessels can occur by simple stresses of motion and the accompanying contacts with physical objects or from ordinary chewing, normal movement and loss of cells in the gastrointestinal tract and so on.

Blood clotting is a complex phenomenon and is triggered on in response to the injury to the blood vessel. The clotting process occurs in a cascade, where each step triggers the following step up to the last step where it terminates in the formation of an insoluble crosslinked fibrin starting from soluble fibrinogen. The course of clotting involves about 20 enzymes (proteases), cofactors and inhibitors. The enzymes are normally called factors and are given a Roman numeral designation. Table 1, gives the common names and functions of the clotting factors. The enzymes of the clotting system circulate in the blood in their inactive zymogen forms, two separate initiating mechanisms operate viz. intrinsic and extrinsic pathways\(^3,4\) which initiate blood clotting. The intrinsic has all its components in the blood, whereas the extrinsic requires a
factor thromboplastin present in exposed tissues. Both the mechanisms converge at factor X and thereon go by a common pathway to the formation of highly crosslinked fibrin. The crosslinks give rigidity to the clot, making a lattice in which varying numbers of platelets, erythrocytes and leukocytes are trapped. Fig. 1 illustrates schematic overview of the fibrin cascade. It depicts the sequential amplification of the clotting mechanism and the interdependencies of the different factors and cofactors. It can be noted that several of these coagulation factors, prothrombin, factor VII, factor IX and factor X are involved in complexes with phospholipid and calcium ions, their calcium binding properties are crucial for phospholipid binding and subsequent biological action.

Vitamin K mediates the formation of calcium binding sites (active sites) in these factors and in absence of vitamin K these factors fail to bind calcium ions. These factors are thus termed as vitamin K dependent factors. Vitamin K activates these clotting factors by increasing their calcium binding ability. This is achieved by vitamin K, acting as a cofactor for a specific carboxylase enzyme which inserts a carboxyl group in the position of glutamyl residues in the polypeptide chain to give carboxyl glutamic acid. This is a much stronger chelating agent as compared to glutamic acid.
Table 1: Factors in Blood Clotting, Common names and functions of the well defined coagulation factors.

<table>
<thead>
<tr>
<th>Coagulation Factor</th>
<th>Function of Active Form</th>
</tr>
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<tbody>
<tr>
<td>Roman numeral</td>
<td>Common names</td>
</tr>
<tr>
<td>designation</td>
<td></td>
</tr>
<tr>
<td>Factor I</td>
<td>Fibrinogen</td>
</tr>
<tr>
<td>Factor II</td>
<td>Prothrombin</td>
</tr>
<tr>
<td>Factor III</td>
<td>Tissue factor, thromboplastin</td>
</tr>
<tr>
<td>Factor IV</td>
<td>Calcium ions</td>
</tr>
<tr>
<td>Factor V</td>
<td>Proaccelerin</td>
</tr>
<tr>
<td>Factor VII</td>
<td>Proconvertin</td>
</tr>
<tr>
<td>Factor VIII</td>
<td>Antihemophilic factor</td>
</tr>
<tr>
<td>Factor IX</td>
<td>Christmas factor</td>
</tr>
<tr>
<td></td>
<td>(Plasma thromboplastin component)</td>
</tr>
<tr>
<td>Factor X</td>
<td>Stuart factor</td>
</tr>
<tr>
<td>Factor XI</td>
<td>Plasma thromboplastin antecedent</td>
</tr>
<tr>
<td>Factor XII</td>
<td>Hageman factor</td>
</tr>
<tr>
<td>Factor XIII</td>
<td>Protramyoglobinase</td>
</tr>
<tr>
<td></td>
<td>(Fibrin stabilizing factor)</td>
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</tbody>
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Precursor of fibrin lattice  
Hydrolyses fibrinogen  
Hydrolyses factor VII  
Cationic cofactor  
Protein cofactor for factor Xa  
Hydrolyses factor X  
Protein cofactor for factor IXa  
Hydrolyses factor X  
Hydrolyses prothrombin  
Hydrolyses factor IX  
Hydrolyses factor XI  
Forms crosslinks in fibrin lattice
The oral anticoagulants act as vitamin K antagonist. They interrupt the fibrin cascade by inhibiting the \( \gamma \) carboxylation of the vitamin K dependent factors thereby reducing their calcium binding ability and rendering them inactive. This is the basis of oral anticoagulant inhibition action\(^{10} \).

**Action of oral anticoagulants: Molecular mechanism:**

Considerable progress has been made in understanding the action of oral anticoagulants principally through elucidation of the role of vitamin K in blood clotting.

The activation of the vitamin K dependent clotting factors by \( \gamma \) carboxylation is coupled to physiologically important vitamin K - Vitamin K epoxide cycle by which the active hydroquinone form of vitamin K is recycled via its epoxide and quinone forms\(^{11} \). Fig. 2, depicts vitamin K - vitamin K epoxide cycle.

The action of the 3-substituted 4-hydroxycoumarins 1 and the indanedione 2 group (fig.5) of oral anticoagulants is to interrupt this cycle by inhibiting the conversion of vitamin K epoxide to active vitamin K mediated by the enzyme vitamin K epoxide reductase\(^{12} \). This inhibition which is the basis of oral anticoagulant therapy leads to an accumulation of vitamin K epoxide\(^{13,14} \), and thus act as vitamin K antagonist. *Invitro* experiments\(^{15} \) using 3-substituted 4-hydroxycoumarin anticoagulants on rat liver microsomal
INTRINSIC PATHWAY

HMWK \rightarrow HMWKA

Kallikrein

Prokallikrein

Surface Activation

XI \rightarrow XIa

HMWKA

XII

VIII \rightarrow (VIIIa+IXa)

VWF

Fibrinogen

Thrombin

Prothrombin

Ca^{2+}, PL

V \rightarrow (Va+Xa)

Ca^{2+}, PL

Crosslinked Fibrin

EXTRINSIC PATHWAY

a = activated form of clotting factor,

HMWK = high molecular weight Kininogen, PL = Phospholipid,

VWF = Von Willebrand factor, TP = thromboplastin.

FIG. 1: Schematic Overview of Coagulation (Fibrin Cascade).
vitamin K dependent carboxylase, vitamin K epoxidase, vitamin K epoxide reductase and cytosolic vitamin K epoxide reductase, demonstrated that millimolar concentration of coumarin derivatives are sufficient to inhibit the carboxylase, epoxidase and vitamin K epoxide reductase activity. The vitamin K epoxide reductase being the most sensitive to coumarin inhibition and is the preferred site of action of coumarin anticoagulants. Whitleon et al also observed a strong inhibition of vitamin K epoxide reductase by warfarin 3, (fig. 5), a coumarin anticoagulant.

Thus any vitamin K antagonist like the 3-substituted 4-hydroxycoumarin anticoagulants may block this cycle and inhibit the clotting of blood.

R.B.Silverman proposed a model for the molecular mechanism of oral anticoagulants, which shows that these anticoagulants are mechanism based inactivators of the enzyme vitamin K epoxide reductase. A mechanism based inactivator is an unreactive molecule which bears a structural similarity to the substrate or product of the target enzyme and it is converted by the enzyme into a reactive molecule which then forms a covalent bond with an essential moiety of the enzyme. The mechanism for the reduction of vitamin K epoxide to vitamin K is not known. However, the enzyme requires added dithiothreitol (thiol) for activity and this requirement could not be substituted by NADH. The mechanism is shown in Scheme 1. The oxidised
Glutamate → O₂ → CO₂ → γ-Carboxy Glutamate

Vitamin KH₂

Glutamate → O₂ → γ-Carboxy Glutamate

DTT = Dithiothreitol, \( R = \text{Phytly} \)

FIG. 2
enzyme 4 is converted into the active reduced form 4a by added thiol. Once the enzyme is reduced, vitamin K epoxide 5 binds to the enzyme and is activated by protonation of the epoxide oxygen 6 which can undergo backside attack by a sulfhydryl group, leading to enzyme bond α-hydroxy sulfide 7, the protonation and sulfhydryl attack are probably concerted. Protonation of the α-hydroxy group generates an active leaving group for reductive elimination which is promoted by another active site sulfhydryl group 8 thus yielding vitamin K 9 and regenerating the oxidised enzyme. This step is analogous to the reductive elimination of α-ketosulfides to ketones, which occur when they are treated with thiols18.

The structure of 3-substituted 4-hydroxycoumarins 1 is quite similar to that of vitamin K 9, the product yielded by vitamin K epoxide reductase. Thus it is reasonable that these compounds would bind to this enzyme. Analogous to the proposed enzyme catalysed protonation of the epoxide of the substrate, the double bond of the hydroxycoumarins could be protonated to give 3 substituted chromandiones,10(Scheme 2), the unstable tautomeric form. These compounds, substituted o-ketophenyl lactones, would be expected to be susceptible to nucleophilic attack at the lactone carbonyl group. These reactive compounds, then, can covalently modify the enzyme to give 11 or 12 by pathways 'a' or 'b' respectively, by acylation of an active site thiol or another active site nucleophile,
thereby rendering the enzyme unavailable for vitamin K epoxide reduction to vitamin K.

Numerous 3-substituted 4-hydroxycoumarins have been prepared and tested for their anticoagulant activity\textsuperscript{19,20}. The potency of the compounds depends upon the structure of the 3-substituent. It is believed that this substituent is important to the stability of E-I complex, but is not involved in the chemistry of inhibition of the enzyme\textsuperscript{21}. Model chemical studies were carried out on 3-substituted 4-hydroxycoumarins and were in agreement with the proposed mechanism, thus providing chemical support for a molecular mechanistic hypothesis that the anticoagulant activity of 3-substituted 4-hydroxycoumarin drugs is a result of mechanism based inactivation of vitamin K epoxide reductases. This hypothesis is also consistent with certain physiological observations reported. Because of the relatively slow uptake of vitamin K in cell, it has been suggested\textsuperscript{22} that the cell biosynthesises vitamin K epoxide for intracellular storage of an inactive form of the vitamin. When the cell needs vitamin K rapidly, it would have ready access to it via reduction of the inactive epoxide, catalysed by vitamin K epoxide reductase. This process of converting the epoxide into active vitamin K could be regulated by the cell just by controlling whether the enzyme was in the inactive disulfide form 4 or the active dithiol\textsuperscript{4a} form as proposed in Scheme 1. If the epoxide were just the storage form of vitamin K, then
in the presence of vitamin K, the cell temporarily would have no need for vitamin K epoxide reductase. Therefore, if an animal were treated with warfarin, which inactivates vitamin K epoxide reductase, the anticoagulant effect would be reversed and a coagulation response observed by administering vitamin K. This is, in fact the case. It also has been found that, after treatment with warfarin, blood coagulability rises slowly over several days until it reaches the normal level. This slow recovery could arise from regeneration of the clotting factors as a new protein biosynthesis or could arise from the regeneration of vitamin K epoxide reductase activity as a result of slow hydrolysis of the proposed acyl enzyme or both.

**Anticoagulant Rodenticides: A survey.**

Several compounds exhibit anticoagulant activity, some of which are naturally occurring and other synthetic. Normal blood contains proteins like antithrombin III, which interacts with thrombin, factor Xa and VIIa to form an inert enzyme substrate complex and prevent clotting. Heparin, present in plasma increases the reactivity of antithrombin III by combining with it. Besides these proteins, Hirudin from the salivary glands of leech, glandular products from certain vampires and certain snake venoms have been found to act as anticoagulants. The various synthetic compounds studied include a wide variety of coumarin derivatives, salicylic acid derivatives and the indanedione group of
compounds. All the synthetic anticoagulants are known to act as vitamin K antagonist and interfere in the vitamin K dependent reactions of blood clotting.

The credit for development of synthetic anticoagulants goes to K.P. Link and coworkers\textsuperscript{28}. They were trying to unveil the cause of a mysterious disease plaguing the cattle in North Dakota and Canada called as "sweet clover disease" whose principal symptom was a severe bleeding tendency. The poisonous substance that caused the bleeding and hypothermominemia was identified by Link as 3,3'-methylene bis (4-hydroxycoumarin), commonly known as dicoumarol \textsuperscript{13} (fig.5). The first clinical application of dicoumarol as anticoagulant was made by Bingham et al.\textsuperscript{29}. Link latter demonstrated that compounds similar to dicoumarol also exhibit anticoagulant effect\textsuperscript{19}. These findings marked a turning point in the 4-hydroxycoumarin chemistry and many research groups put their efforts to study the synthesis and biological testing of 4-hydroxycoumarin derivatives. Coumarin type anticoagulants thereafter emerged as one of the most important drug in medicine and even today is the major weapon in the arsenal of cardiac specialists\textsuperscript{30}. These anticoagulants were demonstrated to exhibit antivitamin K activity.

On the basis of extensive study of coumarin derivatives, Link and coworkers\textsuperscript{19} concluded that the minimum requirement for anticoagulant activity is an intact 4-
hydroxycoumarin with a substituent at position 3. For higher activity a 1,5 spatial relationship between the enolic hydroxyl group of 4-hydroxycoumarin and a keto group is specifically required. Mentzer et al\textsuperscript{31} too confirmed Link et al's theory. Chmielewska and Cieśliak\textsuperscript{32} also analysed the structural requirements for anticoagulant activity from the point of their vitamin K antagonism. They proposed that for a compound to act as vitamin K antagonist it should have identical anchoring group to interact with the chemoreceptor of vitamin K. The active form of vitamin K can be represented as 14,15 (fig.4). To exhibit antivitamin K activity the anticoagulant should have structures 16,17 (fig.4) which are cyclic hemiketals of appropriately 3-substituted 4-hydroxycoumarin containing a carbonyl group or potential carbonyl group in position 2' or 3' of the side chain. Thus both the theories demanded same structural requirements for the anticoagulant activity.

In 1947 O'Connor J.A.\textsuperscript{33} demonstrated the use of anticoagulant dicoumarol for rodent control. From then on, the coumarin anticoagulants particularly warfarin, developed into effective and widely used rodenticide. This was a major contribution to the rodent control program. The anticoagulant rodenticides also termed chronic rodenticides offered many advantages over the then routinely used acute poisons (single dose rodenticides) like red squill, pyrinuron, zinc phosphide, norbormide, strychnine, alpha-naphthyl-thiourea etc.
The acute poisons have many shortcomings, two of which deserve mention: (a) they are hazardous to nontarget animals (lack of specificity), which could lead to secondary poisoning (b) their acceptance and reacceptance by the rodents is very poor (bait shyness), leading to ingestion of sublethal doses, which leads to inadequate control. The anticoagulant rodenticides on the other hand have none of the above shortcomings and they possess general properties which are their assets as rodenticides:

1) Relatively nontoxic to domestic animals and man.
2) Bait shyness does not occur as the death from the ingestion occurs after three to ten days of the first contact, so animal ingests bait until death.
3) These are used in baits at concentrations far lower (0.002 - 0.05%) than those of acute poisons (0.5 - 5%), so the primary and secondary hazards to nontarget species are minimised.
4) Accidental poisoning can be controlled by administration of vitamin K.
5) 90% rodent control can be achieved by inexpert users as against acute poisons which always require skilled personnel for use.

These assets proved to be a booster for the use of anticoagulants as alternate rodenticides. Warfarin was the first to enter the anticoagulant rodenticide market and made a great impact in rodent control programme. This was followed
by the development of a number of 4-hydroxycoumarin and indanedione based compounds viz. pindone 18, phenyl indanedione 19, diphacinone 20, chloropacinone 21, coumatetralyl 22, coumafuryl23, coumachlor 24 (fig.5), which are collectively referred to as 'first generation anticoagulants' and are claimed by specialists as the panacea for most rodent control operations. The introduction of anticoagulants in the early 1950's resulted in a general decline in the use of acute rodenticides and over a few decades over 90% rodenticides used all over the world accounted for coumarin based compounds. All these have certain similarity to vitamin K, but differ from one another in terms of toxicity, rapidity of action, and acceptance by rodent species. Although, most of these rodenticides are effective, some are discernibly better than others for the control of certain species. In view of these differences in effectiveness, the anticoagulant usage varies from country to country depending on the rodent infestation.

Warfarin has proved to be very effective against commensal rodents worldwide and satisfactory control has been reported on long term baiting. Rattus norvegicus and bandicota bengalensis were found to be highly susceptible to warfarin, whereas some species such as Mus booduge, Rattus rattus, Tatera indica, required 8 to 14 days and the house mouse Mus musculus, required 21 to 28 days for total control. The other first generation anticoagulants,
chlorophacinone and diphacinone were more effective in forest control because they are more active per unit weight, and required fewer multiple feedings\textsuperscript{38}. They additionally could control \textit{Mus musculus}, \textit{R. rattus} and \textit{T. indica}, much better than the 4-hydroxycoumarins. However, it is reported that these indanediones are equally toxic to non target animals\textsuperscript{39}.

Apart from the lower performance by the first generation anticoagulants against certain rodent species, the problem of rodent control became complex with the emergence of resistance to some of these anticoagulant rodenticides. The so called 'super rats' resistant to anticoagulant, first appeared in 1958 in Great Britain\textsuperscript{40} and thereafter the reports of resistance started pouring in from all over the globe\textsuperscript{41,42}. Most of these reports were about commonly encountered rodents such as \textit{R. norvegicus}, \textit{R. rattus}, \textit{M. Musculus} and \textit{B. bengalensis}. The resistance seems to be inherited as a single autosomal dominant gene, and resistant rat have also been observed to have a significantly greater requirement of vitamin K\textsuperscript{43}, because of defect in vitamin K metabolism. It is indicated that the resistance observed is because of the change in the binding properties of the enzyme vitamin K epoxide reductase that converts vitamin K epoxide to active vitamin K\textsuperscript{44}. Warfarin is known to bind to this enzyme and inhibit blood clotting in case of susceptible rats. But in case of resistant rats the conversion of vitamin K epoxide to the active vitamin K takes place even in
the presence of anticoagulants. The resistance to warfarin did embrace the other anticoagulants too, and subsequently cross resistance to all first generation anticoagulants developed quickly making the rodent pest management more complex.

Not surprisingly, this gave impetus to the synthesis of novel anticoagulants with a view to improve their activity and to overcome the resistance problem. A breakthrough in the search of anticoagulant rodenticides capable of acting on warfarin resistant rodents and of higher activity came with the advent of new coumarin anticoagulants viz. difenacoum, bromadiolone, bromifacoum, brodifacoum, and clocoumarol (fig.6), and difethialone (thio analog of 27). These newer group also called 'second generation anticoagulants' have been developed with the properties of both acute and chronic poisons minus their disadvantages. The basic physiological effect of these new anticoagulants is similar to that inflicted by the first generation, but are more potent against susceptible as well as warfarin resistant species. These are particularly characterized by having a higher (0.22 - 1.8 mg/kg) acute toxicity. As for conventional anticoagulants, vitamin K serves as the antidote. Their activity studies and field trials put them as the best alternatives for warfarin and coumatetrayl resistant and nonresistant species as they exhibited higher efficacy against a wide spectrum of rodent pests. These second
generation anticoagulants have taken over a considerable part of the rodenticidal market all over the world during the last 10 to 12 years. However, resistance of practical importance has been encountered to difenacoum and bromodiolone in Europe and to brodifacoum in Canada\textsuperscript{51}. Although it has been found that the resistance is not as penetrative as that of warfarin, it is a matter of concern. Additionally because of their higher toxicity, they carry a slight but significant risk to nontarget organisms like poultry\textsuperscript{52}.

The rodent control program has also utilized some alternative strategies to improve the efficacy of these rodenticides, noteworthy are:- improvement in bait formulations, whereby the baits can have improved activity compared to anticoagulant itself. The activity of warfarin is increased by addition of bait sweetners, or attractants such as meat or ethylvanillin, sodium glutamate and phenobarbitol\textsuperscript{53}. The increase in activity is also achieved by addition of antibacterial agents like the penicillin G, Sulfaquinoxalin and terramycin to the bait\textsuperscript{54}. Hydroxycoumarin rodenticidal activity could be potentiated by alkene glycols\textsuperscript{56b} and l-histidine\textsuperscript{56c}. Studies indicate that calciferol, with or without the combination of warfarin, alphachloralose and the chloro analog of vitamin K were shown to be effective against anticoagulant resistant or susceptible rodents especially mice population\textsuperscript{55,56a}. 
Finally though these anticoagulant rodenticides form the backbone of most rodent control operation, the occurrence of resistance to the first generation anticoagulant rodenticides and to a lesser degree to second generation anticoagulant rodenticides, and the risk to non target species poses a significant need for designing newer rodenticides which will be able to overcome these problems. These new materials could be held as backup reserves which could be utilised when resistance develops to the older anticoagulants. This strategy could help in prolonging the possible development of resistance to the new compounds.

**General Synthetic Methods**

The practical utility of the coumarin group of anticoagulants in medicine and their very important role as rodenticides, led to the hectic activity of chemist to synthesize anticoagulants of this group. In the following pages an attempt is made to highlight some of the general synthetic methods used to prepare these coumarin derivatives.

In case of warfarin and its analogs the method usually employed is, Michael condensation of substituted or unsubstituted 4-Hydroxycoumarin with different $\alpha,\beta$-unsaturated ketones or potential $\alpha,\beta$-unsaturated ketones (scheme 3 & 4)$^{57,58a}$. Based on the reactants, a variety of bases, solvents and reaction conditions have been used$^{58b}$. Similar products as above are also reportedly obtained on
**SCHEME 3**

```
O^O
PhCH=CHCOCH_3

O
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**SCHEME 4**

```
O^O
Cl
\text{Dioxane Piperidine}
Reflex

O
```

**SCHEME 5**

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O^O
PhCH=CHCOR'

O
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**SCHEME 6**

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O^O
Ar\text{CN}
1) \Delta
2) HCl

O
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**SCHEME 7**

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O^O
\text{NCH}_2\text{COR'}\text{HCl}^-

O
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The images contain chemical structures and reactions, likely from a scientific document, possibly related to organic chemistry. The reactions are depicted with arrows indicating the transformation of reactants into products.
reaction of 4-hydroxycoumarin with alkynols such as PhCH(OH)CH₂CH=CH₂, in the presence of AcOH, H₂SO₄ or AcOH-BF₃ (scheme 5). Michael reaction is also utilized to prepare 3-(4-hydroxy-3-coumarinyl)-β-substituted propionates by refluxing 4-hydroxycoumarin with Ethyl arylidene cyanoacetate followed by acid treatment (scheme 6)⁶⁰.

The other less commonly employed methods include condensation of 4-hydroxycoumarin with appropriate amine hydrochloride of a ketone (scheme 7)⁶¹; refluxing ethylbenzoyl acetate with 3-bromoethyl-4-acetoxy coumarin (scheme 8)⁶¹. Reaction of Mannich base of 4-hydroxycoumarin with appropriate ketone (scheme 9)⁶² to yield the required warfarin analogs.

Another class of 4-hydroxycoumarin anticoagulants are the 3-alkyl or 3-aryl 4-hydroxycoumarins. These are generally prepared by

1) Condensing respective halides with 4-hydroxycoumarin (scheme 10)⁶³.

2) Direct coupling in acid, of 4-hydroxycoumarin with a diazotized aniline (scheme 11)⁶⁴.

3) Heating anhydrous phenol with ethyl 2-(p-methoxyphenyl) malonate at 290°C under N₂ for 6-7hrs⁶⁵a or heating phenol with malonic acid in presence of POC₃ followed by cyclization with AlCl₃ (scheme 12)⁶⁵b.

4) Condensation of arylsalicylic acid esters o-ORC₆H₄CO₂R¹ (where R=Ac; R¹= monovalent hydrocarbon group) in high
\[ \text{Scheme 8} \]

\[ \text{Scheme 9} \]

\[ \text{Scheme 10} \]

\[ \text{Scheme 11} \]

\[ \text{Scheme 12} \]

\[ \text{Scheme 13} \]
boiling hydrocarbon in presence of alkalimetal, alkoxide, or alkalimetal amide (scheme 13).66.

v) Heating 4-hydroxycoumarin with different dihydronapthalenes in presence of acid catalyst to yield 3-substituted tetrahydronaphth-1-yl-4-hydroxycoumarin (scheme 14).67.

vi) Reacting 4-hydroxycoumarin with p-HOCHR\textsuperscript{1}C\textsubscript{6}H\textsubscript{4}CH\textsubscript{2}CH\textsubscript{2}R in the presence of AcOH and conc. H\textsubscript{2}SO\textsubscript{4} for 3 hrs at 81-2°C (scheme 15).68.

Pyridyl and quinononyl ketones of 4-hydroxycoumarin are synthesised by condensing Pyridynyl or quinonyl carboxylic acids with 4-hydroxycoumarin in presence of POCl\textsubscript{3} (scheme 16).69,70.

Warfarin analogs are converted to their hemiketals as 3,4 dihydropyrano coumarins by treatment with an alcohol in presence of acid catalyst (scheme 17).71 The side chain carbonyl is generally reduced, using NaBH\textsubscript{4} or Al(iPrO)\textsubscript{3}/iPrOH, to the alcohol.

The dicoumarol group of compounds are generally prepared by condensing 2 moles of 4-hydroxycoumarin with appropriate aliphatic or aromatic aldehyde\textsuperscript{1}9 to yield the 3,3'-alkylene or aryllidene bis (4-hydroxycoumarin) respectively in good yields (scheme 18). The other derivatives are prepared by condensing 4-hydroxycoumarin with a solution of OHCCOOH (2.5%) which are further esterified with different alcohols in the presence of conc. H\textsubscript{2}SO\textsubscript{4}/HCl.
SCHEME 14

SCHEME 15

SCHEME 16

SCHEME 17
SCHEME 18

SCHEME 19

SCHEME 20

SCHEME 21

FIG. 6
These esters are also prepared in one step by refluxing 4-hydroxycoumarin with OHCCOR.

3,3' bis 4-hydroxycoumarin acetone is prepared by refluxing 4-hydroxycoumarin and a dihaloacetone (scheme 20). Cyclisation of 3-(2-salicyloylethyl)-4-hydroxycoumarin or a derivative in the presence of dry ethylformate and metallic sodium, or sodium alcoholate gives 3-chromanyl-(4-hydroxy-3-coumarinyl) methane (scheme 21). A slight modification utilizes sodium in dry ethylformate followed by acidification with PH₃.

4-Hydroxycoumarin and acetals of alkoxyaldehydes give rise to 1-alkoxy-2,2-bis(4-hydroxycoumarin), diethyl bis (4-hydroxy-3-coumarinyl) acetate is prepared by refluxing 4-hydroxycoumarin with 2,2 diethoxyethyl acetate.

An Overview of Structure Activity Studies

The very important discovery of anticoagulant activity in dicoumarol and its further application as chronic rodenticide resulted in a spurt of activity towards the synthesis of analogous compounds. A large number of compounds were thus prepared, and screened for their anticoagulant and/or rodenticidal activity. Many groups also made attempts to correlate the biological activity in terms of structural features. Some of these correlations are noteworthy, and the conclusions drawn are helpful to understand the structure activity relationships (SAR) of coumarin anticoagulants.
Overman et al. were the first to synthesise a large group of coumarin analogs and show the relationship of the structure of coumarins to their anticoagulant activity. This was the first major work in studying the SAR in coumarin anticoagulants. Dicoumarol was taken as the standard anticoagulant and the relative anticoagulant index for the analogs was calculated. The study included 105 compounds, and the structure activity data was interpreted as follows:

1) In case of 3,3'-methylene bis (4-hydroxycoumarin) (dicoumarol, 13; fig. 5), the replacement of methylene H by carbon atom residue leads to decrease in activity. An increase in the chain length and the branching in the chain leading to the greater loss in the activity. An aryl substitution reduces the activity to 1/300-500 that of unsubstituted. Replacement of methylene by sulfur leads to decrease in activity twenty fold.

2) In case of 3 substituted 4-hydroxycoumarin, generally the size of the 3 substituent determines activity and the activity is reduced with decrease in the size of the 3-substituent in a homologous series. Cyano, bromo, oximino and nitro as 3 substituents are found to be inactive. Whenever the 3 substituent has a keto group in a 1,5 arrangement with respect to the 4-hydroxy group its potency is quite high and approaches that of 3,3'-methylene bis(4-hydroxycoumarin). Further the activity depends upon the substituents on the side chain.
iii) The masking of the 4-hydroxy group as ether or ester reduces the anticoagulant potency, the potency decreasing progressively with increase in chain length and branching. These esters and ethers of 4-hydroxycoumarin anticoagulants are found to show their effect after a time lag, this was explained on the basis that these esters and ethers act as anticoagulants after invivo hydrolysis to the active compound. The cyclic ketals range in activity close to the 4-hydroxycoumarin anticoagulants, or sometimes higher than the parent, this increase in activity was explained on the basis of more efficient absorption of the ketals than the parent compound, followed by invivo hydrolysis to the active parent compound.

iv) Substitution in the benzenoid nucleus of coumarins reduces or destroys the anticoagulant activity. A similar observation was also recorded by Andrew, et al. 79b.

v) Removal of a mole of water between the two enolic hydroxy groups in bis(4-hydroxycoumarin) to form substituted anhydrides leads to inactivity. Implying that a intact 4-hydroxycoumarin moiety is a must for its biological activity.

vi) A series of 1,5 dienols, enols and ketones having structural similarities with 4-hydroxycoumarin moiety are also inactive, implying and confirming that an intact 4-hydroxycoumarin is indeed necessary for anticoagulant activity.
vii) Coumarins without a 4-hydroxy group, as 4-aminocoumarin do not exhibit anticoagulant activity indicating that a 4-hydroxy group is an essential requirement.

Arora and Mathur\textsuperscript{79a} also did structure activity relationship studies and they concluded that the anticoagulant activity exhibited by coumarins is governed not by individual structural features but by a combination of several factors. Molecular shape being one of the important factors, eg. cyclic ketals augment anticoagulant activity. A substituent in position 8 of 4-hydroxycoumarin ring is considered important for activity. A hydroxy, methoxy, or benzamido group in position 3 of coumarin leads to a decrease in the anticoagulant activity, similar is the case of a phenyl substituent at position 3 or 4. They further proposed that inhibiting effect of a substituent can be counteracted by an effective combination of several potentiating factors, eg. Callophyllolide 30 (fig. 6) is very active even though it has a 4-phenyl substituent which is known to inhibit the activity. In a series of halogen substituted warfarin, 6-Cl warfarin is found to be more active than the 6-Br analog, suggesting that molecular size of the substituent in 4-hydroxycoumarin is crucial for biological activity\textsuperscript{79b}. The position of the halogens on the phenyl ring also governs the order of activity, being maximum in para followed by ortho, and minimum in meta\textsuperscript{80}. 
Generally the effect of substituents on anticoagulant activity of coumarins has been studied with respect to warfarin and dicoumarol. It is observed that introduction of electrophilic substituents like halogens or NO₂ in the side chain phenyl of warfarin, or in 3,3' benzylidene bis (4-hydroxycoumarin) enhance the activity. A general order of activity in phenyl substitution in 3,3' benzylidene bis(4-hydroxycoumarin) was established by Guminska et al. as p-Cl > o-Cl > m-Cl >> p-NO₂ > m-NO₂ > p-CH₃ > o-CH₃ > p-CH₃ > o-CH₃.

The position of attachment of the substituents also dictates activity e.g. in 3,3'-napthyldiene bis (4-hydroxycoumarin) the α- isomers are active and β-isomers are virtually inactive. A furfurylidene substituted or unsubstituted in furan ring is less active than dicoumarol.

J Boulay investigated a homologous series of analogs of warfarin and found that lengthening of the hydrocarbon side chain in β-position of the α-phenyl- β-acetyl analog by 1 CH₂ group shows sharp drop (50%) in toxicity, a lengthening by 3 CH₂ groups removes the toxicity, substitution of phenyl radical by propyl or isobutyl radical leads to inactivity, whereas by napthyl or biphenyl radicals the toxicity was 20% less than warfarin. These results are controversial with those of Overman et al.
4-Hydroxythiocoumarins also exhibit anticoagulant activity on the same lines as the '0' analogs, and activity is found to be close to the 0 analogs. The thio sulfinyl and sulfonyl analogs of warfarin showed activity close to that of warfarin. As in case of coumarins, the 4-hydroxy group is a must for activity in thiocoumarins, as 4-mercaptothiocoumarin is found to be devoid of activity.

Jongh et al. studied a series of 2,2'-bis (4-hydroxycoumarinyl) ethanol ethers and concluded that the anticoagulant activity decreases in the order Me > Et > Pr > iPr > Bu > iBu > Bz. Lower derivatives are as effective as dicoumarol, whereas aromatic derivatives are weak. In case of dicoumarol if one of the rings is deoxygenated i.e. substitution of chromane for coumarin ring, it causes decrease in activity.

The anticoagulant activity increases in going from 3,3'-benzylidene bis (4-hydroxycoumarin) to 3,3'- (alkylthio) benzylidene bis 4-(hydroxycoumarin). A comparison of the activity in a series of different sulfur analogs viz. thio, sulfoxyl, and sulfonyl derivatives showed that sulfoxyl was most active, while sulfonyl and thio derivatives exhibited weaker anticoagulant activity. 3-methyl 4-hydroxycoumarin is the only compound in the 4-hydroxycoumarin group of compounds which exhibits vitamin K like activity, this is attributed to its structural resemblance with vitamin K₁.
Boschetti and Coworkers found that, on reduction of the side chain carbonyl to alcohol in the warfarin derivatives, the activity is retained or increased. The replacement of the terminal methyl group in the side chain of warfarin by various substituted phenyl groups also produces compounds with higher anticoagulant activity.

Hadler et al., investigating a large group of warfarin analogs, concluded that the requirement for anticoagulant activity is a lipophilic group in the para position of the phenyl group, which provides a point for attachment to a lipophilic site.

A very important point noted in case of enantiomeric coumarin anticoagulants was the difference in activity of the two enantiomers, one having higher activity than the other. This was explained on the basis that isomeric coumarins have a difference in permeability or affinity for receptor site, eg. the anticoagulant potency of S(-) warfarin is 6.6 times greater than R(+) warfarin; R(+)acenocoumarol is several times more potent than S(-) acenocoumarol, which is ineffective. Their rodenticidal activity also shows a similar difference, LD50 for S(-) warfarin is 2 mg/kg and of R(+) is 17.7 mg/kg. Thus implying that one of the conformation of warfarin group of anticoagulants is more effective than the other possible ones, probably because of a better steric fit at the receptor site.
Rowe and Redfern studied coumatetralyl and its derivatives, and found it as a suitable alternative poison to warfarin, for use against mice that are susceptible to anticoagulants, but these were not able to control warfarin resistance population effectively. Hadler and Shadbolt also synthesised substituted coumatetralyl derivatives and found them to be least vulnerable to resistance compared to other group of 4-hydroxycoumarin derivatives. They rationalised the data and explained that the activity of these group of compounds in resistant rats might be due to the manner in which, in the tetralin moiety, the four carbon atoms of the saturated ring are held adjacent to the unsaturated ring, whereas the four carbon atoms in the side chain of warfarin can assume many conformations other than those adjacent to the benzene ring.

They prepared a series of 3(3-p-substituted-1,2,3,4 tetrahydronapthyl) 4-hydroxycoumarin and tested their activity against warfarin in resistant and non resistant R. norvegicus. They further concluded that the presence of a lipophilic group in the para position is very important for anticoagulant activity in both strains of rats, as it provides a point of attachment to a lipophilic site to which warfarin and other coagulants do not bond strongly, cases where less lipophilic substituents exhibited high activity was explained to be because of electronic effects playing their part. The activity was found to be substituent
dependent, for eg. the replacement of tetralin moiety with indanyl, led to loss of activity, which was because of the angle the phenyl group makes with the indanyl group.

Arora et al\textsuperscript{97} prepared some 7-hydroxycoumarin derivatives as alternative rodenticides to 4-hydroxycoumarin derivatives, but these were found to be less active than 4-hydroxycoumarin derivatives. Implying reduction in activity in going from a 4-hydroxy to 7-hydroxy group. The toxicity was found to be independent on the size of the side chain in these derivatives, unlike the 4-hydroxycoumarin derivatives.

Thio analogs of 4-hydroxycoumarin were prepared and their anticoagulant activity was evaluated by Vishnyakova\textsuperscript{98}. These compounds exhibit anticoagulant activity comparable to 4-hydroxycoumarin derivatives but with lower toxicity. Jamkhandi et al\textsuperscript{80} also found that the 1-thio analogs exhibit structure dependent activity and the SAR was on the same lines as of 4-hydroxycoumarin group.

The sodium, thallium, amino and dialkylamino ethanol salts of warfarin did exhibit activity\textsuperscript{99-101} similar to warfarin, indicating that formation of salts does not alter the rodenticidal activity.

These SAR studies have led to some concrete points as to the minimum structural requirements for exhibiting anticoagulant activity. There are still quite a few voids and a thorough understanding of structure activity relationship is yet to emerge.
Part B

SYNTHETIC STUDIES OF NOVEL ANTICOAGULANT RODENTICIDES DERIVED FROM 4-HYDROXYCOUMARIN
This part deals with the synthetic studies of novel anticoagulant/rodenticides derived from 4-hydroxycoumarin, such as heterodicoumarols, thio analogs of warfarin and optically active terpene based warfarin analogs.

HETERODICOUMAROLS

INTRODUCTION:

In 1940 Link and coworkers made a very important discovery, when they were looking into the cause of haemorrhagic disease of cows that had been fed on spoilt clover. They isolated toxic chemical substance whose structure was established as 3,3'-methylen bis (4-hydroxycoumarin) \textsuperscript{31,28,102,110} (fig.7), generally called dicoumarol. It was extraordinary that clover hay which normally contains coumarin had developed the new compound under improper conditions of storage wherein the clover got spoiled. Dicoumarol has been shown\textsuperscript{103} to be biosynthesized from o-coumaric acid \textsuperscript{33} by way of 4-hydroxycoumarin \textsuperscript{32} in presence of atmospheric formaldehyde or from formaldehyde present in hay (scheme 23).

The interesting part is that this toxic compound in due course turned out to be a very useful drug. It functions as an anticoagulant and is thus used\textsuperscript{19,104}. It proved to be a lead structure for the family of oral anticoagulants and anticoagulant rodenticides which are very much in use to date.
Dicoumarol was usefully introduced into medicine to lower the blood coagulability in for eg. the treatment and prophylaxis of thromboembolic disorders in veins and arteries. However, a slow onset of action and an idiosyncratic variation in absorption and metabolism made dicoumarol a less than ideal drug. Later O'Connor reported the successful use of dicoumarol as rodenticide.

It is also an antibacterial agent being specifically active against gram positive organisms. Dicoumarol derivatives are also useful as intermediates in the preparation of some pharmaceuticals.

Shortly after it was established that 3,3'-methylenebis (4-hydroxycoumarin) is the causative agent of the haemorrhagic sweet clover disease in cattle, it was found that the hypoprothrombinaemia that characterizes this disease could also be induced by analogous compounds.

A large amount of research has been done to prepare derivatives with better activity and less side effects. Many dicoumarol derivatives have been reported that differ from the parent substance. These compounds fall into two main groups: i) those bearing substituent on the bridge methylene group (34, R= alkyl or aryl, X=O, Y=H, fig. 7) ii) symmetrically disubstituted on the aromatic ring (34, X=O Y= substituent, R=H, fig. 7), as well as combinations of i and ii, (34, R=substituents, X=O, Y=substituents, fig. 7).
All these compounds are reported\textsuperscript{19} to be less active than the parent compound dicoumarol (34, $X=O$, $R=H$, $Y=H$, fig.7). Similarly 3,3'-methylenbis (4-hydroxythiocoumarin) (34, $X=S$, $R=H$, $Y=H$, fig. 7) also exhibits anticoagulant activity\textsuperscript{111} and its analogs bearing alkyl or aryl groups on the methylene bridge and/or substituents on the aromatic ring have been reported\textsuperscript{112}.

Apart from these, a few unsymmetrically substituted dicoumarols 35 (Y= substituents, fig. 7) have also been reported\textsuperscript{113-115}.

**PRESENT WORK**:

The above reports indicate, that placing methylene hydrogen with substituents in 31 decrease activity and also symmetrical disubstituted derivative of 31 show less activity. Therefore we attempted newer variation in the parent molecule and a series of novel unsymmetrically substituted heterodicoumarols 36, (fig. 7) bearing a 4-hydroxycoumarin and a 4-hydroxythiocoumarin moiety on the methylene bridge were synthesized (table 3), expecting them to be potential anticoagulants/rodenticides which would probably express greater activity.

Analogous compound 37 (fig. 7) having an indanedione and a 4-hydroxycoumarin/4-hydroxythiocoumarin moiety on the methylene bridge have been reported to posses anticoagulant activity\textsuperscript{116}. 

\textsuperscript{Th7852}
Apart from these heterodicoumarols, we also synthesized a few unsymmetrically substituted 3,3'-(methylene bis (4-hydroxythiocoumarin) derivatives 36a (fig. 7), which is a newer class of compounds (table 4).

Dicoumarol and its derivatives like those bearing a substituent on the bridge methylene are generally prepared by condensing an aldehyde with two moles of 4-hydroxycoumarin 32 (scheme 24). While symmetrically disubstituted dicoumarols are prepared by condensing a substituted 4-hydroxycoumarin derivative with formaldehyde (scheme 25). Several other reactions are also reported where dicoumarol and its derivatives have been isolated (table 2).

The methylene group at C-3 in 4-hydroxycoumarin is reactive and undergoes Michael addition. It has been suggested that formation of dicoumarol itself from formaldehyde and 4-hydroxycoumarin proceeds by this type of addition. The unstable 3-methylene-2,4-diketo chroman 38 is first formed (scheme 26), which is very reactive and cannot be isolated, it reacts quickly with another molecule of 4-hydroxycoumarin to form dicoumarol 31. This last step involves a Michael type addition. The support for this suggestion was obtained by condensing 4-hydroxycoumarin 32 with an equimolar quantity of salicylaldehyde to give 3-(o-hydroxy benzylidene)-2,4-diketo chroman 39 (scheme 27), which could be isolated and which reacted with 4-hydroxy-6-methylcoumarin 40 to give 41 indicating the stepwise nature of the reaction.
**SCHEME 23**

\[ \text{COOH} \rightarrow \text{OH} \rightarrow \text{COOH} \]

**SCHEME 24**

\[ 2 \text{OH} + R\text{CHO} \rightarrow \text{OH} \]

**SCHEME 25**

\[ 2 \text{OH} + \text{HCHO} \rightarrow \text{OH} \]

**SCHEME 26**

\[ \text{O} \rightarrow \text{O} \rightarrow \text{O} \rightarrow \text{O} \rightarrow \text{O} \]
SCHEME 27

SCHEME 28
### Table 2 Miscellaneous methods for the preparation of dicoumarol 31.

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<th>condition</th>
<th>Ref.</th>
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<td>((\text{CH}_3\text{)}_2\text{SO} + \text{AC}_2\text{O})</td>
<td>Heat, 160°C</td>
<td>119</td>
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<tr>
<td>4-hydroxy coumarin</td>
<td>Heat</td>
<td>120</td>
</tr>
<tr>
<td>4-hydroxy coumarin + HCHO + N_2 HCl</td>
<td>aq. HCl, Heat</td>
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</tr>
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<td>(\text{CH}_2[\text{CH(COOH)}_2]_2)</td>
<td>1) 100°C, 2hrs. 2) 240°C, 1hr.</td>
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*POCl₃*
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<tr>
<th>No.</th>
<th>Z</th>
<th>Y</th>
<th>M.P. °C</th>
<th>C, H %</th>
<th>UV λ max (dioxan)</th>
<th>IR cm⁻¹ (nujol)</th>
<th>Elemental analysis</th>
<th>C%, H% (Required)</th>
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<td>262</td>
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<td>H</td>
<td>272</td>
<td>366</td>
<td>315</td>
<td>1650, 1630, 64.49</td>
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<td>6'-Me</td>
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Table 4 : Physical & Spectral data of 36a.

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<td>(62.82)(3.66)</td>
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<tr>
<td>4.</td>
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This was the first reported example of unsymmetrically substituted derivative of dicoumarol.

For synthesizing unsymmetrically substituted heterodicoumarols the condensation of substituted/ unsubstituted 4-hydroxycoumarin with formaldehyde in presence of a substituted/unsubstituted 4-hydroxythiocoumarin was thought to be unsuitable since the reactive 3-methylene 2,4-diketo chroman 38 (scheme 26) generated insitu would condense with either 4-hydroxythiocoumarin or 4-hydroxycoumarin and would give rise to an inseparable mixture of dicoumarol derivatives.

The unsymmetrically substituted heterodicoumarol 36 were synthesized via Mannich base reaction, such a pathway has been used earlier to prepare the only reported unsymmetrically substituted dicoumarol analogs viz. 35 (Y=6-Me, 6-Cl, 7-OH, 7-Me, fig. 7)

Hence substituted/unsubstituted 4-hydroxycoumarin was reacted with piperidine and formaldehyde\(^{121a,b}\) and the Mannich base 4-hydroxycoumarin-3-N-piperidinomethyl 42 obtained, quarternized with methyl iodide, the salt was then condensed with substituted/ unsubstituted 4- hydroxythiocoumarin (scheme 28) to furnish the unsymmetrically substituted heterodicoumarol derivatives 36.

The crude compound thus obtained included impurities of symmetrically substituted dicoumarols 43 (scheme 29)
formed by partial reversal of the mannich condensation followed by addition of the 4-hydroxycoumarin so formed to the chroman\textsuperscript{121b} \textsuperscript{38} (scheme 29). The product obtained was purified by repeated crystallizations. Compound \textsuperscript{36} (X=0, X'=S, Y=2=H) could also be obtained by forming Mannich base of 4-hydroxythiocoumarin \textsuperscript{121c} (scheme 28, X=S), followed by quarternisation and addition of 4-hydroxycoumarin. The substituted 4-hydroxycoumarin and 4-hydroxythiocoumarin used, were prepared according to the known procedure\textsuperscript{134} (scheme 31).

The product dicoumarol derivatives were characterized by IR, UV, mass and elemental analysis. Their physical constants and spectral data is presented in table 3.

Dicoumarol can exist either in a dichromone \textsuperscript{44} or tautomeric dicoumarin \textsuperscript{31} form. Earlier reports\textsuperscript{123,114} have suggested a dichromone structure for dicoumarol and its derivatives. This was based on the observation that in IR of dicoumarol the -OH stretching band is very weak or absent and the carbonyl stretching band appears at lower frequency. But later studies have shown that dicoumarol and its derivatives have coumarin and not the chromone structure.

It has been shown\textsuperscript{124-127} that dicoumarol persists in coumarin state with strong intramolecular hydrogen bonding as shown in \textsuperscript{45} fig. 9. Such a strong intramolecular hydrogen bonding explained the absence of -OH band and this together with the effect of hyperconjugation of the 3-methylene group.
seemed sufficient to lower the carbonyl frequency in IR. The low carbonyl frequencies of dicoumarol and its derivatives falling between 1650 and 1665 cm$^{-1}$ are unlikely to be due to chromone structures \textsuperscript{44}(fig. 8). Since the presence of strong hydrogen bonding, as indicated in IR, between the two halves of these molecules would be expected to lower the frequency of chromone carbonyl group to well below 1650 cm$^{-1}$ as seen for other enolic chromone compounds\textsuperscript{125}.

Further studies in IR, where the stretching frequency of carbonyl was studied by replacing the carbonyl carbon with \textsuperscript{13}C and the vibration of OH studied by deuteration\textsuperscript{125} have confirmed the structure \textsuperscript{45} (fig. 9). NMR studies\textsuperscript{124} of dicoumarol have suggested the dicoumarin structure \textsuperscript{45} where the downfield signal due to -OH protons was independent of concentration and which could be removed by D$_2$O. These studies\textsuperscript{129} have also indicated the intramolecular exchange between two -OH protons and the restricted rotation around methylene bridge head. The results have been confirmed by \textsuperscript{13}C NMR studies\textsuperscript{130}. The enolic nature of dicoumarol has also been shown\textsuperscript{131} by mass spectral molecular ions of substituted 4-hydroxycoumarins.

A mixed coumarin chromone structure\textsuperscript{132,46} (fig. 8) as suggested by Chmielewska\textsuperscript{32} was considered inconsistent with the observed spectroscopic data\textsuperscript{124}. Even an X-ray crystallographic investigation of a dicoumarol derivative indicates that there is a two fold axis of symmetry
Scheme 29

**FIG 8**

**FIG 9**
consistent with an intramolecularly hydrogen bonded structure 45.

For the earlier reported unsymmetrical dicoumarols 35 (fig. 7), a chromone structure 44 (fig. 8) was proposed. The unsymmetrically substituted heterodicoumarols we synthesized exhibited the IR data similar to that observed for symmetrically substituted 3,3'-methylene bis (4-hydroxycoumarin) and 3,3'-methylene bis (4-hydroxythiocoumarin). The -OH stretching band, which is very weak or absent and the appearance of the carbonyl stretching band between 1650-1670 cm⁻¹ for the derivatives 36 indicates strong intramolecular hydrogen bonding and hence these unsymmetrically substituted heterodicoumarols would be existing in the form 47 as shown in fig. 9.

The mass fragmentation pattern of all the heterodicoumarols 36 resembles that of dicoumarols (scheme 30a). For eg. compound 36 (X=O, X'=S, Y=Z=H) shows peaks in its mass spectrum due to the molecular fragment ions of dicoumarols as well as its thio analogs (with added 16 mass units). The major fragment ion m/e 150, whose genesis is shown in scheme 30b, is the base peak in most of these compounds.
MASS FRAGMENTATION PATTERN OF 33'- METHYLENE BIS(4-HYDROXYCOUMARIN)

SCHEME 30a

SCHEME 30b
THIO ANALOGS OF WARFARIN, 3-(1-ARYL, 3-OXO-3-PHENYLPROPYL) 4-
HYDROXYTHIOCOUMARINS (24).

The thio analogs 52 of the anticoagulant rodenticide, warfarin 50 reported in literature are depicted in table 5. These compounds 52 are known to possess anticoagulant activity which surpasses or is comparable to that of the corresponding 'O'analogs 137,139,80. These reports are indicative that substitution of 'S' in place of 'O' in the coumarin moiety of warfarin derivatives leads to enhancement of anticoagulant activity to a varying degree.

These observations plus our interest to synthesize anticoagulants with improved activity made us embark on a plan to synthesize hitherto unreported new series of thiowarfarin derivatives 54 (table 6).

These thio analogs 3-(1-aryl-3-oxo-3-phenylpropyl)4-
hydroxythiocoumarin 54 (table 6) were prepared by Michael addition of 4-hydroxythiocoumarin to the corresponding α,β -unsaturated ketones (substituted benzalacetophenones) 53 (scheme 32) according to the reported procedure, in presence of dioxane and catalytic amount of piperidine.

All the compounds synthesized were characterized by their elemental analysis, M+, IR & PMR spectral data (table 6). A representative PMR & IR spectra of 54 (spectra 1) is given on pg. 55. An PMR & IR spectra (spectra 2) of a similar 'O' analog is also shown, which provides a good comparision.
Table 5: Known warfarin thioanalogues 52.

<table>
<thead>
<tr>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>H</td>
<td>Me</td>
<td>Ph, p-MeC₆H₄, p-OMeC₆H₄, p-FC₆H₄, p-BrC₆H₄, p-ClC₆H₄, p-NO₂C₆H₄</td>
<td>135, 136</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p-0EtC₆H₄, 2,4-Cl₂C₆H₃, p-OCH₂Me₂C₆H₄, 2-furanyl, α-thienyl</td>
<td>137.</td>
</tr>
<tr>
<td>H</td>
<td>Et</td>
<td>Ph, p-BrC₆H₄, p-ClC₆H₄, p-0MeC₆H₄</td>
<td>137, 138</td>
</tr>
<tr>
<td>Cl,Br</td>
<td>Me</td>
<td>Ph, p-ClC₆H₄, p-0MeC₆H₄</td>
<td>136</td>
</tr>
<tr>
<td>H</td>
<td>Me₃C</td>
<td>Ph</td>
<td>135</td>
</tr>
</tbody>
</table>

Table 6: Physical & Spectral data of 54.

<table>
<thead>
<tr>
<th>No.</th>
<th>R</th>
<th>M.P. °C</th>
<th>M⁺</th>
<th>IR cm⁻¹ (nujol)</th>
<th>Elementa Analysis</th>
<th>Required</th>
<th>Found</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>C% H%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>H</td>
<td>171</td>
<td>386</td>
<td>1675,1595,1560</td>
<td>74.61 4.66</td>
<td>73.51</td>
<td>3.98</td>
</tr>
<tr>
<td>2.</td>
<td>P-Cl</td>
<td>179</td>
<td>420</td>
<td>3280,1670,1580,1550</td>
<td>68.49 4.04</td>
<td>68.39</td>
<td>4.10</td>
</tr>
<tr>
<td>3.</td>
<td>3,4Cl₂</td>
<td>175</td>
<td>454</td>
<td>1665,1600,1555</td>
<td>63.29 3.51</td>
<td>62.96</td>
<td>3.38</td>
</tr>
<tr>
<td>4.</td>
<td>P-Me</td>
<td>230</td>
<td>400</td>
<td>3270,1670,1585,1565</td>
<td>75.00 5.00</td>
<td>74.87</td>
<td>4.93</td>
</tr>
<tr>
<td>5.</td>
<td>P-NO₂</td>
<td>210</td>
<td>431</td>
<td>3285,1675,1590,1575,1515</td>
<td>66.82 3.94</td>
<td>65.29</td>
<td>3.84</td>
</tr>
</tbody>
</table>
WAVELENGTH (cm\(^{-1}\))

TRANSMISSION (%)

Pmr (80 MHz) CDCl\(_3\)

SPECTRA 1
In solution, warfarin and its analogs are reported\textsuperscript{137,140} to exist in an equilibrium mixture of open and cyclic hemiketal forms (figure 10). The complex splitting observed in the 3.5-5$\delta$ region in PMR of all these compounds, could be because of the presence of a mixture of hemiketal and open form.

\begin{center}
\includegraphics[width=\textwidth]{scheme32.png}
\end{center}

\begin{center}
\includegraphics[width=\textwidth]{fig10.png}
\end{center}
OPTICALLY ACTIVE TERPENE BASED ANTICOAGULANTS/ RODENTICIDES

The discovery of resistance among rodents to warfarin and related oral anticoagulant rodenticides resulted in a setback to rodent control operations. This led to a search for effective alternative compounds, the strategies included preparation of various derivatives of warfarin and by restructuring of known anticoagulants in order to improve the rodenticidal value. Most of these compounds were found to be ineffective in the resistant population of rodents. One of the ways to tackle the resistance problem would be to have a radical variation in the warfarin group of anticoagulant rodenticides and this may prove to be an effective alternative.

It has been well established that S(-) warfarin has better anticoagulant/rodenticidal activity than R(+) warfarin and the anticoagulant property of warfarin is because of in vivo formation of a cyclic hemiketal (fig. 10,51). Therefore optically active anticoagulant rodenticides bearing a terpene moiety incorporated with suitable functional groups which could possibly lead to the hemiketal formation in vivo, represents an important class of compounds. These compounds have not been explored in drug design.

Earlier some novel menthone incorporated warfarin analogs (R=H) & (R=H) were reported from our laboratory. The synthesis of (R=H) was accomplished by
refluxing benzyllidene menthone 55 (R=H) with 4-hydroxy-
coumarin in dioxane, using piperidine as a catalyst (scheme
33), the usual conditions for the synthesis of warfarin
analogs 146,57.

As part of the research programme aimed at the
synthesis and biological testing of novel 4-
hydroxycoumarin/4-hydroxythiocoumarin based anticoagulant
rodenticides, we planned to synthesize a series of
substituted menthone based warfarin analogs 56 and to carry
out their biological testing and further use the data for
quantitative structure activity relationship studies. We thus
synthesized a number of substituted benzyllidene menthones 55
by aldol condensation of (-) menthone and substituted
benzaldehydes under basic conditions 147 (scheme 34).

A detailed study on the synthesis and the stereo-
chemistry of these benzyllidene menthones 55 is presented in
chapter 2, part A. The Michael addition of 4-
hydroxycoumarin with these substituted benzyllidene menthones
55 (R=p-Cl, p-CH₃, p-Br) in dioxane and catalytic amount of
piperidine, furnished the product 56 (R=p-Cl, p-CH₃, p-Br)
albiet in minute quantities. All column chromatograhy
purified products were single spot on TLC, gave satisfactory
elemental analysis, M⁺, & IR. The PMR showed them to be a
mixture of isomers/tautomers 44 (open and cyclic forms)
(spectrum 3). Since the product 56 was not obtained in good
yields under the Michael conditions employed (change in
**SCHEME 33**

\[ \text{(-) Menthone} + \text{Diox/Ph, } \Delta \rightarrow 55 + 56 \]

**SCHEME 34**

\[ \text{CHO} + \text{KOH, Ethanol} \rightarrow \text{RT 24 hrs} \]

\[ 55 \]

**SCHEME 35**

\[ \text{58} \]

\[ 58 + \text{59} \]
reaction times also did not improve the yields), a change in reaction conditions was deemed necessary. In a bid to improve the yields of the target compound 56 by utilising easy workup procedures, benzyllidene menthone 55 and 4-hydroxycoumarin were made to react under various different reaction conditions as given in table 7. Unfortunately none of these methods furnished the desired compound 56 in good amount. Either the starting compound was recovered, or along with the product too many inseparable side products were formed in these reactions.

One of these reactions viz. the fusing of 4-hydroxycoumarin with benzyllidene menthone at 140°C (no.18, table 7), which is one of the reported\textsuperscript{148,154} method for the synthesis of warfarin derivatives needs mention. The product isolated in this reaction surprisingly was 3,3' -benzyllidene bis (4-hydroxycoumarin) 59 (R=H) (scheme 35) which was characterized from its IR, elemental analysis and M\textsuperscript{+}. It had identical mass fragmentation pattern as an authentic sample of 3,3'-benzyllidene bis (4-hydroxycoumarin) obtained by refluxing 2 moles of 4-hydroxycoumarin with benzaldehyde in alcohol\textsuperscript{113}. This unusual product obtained, led us to look into its mechanism of formation. The probable mechanism for the formation of 59 in fusion reaction is depicted in the scheme 35. The initial Michael addition gives 57 which undergoes a thermal elimination to yield highly reactive 3-methylene - 2,4-diketochroman 58. This becomes the target of a second
Table 7: Attempted reaction of 4-hydroxycoumarin with benzyllidene menthone under various reaction conditions.

<table>
<thead>
<tr>
<th>No.</th>
<th>Reaction conditions</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Water/triethylamine (reflux)</td>
<td>151,58b</td>
</tr>
<tr>
<td>2</td>
<td>Acetonitrile/piperidine (reflux)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Xylene/piperidine (reflux)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Alcohol 50% piperidine (reflux)</td>
<td>152</td>
</tr>
<tr>
<td>5</td>
<td>Toluene/piperidine (reflux)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Ethanol/Ba(OH)$_2$ (reflux)</td>
<td>153</td>
</tr>
<tr>
<td>7</td>
<td>Ethanol/acidic resin (reflux)</td>
<td>154</td>
</tr>
<tr>
<td>8</td>
<td>Dioxane/K$_2$CO$_3$ (reflux)</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Dioxane/Hexamine (reflux)</td>
<td>161</td>
</tr>
<tr>
<td>10</td>
<td>Ethanol/DABCO (reflux)</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Dioxane/DABCO (reflux)</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>NaOMe/methanol (reflux)</td>
<td>116,155</td>
</tr>
<tr>
<td>13</td>
<td>Pyridine (reflux)</td>
<td>156,157</td>
</tr>
<tr>
<td>14</td>
<td>N,N-dimethyl formamide (reflux)</td>
<td>157</td>
</tr>
<tr>
<td>15</td>
<td>TEBA/Benzene/NaOH 50%</td>
<td>158</td>
</tr>
<tr>
<td>16</td>
<td>TEBA/acetonitrile/K$_2$CO$_3$</td>
<td>159</td>
</tr>
<tr>
<td>17</td>
<td>Na$_3$PO$_4$</td>
<td>160</td>
</tr>
<tr>
<td>18</td>
<td>Fusion at 140°C under nitrogen</td>
<td>148</td>
</tr>
</tbody>
</table>
Michael addition with 4-hydroxycoumarin to yield 59. Michael addition to compounds like 58 are known to yield dicoumarols as has been discussed earlier (Scheme 26, pg. 42).

An attempt to synthesize 56 by rendering C-3 position of 4-hydroxycoumarin more reactive via the formation of enamine\textsuperscript{149}, a good Michael donor, and then effecting Michael addition with benzylidene menthone proved unsuccessful. The enamine of 4-hydroxycoumarin 60\textsuperscript{149a} was prepared by refluxing piperidine and 4-hydroxycoumarin in toluene (scheme 36). This piperidine enamine 60 however, did not react with benzylidene menthone 55 (R=H), under Michael addition conditions. The failure of the above methods utilizing Michael addition to obtain the menthone based anticoagulant rodenticides 56 prompted us to explore a few alternative strategies for their synthesis. Following are some experiments carried out to achieve the target.

a) Reaction of schiff base with 4-hydroxycoumarin:

Benzalaniline 61\textsuperscript{150a} which is formed from the reaction of aniline with benzaldehyde is reported\textsuperscript{150b} to react with acetophenone to yield 62 (scheme 37). On similar lines a synthetic route was planned which involved addition of 4-hydroxycoumarin to benzalaniline 61 to give 63 followed by quaternization to 64. Reaction of menthone/formyl menthone (65) with 64 could yield the target compound 56 (scheme 38).
SCHEME 36

SCHEME 37
SCHEME 39

\[
\text{Scheme 39:}
\]

SCHEME 40

\[
\text{Scheme 40:}
\]
However, on reacting benzalaniline with 4-hydroxy-coumarin under the conditions similar to those employed for the formation of 62 yielded only 3,3'-benzylidene bis (4-hydroxy-coumarin) 59 (scheme 39) which was confirmed from its physical and spectral data.

b) Reaction of thioester with benzylidene menthone:

A retro synthetic route was planned for the synthesis of thio analog of compound 56 such as 68 which is depicted in scheme 40. This route incorporated the addition of dithiophenyldimalonate 66 to benzylidene menthone 55, to give intermediate 67 which would then undergo cyclisation in presence of Lewis acid to furnish the target compound 68. The cyclisation step was based on the routine synthesis of 4-hydroxycoumarin/4-hydroxythiocoumarin (scheme 31). The thio analog was chosen since it would undergo cyclisation in a much facile manner compared to oxygen analog. Thus the dithiophenylmalonic ester was prepared by reacting malonic acid with thiophenol in presence of phosphorus oxychloride. This thio ester 66 on reaction with benzylidene menthone 55 (R=H) in presence of sodium methoxide/methanol yielded 69 (R=H) (scheme 40) instead of the desired product 67. The product 69 (R=H) was characterized from its elemental analysis and its spectral data (table 8).

A similar reaction of thioester 66 with 55 (R=p-Cl) yielded 69 (R=p-Cl), whose synthesis, mechanism of formation and stereochemistry is discussed in chapter 2, part B.
Table 8: Physical and spectral data of 69.

m.p. °C 128
(α)D = -249.14° (CHCl3).
Elemental analysis - required C% 78.40, H% 7.90, S% 9.09.
found C% 78.51, H% 7.90, S% 8.92.
M+ = 351.
IR cm⁻¹ (nujol) 1700.
PMR δ ppm (CDCl3).

0.60 (d) 7.5 Hz 3 H
0.75 (d) 7.5 Hz 3 H
0.95 (d) 7.5 Hz 3 H
4.40 (d) 12 Hz 1 H
7.08 (d) 10 H

\[
\text{SCHEME 38}
\]
EXPERIMENTAL:

Following are the general procedures.

1) Preparation of substituted 4-hydroxycoumarin/4-hydroxythiocoumarin 49 \(^{134}\) (scheme 31).

a) Preparation of diaryl/diarylthio malonate 48 (X=O/S, scheme 31).

A mixture of malonic acid (0.1M), substituted phenol/thiophenol (0.2M) and phosphorus oxychloride (10ml) heated at 110-115°C for 0.5 hr. The reaction mixture decomposed with cold ethanol. Solid filtered and washed with cold ethanol. The diester recrystallised from ethanol.

Compound 48 (X,Y, m.p.°C): O,p-CH\(_3\), 69; O,m-CH\(_3\), 73; O,p-Cl, 122; S,H, 94; S,p-Cl, 109; S,p-CH\(_3\), 74; S,p-Br, 114.

b) Synthesis of substituted 4-hydroxycoumarin/4-hydroxythiocoumarin 49 (X=O/S, scheme 31).

A mixture of diaryl/diarylthio malonate and anhydrous aluminium chloride in equal proportions by weight, heated at 180-90°C for 0.5 hr. The reaction product decomposed with cold dilute (10%) HCl. The solid separated filtered, if filterable, or the supernatant liquid decanted and the residue washed with water. The solid or sticky mass further purified by dissolving in cold 10% NaOH (aq), and charcoal treatment, followed by precipitation with cold dil.HCl. The solid obtained was filtered and washed with cold water and recrystallised from suitable solvent.
2) Synthesis of Heterodicoumarols 36.

a) Preparation of 3-piperidinomethyl-4- hydroxycoumarin/ 4-hydroxythiocoumarin 42 (scheme 28).

A solution of piperidine (0.012 M) and 40% formaldehyde (0.01M), in absolute ethanol (10ml) added to a boiling solution of desired 4-hydroxycoumarin/4-hydroxythiocoumarin (0.012M) in absolute ethanol (20ml) and shaken well. The mixture kept in refrigerator overnight, the solid filtered, washed with little cold ethanol and then with anhydrous ether, dried. Recrystallized from ethanol.

Compound 42 : (X,Y, m.p.'C) : 0,H, 182(dec); 0,6-CH₃, 185(dec); 0,7-CH₃, 173; 0,6-Cl, 163; S,H, 205 (dec).

b) Synthesis of 36 (scheme 28).

A solution of appropriate 4-hydroxy-3-N-piperidinomethyl coumarin 42 (0.001M) in ethanol treated with methyl iodide (1ml) and after heating for few minutes under gentle reflux a solution of desired 4-hydroxythiocoumarin (0.001M) in hot ethanol added, the mixture boiled under reflux for 3 hrs. The solid begins to separate as the reaction proceeds. The product filtered, washed with alcohol and dried. Crude product repeatedly recrystallised from appropriate solvent (dioxane/cyclohexanone).

Spectral and physical data of this series is given in table 3.
3) Synthesis of 36a.

Appropriately substituted 42 (X=S) was condensed with appropriately substituted 4-hydroxythiocoumarin according to the procedure employed for synthesis of 36. Spectral and physical data of this series is given in table 4.

4) Synthesis of 3-[1-aryl-3-oxo-3-phenylpropyl] 4-hydroxythiocoumarin 54 (scheme 32).

a) Preparation of benzalacetophenone (2-propen-1-one, 1-phenyl-4-aryl) 53141.

Acetophenone (0.05M) and substituted benzaldehyde (0.05M) dissolved in 20ml ethanol, 10% NaOH (3ml) added dropwise to this solution with stirring and cooling. Stirred for further 2 hrs. at RT. Solid filtered, and washed with cold ethanol. Crude product recrystallized from ethanol.

b) Synthesis of 54

The appropriate benzalacetophenone (0.01M) and 4-hydroxythiocoumarin (0.01M) dissolved in dioxane (10ml) containing piperidine in catalytic amount. The reaction mixture refluxed gently till completion of the reaction (checked by TLC). Dioxane removed in vacuo and the residue poured in ice/water and left overnight. Solid obtained filtered. (If sticky residue obtained, it was titurated with petroleum ether). Crude product purified by column chromatography over silica gel G with petroleum ether-ethyl acetate solvent mixture as eluent. Spectral and physical data of this series is given in table 6.
5) Reaction of 4-hydroxycoumarin with benzylidene menthone, formation of 56.

General procedure:

The appropriate benzylidene menthone (0.04M) and 4-hydroxycoumarin (0.04M) dissolved in dioxane (50ml) containing catalytic amount of piperidine. The reaction mixture was refluxed gently for 8 hrs. Dioxane removed in vacuo and the residue poured into water/ice with stirring and extracted with chloroform. Usual workup followed by chromatography over silica gel G using petroleum ether -ethyl acetate as eluent yielded the desired product in pure state.

Compound 56:

\( R=p-\text{Cl} \)

\[ M^+ 438, \text{ IR cm}^{-1} \text{ (nujol)} 3420 (\text{OH}), 1710 (\nu \text{C}=\text{O}), 1680 (\text{coumarin C}=\text{O}), 1630, 1610. \]

\( R=p-\text{Br} \)

\[ M^+ 482, \text{ IR cm}^{-1} \text{ (nujol)} 3420 (\text{OH}), 1712 (\nu \text{C}=\text{O}), 1682 (\text{coumarin C}=\text{O}), 1630, 1595. \]

\( R=p-\text{CH}_3 \)

\[ M^+ 418, \text{ IR cm}^{-1} \text{ (nujol)} 3410 (\text{OH}), 1710 (\nu \text{C}=\text{O}), 1685 (\text{coumarin C}=\text{O}), 1635, 1600. \]

6) Reaction of 4-hydroxycoumarin with benzylidene menthone by fusing, formation of 59.

Benzyllidene menthone (0.04M) and 4-hydroxycoumarin (0.04M) heated at 150°C under nitrogen for 6 hrs. Fused solid dissolved in acetone and poured into 2% NaOH (100ml) and the
solution extracted with ether. Ether layer discarded, aqueous layer acidified and further extracted with ether. Removal of ether yielded solid which was recrystallized from acetone.

m.p. 228°C, $M^+$ 412, IR cm$^{-1}$ (nujol) 3060 (OH), 2740, 2520, 1660, 1620, 1570, 1500.

PMR ($\delta$ ppm, CDCl$_3$) 6.1 (s) 3H, 7.16-8.2 (m) 13H, 11.49 (bs) 2H.

7) Enamine of 4-hydroxycoumarin 60.

4-Hydroxycoumarin (0.04M), piperidine (0.04M) and toluene (100ml) containing a pinch of p-toluenesulphonic acid placed in a round bottom flask fitted with a Dean-Stark assembly. The reaction mixture refluxed till no more water was collected in Dean-Stark tube (8hrs.) Toluene and unreacted piperidine distilled out, the gummy residue obtained solidified on keeping at low temperature. Solid product recrystallized from aq. ethanol.

m.p. 110°C,

Elemental analysis : Found C-73.88%, H-6.35%, N-5.69%
Requires C-73.36%, H-6.55%, N-6.11%.

$M^+$ 229, IR cm$^{-1}$ (nujol) 1695 (C=O), 1595, 1545.

8) Reaction of 4-hydroxycoumarin with benzalaniline, formation of 59.

a) Preparation of benzalaniline 61

Benzaldehyde (0.04M) placed in round bottom flask equipped with stirrer, aniline (0.04M) added with rapid stirring. After the addition, reaction mixture allowed to stand for 15 min. and then poured with stirring in alcohol
(10ml). Solid separated out on cooling in ice water filtered and dried in air. m.p. 51°C.

b) Formation of 59.

Benzalaniline (0.01M) and 4-hydroxycoumarin (0.01M) dissolved in chloroform (20ml) and to this was added BF₃ etherate (3ml) slowly with stirring. The solution stirred for 2 hrs. Water (50ml) added to the reaction mixture and the chloroform layer separated out. Aqueous layer extracted with chloroform. Chloroform layers mixed, dried over Na₂SO₄. Solid 59 obtained on removing chloroform, recrystallized from acetone.

m.p.°C 226-27,  
M⁺ 412,  
IR cm⁻¹ (nujol) : 3060 (νOH), 1660, 1620.  
PMR(δppm,CDCl₃): 6.1, (s), 3H; 7.15-8.2, (m), 13H; 11.40, (bs), 2H.

9) Reaction of dithiophenyl malonate with benzylidene menthone, formation of 69.

The procedure is described in part B of chapter II, (pg.202).  
Physical and spectral data of 69 is given in table 8.
PART C

QUANTITATIVE STRUCTURE ACTIVITY RELATIONSHIP (QSAR) STUDIES IN WARFARIN GROUP, EMPLOYING HANSCHE ANALYSIS.
INTRODUCTION

Medicinal chemists have tried to quantify relationship between structure and biological activity since before the turn of the century. However, it was not until the early 1960's, through the joint efforts of Corwin Hansch and his computer, that a workable methodology was developed and the subject that was to become known as quantitative structure activity relationship (QSAR) was born. Since then hundreds of research papers and articles on QSAR have emerged, resulting in the introduction of many new symbols and parameters.

The traditional method of searching for new medicinal compounds has sometimes been described as chemical roulette. A chemical structure, known to have a particular biological activity, is chosen, and attempts are made to improve it by modifications based on chemical intuition and isosteric consideration until a highly active compound with minimal side effects is produced. A plan of probable receptor site is built up, as the number of compounds synthesized and tested increases, and the selection of further new compounds become more rational. Using this approach, one is able to anticipate the shapes of biologically active molecules, and speculate on the types and positions of groups which will bring about the optimal stereochemistry required for activity. Most of the drugs
Drug development using the trial and error methods has proved to be very laborious and expensive.

QSAR has an upperhand over the routine drug development programmes as it takes into consideration the pharmacokinetics, pharmacodynamics and toxic properties before the actual synthesis of chemical compounds. It thus avoids the uneconomical and time consuming path of synthesis of a large number of derivatives before embarking on the structure of significance.

QSAR is basically a method to transform chemical structure of compound into a set of numerical descriptors of the properties relevant to the biological activity. It is a method to establish the quantitative relationship between the physicochemical properties and the biological activities of the compounds. QSAR helps to explain the observed variance in biological response of certain classes of compounds as a function of molecular changes by the substituents.

There are two main applications of QSAR analysis: 1) The predictive aspect: - a) the extrapolation of correlations found between structural parameters and biological activity to propose the synthesis of derivatives with better activity. b) To avoid the synthesis and testing of derivatives with the same equivalent activity and reduce time needed to find
more active derivatives. c) Prediction of physicochemical properties using substituent constants.

2) The diagnostic aspect: - a) To know the rate determining step b) to support a hypothesis for mode of action and to obtain information about the type of binding forces involved. The general expression, for which there are many variations, can be given by the equation-

\[ \text{Biological activity} = f(\text{physicochemical and/or structural parameters}). \]

The objective is to find physicochemical parameters from experiments or theory so that when substituted in the above equation along with biological activities for a series of molecules would give a statistically significant correlation.

Several approaches are used in QSAR studies, these are

1) Hansch approach (LFER mode)\textsuperscript{163,164,170-187,200c},
2) Free wilson or additive method\textsuperscript{167,180,188-192,200d},
3) Discriminant analysis\textsuperscript{180,193,200b},
4) Principle component analysis\textsuperscript{180,194-196},
5) Molecular orbital method\textsuperscript{180},
6) Factor analysis\textsuperscript{180,197},
7) Cluster analysis\textsuperscript{173,180},
8) Minimum topological difference\textsuperscript{186},
9) Comparative molecular field analysis\textsuperscript{200},
10) Pattern recognition\textsuperscript{180,198-200a}. 

Hansch method is the most popular amongst these approaches in QSAR, as it has been able to give better correlation. Other approaches either concentrate on one parameter to the exclusion of other or else parameters are derived that are not fundamental and so cannot be used for prediction or for the diagnosis of mechanism. Without giving insight into mechanism or having predictive value, correlation as an end in itself is of no use to the medicinal chemist. The Hansch approach on the other hand is a multiparameter approach and does not neglect any aspect of group contribution to overall activity.

**Hansch Analysis**

The basic Hansch equation\textsuperscript{170a} was derived by considering the general case of a drug applied to any biological system, be this complex living animal or a simple enzyme *invitro*.

In any biological test only two quantities can be measured, the amount of compound given (the dose) and the biological activity (response) obtained. The response is going to be determined by the structure, that is by the physicochemical properties of the compound, and within a closely related or so called congeneric series of compounds changes in structure can be related to changes in biological activity.
Consider first the problem in an animal system where rates of absorption of the drug, transport to the site of action, metabolic reactions and excretion rates, and intrinsic activity at some unknown receptor site may all contribute to the overall effect which is observed. As regards absorption from the applied phase, and ensuing transport to a sensitive site, it has long been realized that the lipophilic-hydrophilic balance, expressed as partition coefficient, is of paramount importance.

Because metabolic transformations and activity at receptor sites are concerned with enzymes and are much more sensitive to small changes in molecular structure, physicochemical factors involved in these interactions are very important. One speaks loosely of a "hydrophobic pocket" in an enzyme without knowing whether this pocket will have a greater affinity for a cyclohexyl than for a phenyl group, since both groups are lipophilic moieties. One speaks of a cationic binding site without knowing the size of cation that will successfully be accommodated. In fact, rates of enzymic reactions depend very much on affinities for substrate or inhibitor and affinity in turn is controlled much on affinities for substrate or inhibitor, and affinity in turn is controlled by molecular size and shape, electron distribution and lipophilicity, which can all be expressed quantitatively in terms of physicochemical constants.¹⁷⁰b.
Although many factors may be involved in a biological system, Hansch began by assuming that for any congeneric series one particular reaction would be a key reaction and would be rate controlling. The development of his equation is then analogous to the derivation of equations expressing reaction rate from simple kinetic theory.

If $K_X$ is an equilibrium or rate constant for the rate-determining reaction which is possibly, but not necessarily, at the site of action of the drug, $C$ is the applied concentration (dose) and $A$ represents the probability of a molecule reaching this critical site in a given time interval, than equation (6) is an expression for the rate of biological response.

$$\frac{d\text{(response)}}{dt} = ACK_X$$

Fig. 11 expresses this idea; the molecules, after being given as dose $C$, make their way through cellular material by what Hansch terms a "random walk" process, an effective concentration $AC$ accumulating at the critical site.

Hansch related $A$ to log $P$, and changes in $A$ as one compared molecules in a congeneric series, to changes in log $P$. These changes can be expressed in the form of substituent constant which Hansch et al. have termed $\pi$ constants, defining $\pi$ by the Hammett-like relationship as
\[ \Pi = \log \left( \frac{P_X}{P_H} \right) \]

or

\[ \Pi = \log P_X - \log P_H \]

where \( P_X \) and \( P_H \) are the partition coefficients of substituted and parent molecules. Partition, and hence \( \Pi \) coefficients, have all been measured in the octanol-water system as a model representing biological lipid and aqueous phases. A negative \( \Pi \) value thus indicates a change towards greater affinity for the aqueous phase, and a positive value indicates greater affinity for the lipid phase. Just as \( \delta \) is a free-energy related parameter, so too is \( \Pi \) which expresses the relative free-energy change on moving a derivative from one phase to another.

Hansch then chose to assume as a working hypothesis that the probability \( A \) would be related to \( \log P \) for the complete molecule, or to \( \Pi \) for changes in congeneric series, just as for a normal Gaussian distribution expressible by

\[ A = f(\Pi) = a \exp\left[ - \frac{(\Pi - \Pi_0)^2}{b} \right] \]

where \( a, b \) are constants and \( \Pi_0 \) is the value of \( \Pi \) corresponding to the maximum of the distribution. Fig. 12 expresses this idea which, though largely empirical, was based on the fact that in many series of compounds tested in biological systems, as the relative lipophilicity was increased activity rose to maximum, fell off and eventually reached zero. In vivo it is generally to be expected that the molecules which are highly hydrophilic (low or negative \( \log P \)) will not readily
Extracellular phase | Random walk | Critical reaction site

C \rightarrow \text{AC} \rightarrow \text{Response}

FIG. 11

Log $\log P_0(\Pi_0)$

A

Log $P$ or $\Pi$

FIG. 12
partition from water into the lipid of a membrane. If the receptor is within or beyond that membrane, such a molecule will have a low probability of reaching it in the time interval under study. As log P becomes more positive, transport will become more favourable and A will rise to a maximum. Conversely molecules which are highly hydrophobic will readily partition into the first series of lipid membranes but will be held there and thus slowed down in any journey to a remote site of action.

The basic Hansch equation is given as

\[ \log \frac{1}{C} = K_1 \Pi - K_2 \Pi^2 + K_3 \sigma + K_4 \]

where \( C \) is the molar concentration (or dose) that elicits a constant biological response. \( \Pi \) is the partition coefficient of the substituent, \( \sigma \) is substituent electronic effect of Hammett, \( K_1, K_2, K_3, K_4 \) are coefficient derived from statistical curve fitting.

For a congeneric series the set of individual equation forms a set of simultaneous equations which given enough compounds would allow the values of \( K_1, K_2, K_3 \) to be determined. The statistical technique used in Hansch analysis is linear multiple regression\textsuperscript{162,163}, and the least square method is employed to find the equation of 'best fit' with a given combination of parameters. Standard statistical procedures are applied to find the significance of the coefficients, overall significance, closeness of fit of data
to desired equation and the amount of variance in biological data that is explained by the equation.

In a stepwise linear multiregression analysis the physiochemical parameters which have the most statistical significance for the explanation of the observed biological variance undergo evaluation as follows:

\[ \log BA = K_1 \pi + K \] or \[ \text{a log p + K} \]
\[ \log BA = K_1 \sigma + K \]
\[ \log BA = K_1 \varepsilon_s + K \]
\[ \log BA = K_1 \pi + K_2 \sigma + K \]
\[ \log BA = K_1 \pi + K_2 \sigma + K_3 \varepsilon_s + K \text{ etc.} \]

In these equations \( K_1, K_2, K_3 \) are the regression coefficients and \( K \) the intercept obtained by least square method. Biological activity (BA) can be expressed as \( \log 1/C \) where \( C \) is the measure of the drug required to exhibit \( I_{50}, \text{MIC, ED}_{50}, \text{LD}_{50}, \text{EST etc.} \)

This statistical method also provides a measure of
1) whether each coefficient is significantly different from zero
2) the value of \( r^2 \) statistic which is the fraction of the variance in the \( \log 1/C \) data that is explained by the equations and
3) the value of \( S \), which is the standard deviation of the observed \( \log 1/C \) values from those calculated. For the statistical part of a QSAR analysis, one examines the possible equations of a data set to find those of interest, that is, equations that contain only
statistically significant terms that make mechanistic sense and do not overfit the data.

The Hansch equation serves to demonstrate the importance of hydrophobic forces and to clarify the nature of these hydrophobic interactions. Considerable support for the general occurrence of parabolic rather than linear dependence of biological activity on has been accumulated (i.e. incorporation of term in the equation). The special case of linear dependence arises when a one step partitioning process occurs, as in enzymatic studies, or when the range of \( \pi \) values investigated falls in the linear position of the parabolic curve.

The Hansch equation is a linear free-energy relationship. As such parameters other than can be incorporated to express energy of interaction between drug and receptor. Thus not only Hammett\(^{201}\), but also pKa values, spectroscopic values\(^{202,203}\) and constants expressing intermolecular attractive forces such as dipole moment\(\mu\)^\(^{204}\), polarizability, the steric influence of the substituents, which can be expressed by Taft steric constant \(\text{Es}^{205}\), molar refractivity\(^{206}\) (MR), molar volume (MV)^\(^{207}\), have been successfully used to obtain correlations\(^{164-168,172,175,183,208-211}\).

Since the parameters used all have a physical meaning, a significant relationship can often be interpreted to give insight into mechanism of action, location of the critical
site (receptor) and factors underlying transport of the drug. This is the diagnostic value of Hansch analysis.

Also the activity of hypothetical congeneric derivatives can be estimated from the equation, assuming that the same mechanism of action or same rate limiting step still applies. This is the predictive value of Hansch analysis, which can thus be helpful in deciding when to terminate a series or which substituent to introduce for maximum activity, optimal selectivity or favourable transport.

Partition and hence $\pi$ coefficients are normally measured in the octanol-water system as a model representing biological lipid and aqueous phases. An alternate approach to the use of $\pi$ is by splitting the molecule into hydrophobic fragments. One can calculate the log $p$ for a molecule by adding up values for the fragments. Use can be made of literature values of log $p$ for various nuclei and $\pi$ for substitutents which are then summed. The relationship has been found to break down only when strong electronic forces are present, but these can often be taken into account in calculating log $P$ when the molecule is not too complex. $R_m$ values from reverse phase chromatography and retention times (log $k'$) from HPLC can also be related to log $p$ values.

There are few pitfalls to Hansch analysis. First of all there must be sufficient number of compounds included to
enable statistical significance to be reached despite errors in measurements. Secondly, particular parameter, which varies very little in all other compounds in the same set should be avoided. Sometimes certain parameters that may be used in the equation are themselves interrelated and this has to be considered while interpreting the equation.

Failure of the QSAR analysis in the prediction of biological activity of analogues results due to 1) Badly selected series 2) Time dependency of the biological activity 3) Change in the mechanism of action or more than one mode of action 4) Change in the excretion mechanism (structure - pharmacokinetic relationships) 5) Improper selection of substituent constants which do not correctly describe physicochemical properties 6) Competition for binding sites, change in protein structure 7) Limitation in solubility 8) Change in conformation 9) Tautomerism 10) Instability (prodrug).

Failure of regression analysis in the prediction of biological activity of analogs could be due to 1) The prediction is based on a badly selected series or an a statistically incorrect regression equation. 2) The prediction is based on an extrapolation outside of the parameter space covered by the "training set". 3) The conditions for the biological test procedures were not identical.
PRESENT WORK:

Survey of literature indicated that even though the structure activity relationship of different groups of anticoagulant rodenticides (refer pg. 28) has been carried out, there has been no rigorous treatment on quantitative structure-activity relationship (QSAR). The present work was undertaken to study the QSAR in the field of rodenticides belonging to warfarin group by employing Hansch analysis.

Warfarin group of anticoagulant rodenticides were chosen for QSAR studies since, with warfarin (71, \( \text{R}=\text{CH}_3 \), \( \text{X}=\text{H} \), scheme 41) and its analogs, resistance is reported\(^{143}\) to have occurred in some cases. Therefore such an analysis might provide a clue to their mode of action i.e. whether the transport mechanism (if equation incorporates \( \Pi \) parameter) or binding mechanisms (if equation incorporates electronic parameters) is important. This analysis could provide a helpful clue in overcoming the problem of resistance. Hansch analysis would also help in predicting the most active compound among these series, i.e. by determining \( \Pi_0 \), which is optimum partition coefficient and represents maximum activity. One can then synthesize that particular analog having the required substituents which has \( \Pi \) value same as \( \Pi_0 \). These studies would also give an idea as to where a series should be terminated.

For the QSAR studies two series of warfarin analogs I (\( \text{R}=\text{Ph} \)) and II (\( \text{R}=\text{CH}_3 \)) (table 11) were synthesized by known
procedures. The substituents of these analogs were carefully selected so as to provide a range of values as required for good Hansch correlation. Few of these analogs (marked *) which are not reported in literature were characterised from their satisfactory elemental analysis and spectral data.

All the compounds synthesized were subjected to biological screening. The LD50 was determined for both the series, to see their effect as anticoagulants/rodenticides. Some of these compounds were additionally subjected to prothrombin time and clotting time studies. Table 9 & 10 shows the observed LD50, clotting time & prothrombin time respectively, for the compounds. These two series of coumarin derivatives were subjected to Hansch analysis taking LD50 as the measure of biological activity. The substituent parameter values were taken from literature. Table 11 shows the data set that was prepared and subjected for Hansch analysis.

Hansch multiple regression analysis was done by computer using SPOSP programme of Sunderland polytechnic, UK, and different equations were generated involving either individual or a combination of different parameters (\( \pi \), \( \pi^2 \), \( \alpha \), \( \delta \), ES, MR) so as to obtain an equation of 'best fit'.

The output of the computer data was analysed and it was noted that most of these combination did not give a statistically significant correlation. Some of these
correlations did show good 'R', the correlation factor but failed to give a good 'F', significance factor. Thus the attempt to generate a standard equation correlating the activity in these series to the structural changes could not be realized. To achieve a significant correlation it is required to have 10-12 compounds in a series with a range of biological activity. The failure to obtain a good correlation in the present case could probably be due to the reason that even though the number of compounds subjected for Hansch analysis were appropriate, the biological activity nee LD₅₀ data distribution was in three clusters, and not over a wide range. This clustering of the biological data significantly lowered the number of points required for a good curve fit leading to the poor correlation observed.

\[
\begin{align*}
R = \text{ph}, & \quad a = \text{Dioxane/pip, } \Delta. \\
R = \text{Me}, & \quad b = \text{NEt₃, } \Delta.
\end{align*}
\]

SCHEME 41
EXPERIMENTAL:

1) Preparation of chalcone 70.

Various chalcones were prepared as per standard procedure by condensing acetophenone (series I), acetone (series II) with substituted benzaldehydes.

General procedure:

Appropriate ketone (0.06M) and substituted benzaldehyde (0.05M) dissolved in 20ml ethanol, 10% NaOH (2ml) was added dropwise to the solution with stirring, maintaining the temperature below 25°C. Reaction mixture further stirred for 2 hrs. at RT. Water was then added to the reaction mixture and the solid filtered, washed with water and some cold ethanol. The product recrystallized from a suitable solvent. (In few cases where chalcone was liquid or where solid did not separate out on adding water, the mixture was extracted with ether. Ether layer separated, dried and solid/liquid material obtained on removing ether purified by recrystallization /distillation).

Compound 70 - (R,X, m.p./b.p.* C) Ph, H, 56; Ph, p-CH₃, 90; Ph, p-CH₃, 77; Ph, p-iPr, 176*; Ph, p-F, 90; Ph, p-Cl, 112; Ph, p-Br, 120; Ph, p-NO₂, 165; Ph, m-NO₂, 146; Ph, p-CN, 155; Ph, 3,4-Cl₂ 115; CH₃, H, 42; CH₃, p-CH₃, 35; CH₃, p-CH₃, 73; CH₃, p-iPr, -; CH₃, p-F, 51; CH₃, p-Cl, 56; CH₃, p-Br, 84; CH₃, p-NO₂, 110; CH₃, m-NO₂, 95; CH₃, p-CN, 105; CH₃, 3,4 Cl₂, 55.
2) Preparation of 71 (R=Ph).

General procedure:

Equimolar quantities of appropriate chalcone 70 (R=Ph) and 4-hydroxycoumarin mixed in 1,4 dioxane containing catalytical amount of piperidine, and the mixture refluxed for 8-12 hrs. The sticky mass obtained after removal of dioxane poured over ice/water and left overnight. Solid obtained filtered, and purified either by recrystallization from suitable solvent or by column chromatography.

Compound 71 (R,X):

Ph, 3,4Cl₂, -

Elemental Analysis found C-64.92%, H-3.59%

requires C-65.15%, H-3.64%.

M⁺ 438, IR cm⁻¹ 3260, 1710, 1690, 1645, 1620, 1580, 1510.

Ph, p-iPr, -

Elemental analysis found C-78.02X, 5.96%

requires C-78.45%, H-5.82%.

M⁺ 412, IR cm⁻¹ 3230, 1695, 1675, 1615, 1560, 1500.

3) Preparation of 71 (R=CH₃)

General procedure:

Mixture of equimolar quantities of 4-hydroxycoumarin and chalcone 70 (R=CH₃), in water containing catalytical amount of triethylamine was refluxed for 8-12 hrs. Solid separated out, filtered and recrystallized from suitable solvent.
Compound 71 (R, X):

CH$_3$, p-iPr, -

Elemental analysis found C-75.51%, H-6.37%
requires C-75.43%, H-6.29%.

M$^+$ 350, IR cm$^{-1}$ 3385, 1690, 1615, 1515, 1495.

CH$_3$, p-CN, -

Elemental analysis found C-72.20%, H-4.45%
requires C-72.07%, H-4.50%.

M$^+$ 333, IR cm$^{-1}$ 3400, 2230, 1695, 1625, 1575, 1515.

Biological screening

a) Determination of LD$_{50}$:

The LD$_{50}$ studies were carried out in albino mice weighing between 25-30 g. The route of administration used was oral. The least tolerated dose (100% mortality) and most tolerated dose (0% mortality) was calculated by the method of Miller and Trainter$^{220}$. Mice were fasted overnight and separated into groups of six each. The drugs were administered as a suspension of 0.5% gum acacia and toxicity was observed for the first 2 hrs. Thereafter the animals were kept under observation for 24, 48, 72 hrs. duration. Feed and water were provided ad libitum after drug feeding. The approximation of LD$_{50}$ was done on computer.

b) Prothrombin time.

Prothrombin time was calculated by Quick's method$^{221}$. The method involves measuring the time required for clotting
of citrated plasma after the addition of optimal amounts of calcium and brain thromboplastin.

The procedure involved the mixing of 0.1 ml of plasma with 0.2 ml of thromboplastin and observing continuously till the formation of fibrin clot and time required was noted as prothrombin time. All solutions and samples were preincubated for 5 min. at 37°C. The prothrombin time was a mean of 6 readings. Animals (albino mice) were treated with compound for 3 days at 1/20 LD₅₀ dose & 1/5 LD₅₀ dose. The 1/5 LD₅₀ dose was administered and readings were observed after 24 hrs. The compounds were compared with warfarin a known anticoagulant, which was taken as positive control and untreated animals as standard.

Table 10 gives the results of the prothrombin time in mice.

c) Clotting time.

Clotting time was studied in mice using 1/5 of the LD₅₀ dose. Animals were treated with the respective dose of the compound and after a interval, blood from the orbital sinus was taken by means of a capillary tube and the clotting time observed by breaking the capillary every 30 seconds. The clotting time was noted when the capillary broke with a thin fibre attached.

Table 9 summarises the results.
Table 10: Prothrombin time of 71.

<table>
<thead>
<tr>
<th>No.</th>
<th>R</th>
<th>X</th>
<th>LD_{50} time in sec.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1/20</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td>12.3 + 1.80</td>
</tr>
<tr>
<td>Warfarin</td>
<td></td>
<td></td>
<td>53.3 + 30.79</td>
</tr>
<tr>
<td>1. Phenyl</td>
<td>p-iPr</td>
<td></td>
<td>18.3 + 10.58</td>
</tr>
<tr>
<td>2</td>
<td>3,4Cl₂</td>
<td></td>
<td>09.3 + 0.66</td>
</tr>
<tr>
<td>3</td>
<td>p-Br</td>
<td></td>
<td>12.6 + 1.76</td>
</tr>
<tr>
<td>4</td>
<td>p-Cl</td>
<td></td>
<td>12.3 + 2.60</td>
</tr>
<tr>
<td>5</td>
<td>p-CH₃</td>
<td></td>
<td>31.6 + 14.20</td>
</tr>
<tr>
<td>6</td>
<td>p-F</td>
<td></td>
<td>17.0 + 1.52</td>
</tr>
<tr>
<td>7</td>
<td>H</td>
<td></td>
<td>12.5 + 0.50</td>
</tr>
<tr>
<td>8</td>
<td>p-OCH₃</td>
<td></td>
<td>11.0 + 2.08</td>
</tr>
<tr>
<td>9</td>
<td>p-NO₂</td>
<td></td>
<td>12.0 + 0.00</td>
</tr>
<tr>
<td>10</td>
<td>m-NO₂</td>
<td></td>
<td>10.6 + 0.88</td>
</tr>
<tr>
<td>11</td>
<td>p-CN</td>
<td></td>
<td>15.3 + 1.76</td>
</tr>
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</table>

Mean + S.E.
<table>
<thead>
<tr>
<th>No.</th>
<th>R</th>
<th>X</th>
<th>LD₅₀ mg/Kg</th>
<th>Clotting time, sec.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Phenyl</td>
<td>p-1Pr⁺</td>
<td>6986</td>
<td>210.00 + 30.00</td>
</tr>
<tr>
<td>2</td>
<td>&quot;</td>
<td>3,4Cl₂⁺</td>
<td>3555</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>&quot;</td>
<td>p-Br</td>
<td>6986</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>&quot;</td>
<td>p-Cl</td>
<td>405</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>&quot;</td>
<td>p-CH₃</td>
<td>405</td>
<td>143.00 + 18.68</td>
</tr>
<tr>
<td>6</td>
<td>&quot;</td>
<td>p-F</td>
<td>978</td>
<td>180.00 + 17.32</td>
</tr>
<tr>
<td>7</td>
<td>&quot;</td>
<td>H</td>
<td>978</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>&quot;</td>
<td>p-OCH₃</td>
<td>978</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>&quot;</td>
<td>p-NO₂</td>
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<td></td>
</tr>
<tr>
<td>10</td>
<td>&quot;</td>
<td>m-NO₂</td>
<td>405</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>&quot;</td>
<td>p-CN</td>
<td>6986</td>
<td>178.33 + 21.86</td>
</tr>
</tbody>
</table>

1  CH₃  p-iPr⁺  825
2  "      3,4Cl₂  825
3  "      p-Br   825
4  "      p-Cl   681
5  "      p-CH₃  1000
6  "      p-F   1000
7  "      H     1000
8  "      p-OCH₃  825
9  "      p-NO₂  1000
10 "      m-NO₂  1000
11 "      p-CN  1000

* not reported in literature.
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CHAPTER I

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