INTRODUCTION

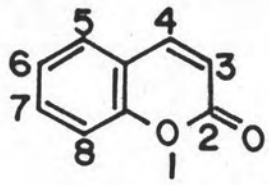
Clausena cambodiana Guill, สมุยหอม (Samui hom)(1), belongs to the plant family Rutaceae, genus Clausena. In Thailand, six species of Clausena have been found and reported, those are

Clausena cambodiana Guill., สมุยหอม (Samui hom)
Clausena excavata Burm.f. หวดหม่อน (Huat non)
Clausena guillauminii Tanaka , ส่องฟ้า (Song faa)
Clausena harmandiana Pierre., ส่องฟ้าดง (Song faa dong)
Clausena lansium Skeels (C.wampi Oliv.) มะไฟจีน (Mafaicheen)
Clausena wallichii Oliv., เพี้ยฟาน (Phia faan)

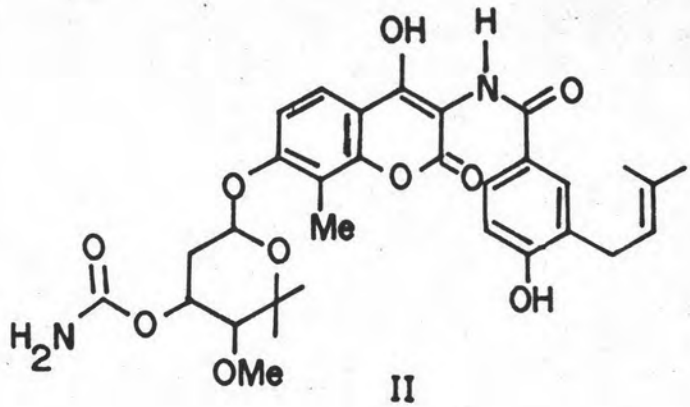
Clausena cambodiana in Thailand is a folkloric medicinal plant and has been told to be used as astringent, stomachica, and for treatment of some dermatology. Coumarin is one of many important classes of natural compounds which are wildly distributed. Over 500 compounds are known to occur in nature and many hundreds more have been synthesized in the laboratory. Indeed, coumarins,being a group of naturally occurring substances in form of phenylpropanoid lactones (I)(Figure 1) exerting a wide range of physiological effects in animals, have been elaborated by many plants and in a few microbial species(2). Novobiocin(II), a commercial antibiotic agent by *Streptomyces nivens* has also a

coumarin nucleus in its structure. Another microbial coumarin type, the aflatoxin(III) has been found to be elaborated by Aspergillus flavus. These metabolites have been reported and so active as potent hepatotoxic (3) and well considered among the most intense carcinogens yet discovered. The hydroxylated C4 coumarin including the well known dicoumarol(IV), functions as anticoagulant. A synthetic compound named 4 hydroxycoumarin has been employed for many years as an effective rat poison under the name warfarin(V) and still being used. Heraclenol obtaining from Parsley seed *(Petroselinum crispnum)* also well known from past and present agricultural experiences, is a water soluble growth inhibitor in higher plants . The linear furanocoumarins, or furocoumarins such as xanthotoxin have been recognized as potent poisonous so being harmful to fish.

The Peruvian plant *Micrandra elata* (Euphorbiaceae) was examined, the chloroform extracted from root bark showed activity against the P-388 lymphocytic leukemia in mice (4). Those compounds were identified as 6,8-dimethoxy-7-hydroxycoumarin (isofraxidin) (VI) which displays marginal cytotoxicity, has now been isolated from the twigs of *M.elata*..



I



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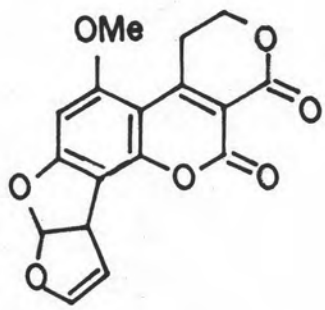

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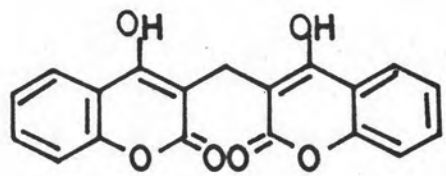

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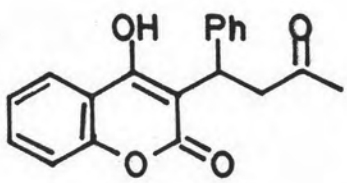
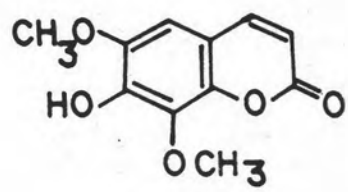


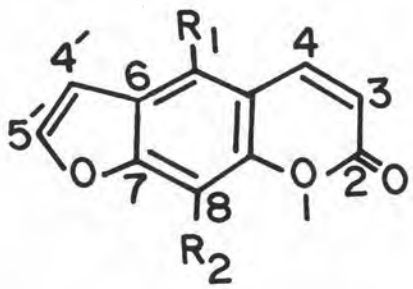
V



VI

Figure 1 Some structure of active coumarin compounds which can be used as drugs and poisonous compounds

Furanocoumarins, derivatives of which are commonly called psoralens, constitute a well-known group of naturally occurring and synthetic substances. The structure of some psoralens and their derivatives are illustrated in Figure 2.



Psoralen: $R_1 = R_2 = H$

Bergapten: R_1 = oMe, $R_2 = H$

Xanthotoxin: $R_1 = H$, R_2 = OMe

Isopimpinellin: $R_1 = R_2$ = OMe

5-Hydroxyxanthotoxin: R_1 = OH, R_2 = OMe

8-hydroxybergapten: R_1 = OMe, R_2 = OH

Bergaptol: R_1 = OH, $R_2 = H$

Xanthotoxol: $R_1 = H$, R_2 = OH

Figure 2 Structure of psoralens and their derivatives illustrated.

Psoralen when added to any of several biological system and irradiated with long-wave uv light (UV-A), produce various biological effects. These effects cannot be obtained with either psoralens or light alone. Psoralen also intercalates into DNA by forming molecular complexes involving weak chemical interaction("dark reaction"). Upon uv-A irradiation of such a system, in vitro or in vivo, formation of

covalent bond between a pyrimidine base and the furocoumarin molecule will definitely take place (5). Such phenomenon have been called C-4 cycloaddition. Psoralen in this reaction can react due to their structure either at their 3,4-double bond or at their corresponding 4',5'-site, yielding monoadducts. Upon absorption of an additional photon, a further chemical reaction yielding a cross-link DNA may occur(Figure 3)

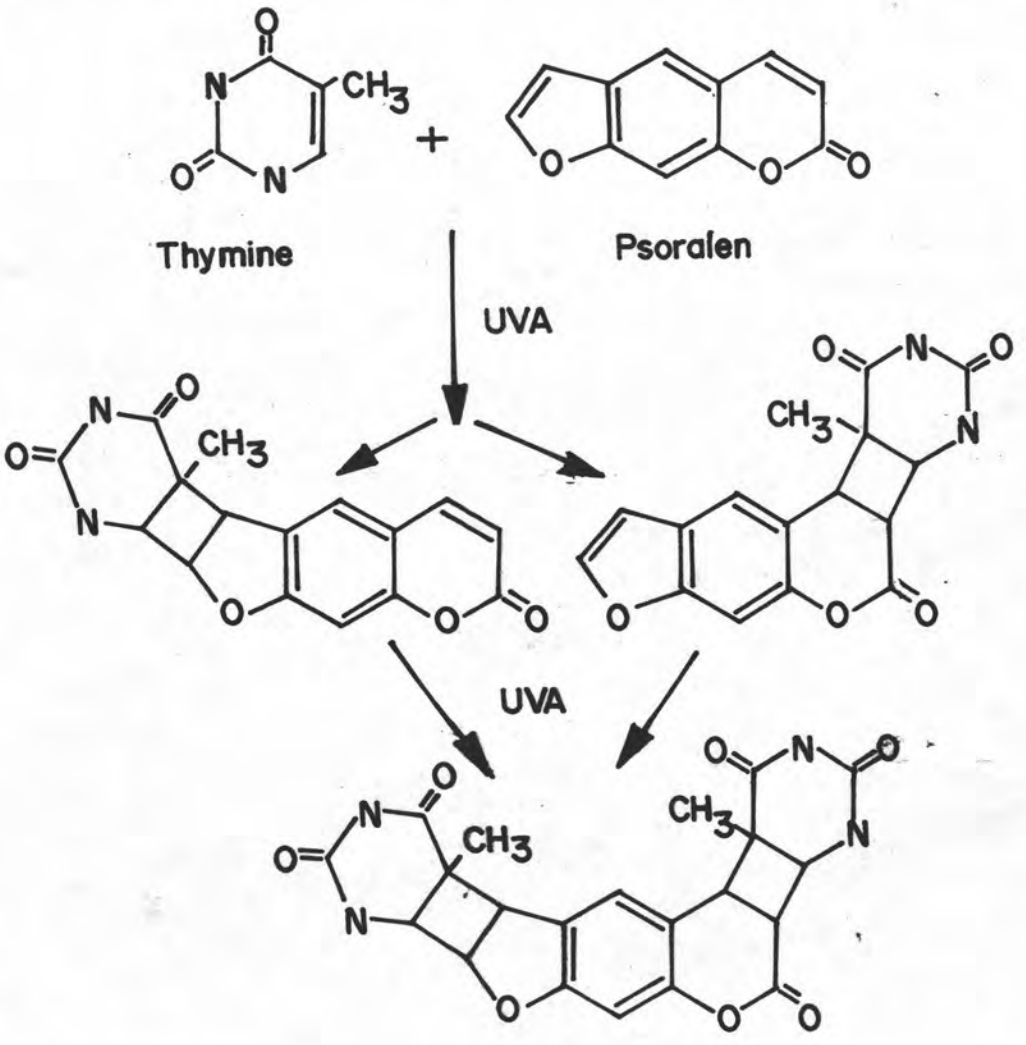


Figure 3 Photoaddition products of psoralen with thymine after UV irradiation in vitro.

Moreover, psoralen can behave as photoreactive bifunctional agents in such manner that one molecule of psoralen reacting with two pyrimidine base in opposite strand of DNA (6). The resulting complex obtained is a cross-linked DNA in which the individual strands cannot be separated by any other standard denaturation conditions known. Both types of these lesions, the monofunctional adduct and the cross-linked product, can be repaired in vivo (Figure 4) The covalent addition of furocoumarins to DNA, particularly the cross-linking reaction, is believe to be responsible for the major effects of psoralen photosensitization. These effect include mutation and lethality to various systems in prokaryotic and eukaryotics, for example inhibition of DNA synthesis, sister chromatid exchange, cutaneous phototoxicity, and carcinogenesis.

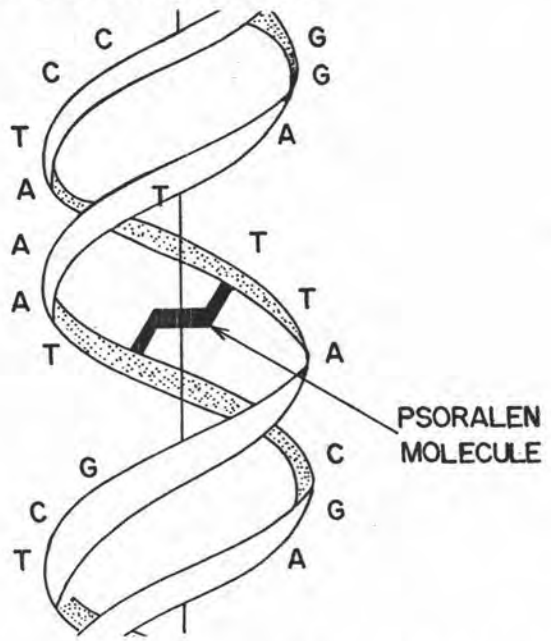
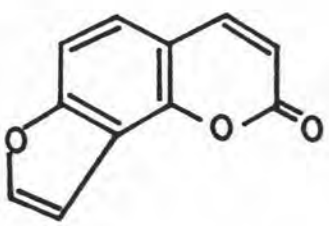


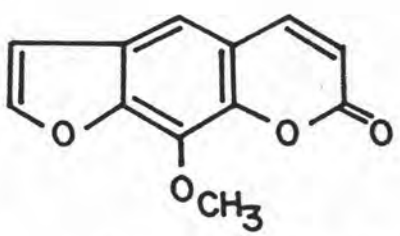
Figure 4 A schematic representation of DNA crosslinked by psoralen molecule is illustrated.

Although furocoumarins are supposed to be the most potent phototoxic compounds known so far, they can be also employed as therapeutic agent. They have been applied clinically to treat vitiligo (leukoderma) and to increase the tolerance of human skin to solar radiation. Methylangelicins, have also been reported recently as a new series of photochemotherapeutic agent for the treatment of psoriasis (7).

Methylangelicins also show evidence of antiproliferative activity, of which a genotoxicity lower than that shown by psoralens. Compound of methyl angelicins generally a lack of skin phototoxicity (8) with the only exception in this connection. That such agent named 4'-methylangelicins which show skin phototoxicity to a measurable extent (Figure 5).



angelicin



8-methoxypsoralen(8-MOP)

Figure 5 Structure of some coumarin were used as photochemotherapeutic agents for treatment of psoriasis.

The recently research showed more and more evidences of a reduction of protein synthesis. This implies in the epidermal cell to devise an effective

treatment for the stubborn and disfiguring skin disease psoriasis(9), which is characterized by a proliferation of these epidermal cells. Oral dose of xanthotoxin, following by controlled exposure to ultraviolet radiation, results in remarkable success in experimental treatment of this disease. In a continuing search for plants having anticancer activity, many research purpose was examine a new compound or a semisynthetic compounds from plant.

The biochemistry of most coumarins are not quite well explored field. Plant biochemists are generally interested in them because of various reasons. Among such reasons are their twofolds effects on the growth and development of plants, the naturally occurring members of the group, and their biosynthesis within the plant. At present the total number of coumarins from natural sources were reported more than six hundred and still rising steadily. Included within this assemblage were compounds of varying biogenetic origin and some biotransformation forms. Most coumarins probably derive their benzopyran nucleus from the cyclisation of a C-2-oxygenated cis-cinnamic acid but significant numbers of them appear to be formed from a mixed cinnamic acid/acetate pathway (4 phenylcoumarins) or totally from acetate (4-n-propylcoumarins) pathway. Simple structures are likely to be derived form cinnamic acid, such as coumarin, umbelliferone and herniarin. They were known

to occur in many plant families. Coumarin, of which structure modified by the addition of C5 units that originating from mevalonic acid, was reported in relatively few families. The resulting simple phenyl coumarins and derived furano- and pyranocoumarins had been reported sporadically in, among others, the Leguminosae, Moraceae and Meliaceae. These compounds, however, have been found commonly in the Umbelliferae and Rutaceae. It is found that most of coumarins in Clausena species are classified in the group of simple coumarin, furanocoumarin (psoralen type, dihydrofurano-coumarin) and pyranocoumarin (xanthyletin type, dihydro-xanthyletin type).

Now the study of coumarin from natural products are considerably interesting due to their structures and chemical reaction for the purposes of synthesizing new compounds which possess more pharmacological action being used as for new drugs. The methylation reaction is a reaction which incorporate methyl group into some molecules and results in either inactive or active compound. There are some recent reports of the regioselective Lewis acid catalyst ortho-Claisen rearrangument of 4'-alloxycoumarate ester. They have successfully applied this approach to the synthesis of naturally occurring linear coumarin, demethylsuberosin , isolated from Ruta graveolens(10).

1. Biosynthesis of coumarin

(a) Biosynthetic pathway to phenylalanine

Among the metabolic pathways of higher plants, the most considerably important pathways for the biosynthesis of hydroaromatic acid is shikimic acid pathway(Figure 6)(11). In the pathway of carbohydrate metabolism to shikimic acid, a four carbon sugar phosphate, D-erythrose-4- phosphate, reacts with phosphoenolpyruvate an the reaction is catalysed by enzyme phospho-2-keto-3-deoxyheptonate aldolase to yield a phosphorylated seven-carbon keto sugar acid so-called 3-deoxyarabinoheptulosonic acid-7-phosphoric acid. On cyclization this compound turn to 5-dehydroquinic acid which has a six carbon aliphatic ring, by the action of enzyme dehydroquinate synthase. This intermediate is then converted into 3-dehydroshikimic acid and undergone simutaneous reduction to shikimic acid catalyse by two enzymes, namely 5-dehydroquinate dehydratase and shikimate dehydrogenase. After which leads via phosphorylated intermediates to chorismic acid and then to quinnonoid compound prephenic acid. Prephenic acid can be aromatized by dehydration and simutaneous decarboxylation to yield phenylpyruvic acid, the precursor of phenylalanine. In the tranamination reaction phenylpyruvic acid is then converted to yield phenylalanine(12)

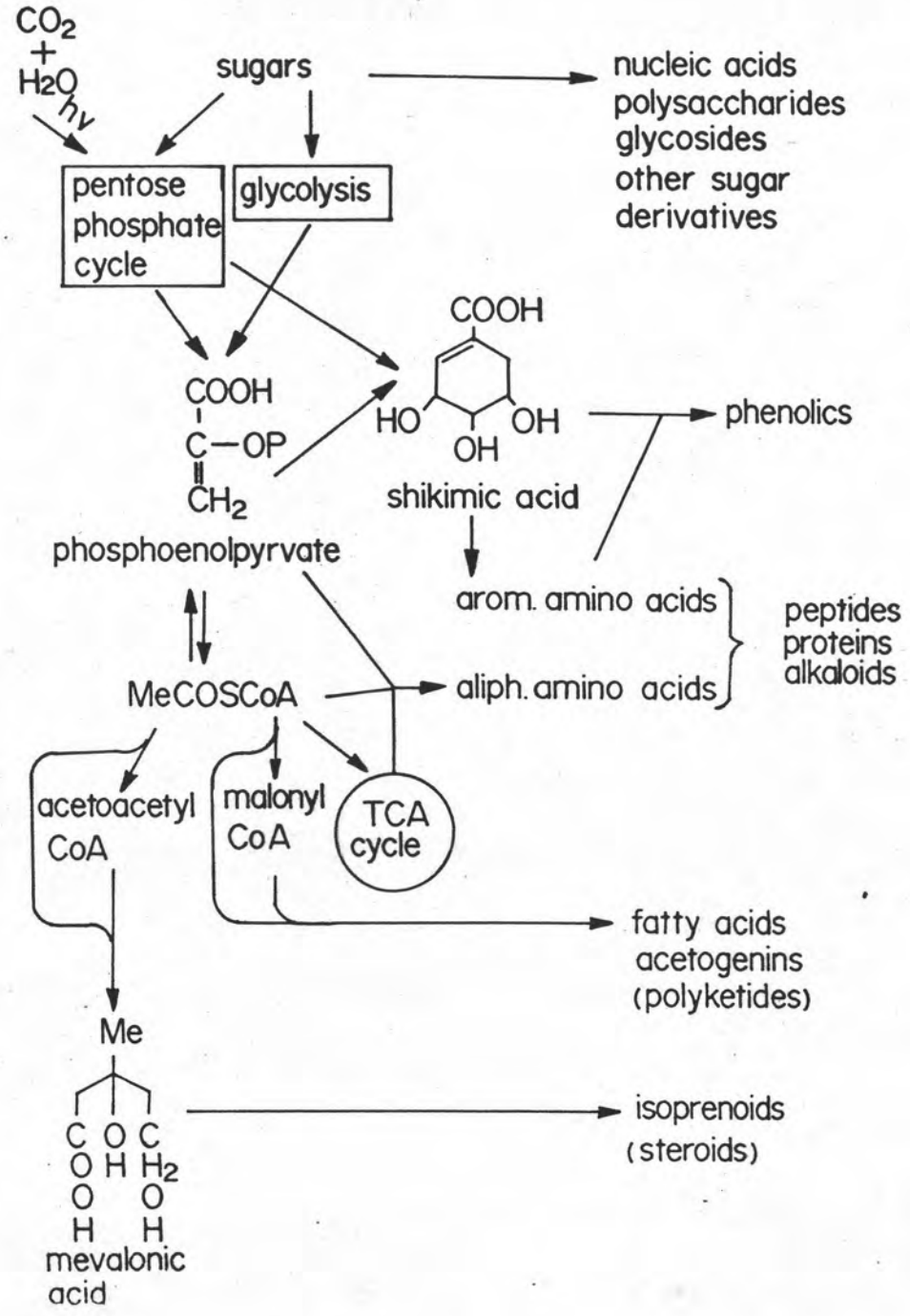


Figure 6 Metabolic pathway of higher plant to synthesis shikimic acid via carbohydrate metabolism.

(b) Conversion of phenylalanine to simple coumarins

Phenylalamine (aromatic amino acid) which came from metabolic pathway can be transformed via a process of deamination by some specific enzymes to corresponding trans-cinnamic acid(Figure 7). The trans-cinnamic acid, formed from the enzyme mediated deamination of phenylalanine undergo ortho oxidation, glucosylation and isomerization to yield the corresponding cis acid(13). These processes may include either enzyme catalysed steps or photochemical phenomena or both. On the basic of tracer investigation, ortho-hydroxylation of trans-cinnamic acid leads to coumarin itself, via a light catalysed trans-cis isomerization, and lactone ring formation which can be formally represented as a dehydration. Para hydroxylation of trans cinnamic acid is a necessary prerequisite for synthesis of the 7-hydroxycoumarins (umbelliferone) respectively, via ortho hydroxylation and lactonization as described before. However, it should be noted that coumarins do not occur in free form in the intact plant cells. Thus coumarins, hermaiarin, and umbelliferone have reportedly all been shown to occur in the form of glycosides of the corresponding cis o-hydroxycinnamic acids(13).

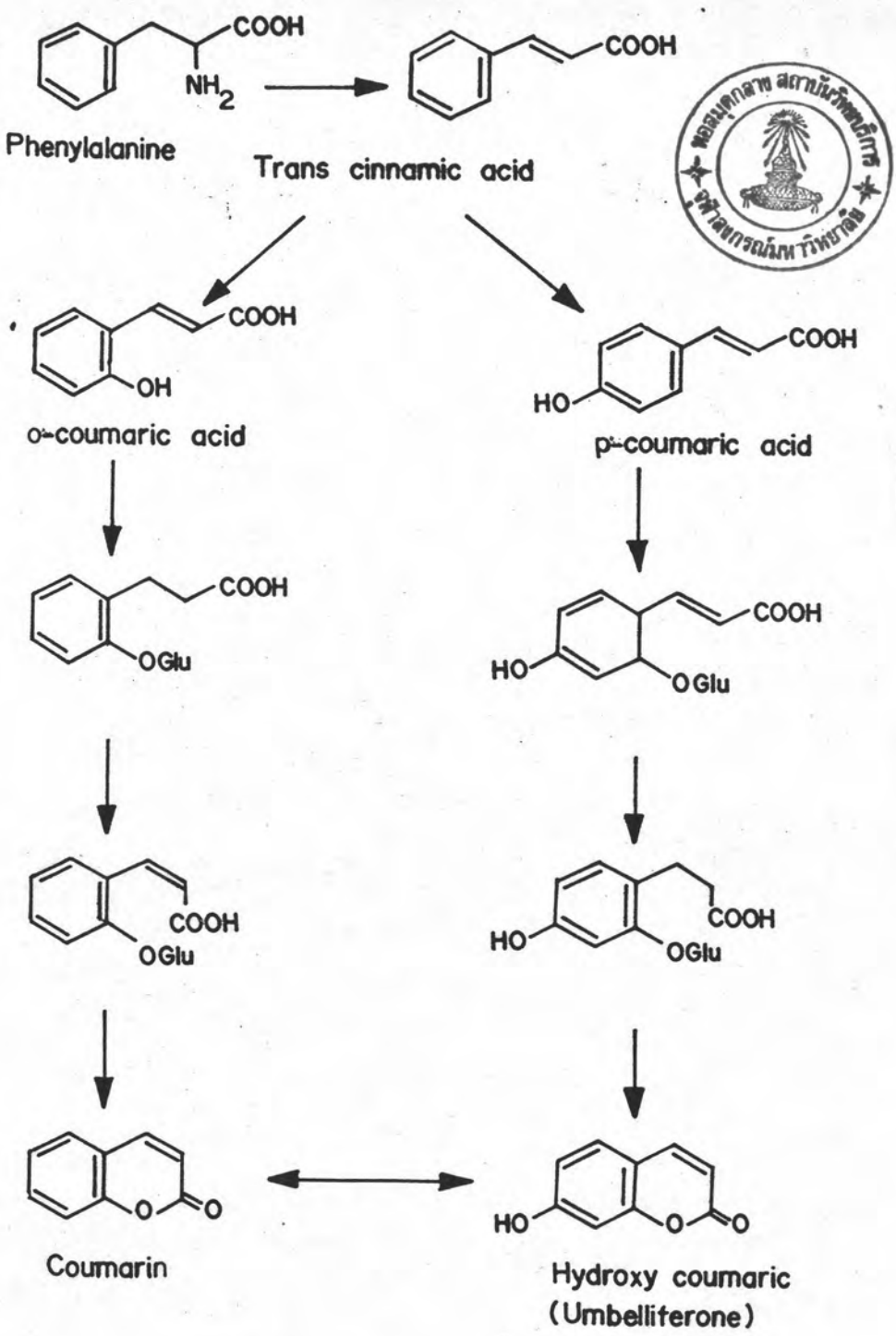


Figure 7 Original formation of simple coumarins.

(c) Formation of furanocoumarins

Umbelliferone (7-hydroxy coumarin) can be regarded as the parent compound of furanocoumarin in plant, and as the base for construction of the furan ring. The addition of dimethyl allyl unit at C-6 appears to be specifically controlled by the enzyme dimethylallylphosphate:umbelliferone transferase. The mechanism of prenylation of umbelliferone is visualized as involving the formation of the stable anion which will permit the electrophilic attract of prenyl carbonium ion at either C-6 or C-8 to yield C-phenyl coumarin or on phenoxide to give o-phrenyl coumarin compounds(Figure 8)

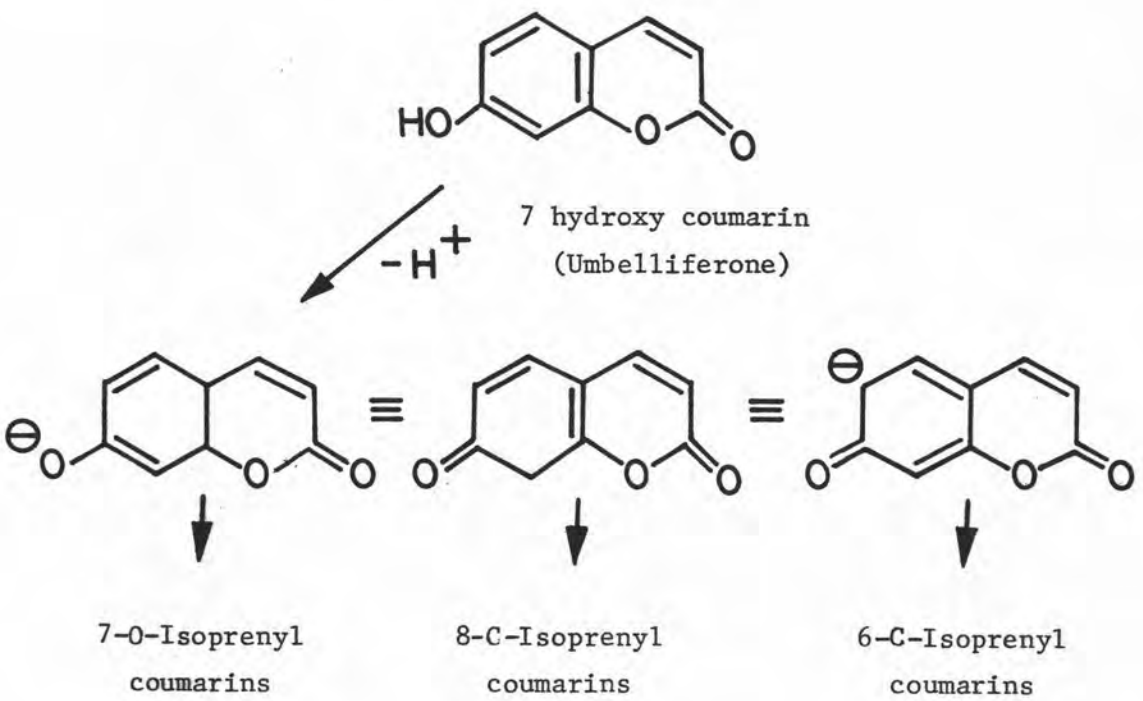
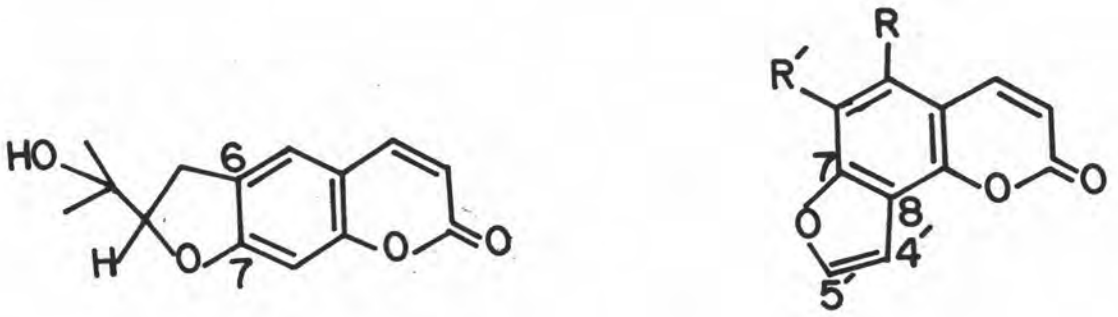


Figure 8 Formation of furanocoumarin from umbelliferone.

Two type of furanocoumarins are recognized : the linear type (a), in which the furan ring is fused at C6 and C7 position of the benzene nucleus (Psoralens) and angular type(b), where fusion is at C7 and C8 positions. (Figure 9)



(a) Linear (b) Angular

Figure 9 Structure of furanocoumarins, linear and angular type.

In Rutaceae and Umbelliferae the reaction of a particulate transferase enzyme able to mediate the prenylation of position 6 of umbelliferone by dimethylallyl pyrophosphate(14), an intermediate well known to be mevalonate derivative, to form the naturally occurring demethylsuberosin (6-dimethylallyl umbelliferone)(Figure 10). This phenylase require a divalent cation (Mn^{2+}) as cofactor in the so-called reaction. Epoxidation of the double bond of the prenyl side chain as refers a prerequisite to cyclization to a dihydrofuranocoumarin (marmesin) formation.

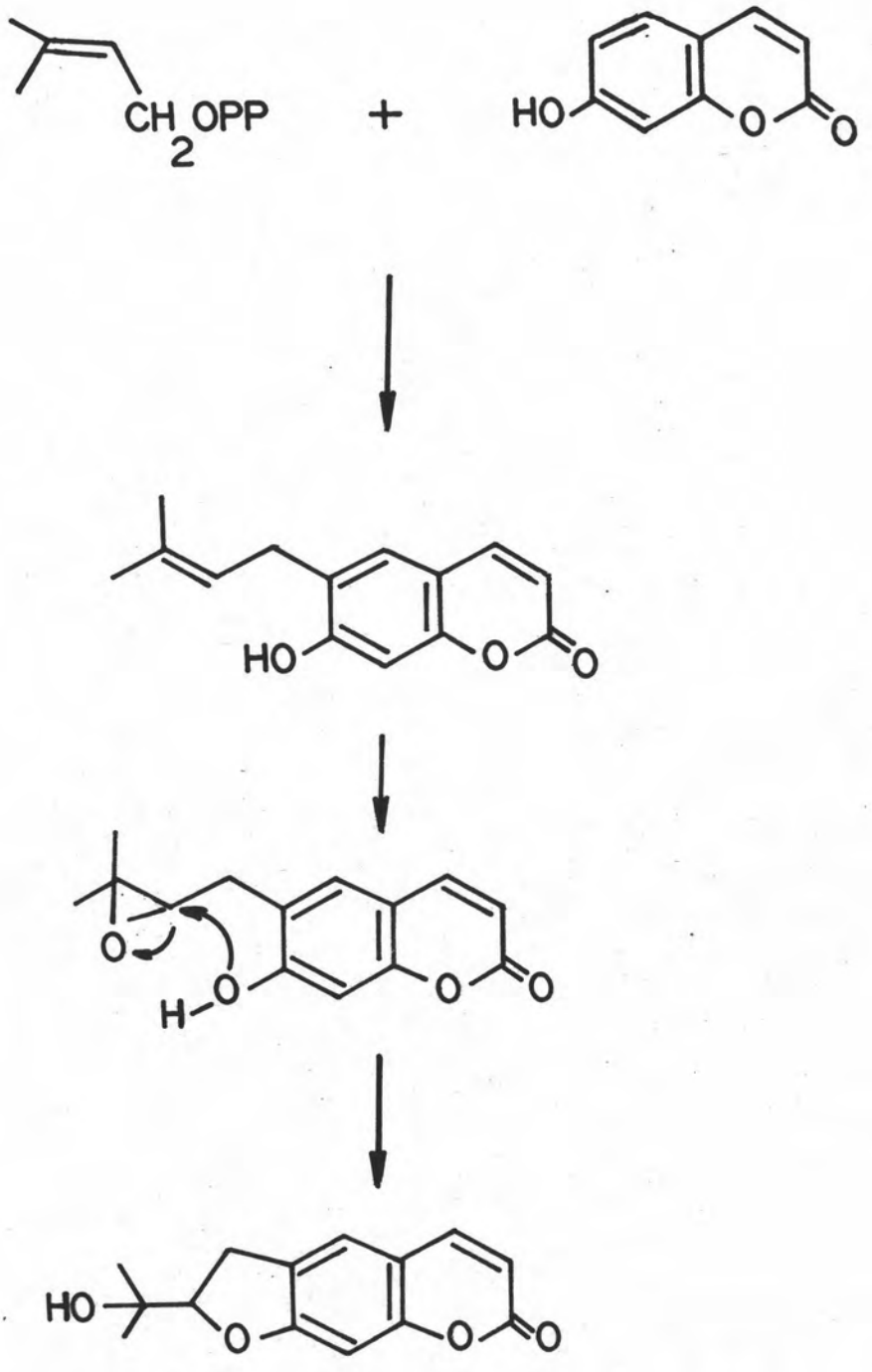


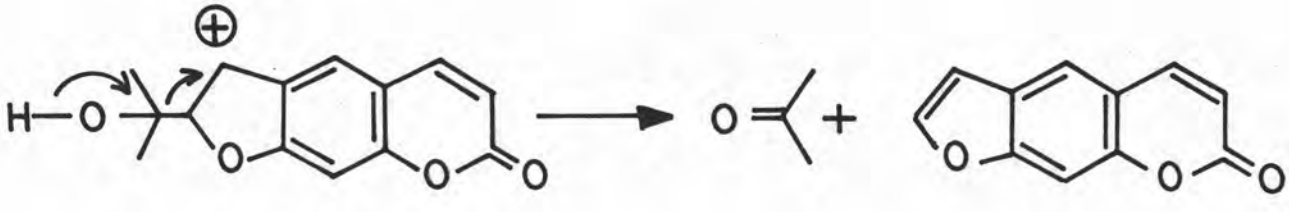
Figure 10 Formation of dihydrofuranocoumarin(marmesin) from umbelliferone and dimethylallyl pyrophosphate.

In 14C-tracer experiments, they have demonstrated that osthenol is an intermediate precursor(15) of angular-type furanocoumarins (columbianetins) (Figure 11).

Osthenol Angular furanocoumarin

Figure 11 Formation of angular furanocoumarin from osthenol

The mechanism is to deal with the procedure, whereby the isopropyl side chain is eliminated from marmesin and columbianetin and the double bond introduced to form the furan ring. Carbonium ion is generated at C-4'position, followed by a 1,3 elimination, as show in Figure 12 . The 3-C side chain bringing in has then been converted to acetone in the process and form double formed afterward to yield bond is furan ring.



Marmesin Psoralen

Figure 12 Mechanism proposed by Birch et al for the conversion of marmesin to psoralen.

It was noted that oxygenetion of benzene ring of simple coumarins can often occure before the benzopyrone nucleus has been elaborated. In *Ruta graveolens*, it has been reported 8-hydroxymarmesin seems to be an intermediate between marmesin and 8-methoxypsoralen (Xanthotoxin), but this reaction appears to be exceptional, as attempts to demonstrate it in other species have failed. Other tracer studies have provided evidence that psoralen, the unsubstituted linear furanocoumarin, is converted in vivo to the mono-and dimethoxypsoralens, and a few species accumulate the corresponding hydroxypsoralens. It has been postulated that psoralen is first hydroxylated, and that a transmethylation reaction completes the biosynthetic pathway to furanocoumarins. In laboratory experiments, its show that the transmethylation reaction would have constituted the final step in the synthesis of such coumarin as bergapten, xanthoxin, and isopimpinellin in the linear series.

This reaction also presumes to be involved in formation of angular furanocoumarins such as isobergopten, sphoolin and pimpinellin

In cell-free extracts experiment, o-methylation of these derivative can be produced many kinds of coumarins(16).

(d) Formation of pyranocoumarins

About the route to synthesis these compounds and isomeric with furanocoumarins, little have been determined. The only available direct experimental evidence is that demethylsuberosin is a very efficient precursor of dihydroxanthyletin in *Coronilla glauca* , suggesting that the pathway up to the cyclization stage coincide with that to furanocoumarins. In the light of in vitro experiments, they have speculated that mode of cyclization may be upon pH at the synthetic site.

Although no detailed investigation into the formation of pyranocoumarins has yet been reported the observation that demethylsuberosin has been incorporated into 3'-4'-dimethylsuberosin structure strongly suggests a pathway analogous to that for furanocoumarins is in option. It has also been noted that, as anticipated, the configuration of the C-prenyl epoxide intermediate is retained during the formation of pyranocoumarins. The usually 2-3-f angular pyranocoumarins are probably the product of cyclization of a C-6 prenyl unit and a free C-5 hydroxy substituent.

The mechanism of cyclization of furan and pyran ring is now understood. The questions to be answered concerns the likely intermediate, the epoxide or the diol. In vitro experiments have shown cyclization to occur spontaneously upon epoxidation of a prenyl group to which there is a free ortho hydroxy substituent and

suggest the epoxide to be the most likely intermediate. The relative paucity of prenyl coumarins isolated (17) with free ortho-hydroxy function from Rutaceae would seem to agree with this concentration. The mechanism of formation of either furano or pyrano coumarin ring is not well understood at present. No enzyme system has yet been found to govern either, and in the spontaneity of cyclization, it seems to be feasible that none needs to exist. In this context the observation that, in vitro, the furan ring is to be formed under neutral and basic condition may well be significant. If this situation is paralleled in vitro then external factors affecting the pH at the side of synthesis will obviously play a primary role in deciding the structures of the coumarin produced.

(e) Formation of phenylcoumarins

The first biosynthetic research on phenylcoumarins was conducted on coumestrol, a 3-phenyl coumarin of *Medicago sativa* with estrogenic properties. By process on isotropic technique, it showed that 4,2',4'-trihydroxychalcone-4'-glucoside was the known precursor of coumestrol. After further investigation on the later stages of this pathway in *Phaseolus aureus* and *Soja hispida* being made, the proposed the reaction sequence has been made as the major route to coumestrol.

Recently report that neoflavonoids have been considered as a precursor of 4-phenylcoumarins. From study in detail the incorporation of [3-14C]-phenylalanine into the calophyllolide elaborated by shoots of *Calophyllum inophyllum* (Guttiferae) and located essentially all the 14C of the isolated coumarin in C-4 has been eliminating the possibility of rearrangement in the phenylpropanoid moiety. These workers later demonstrated incorporation of acetate into the phloroglucinol nucleus. In investigations with the structurally related 4-phenylcoumarin inophyllolide, this research group established that calophyllic acid had eight times the specific activity of inophyllolide after the administration of [3-14C] phenylalanine, a finding consistent with an intermediate function for calophyllic acid in the elaboration of 4-phenylcoumarins. They also showed that the 2-methylbutenoyl side chain of calophylloie have been derived from isoleucine, presumably via tiglic acid and that the isovaleryl substituent in the phloroglucinol ring of the related 4-phenylcoumarin and mammeisin originated in leucine(18).

2. Classification of coumarins

Until now, no complete satisfactory classification of coumarin is made possible. Coumarin is the term applied to a group of naturally occurring compounds possessing a 2-H-1-benzopyran-2-one nucleus(Figure 13) and derivatives. Steck,W. and Mazurek, M.(19) have classified coumarins into two types, firstly, "normal type" have an oxygen function at C-7 and hydrogens at C-3 and C-4 (unsubstituted pyrone ring). This type of coumarin comprises the great majority of natural derivatives. Secondly, the "abnormal type," which either lack the C-7 oxygen or possesses pyrone ring was substituted by other functional group.

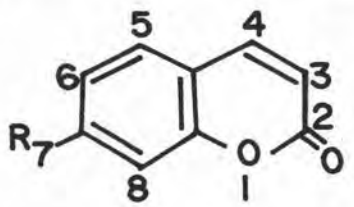
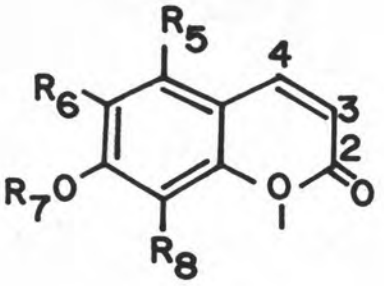


Figure 13 Coumarin nucleus (2-H-1-benzopyran-2-one nucleus)

Naturally occurring coumarins, however, can be generally classified as followings(20):

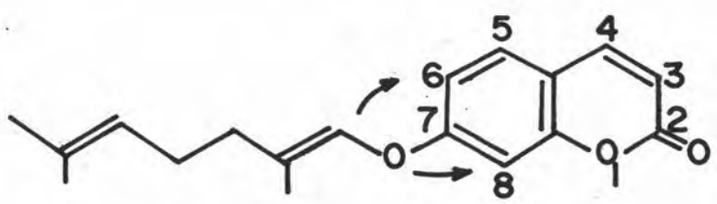
(1) Coumarins substituted with one or more hydroxyl and/or methoxyl group in the benzene ring(Figure 14). This type also called in term of simple coumarin. Umbelliferone, for example, represent the simple coumarin in this type. There are widely distributed of simple coumarin in certain plant family, Rutaceae and Umbelliferae.



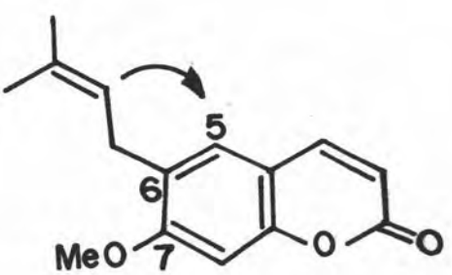
	R_5	R_6	R_7	R_8
1	H	H	H	H (Umbelliferone)
2	H	H	CH_3	H (Herniarin)
3	H	OH	H	H (Esculetin)
4	H	CH_3O	H	H (Scopoletin)
5	H	CH_3O	CH_3	H (Scoparone)
6	H	H	H	OH (Daphnetin)
7	CH_3O	H	CH_3	H (Limettin)
8	H	H	Prn	H
9	H	Prn	H	H (Demethylsuberosin)
10	H	Prn	CH_3	H (Suberosin)
11	H	H	H	Prn (Osthenol)
12	H	H	CH_3	Prn (Osthol)
13	H	OH	Prn	H (Prenyletin)
14	CH_3O	H	CH_3	Prn (Coumurrayin)
15	H	H	CH_3	Prn oxide (Meranzin)
16	H	H	CH_3	Prn diol (Meranzin hydrate)
17	CH_3O	H	CH_3	Prn oxide (Sibiricin)
18	CH_3O	H	CH_3	Prn diol (Mexoticin)

Figure 14 Simple coumarin: Prn=3-methyl-2-butenyl(=prenyl);Prn oxide-3-methyl-2,3-epo-xybutyl; Prn diol=3-methyl-2,3-dihydroxybuty

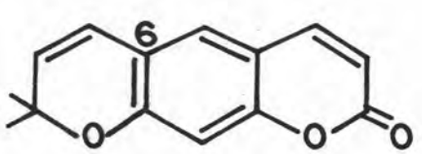
(2) Coumarins substituted, with isoprenoid residues (a,b). These isoprenoids can cyclization to form six-member ring namely pyranocoumarin and dihydro-pyranocoumarin. So coumarins in this group are also have a pyran ring fuse to the nucleus at a position of C6 or/and C7 to form linear type pyranocoumarins(c) or at position C5, C6 / C7, C8 to form angular type pyrano-coumarins(d,e) (Figure 15),some compounds showed in Figure 16.



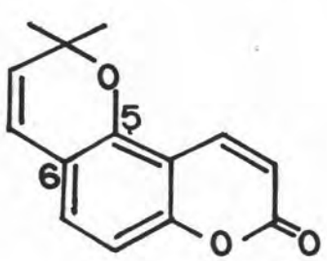
(a) aurapten



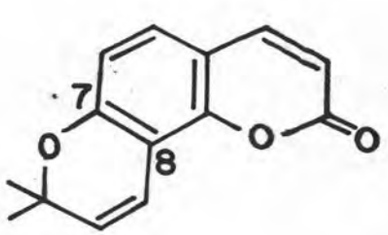
(b) suberosin



(c) linear type (C6,C7)

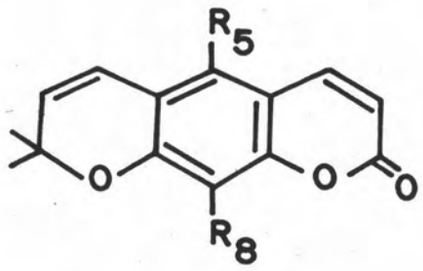


(d) angular type (C5,C6)

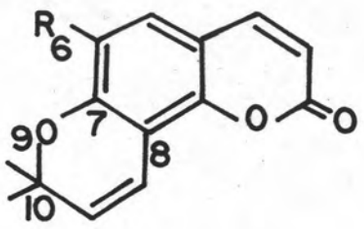


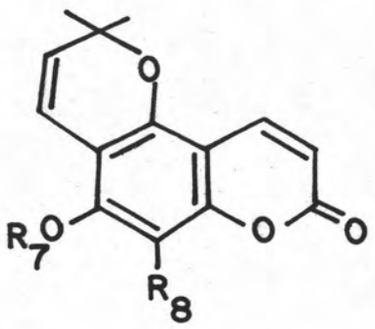
(e) angular type (C7,C8)

Figure 15 Linear and angular type coumarins



	R_5	R_8
19	H	H (Xanthyletin)
20	OCH_3	H (Xanthoxyletin)
21	H	OCH_3 (Luvangetin)



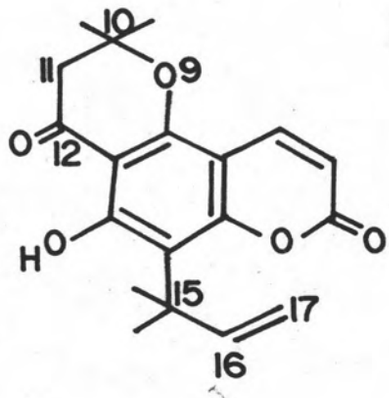


	R_6
22	H (Seselin)
23	OCH_3 (Braylin)

	R_7	R_8
24	H	Prn (Nordentatin)
25	CH_3	Prn (Dentatin)
26	CH_3	H (Xanthoxyletin,allo)

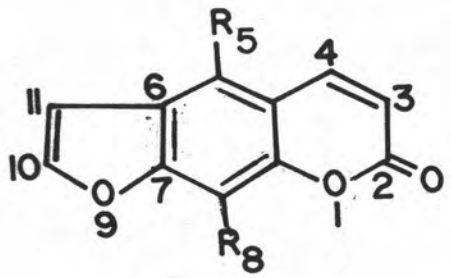
Figure 16 Pyranocoumarin compounds

Naturally pyranocoumarin compounds have been reportedly found in Clausena species. Pyran ring of some pyranocoumarins are oxidized to form carbonyl group at C12 such as clausenidin.



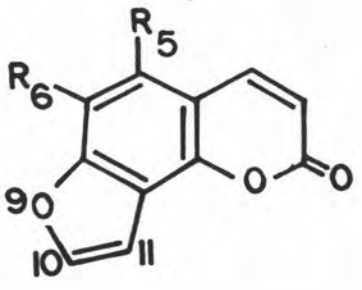
27 clausenidin

(3) Coumarins that consist of the furan ring fused to the coumarin nucleus. They are usually, also called furanocoumarins, which have been classified into two subgroups, linear furanocoumarins (Figure 17) and angular furanocoumarins(Figure 18).



	R_5	R_8
28	H	H (psoralen)
29	OCH_3	H (Bergapten)
30	H	OCH_3 (Xanthotoxin)
31	OCH_3	OCH_3 (Isopimpinelline)
32	O-Prn	H (Isoimperatorin)
33	H	O-Prn (Imperatorin)
34	O-Prn	OCH_3 (Cnidilin)
35	OCH_3	O-Prn (Phellopterin)
36	O-Prn oxide	H (Oxypeucedanin)
37	O-Prn diol	H (Oxypeucedanin hydrate)
38	H	O-Prn oxide (Heraclenin)
39	Prn oxide	OCH_3

Figure 17 Linear furanocoumarins

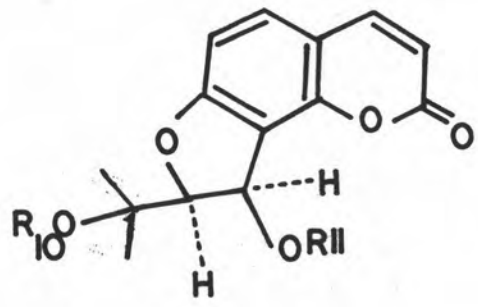


	R_5	R_6
40	H	H (Angelicin)
41	OCH_3	H (Isobergapten)
42	H	OCH_3 (Sphondin)
43	OCH_3	OCH_3 (Pimpinellin)

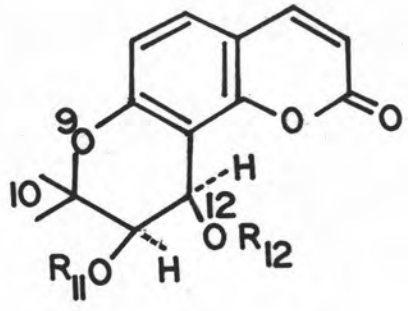
Figure 18 Angular furanocoumarins

(4) Dihydrofurano and Dihydropyranocoumarins

These coumarins have been substituted by hydrogen at C10,C11 (furanocoumarin) and C11,C12 (pyranocoumarin) position(21).Natural occurring dihydrofuranocoumarins usually have an isopropyl type substiuent at C10; and dihydropyranocoumarins usually have an oxygen function at C11. Both classes of compounds include a large number of members with additional oxygen function at C10(furanocoumarin) and C11(pyranocoumarin) (Figure 19)



	R_{10}	R_{11}
44	senecioyl	H
45	H	isovaleryl (vaginidin)
46	isovaleryl	isovaleryl (athamantin)
47	angely	angely (archangelicin)
48	senecioyl	acetyl (peucenidine)



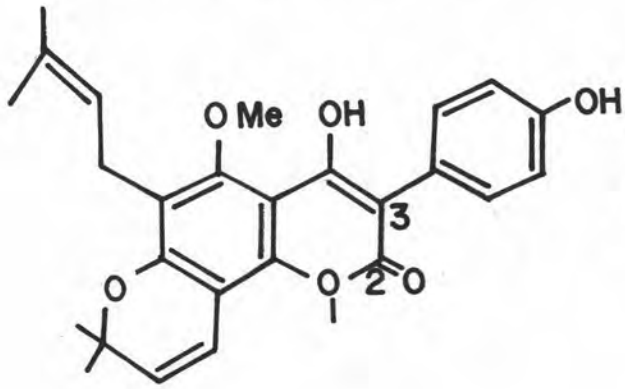
	R_{11}	R_{12}
49	H	H (cis-khellactone)
50	acetyl	acetyl (pteryxin)
51	angelyl	acetyl (isopteryxin)
52	acetyl	isovaleryl(suksdorfin)
53	angelyl	angelyl (anomalin)

Figure 19 Dihydrofurano and dihydropyranocoumarin compounds

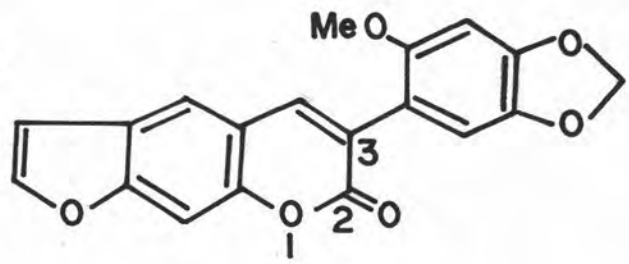
The rest of coumarin which have been substituted on pyrone ring are also called "abnormal coumarins". These types of coumarin comprises the minority of natural derivatives, those are:

(5) Coumarin substituted with phenyl group at C3 or C4 on coumarin nucleus(22). Sometimes they are called phenyl coumarins.

5:1 3-Phenylcoumarins

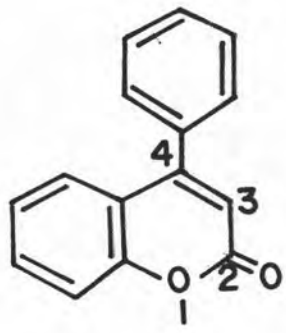


54 scandenin

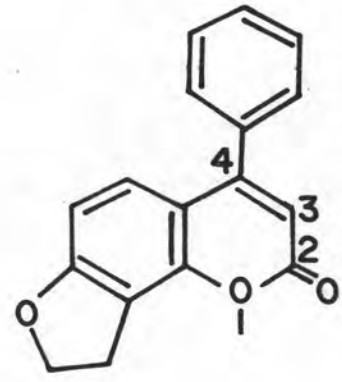


55 pachyrrhizin

5:2 4-Phenylcoumarins

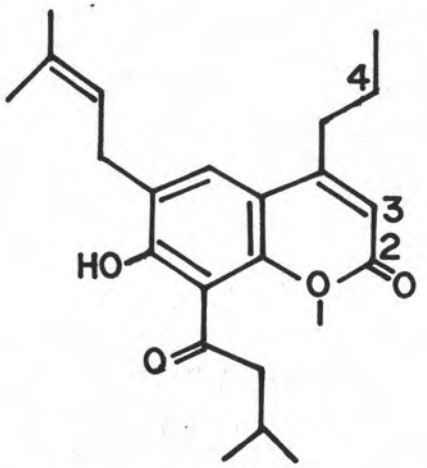


56 4-phenylcoumarin

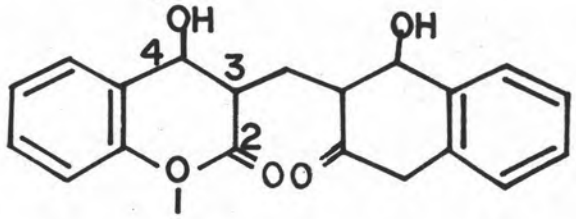


57 4-phenyldihydroangelicin

(6) Substituted coumarin with alkyl group or hydroxyl group at C4 position(23).

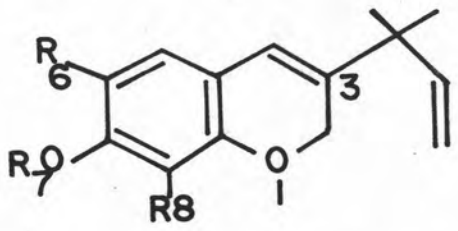


58 mammein



59 dicoumarol

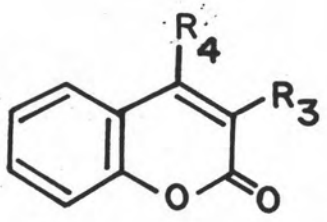
(7) Coumarins subsituted with prenyl (1,1 dimethyl allyl group) on C3 position(Figure 20)



	R_6	R_7	R_8
60	prenyl	H	H(gravelliferone)
61	prenyl	H	OCH (8-methoxygravelliferone
62	CH_3O	CH_3	H(rutacultin)
63	H	CH_3	CH O

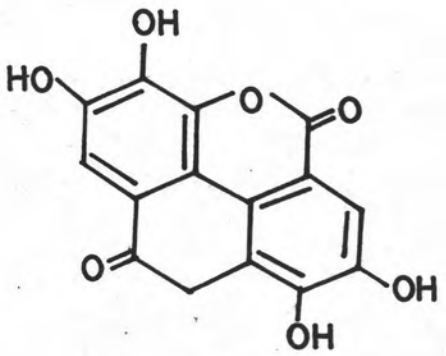
Figure 20 Prenyl substitution coumarins.

(8) Coumarins having pyrone ring substituted with hydroxyl group at C3 or C4.



	R_3	R_4
64	CH_3	OH
65	OH	geranyl

(9) 3,4- benzocoumarins



66 ellagic acid

3. Column chromatography

The chromatography now covers several hightly efficient laboratory procedures. According to the classical technique, a solution is to elute through a powder column of some substances on which a mixture of solution is resolved, by selective adsorption, into its various components. The individual of substances containing in each distinct zone, can be separated by cutting the column or washed fractionally into the eluent or filtrate. The principle of this technique depend on two phenomena, adsorption and partition(24).

The adsorption chromatographic method depends on properties of substances interacting with adsorbent molecules. The intermolecular forces which are throught to be primarily responsible for adsorption in chromatographic system may be classified as the following:

(a) Van der wall force between all surfaces and adsorbed molecules.

(b) Electrostatic force between polar substance and any adsorbed molecules or between nonpolar surface and polar absorbed molecules.

(c) Charge transfer force between strong electron donors and acceptors.

(d) Hydrogen bonding.

The about intermolecular surface forces are for the most part and much weaker than the forces existing between covalently or ionically bounded atoms. Adsorption may involve either the former weak physical forces or the latter strong chemical forces. This give rise to two respective adsorption type, physical adsorption and chemical adsorption. The most distinguishably characteristic feature of physical adsorption is rapid reversibility, which in turn is essential to good chromatographic separation.

Partition chromatography method represents the term of technique for separating substances by the abilities of such compounds which can be partitioning in difference solvent systems . The partition chromatographic column consists of a finely divided solid, called the support, on which the solvent, the immobile, stationary substrate, or partitioner phase is fixed with such tenacity that it will not migrate. Second liquid phase, which is immiscible with the first called the mobile, carrier, or developer phase flows over the support partitioner combination in such a way that the two phases are in contract over a very large interface, and the equilibrium distribution of the solute between the two phases is rapidly attained. The solutes participate in a partition between the stationary phase, where they are held in a fixed position, and the mobile phase, where they migrate. The

solutes are presumed not to be adsorbed by the support.

Liquid column chromatography

The liquid column chromatography method based on partition and adsorption principles has generally been reserved for the preparative separation or precise quantitative analysis of relatively nonvolatile, nonionic organic compounds. This method is a separation technique base on a sample partition between two phase. Stationary phase is usually are solid substances which packed in the glass column. Mobile phase, which is the mobile liquid percolates over stationary phase. In process of column development, it is quite similar to elution. Sample is to be added to the head of a column, and solvent is then allowed to flow into the column until the solvent front reaches to a certain point short of the opposite end. In the course of being washed through the column, the various sample component form bands which migrate at varying distances along the column. The main advantage of column development is the simplicity of the development operation. However, after the separation is completed, determination or recovery of the separated components from the column requires extrusion of the column packing, estimation of the band positions (by inspection in visible or ultraviolet light, or by staining), and mechanical separation of the bands. This procedure, messy and laborious at best is

inapplicable when the long, thin column that provides the better separations are used. Column development has still been recommended, because cutting the bands from the extruded column permits the operator to the correct separation for unevenly developed or sloping bands. However, uneven bands become less of a problem in reasonably narrow columns. Also, column extrusion have been recommended for sample components that are strongly held on the column, but there are vary few cases where such compounds cannot be eluted by the use of a strongly elution solvent. At present, most worker prefer either elution from columns or sheet development to column development.

Thin layer chromatography (TLC)

TLC now is considered an indispensable tool in many laboratories, especially those engaged in research on lipophilic natural products. This technique is easy to learn and its application is comparative inexpensive. It is fast, convenient, and versatile. Thin layer chromatography is applicable on an analytical scale and, especially when used as an adsorption method, as a micro-preparative tool as well(25). The basic equipment, slurries of adsorbents and other solid materials can be applied as thin layers to glass plate or plastic sheet by spreading, pouring, dipping, or spraying. The spreading procedure, which yields the most uniform and best reproducible layers, is usually carried out with a

commercial appicator. Rectangular glass plates, measuring 5x20 cm. or/and 20x20 cm. are most oftenly used; they can be obtained by cutting glass or commercially. The coating material, all type of sorbents formerly used in chromatographic columns are applicable to the preparation of thin layers, but they must be of much smaller particle size. Some of these materials are free of additives, others contain a binder, such as gypsum, or an indicator, such as a fluorescent mineral, or both.

Siliga gel is used more often than any other coating material. It should be noted that preparation from a different manufacturer varies in their separation characteristics. To prepare the coated of plates, coating materials are usually applied as aqueous slurry is made by thoroughly mixing 25 gm. of Siliga Gel G ("Emerck") with 50 ml. of distilled water in mortar until it is of uniform consistency and free of air bubbles. Slurries of Siliga Gel G are then applied as thin layer onto glass plate. In experiments, unknown and standard compounds are applied on TLC plates and developed in suitable solvents system which contain in close chamber tank. The detected spots can be calculate Rf value of substance by equation

$$Rf = \frac{\text{Distance traveled by center of zone}}{\text{Distance simutaneously traveled by developer front}}$$

4. Infrared spectroscopy

Infrared spectra was a physical technique for analysis of organic compounds. The IR spectra of the most organic compounds contain extensive numbers of absorption band due to vibrations of almost all chemical groups in compounds. The infrared spectra are recorded in the 2-50 m range. Infrared radiation refers broadly to that part of the electromagnetic spectrum between the visible and microwave regions.

Infrared radiation of frequencies less than about 100 cm is absorbed and converted by an organic molecule into energy of molecular rotation. The chemical bond of a diatomic molecule can be described in a simplified form as an elastic spring whose stretching and compression simulate vibrations of atoms in the molecule. For the harmonic oscillator the restoring force is proportional to the displacement of nuclei from the equilibrium position and its direction is opposite to the displacement.

$$f = -K \Delta r$$

The coefficient K is known as the force constant of the bond and describe the rigidity of the bond (or elasticity of bond). The law of mechanics yields the following relationship between the frequency ν of the stretching vibration, the force constant K, and the masses m1 and m2 of the atoms in the molecule.

$$\nu = \frac{1}{2\pi} \sqrt{\frac{K}{\mu}}$$

where μ is the reduced mass: $\mu = \frac{m_1 m_2}{m_1 + m_2}$

From this equation shows that the vibration frequency increases with increasing bond strength and decreasing atom masses. By using this equation can estimate the ranges of variation of the vibration frequencies and predict the functional groups of organic compounds.

5. Nuclear magnetic resonance spectroscopy (NMR)

Under definite conditions, the radiofrequency range can also yield information on the fine features of the structure of organic and biological compounds. The fundamental principles of the NNR technique depend on condition of nucleus atom. The atom are known to be comprised of nuclei and electrons. The nuclei have positively charges which are multiples of charge of the hydrogen nucleus (the proton). The nuclei of many isotopes have also the angular momentum known as the spin and characterized by the spin quantum number I (I=0,1/2,1,...). Rotation of the charged nucleus is said to be equivalent to a circular electric current and gives rise to the magnetic field directed along the rotation axis. Thus, the nucleus can be regarded as a micromagnet with the magnetic moment n . The magnetic moment of the nucleus is related to the spin by the following equation:

$$\mu = \gamma \cdot p = \gamma \left(\frac{h}{2\pi} I \right)$$

where μ is the magnetic moment of the nucleus, p

is the spin of the nucleus, γ is a constant describing the type of the nucleus (the gyromagnetic ratio) and h is Plank's constant. The nuclei with even numbers of protons do not possess spins (I= 0) and their magnetic moment is zero. Therefore atoms with even mass and atomic numbers, such as C, O and S have no magnetic properties and do not produce NMR signals. Other nuclei have reportedly nonzero spins ($I \neq 0$).

The nuclear magnetic moment is quantized, that is, its projection on an external uniform magnetic field Ho can have any of 2I+1 discrete values determined by the magnetic quantum number m (m =I, I-1,...,0,..., -I+1,-I). Each value of m corresponds to a certain energy level; the distance between the levels is constant and equal to μHo/I. The nuclei which are most common in organic chemistry (^{1}H, ^{13}C, ^{15}N, ^{19}F, ^{31}P) have the spin I=1/2. In the external magnetic field such nuclei can be in two energy states with the magnetic moment parallel (m=1/2) or antiparallel (m=-1/2) to the external magnetic field. The distance between these levels is determined by the nuclear magnetic moment and the external field:

$$\Delta E = 2\mu H_0 = \gamma \frac{h}{2\pi} H_0$$

Since reorientation of spin is equivalent to transition from one energy level to another the process results in absorption or liberation of energy in the form of the electromagnetic radiation quantum hν:

$$\Delta E = h\nu = \gamma \cdot \frac{h}{2\pi} H_0$$

Hence the frequency

$$\nu = \frac{\gamma}{2\pi} H_0$$

6. Mass spectometry

A mass spectometry also a physical technique which applied to elucidation of chemical compound structure especially organic compound. By bombards the substance under investigation with an electron beam and quantitatively record the result as a spectrum of positive ion fragments. This record is a mass spectrum. Separation of the positive ion fragments is on the basic of mass (strictly, mass/charge, but the majority of ion are singles charged). From this result can related with condition of atoms in that state, applies to identified the organic compounds or to structure. Mass spectrometers for structure elucidation can be classified according to the method of separating the charged particles(26):

1 Magnetic Field Deflection (Direction Focusing)

1.1 Magnetic field only (unit resolution)

1.2 Double focusing (electrostatic field before magnetic field, high resolution)

2 Time of Flight

3 Quadrupole

The importance of mass spectrum for organic compounds is the ability to record the molecular weight of the unknown substances. To determine the resolution of an instrument, consider two adjacent peaks of approximately equal intensity. These peaks should be chosen so that the height of the valley between the peaks is about 10% of the intensity of the peaks. The resolution (R) is

$$R = \frac{M}{\Delta M}$$

Where M = The higher mass number of the two peaks

ΔM = The difference between the two mass numbers.