STUDIES IN THE CHEMISTRY OF CERTAIN PLANT PIGMENTS

A THESIS
SUBMITTED TO
THE UNIVERSITY OF POONA

FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN CHEMISTRY

BY
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NATIONAL CHEMICAL LABORATORY
POONA
1972

C O N T E N T S

CHAPTER I

CHEMICAL INVESTIGATION OF THE TRUNK BARK OF ARTOCARPUS HETEROPHYLLUS

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INTRODUCTION

The genus *Artocarpus*[1] is characterised by its spirally arranged leaves, amplexicaul stipules, annulate scars, gland hairs with 4 to 16 celled head, mesophyll long armed with globase or ellipsoid resin cells. The genus *Artocarpus* consists of evergreen or deciduous trees comprising about 100 species distributed in the Indo-Malayan region and China, and belongs to family *Moraceae*.

There are six species of *Artocarpus*[1] occurring in India of which *A. chaplasha*, *A. hirsutus* and *A. lakoocha* are important timber trees. *A. heterophyllus*, *A. incisa*, *A. lakoocha* and *A. gomezianus* yield edible fruits.

Artocarpus heterophyllus Lamk.

A. heterophyllus[2,3] formerly known as *A. integrefolia* is a large evergreen tree cultivated throughout India, Burma and Ceylon for its fruits. The heartwood is yellow when freshly cut and turns to brown on long exposure. The wood is fairly strong, durable and resistant to fungi and white ants. By virtue of these properties it finds extensive use in carpentry work and in the making of musical instruments. As a mordant dye for wool and silk applied in conjunction with alum it was used for dyeing the robes of Buddhist priests

yellow. In Cambodia the wood is considered to be a sedative and is administered in convulsions. The young leaves are used in skin diseases and the root is used internally in diarrhoea.[4]

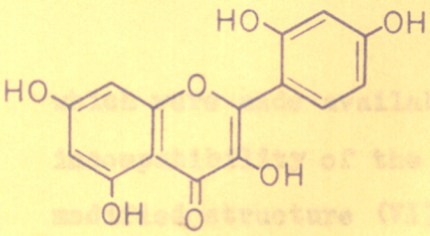
From the heartwood of A. heterophyllus Perkin and Cope[5] isolated morin (I) and cyanomaclurin (II) in 1895, but it was only in 1963[6] that a study of the NMR spectrum of the acetate of cyanomaclurin trimethyl ether led to the correct structure of cyanomaclurin (II). Perkin gave cyanomaclurin its name, because of the intense blue colour it gives with aqueous alkali. Morin is well known as a reagent for aluminium, based on the brilliant bluish green fluorescence produced by the addition of aluminium salt to a solution of morin in ethanol. Due to this property of morin colorimetric determination of aluminium ions are carried out with great accuracy.[7,8] Morin and cyanomaclurin in minute quantities can thus be readily detected and are useful as taxonomic markers.

From the latex of the fruits of A. heterophyllus, a triterpene ketone (cycloartenone) the corresponding alcohol (cycloartenol) and a second triterpenoid alcohol (butyrospermol) have been isolated.[9] From the latex of A. elasticus and A. communis α- and β- amyrin, lupeol and

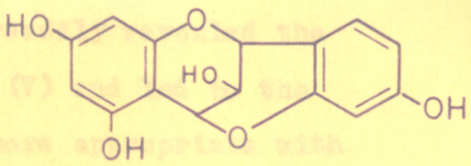
cerotic acid have been isolated.[10] The presence of acetylcholine has been reported from the seeds and leaves of A. integra Merrill.[11] The heartwood of A. lakoocha has been shown to contain 2,4,3',5'-tetrahydroxystilbene[12] (oxyresveratrol). From the bark of A. lakoocha, lupeol acetate and β-amyrin acetate have been isolated.[13] The presence of β-sitosterol in the bark of A. chaplasha[14] has been reported.

By extraction of the powdered heartwood with hexane, Dave and Venkataraman[15] isolated artocarpin. Adapating the classical methods of alkali fusion, alkaline hydrolysis, hydrogenation and ozonolysis, artocarpin was formulated as (III) and later confirmed by the synthesis of its tetrahydrodimethyl ether.[16]

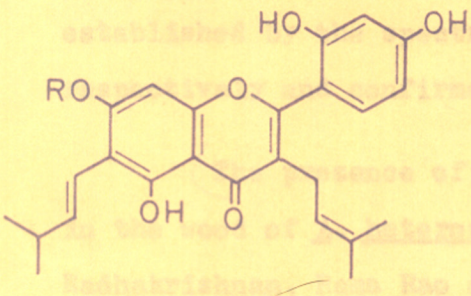
Working on the similar lines isoartocarpin was obtained along with artocarpin in the hexane extract and separated by its sparing solubility in methanol and was deduced as (V).[17] However, an unambiguous synthesis of 6-isoamyl-5,7,2',4'-tetramethoxyflavone[18] and its non-identity with the product of dealkylation of tetrahydro or dihydroisoartocarpin and subsequent methylation, led to suspect the validity of structure (V) and consequently the problem was reinvestigated. The NMR and mass spectral data



MORIN (I)

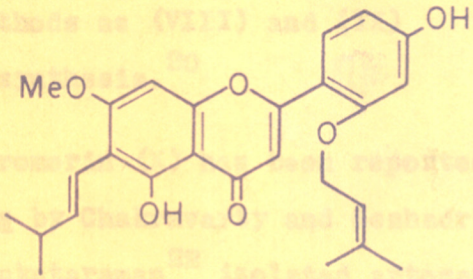


CYANOMACLURIN (II)

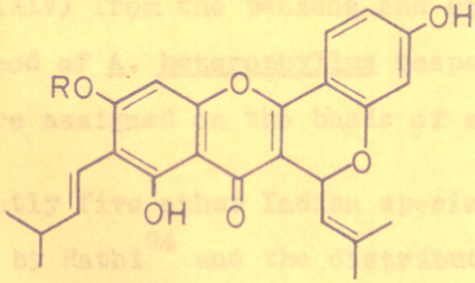


ARTOCARPIN (III); R = Me

NORARTOCARPIN (IV); R = H



ISOARTOCARPIN (V)



CYCLOARTOCARPIN (VI); R = Me

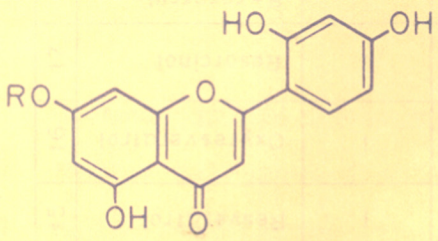
NORCYCLOARTOCARPIN (VII); R = H

which were made available later readily revealed the incompatibility of the structure (V) and led to the modified structure (VI). To be more appropriate with the structure, isoartocarpin has been renamed as cycloartocarpin.[19]

Dave, Telang and Venkataraman[20] isolated artocarpetin and artocarpanone from the benzene extract of the heartwood of A. heterophyllus and their structures established by the spectral methods as (VIII) and (IX) respectively and confirmed by synthesis.[20]

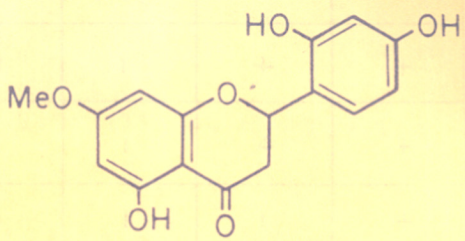
The presence of dihydromorin (X) has been reported in the wood of A. heterophyllus by Chakravarty and Seshadri.[21] Radhakrishnan, Rama Rao and Venkataraman[22] isolated artocarpesin (XI) and norartocarpetin (XII) from the ethyl acetate extract of the A. heterophyllus heartwood, and the structures were assigned on the basis of spectral data. Parthasarathy et al.[23] have isolated cycloartocarpesin (XIII) and oxydihydroartocarpesin (XIV) from the benzene and ethyl acetate extracts of the heartwood of A. heterophyllus respectively, and the structures were assigned on the basis of spectral data.

Recently five other Indian species of Artocarpus were examined by Rathi[24] and the distribution of phenolics in the heartwoods of all six species is shown in Table 1.

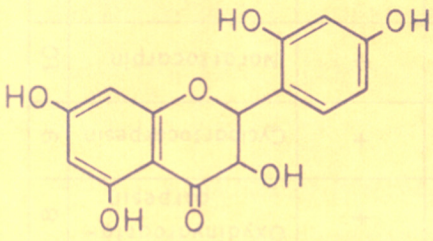


ARTOCARPETIN (VIII); R=Me

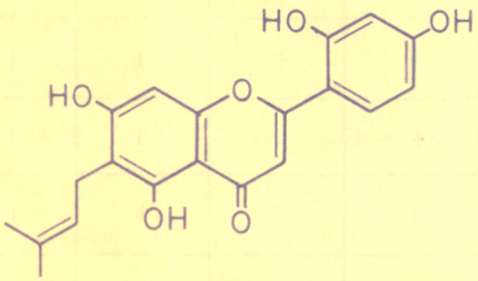
NORARTOCARPETIN (XII); R=H



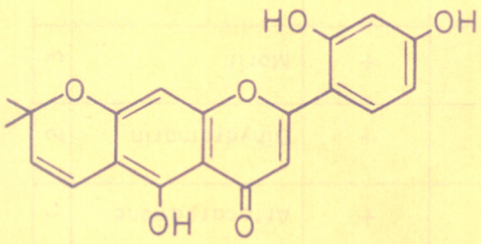
ARTOCARPANONE (IX)



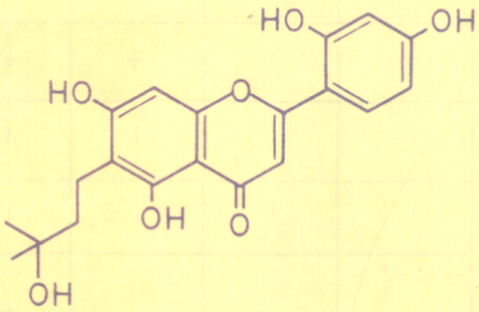
DIHYDROMORIN (X)



ARTOCARPESIN (XI)



CYCLOARTOCARPESIN (XIII)



OXYDIHYDROARTOCARPESIN (XIV)

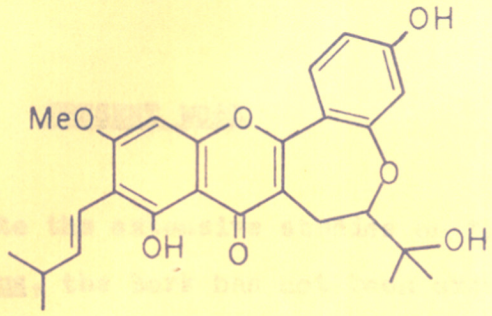
TABLE 1

DISTRIBUTION OF PHENOLICS IN THE HEARTWOOD OF SIX ARTOCARPUS SPECIES

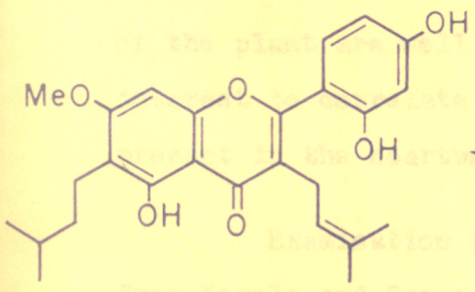
	Artocarpus species	1 Artocarpanone	2 Dihydromorin	3 Morin	4 Cyanomaclurin	5 Norartocarpetin	6 Artocarpetin	7 Artocarpesin	8 Oxydihydroarto-carpesin	9 Cycloartocarpesin	10 Norartocarpin	11 Artocarpin	12 Norcycloarto-carpin	13 Cycloartocarpin	14 Chaplashin	15 Resveratrol	16 Oxyresveratrol	17 Resorcinol	18 β-resorcyl-aldehyde
1	A. heterophyllus	+	+	+	+	+	+	+	+	+	+	+	−	+	−	−	−	−	−
2	A. hirsutus	+	+	+	+	+	+	+	+	+	−	+	−	+	−	−	−	−	−
3	A. gomezianus*	−	−	+	−	+	−	+	−	−	−	+	−	+	−	−	−	−	−
4	A. incisa	−	−	+	−	+	−	−	−	−	−	+	−	+	−	−	−	−	−
5	A. lakoocha	−	−	−	−	−	−	−	−	−	+	+	+	+	−	−	+	+	−
6	A. chaplasha	−	−	−	−	−	−	+	−	+	−	+	−	+	+	+	+	+	+

* MESOERYTHRITOL HAS BEEN ISOLATED FROM THE HEARTWOOD OF THIS SPECIES

Artocarpin and cycloartocarpin occur in all the six species. During his investigation, Rathi isolated norartocarpin (IV) and norcycloartocarpin (VII) from A. heterophyllus and A. lakoocha respectively. The structures of these compounds were assigned on the basis of spectral data. Except for the absence of norartocarpin, A. hirsutus is identical with A. heterophyllus in its flavonoid content; the two species are distinguished from others by the presence of cyanomaclurin and by much greater complexity of flavonoid distribution. Artocarpus gomezianus and A. incisa form a second group with four or five flavones in common. A. lakoocha and A. chaplasha are in a third group, characterised by the presence of resorcinol and oxyresveratrol. A. chaplasha contains β-resorcylaldehyde, resveratrol, and a new uniquely constituted flavone, chaplashin (XV). The structure of chaplashin was demonstrated by spectral methods, and by the synthesis of the racemic dihydrochaplashin (XVIII) from the compound (XVII) obtained by the action of dichlorodicyanobenzoquinone (DDQ) on dihydroartocarpin (XVI).

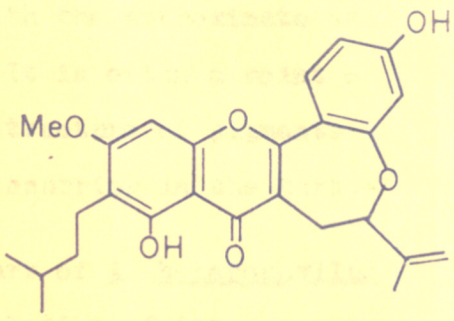


CHAPLASHIN (XV)



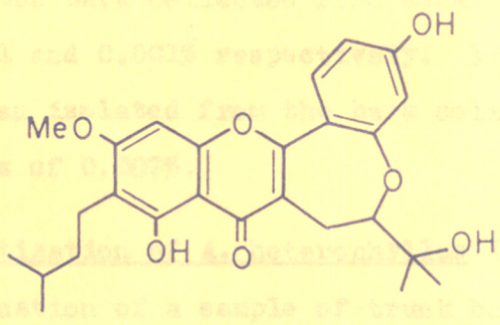
DIHYDROARTOCARPIN (XVI)

DDQ



(XVII)

10% H_2SO_4



DIHYDROCHAPLASHIN (XVIII)

PRESENT WORK

Despite the extensive studies on the heartwood of A. heterophyllus, the bark has not been examined. The deeper layers of wood are decidedly older in age as compared with more superficial layers, because the secondary growth takes place by the activity of cambium which adds layers of wood year after year and in many plants the phenomenon of annual ring formation and its correlation with the approximate age of the plant are well established. It is often a point of interest to correlate the chemical structure of pigments present in the heartwood and those occurring in the bark.

Examination of the trunk bark of A. heterophyllus from Kerala and Poona led to the isolation of three novel flavonoids which are present in very minute quantities and which are different from compounds isolated from the heartwood. Cycloheterophyllin and heterophyllin[25] have been isolated from the bark collected from Kerala in yields of less than 0.01 and 0.001% respectively. Isocycloheterophyllin has been isolated from the bark collected from Poona in yields of 0.007%.

Chemical investigation of A. heterophyllus (trunk bark)

Examination of a sample of trunk bark of A. heterophyllus obtained from Kerala revealed the presence of two

flavones cycloheterophyllin (XIX) and heterophyllin (XX).

The presence of these pigments were first brought to light by the examination of the crude acetone extract of the bark by thin layer chromatography on silica gel using benzene acetone (4:1) as solvent system and iodine vapour as developer.

The residue from the hexane and benzene extracts of the bark were dissolved in minimum amount of methanol and allowed to stand when a colourless crystalline compound, m.p. 316° was obtained. It showed a single spot by TLC on silica gel and a violet colour with the Liebermann-Burchard reagent. There was no specific absorption in the UV region (200-400nm). The IR spectrum showed peaks characteristic of hydroxyl (3600 cm^{-1}), carboxylic acid (1720 cm^{-1}) and vinylidene (1645 and 890 cm^{-1}) groups. The compound was identified as betulinic acid.[11] (Superposable IR and mixed m.p. with an authentic sample).

After the removal of betulinic acid the filtrate was concentrated and adsorbed on a polyamide column, and eluted with hexane and benzene. The hexane fractions showed the presence of two compounds (on a TLC silica gel plate), but the pigment in pure form could not be isolated.

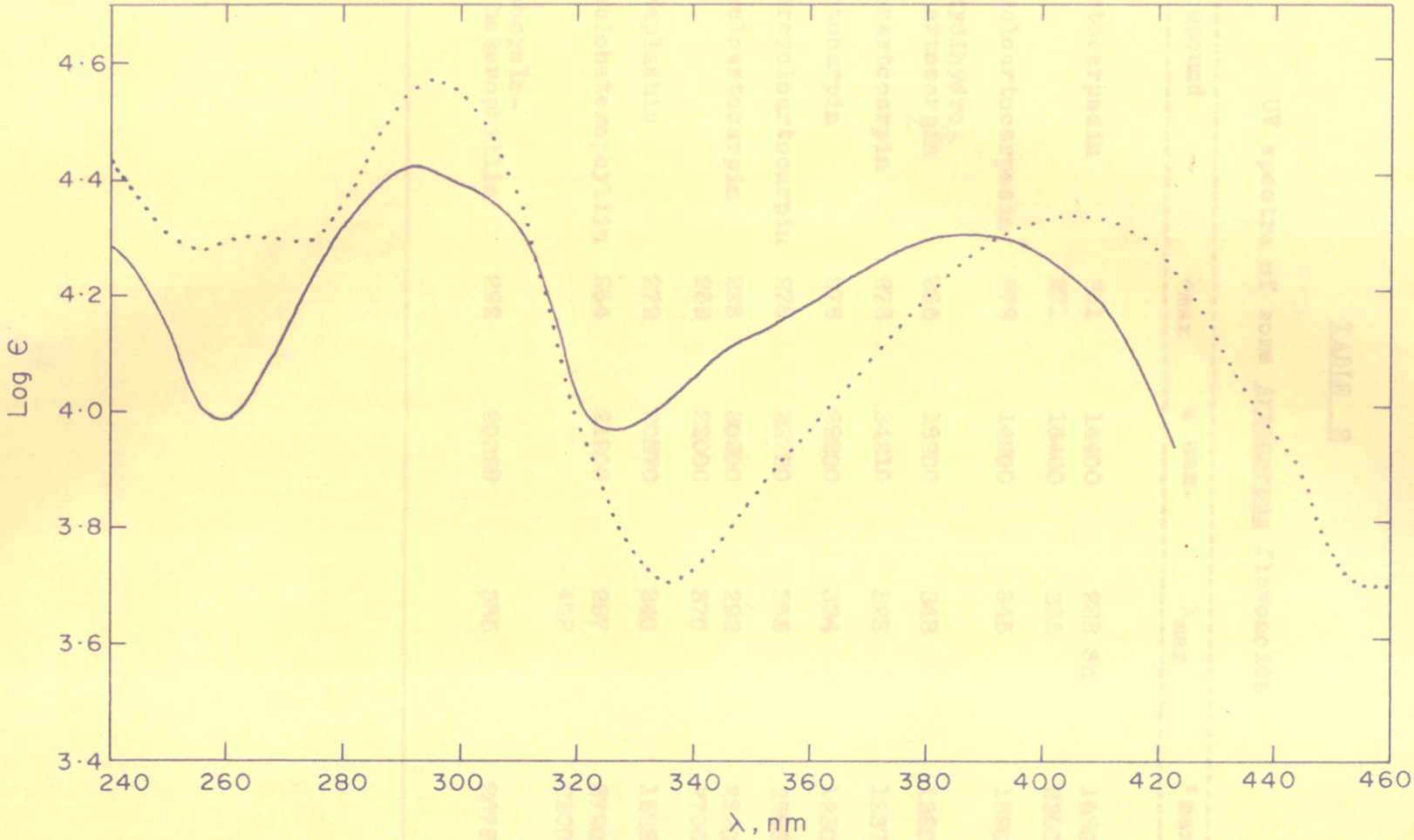
The benzene eluate was concentrated to a small volume and chromatographed on a column of silica gel, monitoring the separation on a TLC using benzene-acetone as a solvent system. Initial fractions eluted by benzene contained a yellow compound in very small amount identified by its R_f value (silica gel) as cycloartocarpin.[19] The next fractions gave cycloheterophyllin which crystallised from methanol in yellow needles, m.p. 205-206°. The next fractions gave a mixture of three compounds. One of the compounds was identified by its R_f value (TLC silica gel) as artocarpin.[15] A minor compound moving slightly faster than artocarpin could not be isolated. The other compound moving slower than artocarpin could be isolated in small quantities, but this compound, heterophyllin, was contaminated with traces of artocarpin.

Cycloheterophyllin gives a wine red colour in the Shinoda test and green colour with alcoholic ferric chloride. The elemental analysis and molecular weight (M^+ 502) are in agreement with the molecular formula, $C_{30}H_{30}O_7$. It forms a dimethyl ether (M^+ 530) with ethereal diazomethane; a green colouration with alcoholic ferric chloride indicates the presence of a bonded hydroxyl group. On prolonged treatment with excess dimethylsulphate and potassium carbonate

in boiling acetone, a trimethyl ether (M^+ 544) was obtained. Hydrogenation of trimethyl ether in acetic acid in the presence of Adams catalyst yielded a hexahydro derivative (M^+ 550), showing the presence of three ethylenic bonds.

In its UV spectrum (Fig. 1) [$\lambda_{max}^{ethanol}$ nm (log ε), 264 (4.32), 297 (4.57), 402 (4.34)] cycloheterophyllin closely resembles cycloartocarpin[14] (VI) rather than artocarpin[10] (III), the two major flavonoid pigments isolated from the heartwood. Table 2 gives UV data of some of the prenylated flavonoids isolated from Artocarpus. The long wavelength absorption in artocarpin and cycloartocarpin is at 324 and 370 nm respectively, and the large bathochromic shift in the latter was explained[26] by the coplanarity of rings B, C and D, resulting in maximum conjugation between the chromone and phenyl rings. In artocarpin the 3-γ,γ-dimethylallyl substituent forces the 2-phenyl group out-of-plane with the chromone ring. The presence of a free 4'-hydroxyl group as in all the other Artocarpus pigments was established by the shift of 402 nm band to 440 nm with a marked increase in intensity on adding sodium ethoxide to the ethanolic solution.[27]

The IR spectrum (nujol) of cycloheterophyllin showed characteristic features for the 2,2-dimethylchromene system[28] with bands at 1375 and 1360 cm^{-1}



FIG·1 UV SPECTRA QF CYCLOHETEROPHYLLIN (·····) AND ISOCYCLOHETEROPHYLLIN (——) IN ETHANOL

TABLE 2

UV spectra of some Artocarpus flavonoids

Compound	λ_{max}	ε max.	λ_{max}	ε_{max}
Artocarpesin	251	14400	288 Sh	10800
	271	16400	355	23000
Cycloartocarpesin	278	14200	345	15200
Oxydihydro-artocarpin	265	18200	348	13800
Norartocarpin	278	34210	323	12370
Artocarpin	278	29200	324	12300
Norcycloartocarpin	276	26720	365	25860
Cycloartocarpin	225	30200	292	28200
	259	23000	370	27000
Chaplashin	272	20870	340	15280
Cycloheterophyllin	264	21200	297	37200
			402	22000
Isocyclo-heterophyllin	292	20329	385	26759

attributable to the gem-dimethyl groups and a -C=C- band at 1630 cm^{-1}. In addition, bands were observed at 3460 (phenolic OH) and 1645 cm^{-1} (C=O in a 5-hydroxy flavone).

The NMR spectra of cycloheterophyllin and its derivatives gave much more detailed structural data. The NMR spectrum of cycloheterophyllin (solvents DMSO and pyridine; Fig. 2) indicates the absence of alkoxyl groups and the presence of three dimethylallyl groups. Two vinyl doublets at 3.42 and 4.27 (chemical shifts on the τ scale) in conjunction with a six proton singlet at 8.53 correspond to the olefinic protons and the methyl groups in a chromene system. Two vinylic methyls at 8.03 and 8.37, together with doublets (J = 9.5 Hz) at 3.93 and 4.58, suggest that the 2'-hydroxyl of the B-ring in a flavone has oxidatively cyclized with the doubly allylic methylene of a prenyl chain in the 3-position as in cycloartocarpin.[19] Further both these methyls are split by the vinylic protons which appear as a broad doublet at 4.58, while the homoallylic interaction of the proton of the ring D is sterically suppressed. Confirmation of the presence of the cycloartocarpin ring system is provided by the fact that the hydrogen of ring D in the trimethyl ether (solvent $CDCl_3$) (Fig. 3) appears as a doublet at 3.68 and shifts to 4.42 as a quartet in the corresponding hexahydro derivative,

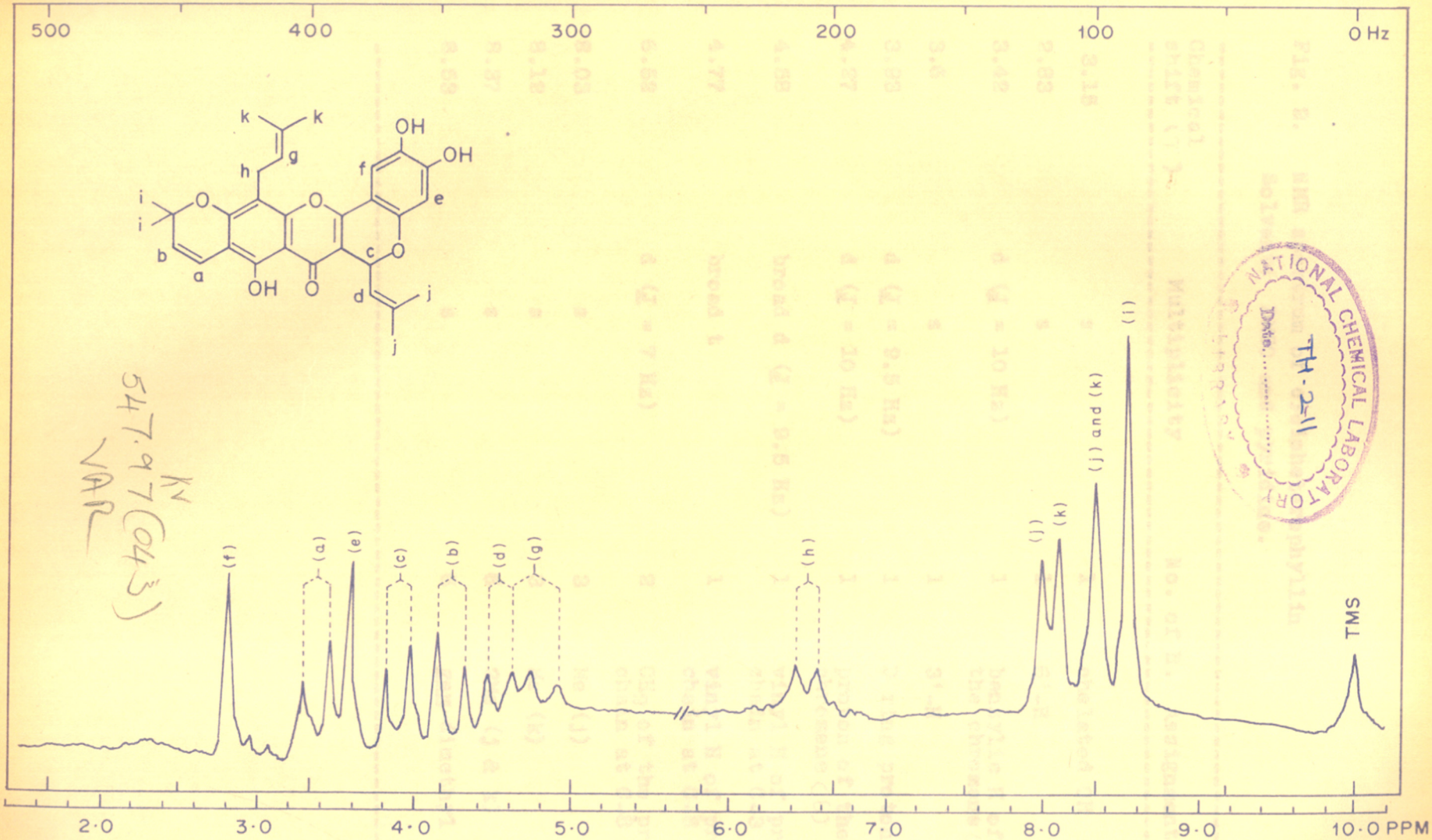


FIG. 2 NMR SPECTRUM OF CYCLOHETEROPHYLLIN IN DMSO AND PYRIDINE

Fig. 2. NMR spectrum of cycloheterophyllin
Solvent: DMSO and pyridine.

Chemical shift (τ)	Multiplicity	No. of H.	Assignment
- 3.15	s	1	chelated OH
2.83	s	1	6'-H
3.42	d (J = 10 Hz)	1	benzylic H of the chromene (a)
3.6	s	1	3'-H
3.93	d (J = 9.5 Hz)	1	D ring proton
4.27	d (J = 10 Hz)	1	proton of the chromene (b)
4.58	broad d (J = 9.5 Hz)	1	vinyl H of prenyl chain at C-3
4.77	broad t	1	vinyl H of prenyl chain at C-8
6.52	d (J = 7 Hz)	2	CH_2 of the prenyl chain at C-8
8.03	s	3	Me (j)
8.12	s	3	Me (k)
8.37	s	6	2Me (j & k)
8.53	s	6	gem-dimethyl group (c)

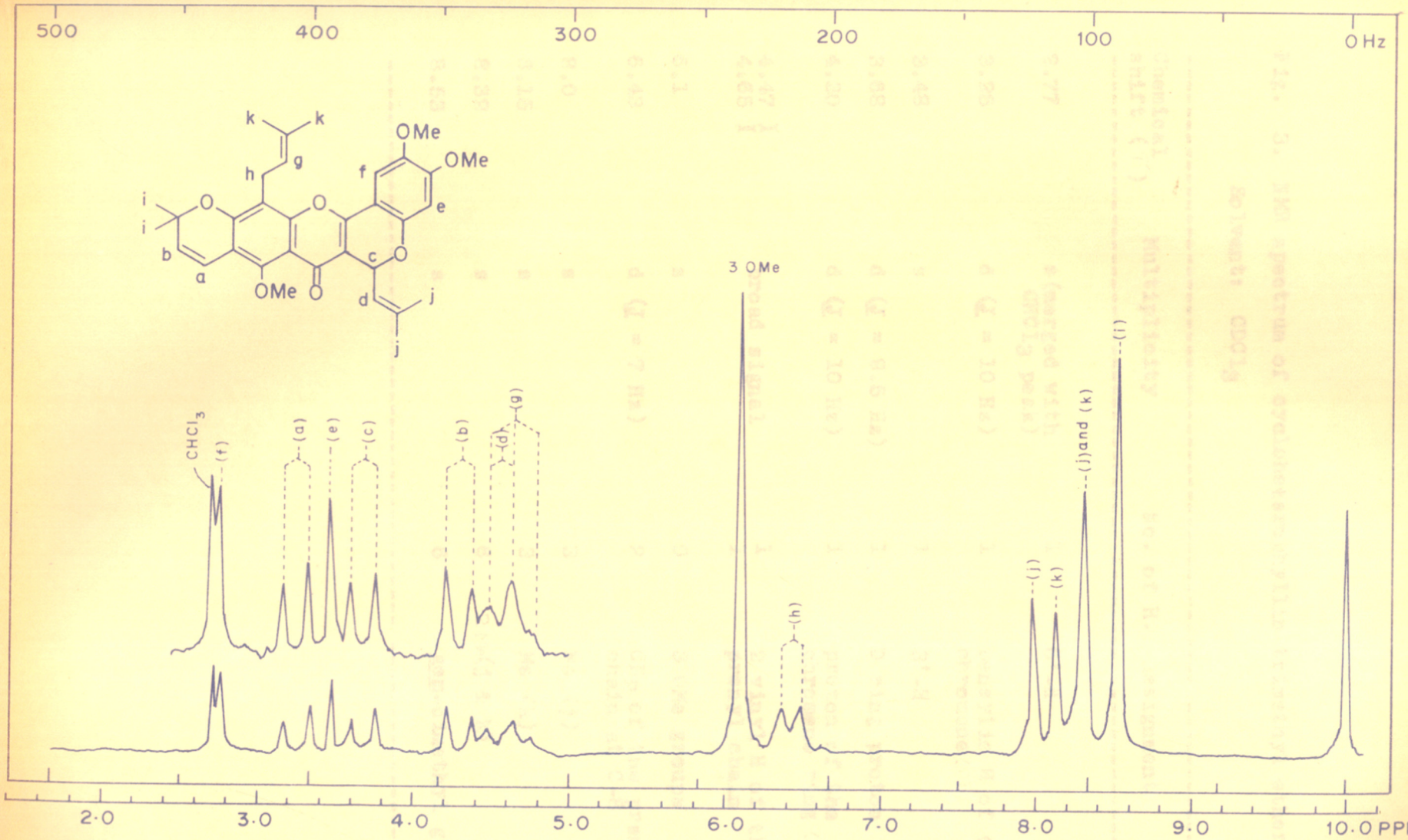


FIG. 3 NMR SPECTRUM OF CYCLOHETEROPHYLLIN TRIMETHYL ETHER IN $CDCl_3$

Fig. 3. NMR spectrum of cycloheterophyllin trimethyl ether.

Solvent: $CDCl_3$

Chemical shift (τ)	Multiplicity	No. of H.	Assignment
2.77	s (merged with $CHCl_3$ peak)	1	6'-H
3.25	d (J = 10 Hz)	1	benzylic H of the chromene (a)
3.48	s	1	3'-H
3.68	d (J = 9.5 Hz)	1	D ring proton
4.30	d (J = 10 Hz)	1	proton of the chromene ring (b)
4.47 4.65	broad signal	1 1	2 vinyl H of the prenyl chain
6.1	s	9	3 OMe groups
6.43	d (J = 7 Hz)	2	CH_2 of the prenyl chain at C-8
8.0	s	3	Me (j)
8.15	s	3	Me (k)
8.32	s	6	2 Me (j & k)
8.53	s	6	gem-dimethyl group (i)

because this hydrogen is on an asymmetric carbon and the adjacent methylene group in the hydrogenated product forms an AB system.[29,30] The third prenyl unit is in the form of a γ,γ - dimethylallyl group attached to an aromatic nucleus as shown by the signals at 8.12 and 8.37 (methyls), a two-proton doublet (J = 7 Hz) at 6.52 (methylene, benzylic and allylic) and a broad one proton triplet at 4.77. Two singlet signals at 2.83 and 3.60, not disappearing in the hexahydro derivative (Fig. 4) can be associated with two aromatic hydrogens. Except for cycloheterophyllin, all the flavones so far isolated from the heartwood of A. heterophyllus have the unique feature of having the B-ring oxygenated at the 2'- and 4'- positions only, and the aromatic hydrogens of the B-ring showed the characteristic ABC pattern of the β-resorcylic acid type. In all these compounds 6'-H appeared as a doublet (J = 9 Hz) around 2.2-2.4 normally expected in a flavone due to the deshielding influence of the 2,3-double bond on this proton; a notable exception was artocarpin in which it appeared at 2.9 because of the B-ring being non-planar and out of conjugation with the chromone ring. Since the UV absorption and the NMR data concerning the prenyl group attached to the 3-position of the flavone nucleus in cycloheterophyllin proved beyond doubt that cyclization of the 3-prenyl group as in cyclo-artocarpin has taken place, the appearanceof 6'-proton at

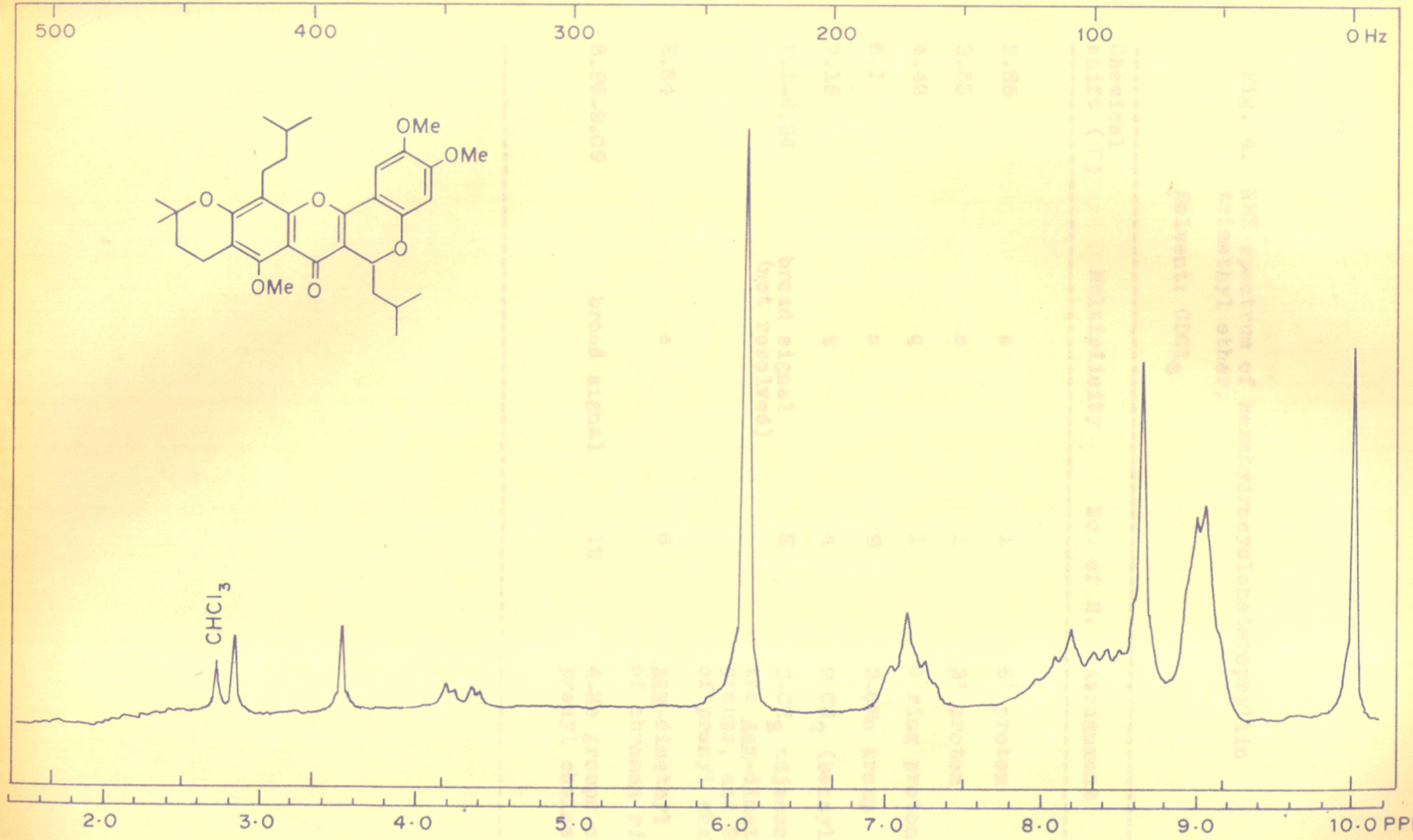


FIG. 4 NMR SPECTRUM OF HEXAHYDROCYCLOHETEROPHYLLIN TRIMETHYL ETHER IN $CDCl_3$

Fig. 4. NMR spectrum of hexahydrocycloheterophyllin trimethyl ether.

Solvent: $CDCl_3$

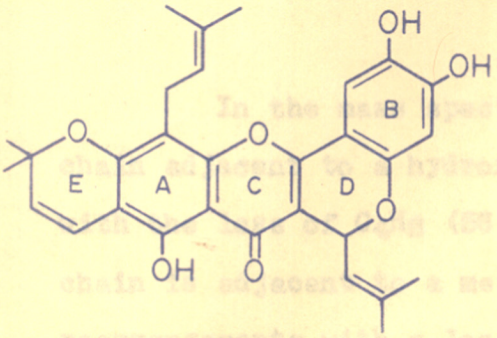
Chemical shift (τ)	Multiplicity	No. of H.	Assignment
2.85	s	1	6' proton
3.53	s	1	3' proton
4.42	q	1	D ring proton
6.1	s	9	3-OMe group
7.15	t	4	2 CH_2 (benzylic)
8.1-8.34	broad signal (not resolved)	8	3-CH_2 adjacent to the gem-dimethyl groups, and 2-CH of prenyl chains.
8.64	s	6	gem-dimethyl group of chroman ring
8.96-9.09	broad signal	12	4-Me groups of prenyl chains.

2.83 as a singlet (about 0.5 ppm upfield in comparison with the 6'-proton in cycloartocarpin appearing as a doublet) shows that it is flanked by a hydroxyl rather than an alkyl group in the 5'- position. Cycloheterophyllin can then be represented by the partial structure (XXI) or the isomer with the angular alignment of rings D and E, assuming that the A-ring has the phloroglucinol hydroxylation pattern as in other Artocarpus flavonoids, and remembering that there is a free hydroxyl group in the 5'-position. The remaining problem is to decide if the third prenyl group is in the 3'- or 6- or 8-position. The singlet at 3.60 is in the region of an aromatic proton between two carbon atoms carrying oxygen substituents, and may represent 8-H or 3'-H, but it can be assigned to the latter for two reasons: (a) in cycloartocarpin and artocarpin 8-H appeared at a slightly higher field (3.8), and (b) no coupling (J = 0.6-0.7 Hz) has been observed between the olefinic proton of the chromene ring (Hα) and 8-H in cycloheterophyllin or its derivatives, which is to be expected if the 8-position is unsubstituted.[31]

The position and relative orientation of the methoxyl groups in cycloheterophyllin dimethyl ether can be inferred from the benzene-induced solvent shifts of the methoxyl resonances. Two methoxyls appear as a single signal at 6.1 in $CDCl_3$, and on the addition of benzene it shifts

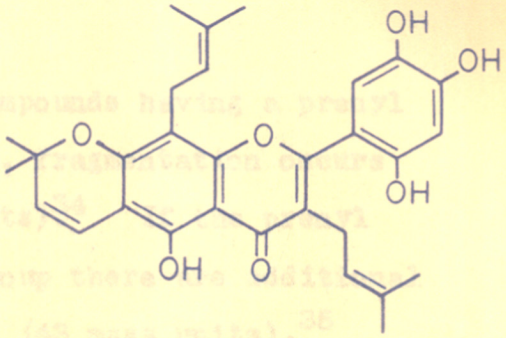
upfield as two signals at 6.35 and 6.63, indicating that at least one position adjacent to each methoxyl group is unsubstituted. The latter signal can be assigned to 4'-OMe and the larger shift it suffers compared with the methoxyl shift in anisole (0.46 ppm) is because of its being in conjugation with a C=O group; there is a consequent decrease in π-electron density at the oxygen atom in the 4'-position and an enhancement of its association with the benzene molecule.[32] The comparatively smaller upward shift suffered by the 5'-OMe can be explained by the presence of electron-donating ortho and para substituents.[33]

The angular structure (XXII) for cycloheterophyllin is ruled out by a consideration of the changes in chemical shift of the chromene olefinic protons in its dimethyl ether compared with the corresponding acetyl derivative. In the dimethyl ether, the two protons marked α- and β- appear at 3.25 and 4.40 and in the corresponding acetyl derivative at 3.45 and 4.24 respectively. These changes are of same sign and similar order of magnitude as those observed by Merlini and his associates[31] for several 2,2-dimethylchromenes in which the hydroxyl group is in the 5-position and, therefore, peri to the α-hydrogen.



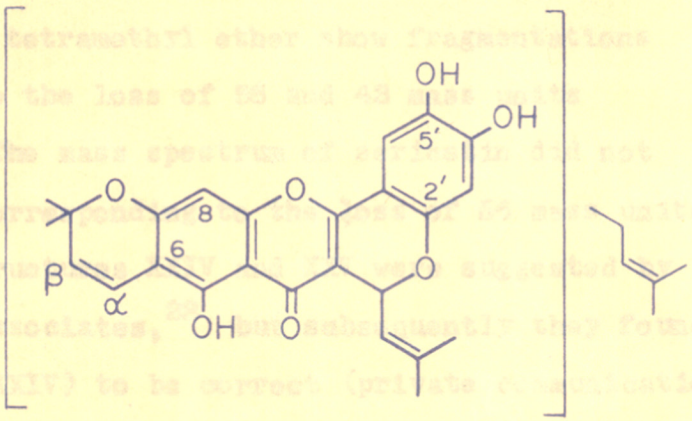
(XIX)

CYCLOHETEROPHYLLIN

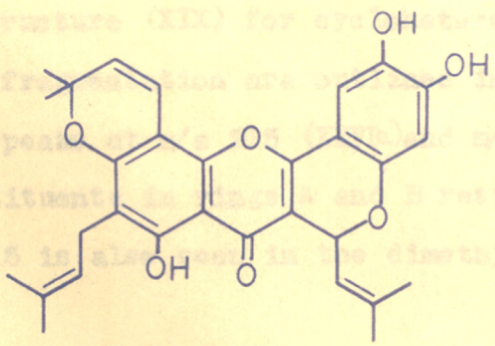


(XX)

HETEROPHYLLIN



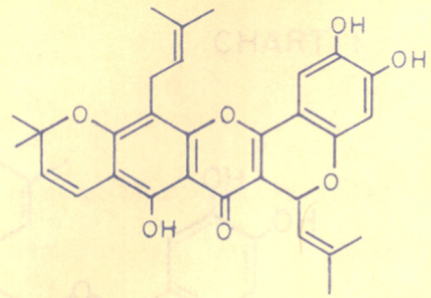
(XXI)



(XXII)

In the mass spectra of compounds having a prenyl chain adjacent to a hydroxyl group, fragmentation occurs with the loss of C_4H_8 (56 mass units).[34] If the prenyl chain is adjacent to a methoxyl group there are additional rearrangements with a loss of C_3H_7 (43 mass units).[35]

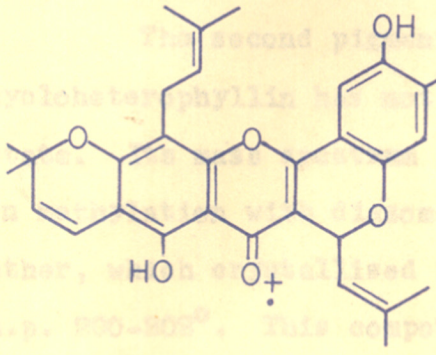
These observations are in general agreement with the mass spectra of a number of flavones isolated from Artocarpus and Morus species.[24] For example mulberrin (XXIII) and its tetramethyl ether show fragmentations corresponding to the loss of 56 and 43 mass units respectively. The mass spectrum of sericetin did not show any peak corresponding to the loss of 56 mass units. Earlier, two structures XXIV and XXV were suggested by Ollis and his associates,[28] but subsequently they found the structure (XXIV) to be correct (private communication from Prof. W.D. Ollis). The mass spectra of cycloheterophyllin (Fig. 5) and its trimethyl ether have not shown any peaks corresponding to M-56 or M-43 ions respectively, thus supporting structure (XIX) for cycloheterophyllin. The main paths of fragmentation are outlined in Chart 1 (Fig. 5). The two peaks at m/e 215 (XXVIa) and m/e 216 (XXVIb) indicate the substituents in rings A and B respectively. The peak at m/e 215 is also seen in the dimethyl ether.


OH
OH
O
O
O
O
OH
O

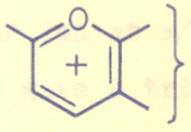
RELATIVE INTENSITY
100
80
60
40
20
0
447
216
487
502
236
215
236·5
243
237
243·5
431
433
459
100
200
220
240
250
420
440
450
460
480
500
510
m/e

FIG. 5

CHART 1



m/e 502 (35%)



m/e 487 (39%)

m/e 243·5 (5%)

$-CH_3$

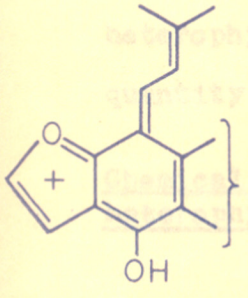
$-C_3H_4O$

m/e 431 (6%)

$-C_5H_9$

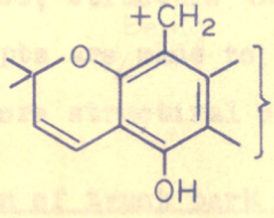
m/e 433 (4%)

$-C_3H_7$

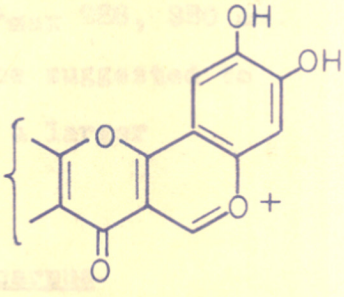


m/e 459 (6%)

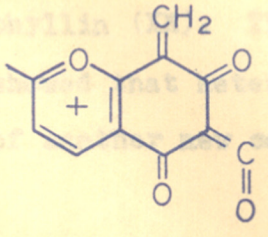
$-C_4H_7$



or

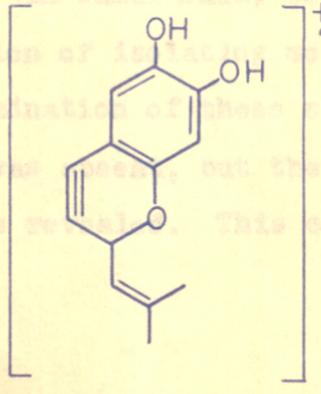


m/e 447 (100%)



m/e 215 (10%)

(XXVI a



m/e 216 (42%)

(XXVI) b

Heterophyllin

The second pigment, heterophyllin, accompanying cycloheterophyllin has not yet been obtained in a pure state. Its mass spectrum shows the molecular ion at m/e 504. On methylation with diazomethane in ether it gave a trimethyl ether, which crystallised from methanol in pale yellow needles, m.p. 200-202°. This compound gives a green colour with alcoholic ferric chloride. The mass spectrum of the trimethyl ether of heterophyllin shows the molecular ion at m/e 546, indicating that cyclization to one of the rings, D or E, of cycloheterophyllin has not taken place. Electronic spectrum of this compound shws peaks at λ_{max} 286, 380 nm. From the above evidence, structure (XX) can be suggested to heterophyllin. Attempts are being made to isolate a larger quantity and obtain more structural data.

Chemical investigation of trunk bark of Artocarpus heterophyllus from Poona

The orange-red powder scraped from the underside of the bark of A. heterophyllus collected from Pashan Village (near Poona) and Kumbakonam (in Tamil Nadu) were separately examined with the intention of isolating more of heterophyllin (XX). The TLC examination of these crude extracts showed that heterophyllin was absent, but the presence of another new compound was revealed. This compound

This compound isocycloheterophyllin, on a TLC plate moves faster than cycloheterophyllin (XIX) and artocarpin (III), but slower than cycloartocarpin (VI) (solvent system: 1:4; acetone-benzene).

The benzene soluble portion of the acetone extract of the bark from Poona was extracted with 5% sodium hydroxide solution. The phenolic fraction was recovered by acidification. The sodium hydroxide soluble portion was chromatographed on a column of silica gel using benzene and benzene-acetone mixture (with increasing percentage of acetone upto 30%) as the eluant. Fractions were collected and monitored by TLC using benzene-acetone as solvent system and iodine vapour as developer. Initial fractions gave a yellow compound in very minute quantity identified by its R_f value (silica gel, TLC) as cycloartocarpin. The next fraction gave isocycloheterophyllin which crystallised from benzene in yellow needles, m.p. 231°.

Further fractions gave cycloheterophyllin identified by its R_f value (silica gel TLC).

Isocycloheterophyllin

Isocycloheterophyllin gives a green colour with alcoholic ferric chloride. The elemental analysis and molecular weight (M^+ 502) are in agreement with the

molecular formula, $C_{30}H_{30}O_7$, and showed that it is an isomer of cycloheterophyllin. Isocycloheterophyllin forms a dimethyl ether, m.p. 250-252°, (M⁺ 530) with ethereal diazomethane. It gives a green colouration with alcoholic ferric chloride indicating the presence of a bonded hydroxyl group. On prolonged treatment with excess of dimethyl sulphate and potassium carbonate in boiling acetone a trimethyl ether is formed, m.p. 180-82°. In its UV spectrum (Fig. 1) isocycloheterophyllin shows maxima at λ_{max} log ε, 292 (4.43), 385 (4.30) nm. A comparison of the UV data of Artocarpus pigments (Table 2) shows that isocycloheterophyllin resembles cycloartocarpin and cycloheterophyllin.

The IR spectrum (nujol; Fig. 6) of isocycloheterophyllin showed bands at 3540, 1645 and 1630 cm^{-1} (phenolic hydroxyl, C=O and C=C respectively). Bands at 1355 and 1370 cm^{-1} indicate the gem-dimethyl group of a 2,2-dimethylchromene system.[28]

The NMR spectrum of isocycloheterophyllin trimethylsilyl ether in CCl_4 showed the absence of alkoxyl groups. The NMR spectrum of the trimethyl ether of isocycloheterophyllin in $CDCl_3$ (Fig. 7) shows the characteristic signals of a 2,2-dimethylchromene system (two vinyl doublets (J = 10 Hz) at 3.23 and 4.3 in conjunction with a six-proton singlet at 8.52. A γ,γ-dimethylallyl group attached to an aromatic

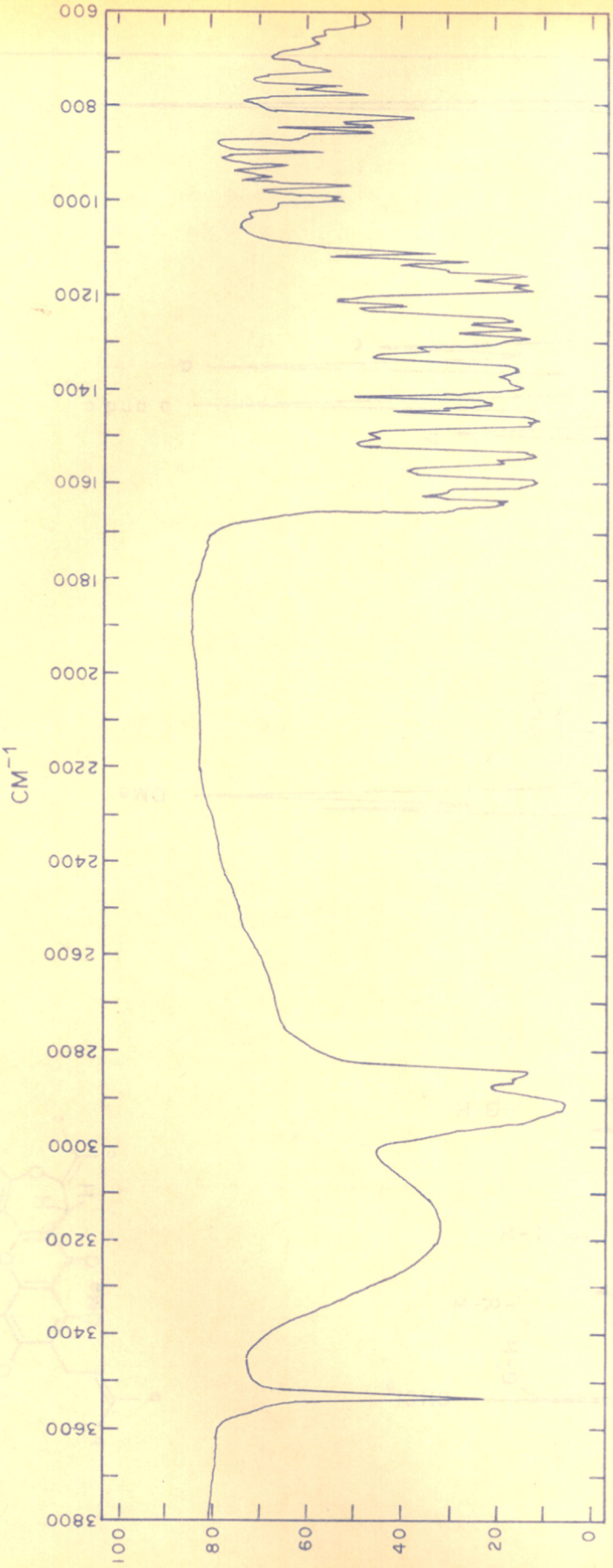
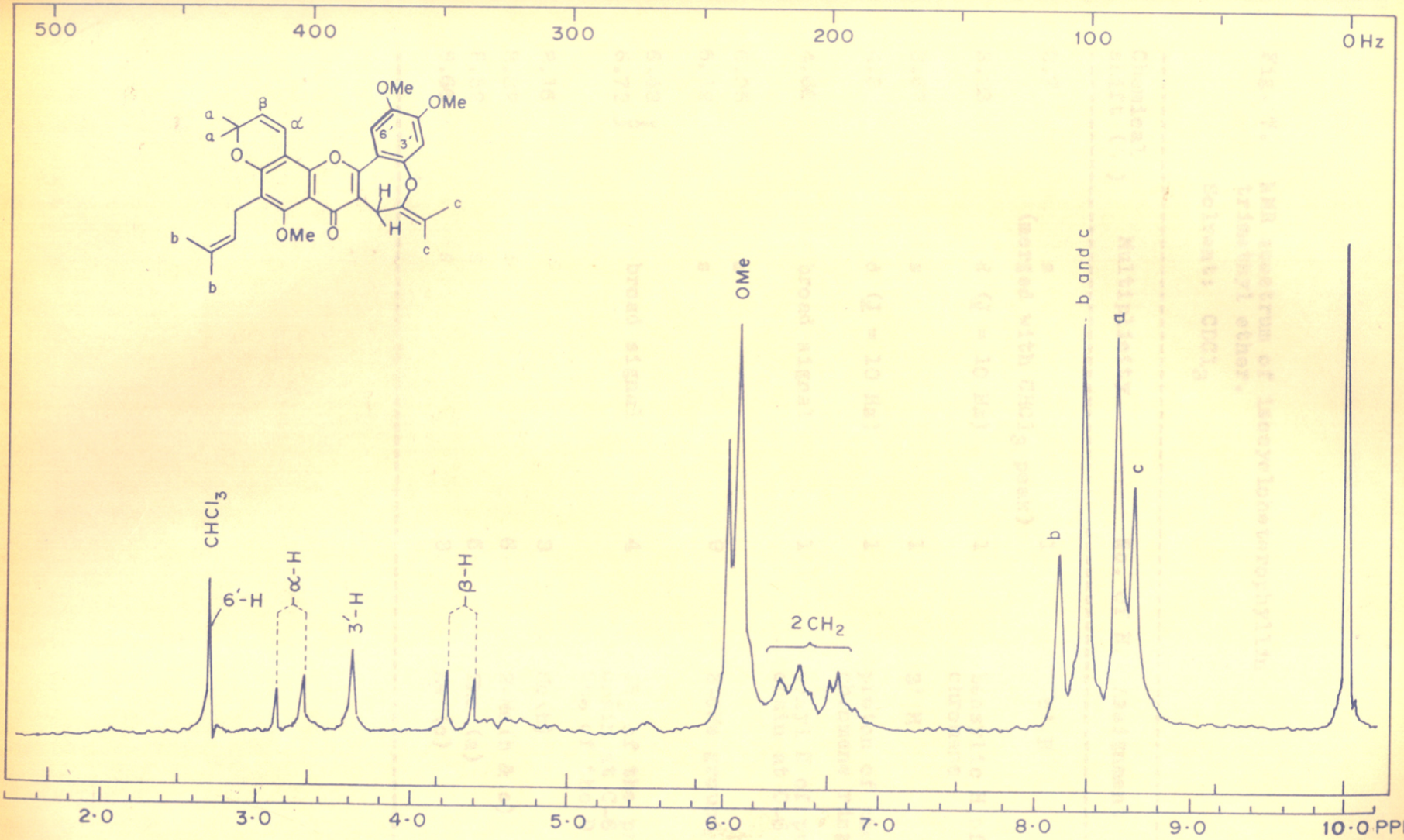


FIG.6 IR SPECTRUM OF ISOCYCLOHETEROPHYLLIN IN NUJOL



FIG·7 NMR SPECTRUM OF ISOCYCLOHETEROPHYLLIN TRIMETHYL ETHER IN $CDCl_3$

Fig. 7. NMR spectrum of isocycloheterophyllin trimethyl ether.

Solvent: $CDCl_3$

Chemical shift (τ)	Multiplicity	No. of H.	Assignment
2.7	s (merged with $CHCl_3$ peak)	1	6'-H
3.23	d (J = 10 Hz)	1	benzylic H of the chromene
3.62	s	1	3' H
4.3	d (J = 10 Hz)	1	proton of the chromene ring
4.66	broad signal	1	vinyl H of prenyl chain at C-6
6.05 6.12	s s	9	3-OMe groups
6.43 ⌇ 6.73 ⌇	broad signal	4	CH_2 of the prenyl chain at C-6 and CH_2 of the D ring
8.16	s	3	Me (b)
8.32	s	6	2-Me (b & c)
8.52	s	6	2Me (a)
8.66	s	3	Me (c)

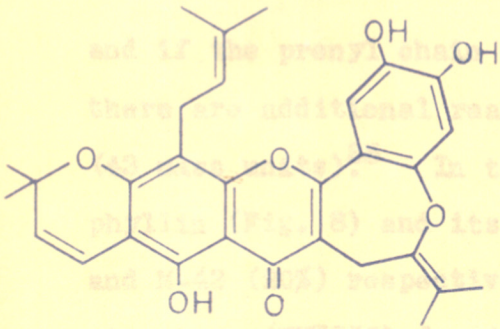
nucleus is shown by the signals at 8.16 and 8.32 (methyls), a two-proton doublet (J = 7 Hz) at 6.43 (methylene, benzylic and allylic) and a broad one-proton signal at 4.66 (vinyl-H). In addition to these signals, a methylene group is seen as a broad multiplet centered at 6.73. The aromatic region of the spectrum shows two singlet signals at 2.67 (merged with the $CHCl_3$ peak) and 3.57 which can be assigned to 6' and 3' protons respectively.

From the above data, it can be concluded that isocycloheterophyllin resembles cycloheterophyllin in many respects except some variation in the D-ring. This was depicted by the absence of the lone proton of the six-membered ring (which appears at 3.68 in cycloheterophyllin) and also having one vinyl hydrogen less compared to cycloheterophyllin. Based on the available evidence, isocycloheterophyllin can be represented by either structures (XXVII) or (XXVIII). Evidence of the 7-membered character of the ring D was provided by the broad 2-proton signal at 6.73 representing the CH_2 group and by the absence of characteristic CH proton of the ring D as in cycloheterophyllin trimethyl ether. The only abnormality in the NMR data is the appearance of one of the vinyl methyls at 8.66. This may be due to the shielding of the methyl group by the dihydro-oxepine ring system.

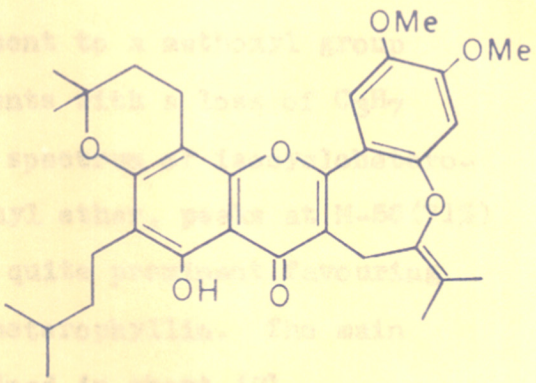
The position and relative orientation of the methoxyl groups in the methyl ether of isocycloheterophyllin can be inferred from benzene induced solvent shifts of the methoxyl resonances. Two methoxyl signals appear at 6.05 and 6.12 and on addition of benzene very slight upfield shift (6.27) was observed. The signal at 6.12 shifts upfield as two signals at 6.55 and 6.64 indicating that atleast one position adjacent to each methoxyl group is unsubstituted.

Isocycloheterophyllin on prolonged hydrogenation in acetic acid in the presence of Adams catalyst yielded only a tetrahydroderivative (M^{+} 506). The dimethyl ether of the tetrahydroderivative was prepared by methylation with etheral diazomethane. The NMR spectrum of tetrahydroderivative of the dimethylether in $CDCl_3$ showed the disappearance of chromene doublets and the vinyl hydrogen of the γ,γ-dimethylallyl group. The other changes in the NMR spectrum are consistant with structure (XXVIIa) or its isomer for the tetrahydroisocycloheterophyllin dimethyl ether.

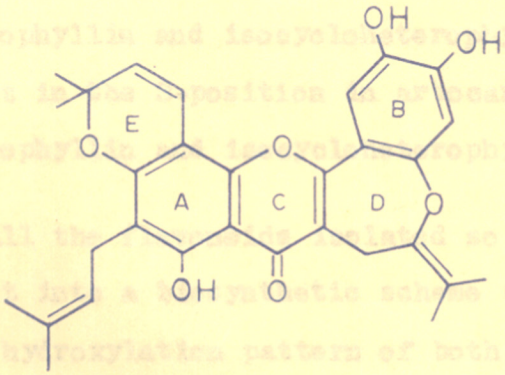
Structure (XXVIII) has been prefered to isocycloheterophyllin on the basis of mass spectral evidence. As discussed in case of cycloheterophyllin, the compounds in which a prenyl chain is adjacent to a hydroxyl group, fragmentation occurs with the loss of C_4H_8 (56 mass units)[34]



(XXVII)



(XXVII a)



(XXVIII)

ISOCYCLOHETEROPHYLLIN

and if the prenyl chain is adjacent to a methoxyl group there are additional rearrangements with a loss of C_3H_7 (43 mass units).[35] In the mass spectrum of isocycloheterophyllin (Fig. 8) and its trimethyl ether, peaks at M-56(71%) and M-43 (20%) respectively are quite prominent favouring structure (XXVIII) for isocycloheterophyllin. The main paths of fragmentation are outlined in chart (2).

Biogenetic aspects

The biogenesis of the Artocarpus pigments is of special interest because of their unique structural features; the β-resorcylic acid orientation of hydroxyl groups in the B-ring in all the compounds (with an additional hydroxyl in cycloheterophyllin and isocycloheterophyllin) and the C_5 substituent in the 3-position in artocarpin, cycloartocarpin, cycloheterophyllin and isocycloheterophyllin.

All the flavoneids isolated so far from A. heterophyllus fit into a biosynthetic scheme (Charts 3, 4 and 5) in which the hydroxylation pattern of both the A and B rings is fixed at the chalcone stage; but cycloheterophyllin and isocycloheterophyllin are exceptional to the above rule.

The only other flavones having 2',4'-hydroxylation are morin, the colouring matter of 'old fustic' (Morus tinctoria)[36] which also occurs in M. alba,[37] M. bambycis[38]

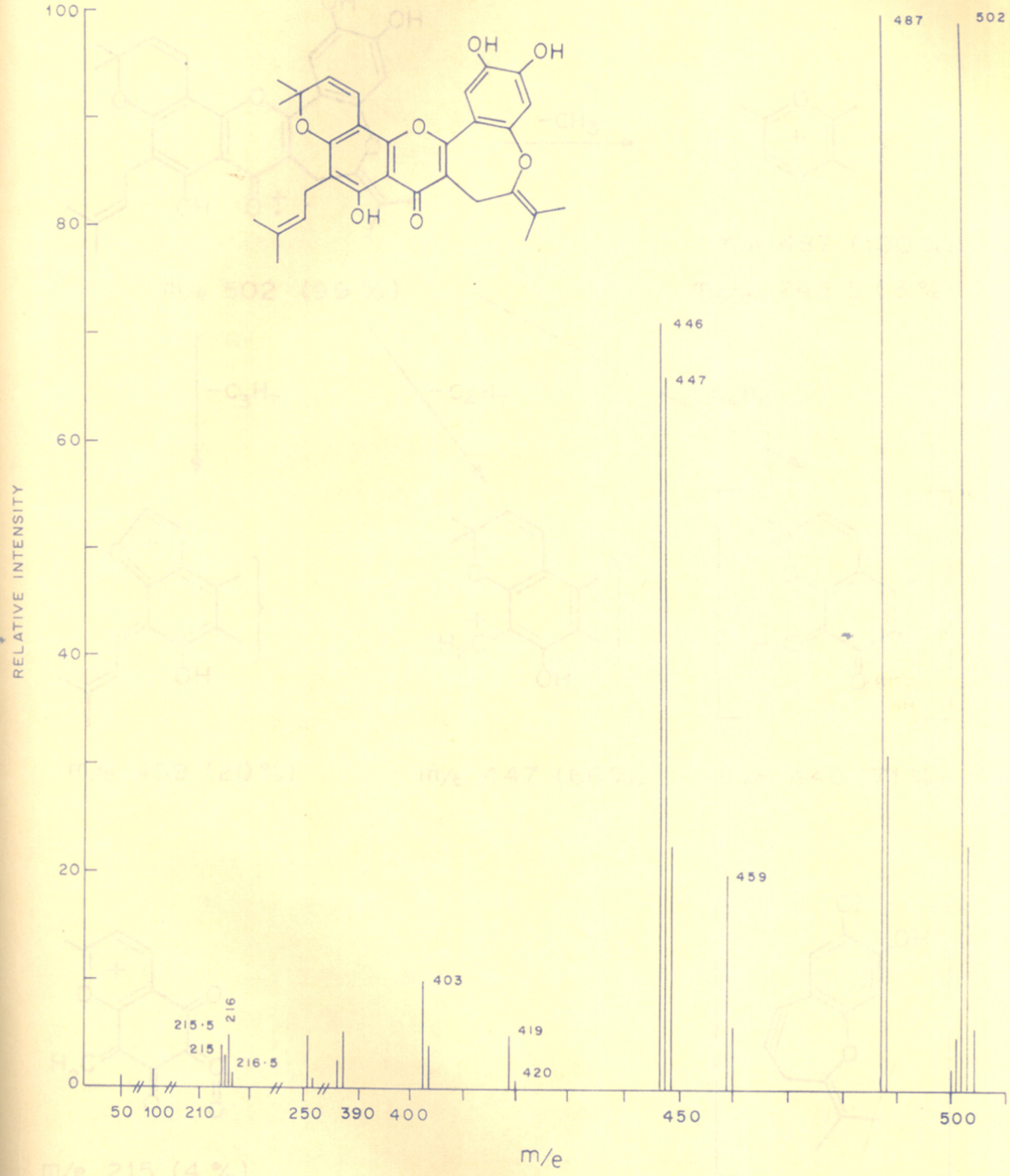
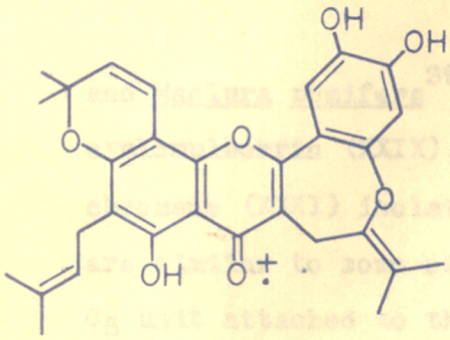
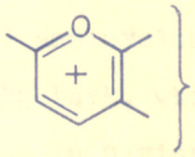

100
80
60
40
20
0
RELATIVE INTENSITY
OH
OH
OH
O
O
O
O
487
502
446
447
459
403
419
420
215·5
216
215
216·5
50 100 210
250 390 400
450
500
m/e

FIG. 8



m/e 502 (99 %)

$-CH_3$



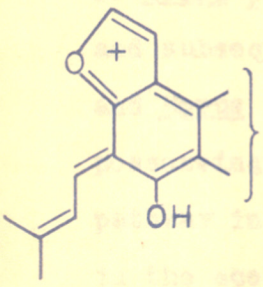
m/e 487 (100 %)

m/2e 243·5 (3 %)

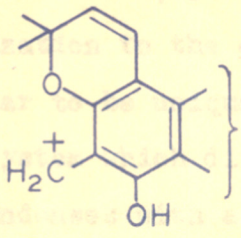
$-C_3H_7$

$-C_4H_7$

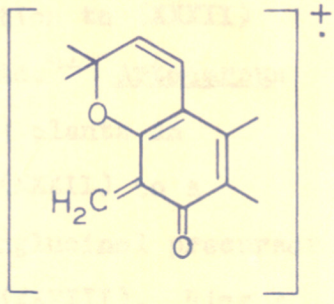
$-C_4H_8$



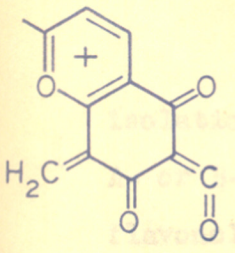
m/e 459 (20 %)



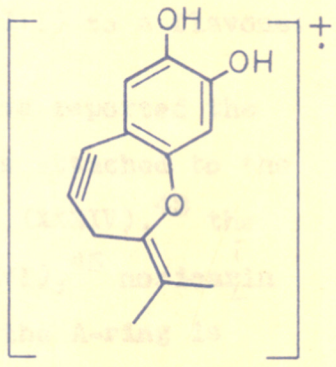
m/e 447 (66 %)



m/e 446 (71 %)



m/e 215 (4 %)

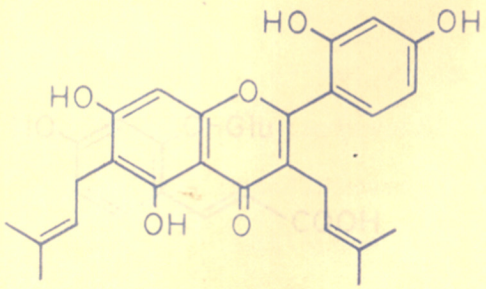


m/e 216 (5%)

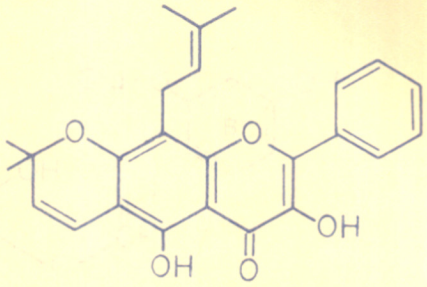
and *Maclura pomifera*[39] and the new flavones mulberrin (XXIII), cyclomulberrin (XXIX), mulberrochromene (XXX) and cyclomulberrochromene (XXXI) isolated recently from *M. alba* bark, which are similar to some of the *Artocarpus* pigments in having C_5 unit attached to the 3- and 6-positions of the chromone ring.[40] *Morus* and *Artocarpus* belong to the same family, *Moraceae*.

It has been shown that the biosynthesis of umbelliferone (7-hydroxycoumarin) involves the *ortho*-hydroxylation of *trans*-*p*-hydroxycinnamic acid, glucosylation to (XXXII) and subsequent isomerization to the *cis*-acid.[41] *Artocarpus* and *Morus* species appear to be unique among plants in possessing an enzyme system which directs (XXXII) to a pathway in which it condenses with a phloroglucinol precursor in the acetate route to form the chalkone (XXXIII). Ring A in (XXXIII) may react with γ,γ-dimethylallyl pyrophosphate[42] at this or a later stage, but the introduction of the second γ,γ-dimethylallyl group for the formation of artocarpin must be effected before the cyclisation of (XXXIII) to a flavone.

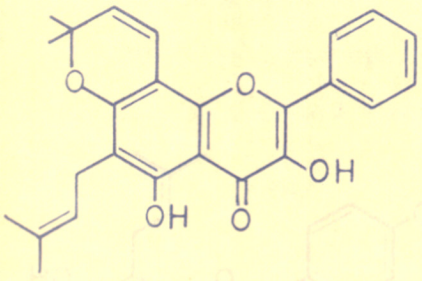
In recent years several groups have reported the isolation of flavonoids with isoprene units attached to the A- or B-ring (see Table 3) such as flavone (XXXIV),[43] the flavonols icaritin (XXXV),[44] amurensin (XXXVI),[45] noricarin (XXXVII)[45] and sericetin (XXXIV),[28] in which the A-ring is



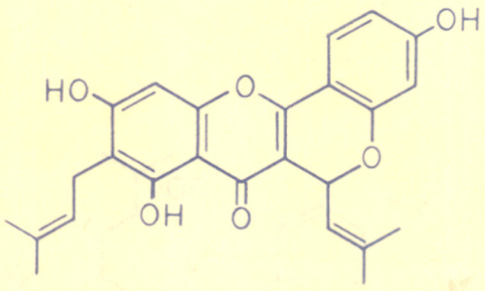
(XXIII)



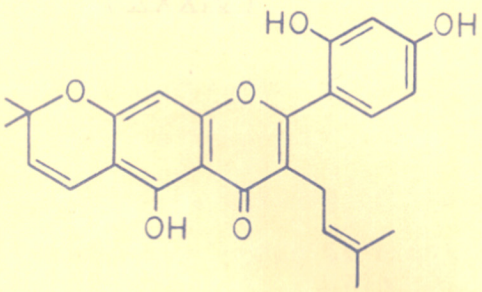
(XXIV)



(XXV)



(XXIX)



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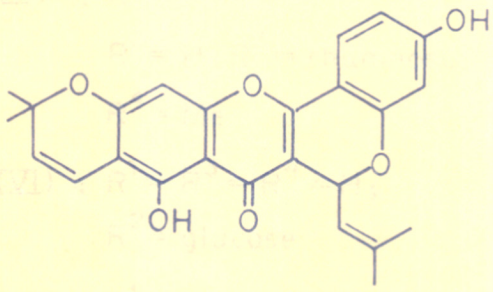
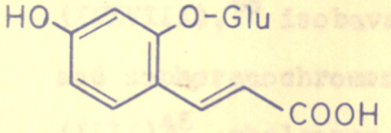
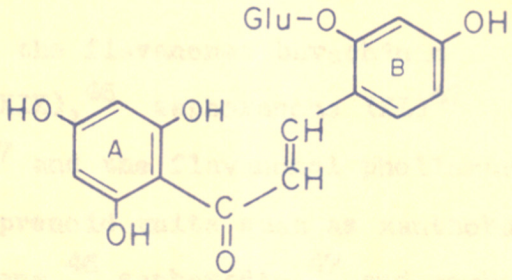


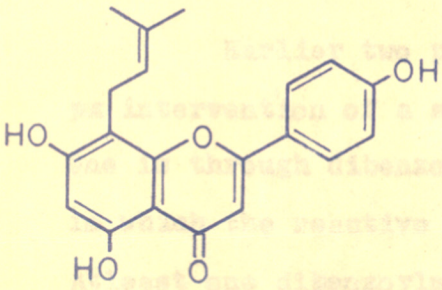
(XXXI)



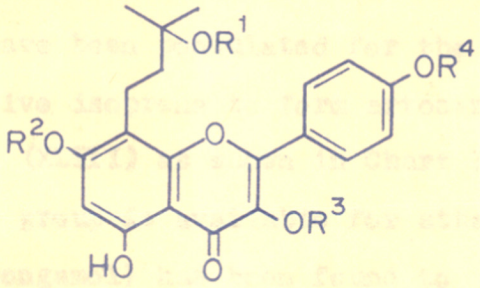
(XXXII)



(XXXIII)



(XXXIV)



(XXXV) ; R^1 = glucose;
R^2 = H; R^3 = rhamnose;
R^4 = Me

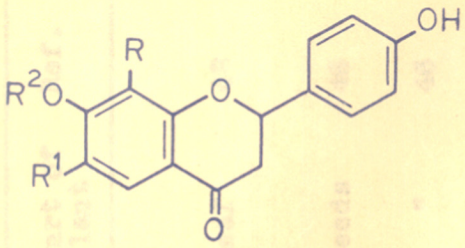
(XXXVI) ; $R^1 = R^3 = R^4 = H$;
R^2 = glucose

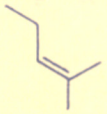
(XXXVII) ; R^1 = glucose;
$R^2 = R^3 = R^4 = H$

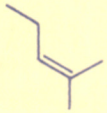
(XLII) ; Dihydro derv. of (XXXVI)

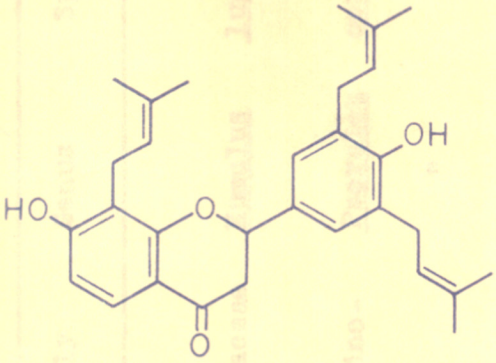
substituted by two C_5 units, the flavanones bavachinin (XXXVIII),[46] isobavachin (XXXIX),[46] saphoranone (XL)[47] and sophoranochromene (XLI)[47] and the flavanonol phellamurin (XLII);[45] chalcones with isoprenoid units such as xanthohumol,[48] bavachalcone,[46] isobavachalcone,[46] saphoradin,[47] and sophoradochromene[47] are also known. Flavanones having the isopentenyl group in the form of an ether linkage at the 4'-position were also encountered.[49,50] Several isoflavones of this type have also been isolated in recent years in addition to the closely related rotenoids.

Earlier two pathways have been postulated for the intervention of a second active isoprene to form artocarpin.[51] One is through dibenzoylmethane (XLIII) as shown in Chart III, in which the reactive methylene group is available for attack. Atleast one dibenzoylmethane (pongamol) has been found to occur in nature.[52] Another route involves isomerization (XLIV) to a flavanone in which the methylene group adjacent to the chromone carbonyl can conceivably be attacked by "active isoprene". The first route appears to be more probable, because of the much greater reactivity of the methylene group in a dibenzoylmethane; further, no flavanone with a C_5-unit in the 3-position has been encountered so far among the Artocarpus or Morus pigments or in other plants from which flavanones with C_5 units attached to the A- or B-

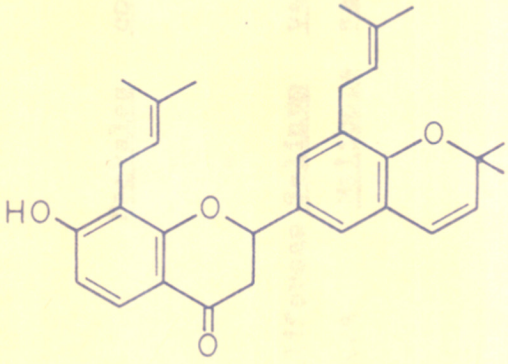


(XXXVIII); R = H; R^1 =  ; R^2 = Me

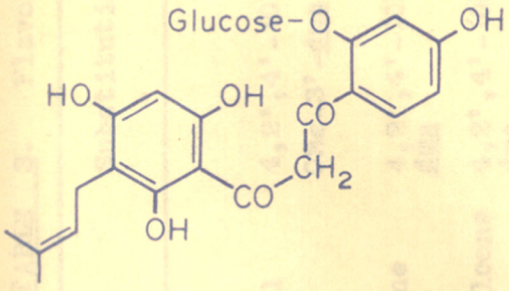
(XXXIX); R^1 = H; R =  ; R^2 = H



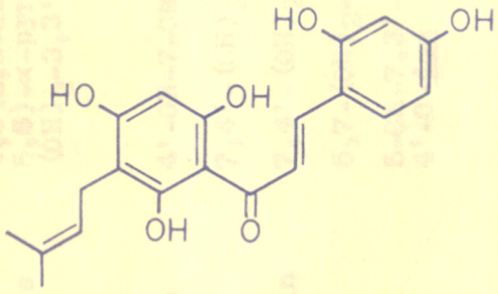
(XL)



(XLI)



(XLIII)



(XLIV)

TABLE 3. Flavonoids (other than isoflavonoid) with isoprenoid substituents

		Substitution	Family	Genus	Species	Part of plant	Ref.
	Chalcones:						
1.	Xanthohumol	4,2',4'-$(OH)_3$-6'-OMe-3'-dma	Urticaceae	Humulus	lupulus	Leaf	48
2.	Bavachalcone	4,2',4'-$(OH)_3$-5'-dma	Leguminoseae	Psoralea	corylifolia	Seeds	46
3.	Isobavachalcone	4,2',4'-$(OH)_3$-3'-dma	"	"	"	"	46
4.	Sophoradin	4,2',4'-$(OH)_3$-3,5,3'-$(dma)_3$	"	Saphora	subprostrata	Root	47
5.	Sophoradochromene	4,5(2,2-dimethyl-5,6)-α-pyrano-2',4'-$(OH)_2$-3,3'-$(dma)_2$	"	"	"		47
	Flavanones						
1.	Bavachinin	4'-OH-7-OMe-6-dma	"	Psoralea	corylifolia	Seeds	46
2.	Bavachin	7,4'-$(OH)_2$-6-dma	"	"	"	"	46
3.	Isobavachin	7,4'-$(OH)_2$-8-dma	"	"	"	"	46
4	Selinone	5,7-$(OH)_2$-4'-O-dma	Umbellifereae	Selinum	vaginatum		46
5.		5-OH-7,3'-$(OMe)_2$-4'-O-dma	Rutaceae	Melicope	sarcococca		27

6.	5-OH-7,3'-$(OMe)_2$-4'-O-$CH_2CH=\overset{Me}{C}-CH_2CH_2CHMe_2$	Rutaceae	Melicope	Sarcococca	Root	50
7. Sophoranone	7,4'-$(OH)_2$-8,3',5'-$(dma)_3$	Legumi-noseae	Sophora	subprostrata	"	47
8. Sophorano-chromene	7-(OH)-4',5'(2,2-dimethyl-5,6)-α-pyrano-8,3'-$(dma)_2$	"	"	"	"	47
Dihydroflavonol						
1. Phellamurin	3,5,4'-$(OH)_3$-7-O-Gl-8(3-hydroxy)-isoamyl	Rutaceae	Phelloden dron	amurense	Leaves	45
Flavones						
1. Amurensin	"	"	"	"	"	45
2. Icariin	5,7-$(OH)_2$-4'-OMe-3-O-rhamnosidyl-8(3-O-Gl)-isoamyl	Berberi-daceae	Epimedium	3 spp.	Root & leaves	44
3. Noricariin	4'-OH in icariin	"	"	macranthum	Roots	45
4. Noricariside	3,5,7,4'-$(OH)_4$-8-(3-O-Gl)-isoamyl	Rutaceae	Phello dendron	6 spp.		56
5. Sericetin	3,5-$(OH)_2$-7,6(2,2-dimethyl-5,6)-α-pyrano-8-dma	Legumi-noseae	Mundulea	sericea	Root bark	28
6. Artocarpus pigments		Moraceae	Artocarpus	6 spp.	Heartwood	
7. Morus pigments		"	Morus	5 spp.	Bark	

dma = γ,γ - dimethylallyl O-Gl = glucosidoxy.

rings were isolated. Finally it is likely that artocarpin (III) is converted to cycloartocarpin (VI) through a dehydrogenation involving the doubly allylic methylene group.

Biosynthesis of cycloheterophyllin

In the biosynthesis of cycloheterophyllin the attack of the third γ,γ-dimethylallyl group may occur at any stage; but the additional hydroxylation in the B ring probably represents the final step, because no other Artocarpus or Morus pigment has such substitution in the B ring. The only other flavone believed to have the 2',4',5'-oxygenation pattern in the B ring is oxyayanin A (5,2',5'-trihydroxy-3,7,4'-trimethoxyflavone),[52] but Jain et al.[53] synthesised a compound with this structure and found that it was different from natural oxyayanin A. Dreyer and Bertelli[54] have commented on the properties of oxyayanin A, which are similar to those of zapotin in the (a) remarkable stability towards refluxing 20% KOH, and (b) the very low intensity of the long wave length UV absorption band; and they have suggested the possibility of a 2',6'-substitution in the B ring.

Biogenesis of isocycloheterophyllin

Isocycloheterophyllin is the second flavone ~~acid pigment~~ having an additional oxygen-containing seven-membered

CHART 3 BIOGENESIS OF ARTOCARPUS AND MORUS FLAVONOIDS

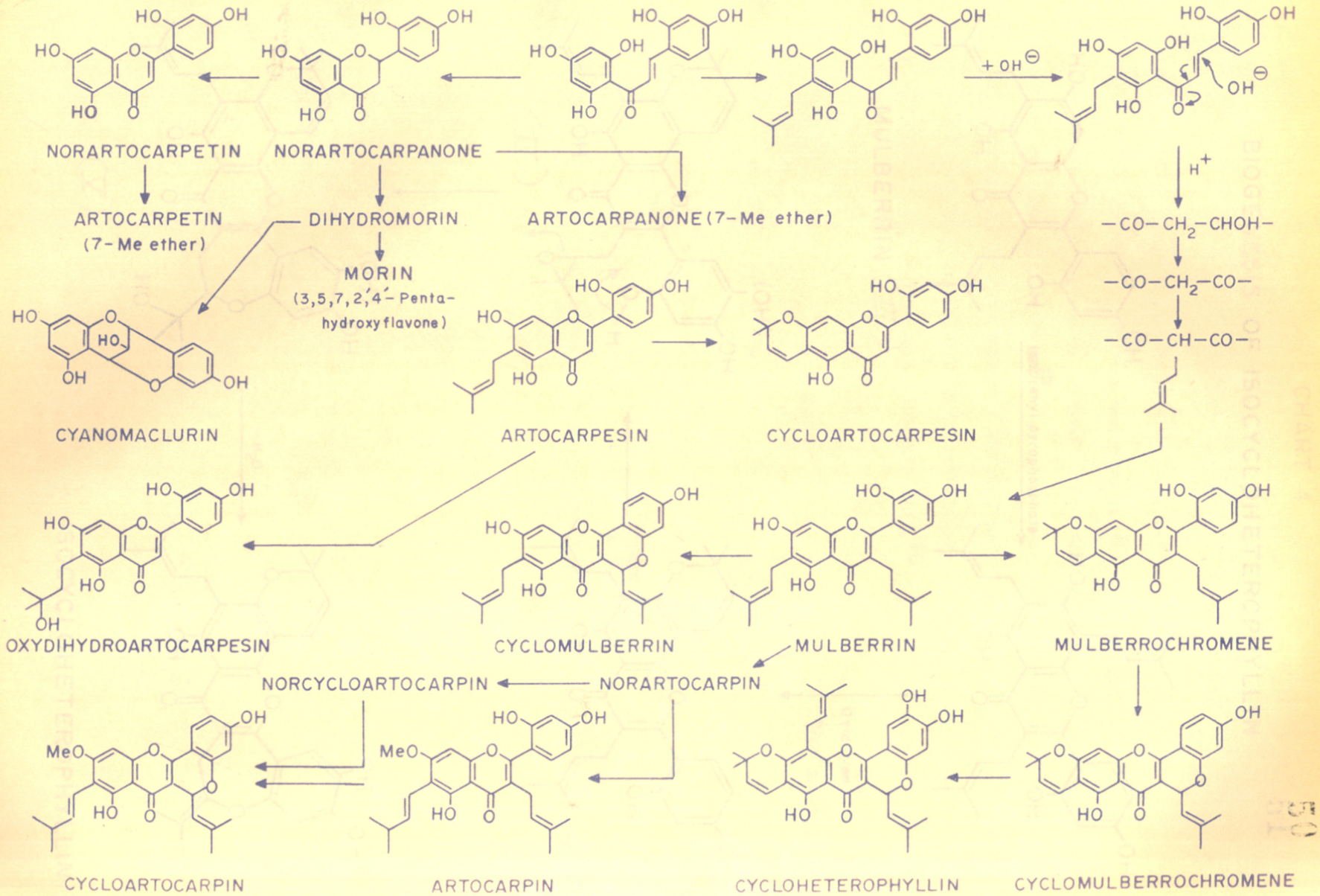
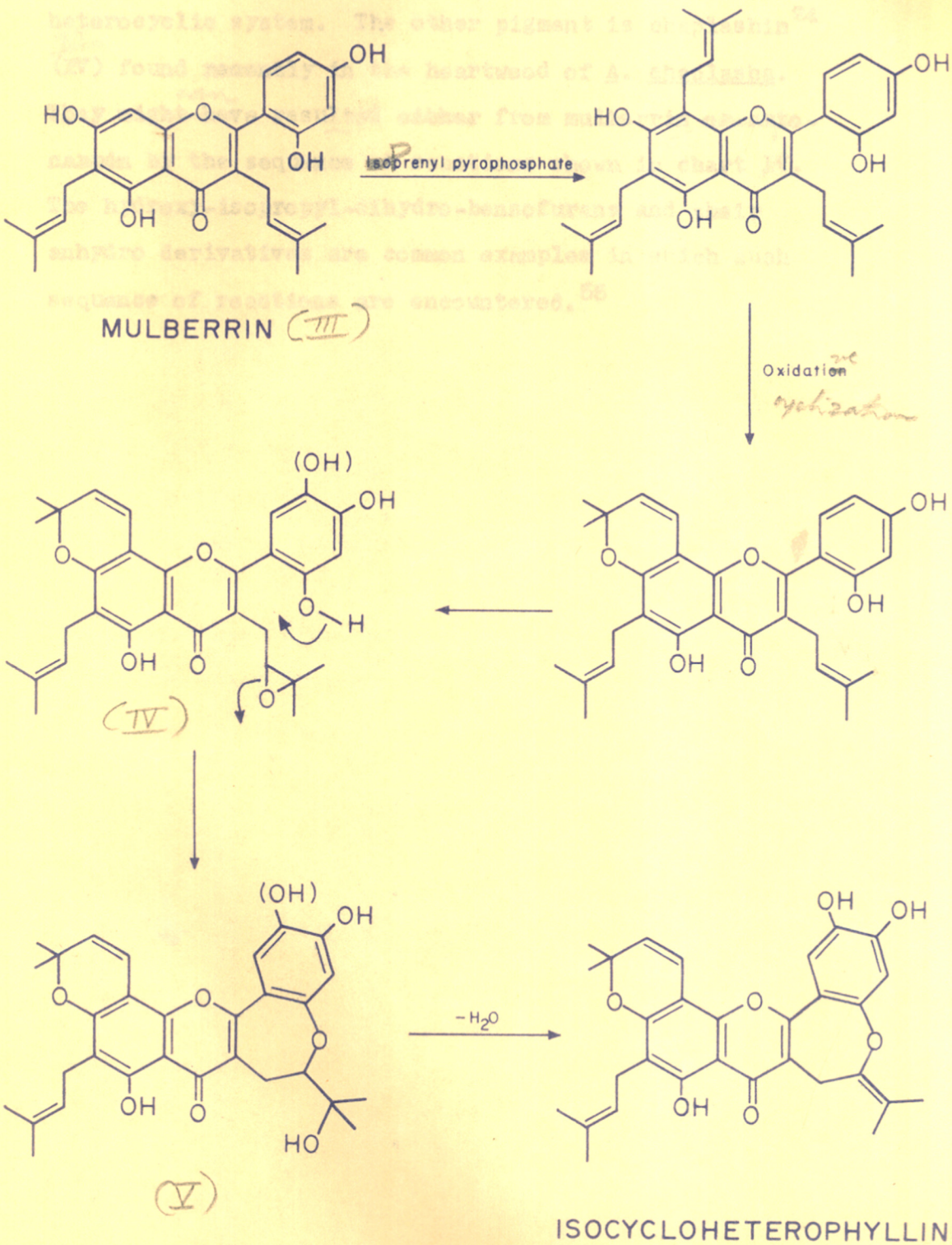


CHART 4

BIOGENESIS OF ISOCYCLOHETEROPHYLLIN



heterocyclic system. The other pigment is chaplashin[24] (XV) found recently in the heartwood of A. chaplasha. They might have resulted either from mulberrin or artocarpin by the sequence of reactions shown in chart IV. The hydroxy-isopropyl-dihydro-benzofurans and their anhydro derivatives are common examples in which such sequence of reactions are encountered.[55]

EXPERIMENTAL

Melting points are uncorrected and have been taken in capillaries. Ultraviolet spectra were taken in ethanol on a Perkin-Elmer Model 350 spectrophotometer. Infrared spectra were recorded as nujol mulls unless otherwise stated, on a Perkin-Elmer Model 221 spectrophotometer or Perkin-Elmer infracord. The maxima are reported in cm^{-1}. Proton magnetic resonance spectra were recorded on a Varian A-60 or T-60 spectrometers using tetramethyl silane as the internal standard. Mass spectra were recorded in a CEC-21-110B double focussing mass spectrometer operating at 70 ev using a direct inlet system. Optical rotations were determined on a Carl Zeiss polarimeter.

EXPERIMENTAL

Extraction of Artocarpus heterophyllus bark

The coarsely powdered bark (1 kg) of Artocarpus heterophyllus from Kerala State was extracted in a soxhlet with acetone for 48 hrs. The extract was concentrated to a small volume, mixed with the exhausted bark powder and successively extracted with hexane and benzene. The hexane extract (10 g) and benzene extract (15 g) showed the same spots on TLC silica gel plates using benzene-acetone (4:1) as solvent system. The residue from hexane and benzene extracts was dissolved in minimum amount of methanol and allowed to stand in cold when a white crystalline compound (4 g), m.p. 316-318^{o} separated. It showed a single spot by TLC on silica gel, a violet colour with the Liebermann-Burchard reagent and a yellow colour with tetranitromethane. The substance was identified as betulinic acid (superimposable IR and m.m.p. with an authentic sample).

Isolation of cycloheterophyllin

After removing betulinic acid the filtrate was concentrated, adsorbed on a polyamide column and eluted successively with hexane, benzene and acetone. The hexane fractions (5 g) along with faster moving waxes showed two

spots on a TLC silica gel plate (benzene acetone 4:1), but no pigment was isolable in pure form. The benzene eluate (12 g) was concentrated to a small volume and loaded on a column of silica gel (300 g). The first 15 fractions (15 ml each) contained a yellow compound in very small amount. It was identified by its Rf value on silica gel TLC as cycloartocarpin.

The twelve later fractions monitored on TLC plates (silica gel) showed a single ≈ yellow spot, which was slightly faster than artocarpin. All these fractions were mixed, the solvent removed, and cycloheterophyllin crystallised from methanol in yellow needles (0.104 g), m.p. 205-206°. It gave a green colouration with alcoholic ferric chloride and pink colour in the Shinoda test.

The next fractions (3 g) gave a mixture of three compounds. One of the compounds was identified by its Rf value on a TLC silica gel plate as artocarpin. A minor compound moving very slightly faster than artocarpin was not isolable. The other compound moving slower than artocarpin was separated from artocarpin by PLC (silica gel-benzene-acetone 4:1). The slower moving band was eluted with acetone. Removal of solvent and crystallisation from methanol gave heterophyllin (0.013 g), which was contaminated with traces of artocarpin (m.p. of the impure product 180-84°).

This compound also gives green colour with alcoholic ferric chloride and red colour with magnesium and hydrochloric acid.

The hexane benzene insoluble and acetone soluble of the bark contained mainly tannins.

Cycloheterophyllin trimethyl ether

A solution of cycloheterophyllin (0.1 g) and dimethyl sulphate (0.5 ml) in acetone (50 ml) was refluxed with anhydrous potassium carbonate (2 g) for 18 hrs. Distillation of acetone and treatment of the residue with water yielded a solid free from ferric colour. The methyl ether gives a red colour in Shinoda test. It was crystallised from methanol (0.07 g) in yellow needles, m.p. 168-69°. (Found: C, 73.0; H, 6.7. $C_{33}H_{36}O_7$ requires C, 72.8; H, 6.6%). It is optically active $(\alpha)_D$ = + 20° (C = 1.0 g/100 ml $CHCl_3$).

Hexahydrocycloheterophyllin trimethyl ether

Cycloheterophyllin trimethyl ether (0.04 g) in glacial acetic acid (15 ml) was hydrogenated, using platinum-oxide (0.01 g). The absorption was rapid and the compound took 3 moles of hydrogen in 2 hr. The catalyst was filtered off, the solvent removed under reduced pressure, and the yellow solid crystallised from methanol in pale yellow needles (0.035 g), m.p. 149-150°.

Cycloheterophyllin dimethyl ether

To a solution of cycloheterophyllin (0.1 g) in dry ether, a solution of ethereal diazomethane (obtained from 2 g. of nitrosomethyl urea) was added and left in a refrigerator for 15 hrs. The excess diazomethane was decomposed by the addition of few drops of acetic acid. The solvent was removed and the yellow solid showed two spots on a TLC plate (solvent system 9.9:0.1 benzene-acetone). The two fractions were separated by PLC on silica gel plates using the same solvent system. The two fractions were collected and crystallised from methanol. The major faster moving fraction was identified as the dimethyl ether (0.045 g) m.p. 218-219° (M^+ 530). (Found: C, 72.0; H, 6.6. $C_{32}H_{34}O_7$ requires C, 72.4; H, 6.4%). The slow moving minor fraction (0.02 g), m.p. 210-212° is a monomethyl ether (M^+ 516) and is probably the 4'-methyl ether as shown by the UV spectral data ($\lambda_{max}^{ethanol}$ 266, 284 sh, 298 and 390 nm). The band at 390 nm was shifted to 415 nm with less intensity showing the presence of 3'-hydroxyl and 4' methoxyl group. (Found: C, 71.8; H, 6.5. $C_{31}H_{32}O_7$ requires C, 72.0; H, 6.2%).

Acetate of cycloheterophyllin dimethyl ether

Dimethyl ether of cycloheterophyllin (0.04 g) was refluxed with acetic anhydride (1 ml) and pyridine (1 ml)

for 4 hrs. The product was poured on ice and filtered. The filtered solid was crystallised from methanol in pale yellow needles (0.027 g), m.p. 198-201°.

Trimethyl ether of heterophyllin

To a solution of heterophyllin (0.01 g) in dry ether, a solution of ethereal diazomethane was added and left in a refrigerator. The product was worked out as usual, and crystallised from methanol in yellow needles (0.006 g), m.p. 200-201° (M^+ 546). The electronic spectrum of heterophyllin trimethyl ether in ethanol shows maxima at 286 and 380 nm.

Extraction of Artocarpus heterophyllus bark from Poona

The orange red powder (0.4 kg) scraped from the underside of the bark of A. heterophyllus was extracted with acetone in a soxhlet for 40 hrs. The extract (15 g) was concentrated to a small volume mixed with the exhausted bark powder and successively extracted with hexane and benzene. The hexane soluble portion (0.3 g) was mainly waxes and was not further investigated. The benzene extract (10 g) when spotted on a TLC silica gel plate (solvent system 1:4 acetone-benzene) showed the presence of cycloartocarpin, cycloheterophyllin and artocarpin. A new compound moving

slightly faster than cycloheterophyllin was detected in the silica gel plate. The benzene extract was dissolved in ether and was shaken with 5% sodium bicarbonate solution to remove the acidic impurities. Then it was shaken up with 5% sodium hydroxide solution. The phenolics were removered by acidification (1.2 g). The sodium hydroxide soluble portion (1.2 g) was chromatographed on a column of silica gel (40 g) using benzene and benzene-acetone as the eluent. Fractions (50 ml) were collected and after checking the TLC behaviour similar fractions were pooled together. First fraction gave a yellow compound in very minute amount identified by its Rf value (silica gel TLC) as cycloartocarpin. The next fraction gave isocycloheterophyllin crystallised from benzene in yellow needles, m.p. 231° (0.03 g) (Found: C, 71.1; H, 6.5. $C_{30}H_{30}O_7$ requires C, 71.7; H, 5.9%). Further fractions gave cycloheterophyllin identified by its Rf value on a silica gel plate.

Isolation of isocycloheterophyllin from the bark of A. heterophyllus from Kumbakonam (Tamil Nadu)

The orange red powder (0.8 kg) scraped from the underside of the bark was extracted in the usual way as explained in the previous experiment and 0.08 g. of isocycloheterophyllin was isolated and the presence of cycloartocarpin, artocarpin and cycloheterophyllin were detected.

Isocycloheterophyllin trimethyl ether

A solution of isocycloheterophyllin (0.06 g) and dimethyl sulphate (0.4 ml) in acetone (50 ml) was refluxed with anhydrous potassium carbonate (2 g) for 24 hrs. Distillation of acetone and treatment of the residue with water yielded a solid free from ferric colour. It crystallised from methanol in pale yellow needles (0.03 g), m.p. 180-82° (Found: C, 73.0; H, 6.5. $C_{33}H_{36}O_7$ requires C, 72.8; H, 6.6%).

Isocycloheterophyllin dimethyl ether

To a solution of isocycloheterophyllin (0.03 g) in dry ether a solution of ethereal diazomethane (obtained from 2 g. of nitrosomethyl urea) was added and left in refrigerator. The excess of diazomethane was decomposed by the addition of a few drops of acetic acid. After removal of solvent the compound crystallised from methanol in yellow needles (0.02 g), m.p. 262-64°, The compound gives a green colour with alcoholic ferric chloride (Found: C, 72.0; H, 6.5. $C_{32}H_{34}O_7$ requires C, 72.4; H, 6.4%).

Tetrahydroisocycloheterophyllin

Isocycloheterophyllin (0.025 g) in ethanol(15 ml) was hydrogenated, using platinum oxide (0.01 g) till there was no absorption of hydrogen. After filtration off the

catalyst the yellow compound was tried to crystallise, but it resisted crystallisatinn (0.02 g), m.p. 215-219° (M^+ 506)

Tetrahydroisocycloheterophyllin dimethyl ether

To a solution of tetrahydroisocycloheterophyllin (0.02 g) in dry ether a solution of ethereal diazomethane was added and left in refrigerator. The product was worked out as usual. The yellow solid obtained had a m.p. of 200-204° and had no tendency to crystallise.

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CHAPTER II

THE PHENOLIC CONSTITUENTS OF THE BARK AND HEARTWOOD OF TOXYLON POMIFERUM

***

INTRODUCTION

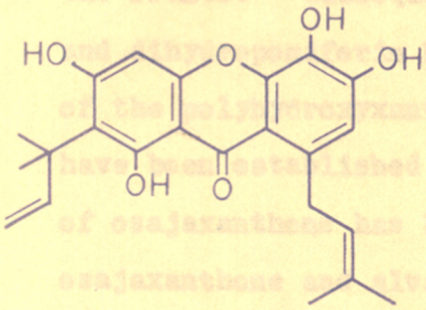
Toxylon pomiferum Rahn (Maclura pomifera Schneid; M. aurantica Nutt; Osage orange) is classified under genus Maclura and family Moraceae.[1,2] Maclura, Nutt is a monotypic genus of trees, native in the Arakansas to Texas region. Maclura consists of deciduous trees 50-60 ft. high, with glabrous twigs, axillary spines, and entire long-pointed oblong-lanceolate glabrous slender-petioled leaves. Flowers are very small and dioecious. The staminate is in short racemes with 4-parted perianth and 4-inflexed stamens. The pistillate is in a small dense globular head with 4-parted perianth including the ovoid sessile ovary, styles filiform and exserted. The fruit is a syncarp formed of the enlarged fleshy perianths in which the drupelets are embedded.[1]

T. pomiferum[3] is a spiny tree with spreading branches. It is hardy against frost and drought, and thrives well in deep soil. This is chiefly grown as a hedge plant, also planted as an ornamental tree for its handsome bright green foliage and the conspicuous orange like fruits. The leaves of the plant is used for rearing mulberry worms. The fruit is inedible and is suspected to be poisonous, but conclusive results have not been

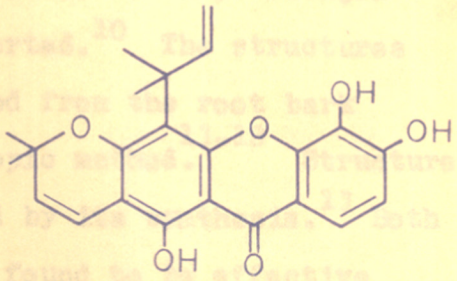
obtained. The resins present in the fruit are suitable for paints and adhesives. The fruit extract is useful as a cardiac stimulant.

The wood of the plant is hard and is bright orange in colour becoming brown on exposure. The wood is noted for its great durability and resistance to decay. It is rather difficult to work, but finishes to a smooth surface. It can be used for rims of wagon wheels, spokes, insulator pins, tree nails, walking sticks, golf shafts, fense posts and agricultural implements. An extract of the wood have been used for dying and tanning.

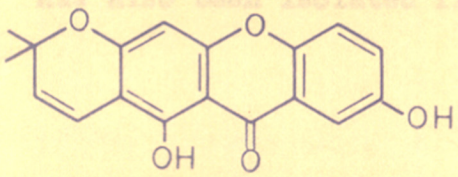
Earlier from the heartwood of Toxylon pomiferum Morin[4] (5,7,2',4'-tetrahydroxyflavonol), dihydromorin,[5] dihydroquercetin,[5] dihydrokaempferol[5] and its 7-glucoside[6] and the stilbene oxyresveratrol[4] have been isolated. Kaempferol-7-glucoside[6] has also been reported from the bark, leaves, flowers and fruits[6] of T. pomiferum. According to Barnes and Gerber[4] the presence of oxyresveratrol (about 1%) is responsible for the remarkable resistance of the wood to decay. Recently Bhat and Wolfrom[7] have reported the presence of 1,3,6,7-tetrahydroxyxanthone in the heartwood. Wolfrom and his colleagues isolated alvaxanthone (I), macluraxanthone (II) and osajaxanthone (III) from the root bark,[8] and the isoflavones osajin (IV) and pomiferin(V) from



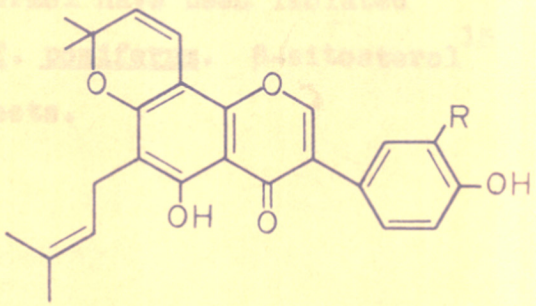
ALVAXANTHONE (I)



MACLURAXANTHONE (II)



OSAJAXANTHONE (III)



OSAJIN (IV) ; R = H

POMIFERIN (V) ; R = OH

the fruits.[9] Subsequently the synthesis of dihydroesajin and dihydropomiferin have been reported.[10] The structures of the polyhydroxyxanthones isolated from the root bark have been established by spectroscopic method.[11-13] Structure of osajaxanthone has been confirmed by its synthesis.[11] Both osajaxanthone and alvaxanthone are found to be effective fish poisons and were toxic to mosquito larvae.[8] Maclura-xanthone is believed to be the first case of a natural phenolic compound substituted with an isoprenoid unit in the form of a 1,1-dimethylallyl group.[12] This pigment is found to be an unusually good antitermite agent.[12]

Besides these phenolics, the triterpenes, lupane-3β-2-o-diol, lupeol, and butyrospermol have been isolated from the fruits[14] and roots[15] of T. pomiferum. β-sitosterol[15] has also been isolated from the roots.

PRESENT WORK

Despite the extensive studies on the heartwood of T. pomiferum, the only reference to the stem bark in the literature is the isolation of kaempferol-7-glucoside.[6] In the present work a systematic examination of the stem bark of T. pomiferum obtained from the United States has been carried out, and three known xanthones and four new xanthones have been isolated.

Soxhlet extraction of the stem bark with acetone yielded a yellow solid, which was mixed with the exhausted bark powder and soxhleted with hexane and benzene and then with acetone. The hexane and benzene extracts contained the same compounds, which separated as six distinct spots when examined on a TLC silica gel plate, using acetone-benzene (1:4) as the solvent system and exposing the plate to iodine vapour. The residue from the hexane extract was dissolved in the minimum amount of hot methanol and left at room temperature when a colourless solid separated which gave a positive Liebermann-Burchard test for triterpenoids. The product obtained on removal of methanol was dried and dissolved in benzene, and chromatographed on a silica gel column using benzene and benzene-acetone for development and elution. The acetone concentration in benzene was gradually increased. Fractions were collected and monitored on silica gel TLC plates.

Like fractions were mixed and further purification was effected by PLC on silica gel. Seven compounds were thus isolated and were numbered (1) to (7) in the order of decreasing R_f values on TLC plates. The R_f values and the yields of the compounds isolated are given in Table 1.

Table 1.

Compound	R_f value (silica gel. solvent system: acetone-benzene 1:4)	Yield per cent
Xanthone 1	0.90	0.004
Xanthone 2	0.84	0.004
Xanthone 3	0.76	0.034
Xanthone 4	0.74	0.014
Xanthone 5	0.54	0.04
Xanthone 6	0.41	0.12
Xanthone 7	0.33	0.016

All of them gave a green colouration with alcoholic ferric chloride. Xanthones 6 and 7 in sodium hydroxide gave red colouration with violet tinge with alcoholic o-dinitrobenzene diagnostic of a catechol grouping,[16] but xanthones 1,2,3,4 and 5 did not respond to this test. UV spectral properties of these compounds are characteristic of xanthones (Fig.1; Table 2).

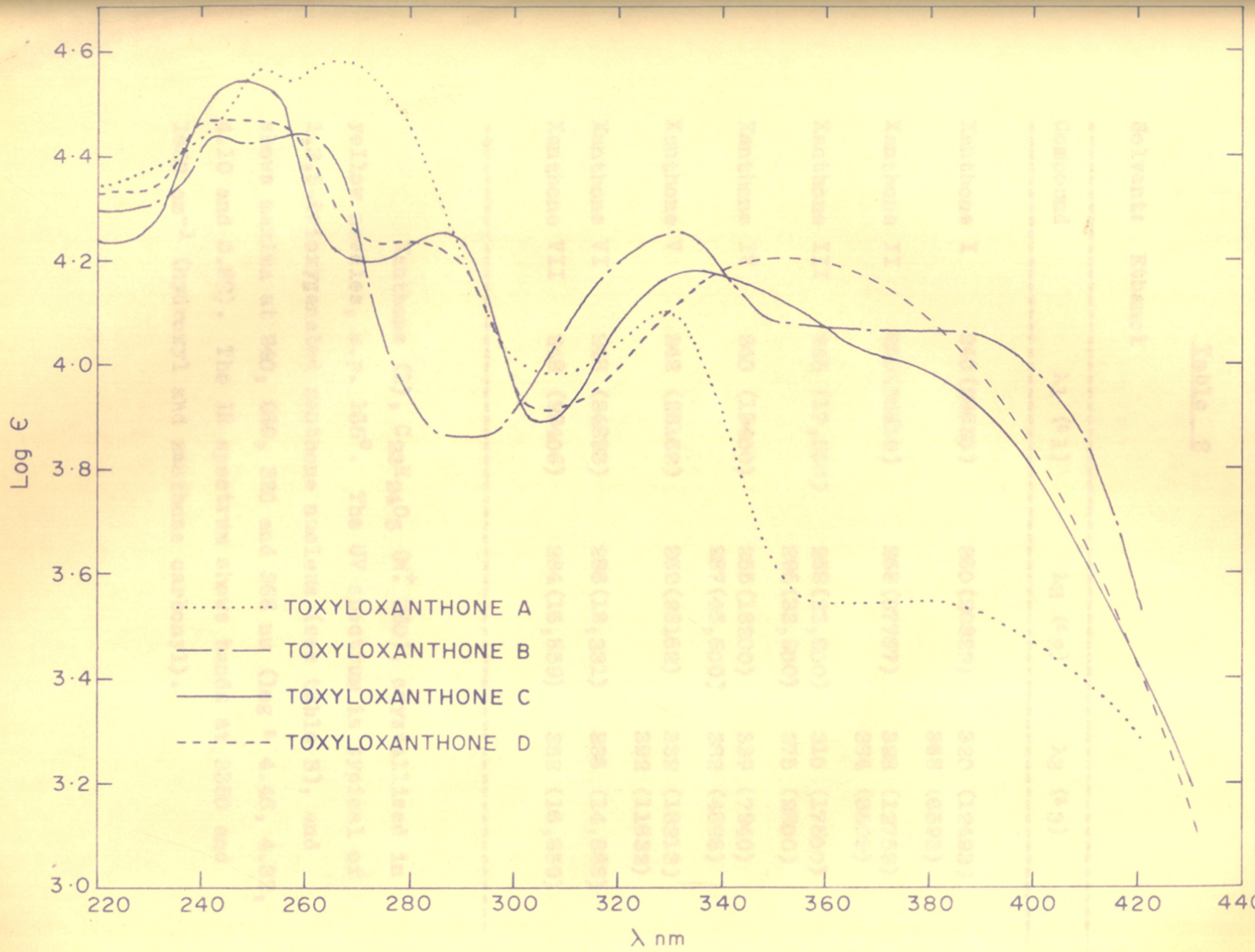


FIG. 1 UV SPECTRA OF TOXYLOXANTHONES A, B, C and D IN ETHANOL

Table 2

Solvent: Ethanol

Compound	λ_1 (ε_1)	λ_2 (ε_2)	λ_3 (ε_3)
Xanthone I	240 (28630)	260 (20822)	320 (12493) 368 (6593)
Xanthone II	252 (36810)	268 (37767)	328 (12759) 384 (3509)
Xanthone III	245 (17,500)	268 (23,800) 296 (33,900)	310 (17500) 375 (2800)
Xanthone IV	240 (18400)	255 (18200) 287 (46,600)	339 (7940) 382 (4886)
Xanthone V	242 (28162)	262 (28162)	332 (18213) 392 (11632)
Xanthone VI	248 (34893)	286 (18,331)	326 (14,888)
Xanthone VII	248 (29406)	284 (16,859)	352 (16,859)

Xanthone (1), $C_{23}H_{24}O_5$ (M^+ 380), crystallised in yellow needles, m.p. 165°. The UV spectrum is typical of 1,3,5-trioxygenated xanthone nucleus (see table 3), and shows maxima at 240, 260, 320 and 368 nm (log ε 4.46, 4.32, 4.10 and 3.82). The IR spectrum shows bands at 3350 and 1650 cm^{-1} (hydroxyl and xanthone carbonyl).

Table 3

UV spectra of tri- and tetra-oxygenated xanthones

Compound	$\lambda_1(E_1)$	$\lambda_2(E_2)$	$\lambda_3(E_3)$	$\lambda_4(E_4)$	Sol.	Ref.
Xanthone	239(3900)	261(12600)	287(4200)	337(6350)	b	17
1,3,5-trihydroxyxanthone	246(28000)	314(12400)	350(7400)	-	b	18
1-Hydroxy-3,5-dimethoxy xanthone	245(40200)	306(19100)	355(5600)	-	a	19
6-Desoxyjacareubin	243(16500) 270(24700)	296(33900)	310(16800)	370(2400)	a	19
6-Dehydrojacareubin	240(1900) 250(18900)	286(42600)	309(19600)	369(4000)	b	8
8-Desoxygartanin	244(29510)	260(23440)	324(14790)	375(3548)	b	20
1,3-Dihydroxy-7-methoxyxanthone	235(28180)	259(31620)	311(13800)	369(6310)	b	21
Osajaxanthone	240(18620)	249(18200)	285(46770) 339(7943)	382(4786)	b	8
1,3,5,6-tetrahydroxyxanthone	253(48980)	281(12880)	326(21380)	-	a	19
1,3,5,6-tetrahydroxy-2-3,3-dimethyl allyl xanthone	251(26300)	283(6457)	324(16220)	-	a	19
Jacareubin	240(12300)	279(40740)	334(18200)	-	b	21
Maclura-xanthone	242(20420)	283(43650)	338(19050)	-	b	8

Table 3 contd.

Alva-xanthone	257(75860)	280(8710)	332(23990)	-	b	8
γ-Mangostin (1,3,6,7-tetrahydroxy-2,8-3,3-dimethylallyl xanthone)	247(26920)	261(29510)	314(21880)	364(7943)	b	22
1,3,7-Trihydroxy-6-methoxy-xanthone	239(21880)	256(31620)	310(14130)	362(9772)	b	22
Mangostin (1,3,6-trihydroxy-7-methoxy-2,8-3,3-dimethyl-allylxanthone)	243(34670)	259(27540)	318(23990)	351(7244)	b	23

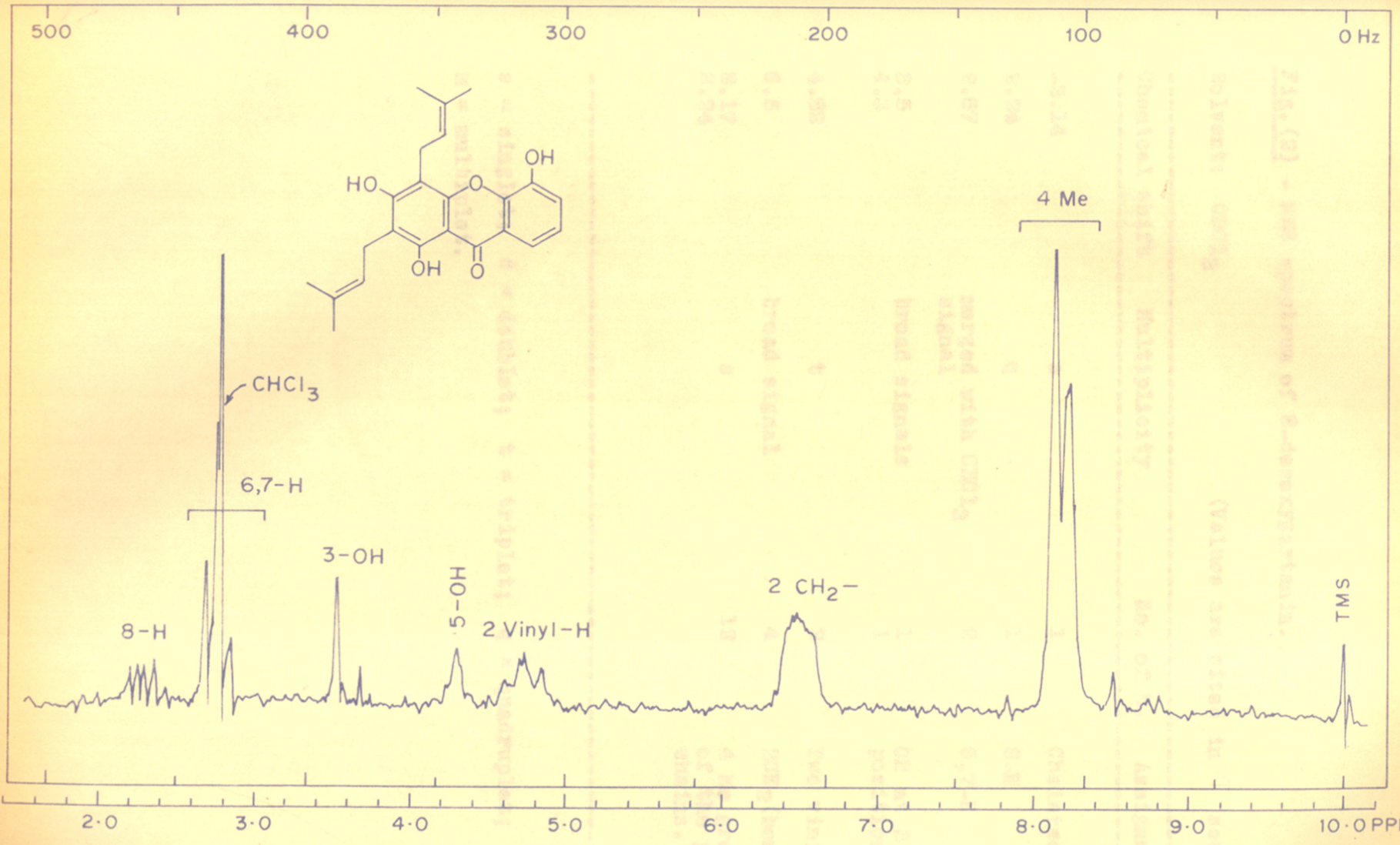
Solvent :

a = Methanol

b = Ethanol.

The NMR spectrum in $CDCl_3$ (Fig. 2) shows absorptions due to three hydroxyl groups at 3.54, 4.3 and a sharp signal at -3.14 (hydroxyl bonded to the xanthone carbonyl group) all of which disappeared on shaking the solution with a few drops of D_2O (chemical shifts on the τ scale). In the region between 4 to 9, the absorption pattern is consistent with two 3,3-dimethylallyl groups attached to an aromatic ring system: two signals at 8.17 and 8.24 for four methyl groups, a broad signal (not resolved due to overlapping) at 6.5 for two methylene groups, and a broad triplet centered at 4.82 for two vinyl hydrogens. In the aromatic region a single-proton quartet centered at 2.24 and a two-proton multiplet around 2.67, indicate that one ring of the xanthone molecule is fully substituted. From biogenetic considerations it can be assumed that two hydroxyl groups are at 1,3-positions and the third is probably in the 5-position, suggesting structure (VI) for xanthone (1). Very recently Govindachari *et al.*[20] have assigned structure (VI) to 8-desoxygartanin, a minor component of very ripe fruits of *Garcinia mangostana*. All the properties of xanthone (1) agree with those of 8-desoxy-gartanin.

Xanthone (2) (Toxyloxanthone A) was obtained as yellow needles, m.p. 165-66°. Elemental analysis and molecular weight (M⁺ 378) suggested the molecular formula $C_{23}H_{22}O_5$; λ_{max} 252, 268, 328, 384 nm; (log ε 4.56, 4.58, 4.11, 3.54). Comparison with the UV spectra of known xanthones



FIG·2 NMR SPECTRUM OF 8-DESOXYGARTANIN IN $CDCl_3$

Fig.(2) - NMR spectrum of 8-desoxygartanin.

Solvent: $CDCl_3$ (Values are cited in τ scale)

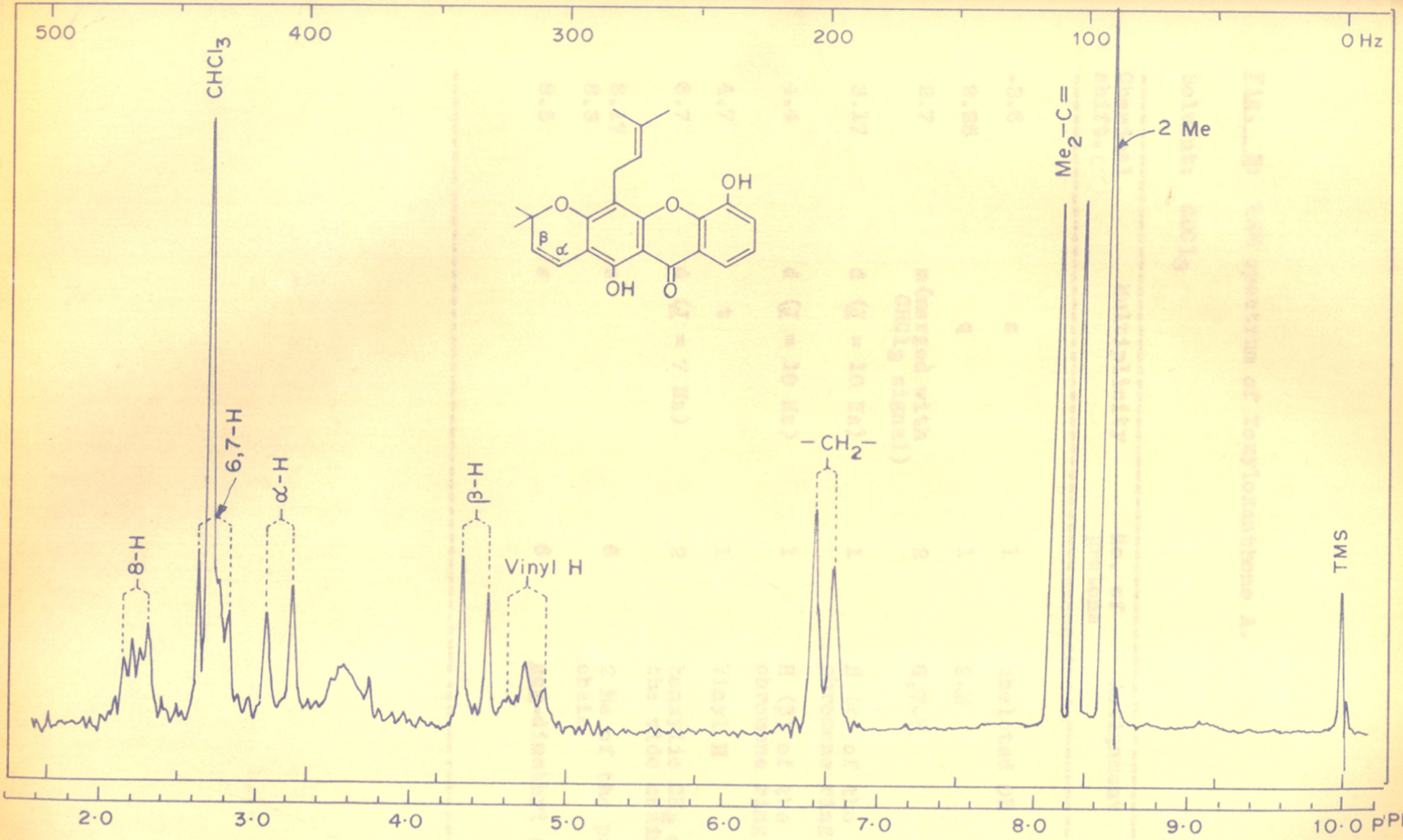
Chemical shift (τ)	Multiplicity	No. of H.	Assignment
-3.14	s	1	Chelated 6H
2.24	q	1	8-H
2.67	merged with $CHCl_3$ signal	2	6,7-H
3.5 4.3	broad signals	1 1	OH at 3 & 5 positions.
4.82	t	2	Two vinyl H
6.5	broad signal	4	$2CH_2$(benzylic)
8.17 8.24	s	12	4 Me groups of the prenyl chains.

s = singlet; d = doublet; t = triplet; q = quadruplet;

m = multiplet.

indicate that xanthone (2) has a 1,3,5-trioxygenated system.

The IR spectrum shows bands at 3500 and 1650 cm^{-1}; characteristic of hydroxyl and carbonyl groups. The NMR spectrum in $CDCl_3$ (Fig. 3) shows a sharp signal in the downfield region at -3.16 (bonded OH). Methoxyl singlets are absent. The spectrum shows absorptions typical of a 3,3-dimethylallyl group and a 2,2-dimethylchromene ring system. Thus two doublets centered at 3.17 (J = 10 Hz) and 4.40 (J = 10 Hz), together with a six-proton singlet at 8.5, represent respectively the two vinyl and two methyl groups of the chromene system. Two broad singlets at 8.17 (3H) and 8.3(3H), together with a doublet at 6.7 (2H; J = 7 Hz) and a triplet at 4.7, are consistent with a 3,3-dimethylallyl group attached to an aromatic ring. In the aromatic region the spectrum is identical with that of xanthone (1): a quartet at 2.25 (1H) and a multiplet at 2.7 (2H), indicating that xanthones (1) and (2) are substituted in the same positions. Two structures (VII) and (VIII) can be written for xanthone (2), but (VII) is favoured by mass spectral evidence. If the prenyl chain is adjacent to a hydroxyl group as in structure (VIII), fragmentation should occur with the loss of C_4H_8 (56 mass units),[24] but no such fragmentation is seen. A peak corresponding to the loss of C_4H_7 supports structure (VII); xanthone (2), a new compound, is designated as toxyloxanthone A.

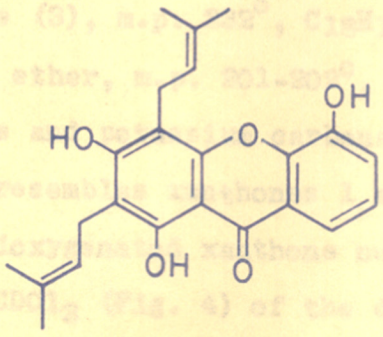


FIG·3 NMR SPECTRUM OF TOXYLOXANTHONE A IN $CDCl_3$

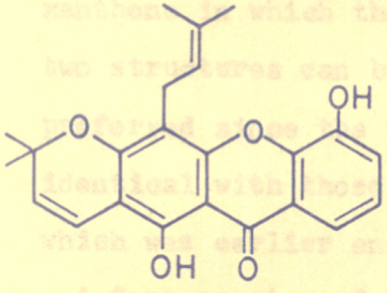
Fig. 3: NMR spectrum of Toxyloxanthone A.

Solvent: $CDCl_3$

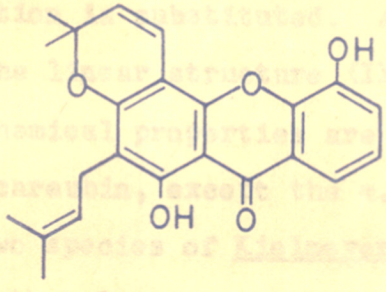
Chemical shift. (τ)	Multiplicity	No. of protons	Assignment
-3.6	s	1	chelated OH
2.25	q	1	8-H
2.7	m(merged with $CHCl_3$ signal)	2	6,7-H
3.17	d (J = 10 Hz)	1	H (∝) of the chromene ring.
4.4	d (J = 10 Hz)	1	H (β) of the chromene ring
4.7	t	1	Vinyl H
6.7	d (J = 7 Hz)	2	benzylic CH_2 of the side chain.
8.17 8.3	s	6	2 Me of the prenyl chain
8.5	s	6	gem-dimethyl group.



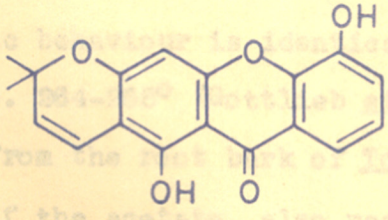
8-DESOXYGARTANIN (VI)



TOXYLOXANTHONE A (VII)



(VIII)



6-DESOXYJACAREUBIN (IX)

Xanthone (3), m.p. 232°, $C_{18}H_{14}O_5$ (M^+ 310). It forms a dimethyl ether, m.p. 201-202°, on treatment with dimethyl sulphate and potassium carbonate in boiling acetone. The UV spectrum resembles xanthones 1 and 2, and is characteristic of 1,3,5-trioxygenated xanthone nucleus (Table 3). The NMR spectrum in $CDCl_3$ (Fig. 4) of the dimethyl ether shows two methoxyl groups and absorptions typical of a 2,2-dimethylchromene ring system. In the aromatic region, compared with the spectra of xanthones (1) and (2), a singlet single-proton signal at 3.7 corresponds to the lone proton of a 1,3-dihydroxyxanthone in which the 2/6- or 4/8-position is substituted. Although two structures can be suggested, the linear structure (IX) is preferred since the physical and chemical properties are identical with those of 6-desoxyjacareubin, except the m.p. which was earlier encountered in two species of Kielmeyera and four species of Calophyllum, both belonging to the family Guttiferae.[25]

Xanthone (4), $C_{18}H_{14}O_5$ (M^+ 310), m.p. 241-243°, obtained in minute amounts, was identified as slightly impure osajaxanthone (III), contaminated with 6-desoxyjacareubin. Its chromatographic behaviour is identical with that of osajaxanthone, m.p. 264-265° (Gottlieb et al.[38] m.p. 249-52°) isolated earlier from the root bark of Toxylon pomiferum.[8] The NMR spectrum of the acetate, also recorded by Wolfrom et al.[11]

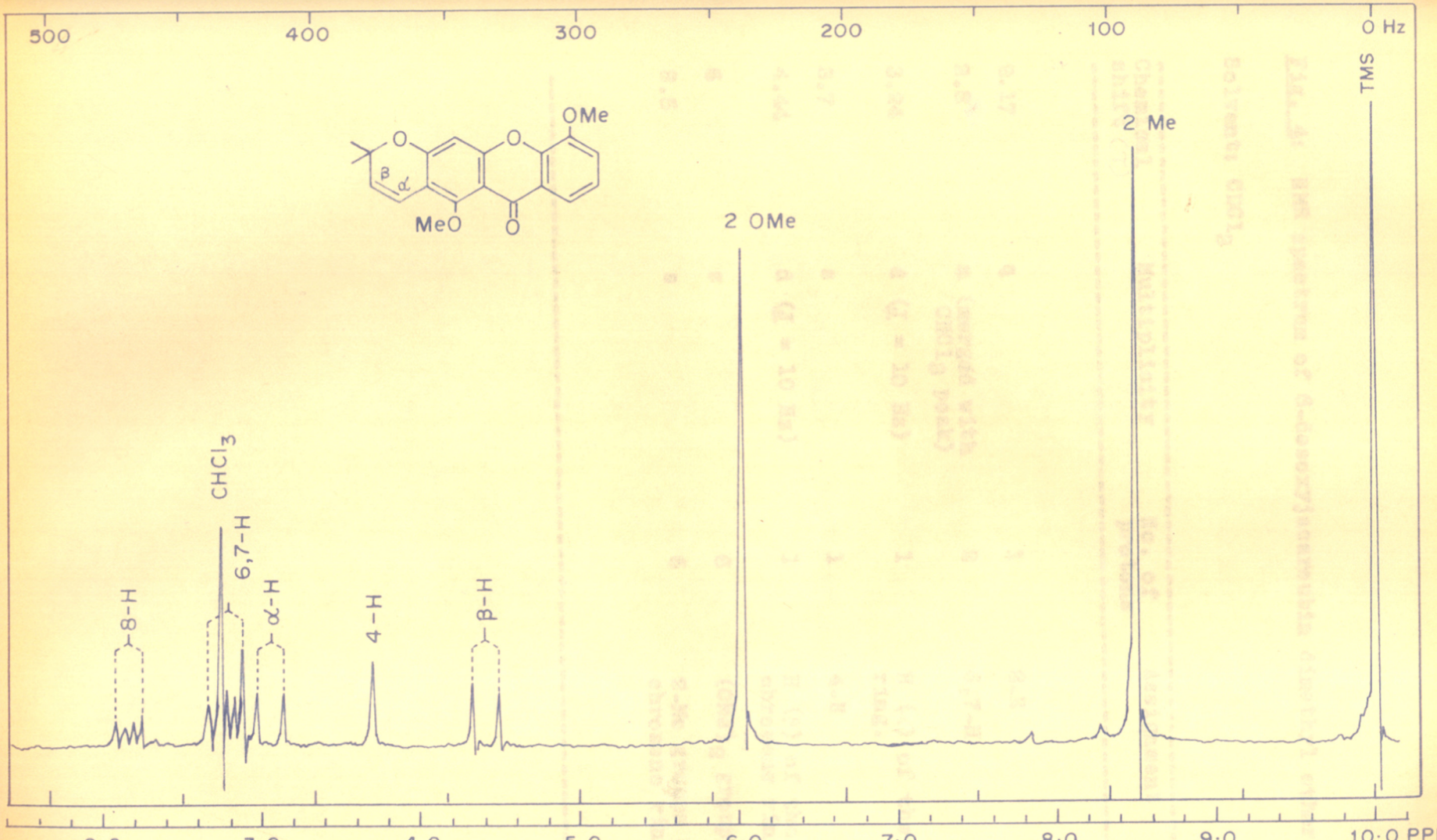


FIG. 4 NMR SPECTRUM OF 6-DESOXYJACAREUBIN DIMETHYL ETHER IN $CDCl_3$

Fig. 4: NMR spectrum of 6-desoxyjacareubin dimethyl ether.

Solvent: $CDCl_3$

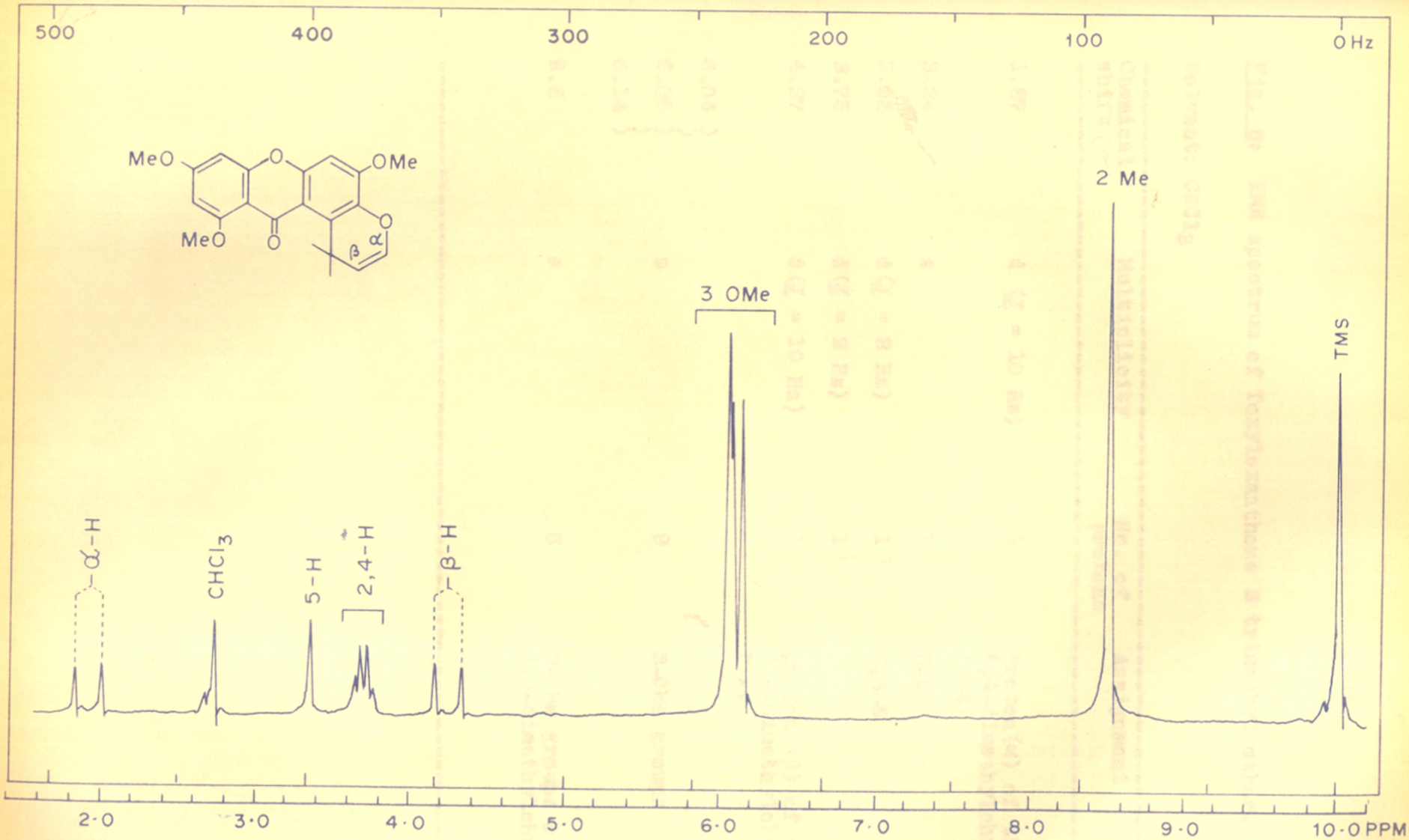
Chemical shift (τ)	Multiplicity	No. of protons	Assignment
2.17	q	1	8-H
2.8	m (merged with $CHCl_3$ peak)	2	6,7-H
3.24	d (J = 10 Hz)	1	H (α) of the chromene ring.
3.7	s	1	4-H
4.44	d (J = 10 Hz)	1	H (β) of the chromene ring
6	s	6	$(OMe)_2$ groups
8.5	s	6	2-Me groups of the chromene ring.

shows in addition to the absorptions for the 2,2-dimethylchromene ring system, two protons as a multiplet centered at 2.6 and a single-proton singlet at 3.7. The lowest signal appears at 2.1 as a meta-coupled doublet (J = 2.0 Hz), indicating substitution at the 7-position. The linear structure (V) was suggested by Wolfrom et al. on the basis of the Gibbs test.[11] Recently osajaxanthone has also been reported in two species of Kielmeyera and two species of Calophyllum.[25]

Xanthone (5) (Toxyloxanthone B), $C_{18}H_{14}O_6$ (M^+ 326), crystallised from acetone-benzene in yellow needles, m.p.300°. The molecular formula and the UV spectrum (λ_{max} 242, 262, 332, 392 nm; log ε 4.45, 4.45, 4.26, 4.06) suggested a tetrahydroxyxanthone with an extended chromophore. The IR spectrum shows phenolic hydroxyl groups and a xanthone carbonyl(3400 & 1650 cm^{-1}).

A trimethyl ether (M^+ 368), m.p. 192-193°, was obtained by treatment with dimethyl sulphate and potassium carbonate in boiling acetone, and a dimethyl ether with ethereal diazomethane, m.p. 210-212°, indicating the presence of three phenolic hydroxyl groups, one of which is strongly bonded with the xanthone carbonyl group.

The NMR spectrum of the trimethyl ether (solvent $CDCl_3$; Fig. 5) showed the presence of three methoxyl groups (singlets at 6.04, 6.06 and 6.14, corresponding to 9H) and a sharp singlet at 8.5(6H). In the aromatic region, there are two single-proton doublets (J = 10 Hz) centered at 1.87

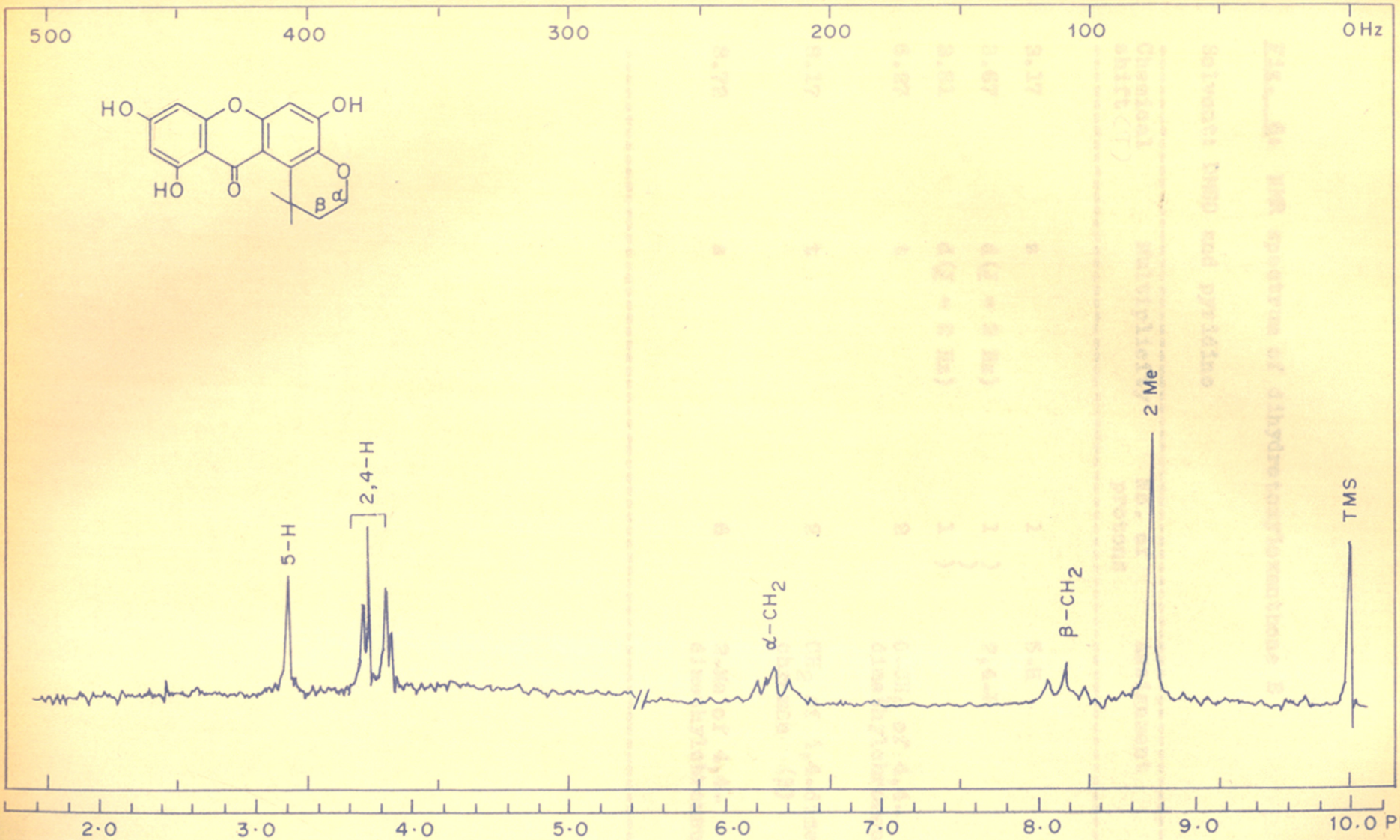


FIG·5 NMR SPECTRUM OF TOXYLOXANTHONE B TRIMETHYL ETHER IN $CDCl_3$

Fig. 5: NMR spectrum of Toxyloxanthone B trimethyl ether.

Solvent: $CDCl_3$

Chemical shift (τ)	Multiplicity	No. of protons	Assignment
1.87	d (J = 10 Hz)	1	Proton(α) of the 4,4-dimethylchromene ring.
3.34	s	1	5-H
3.65	d (J = 2 Hz)	1	2,4-H
3.75	d (J = 2 Hz)	1	
4.27	d (J = 10 Hz)	1	proton (β) of the 4,4-dimethylchromene ring
6.04 6.06 6.14	s	9	3-OMe groups
8.5	s	6	2- Me groups of 4,4-dimethylchromene



FIG·6 NMR SPECTRUM OF DIHYDROTOXYLOXANTHONE B IN DMSO AND PYRIDINE

Fig. 6: NMR spectrum of dihydrotoxyloxanthone B

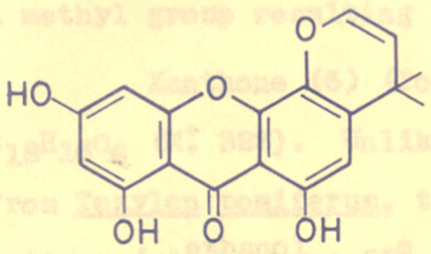
Solvent: DMSO and pyridine

Chemical shift (τ)	Multiplicity	No. of protons	Assignment
3.17	s	1	5-H
3.67	d (J = 2 Hz)	1)	2,4-H
3.81	d (J = 2 Hz)	1)	
6.27	t	2	O-CH_2 of 4,4-dimethylchroman (α)
8.17	t	2	CH_2 of 4,4-dimethylchroman (β)
8.72	s	6	2-Me of 4,4-dimethylchroman

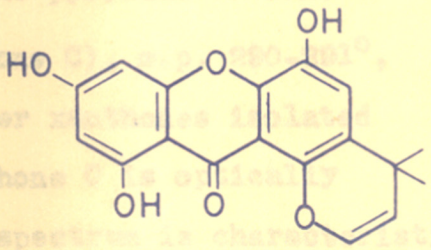
and 4.27, a singlet at 3.34 and two meta-coupled doublets (J = 2 Hz) at 3.65 and 3.75. The latter two signals suggest that one of the benzene rings is substituted at 1,3-positions by methoxyl groups. Of the four oxygens, other than the two which form part of the xanthone ring system, three are present as methoxyl groups; the remaining oxygen must therefore be involved in an ether linkage, probably with an adjacent C_5 group. The possibility of a 2,2-dimethylchromene ring system, which is very common in natural products, is ruled out as two vinyl hydrogens should then appear at about 3.3 and 4.3. However, the chemical shifts of the two doublets (1.87 and 4.27), together with the six-proton singlet at 8.5, suggest the other alternative of a 4,4-dimethylchromene ring system. All the chromenes so far isolated as natural products are 2,2-dimethylchromenes formed by oxidative cyclisation of a 3,3-dimethylallyl group with an adjacent phenolic hydroxyl group. Toxyloxanthone B is therefore unique as a 4,4-dimethylchromene. This might have resulted by the oxidative cyclisation of a 1,1-dimethylallyl group with an adjacent hydroxyl group by a radical mechanism. The cyclisation of an o-1,1-dimethylallyl phenol under acid(ionic)conditions is expected to yield a dihydrobenzofuran.[26] Toxyloxanthone B, on hydrogenation over Adams catalyst, absorbed one molecule of hydrogen, and the dihydro derivative (M^+ 328) showed in its NMR spectrum (Fig. 6) two two-proton triplets at 6.27 and 8.17 corresponding to $O\text{-}CH_2$- and $\text{-}CH_2$- groups.

Since ring A of xanthone (5) is substituted by hydroxyl groups at 1,3-positions, ring B is left with a single position unsubstituted. Natural xanthones, particularly in higher plants, are formed by a mixed shikimate-acetate biogenetic pathway. Ring B, the part derived from shikimate, is substituted by two oxygen functions, which can be in the 5,6-, 6,7- or 5,8- positions, but not in the 5,7-positions.[25] Five structures (X to XIV) can then be considered for xanthone (5). Since there is only one bonded hydroxyl group, structure (X) is excluded. Benzene-induced solvent shifts of the methoxyl groups in the trimethyl ether indicate that all the OMe groups have at least one adjacent aromatic position unsubstituted. The methoxyl signals at 6.04, 6.06 and 6.14 in $CDCl_3$ are shifted in benzene to 6.5 (3H) and 6.62 (6H), indicating that none of the OMe groups is sandwiched between two substituents. Structure (XIV) is thus eliminated. A choice between the three structures (XI, XII and XIII) can be made on the basis of the chemical shift of the singlet, which is at 3.34, suggesting that this proton is flanked by two oxygen functions. In structures (XIII) and (XI) the proton o- or p- to the carbonyl group will suffer a paramagnetic shift in comparison with the m-protons. The structure of toxyloxanthone B must therefore be (XII). One feature in the NMR spectrum of the trimethyl ether which may be regarded as not in agreement with structure (XII) for toxyloxanthone B is the appearance of the two methyl groups of the 4,4-dimethylchromene ring as a singlet, rather than two separate signals. Normally one would expect the two methyl groups to be magnetically non-equivalent, because they are in close proximity to a carbonyl group.

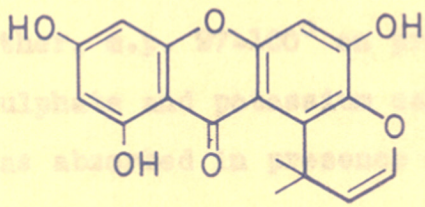
In the mass spectrum of toxyloxanthone B the base peak, as in 2,2-dimethylchromenes, is formed by the loss of



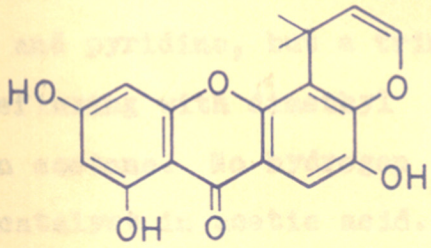
(X)



(XI)

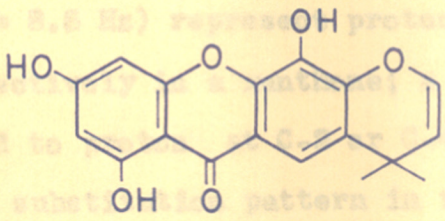


(XII)



(XIII)

TOXYLOXANTHONE B



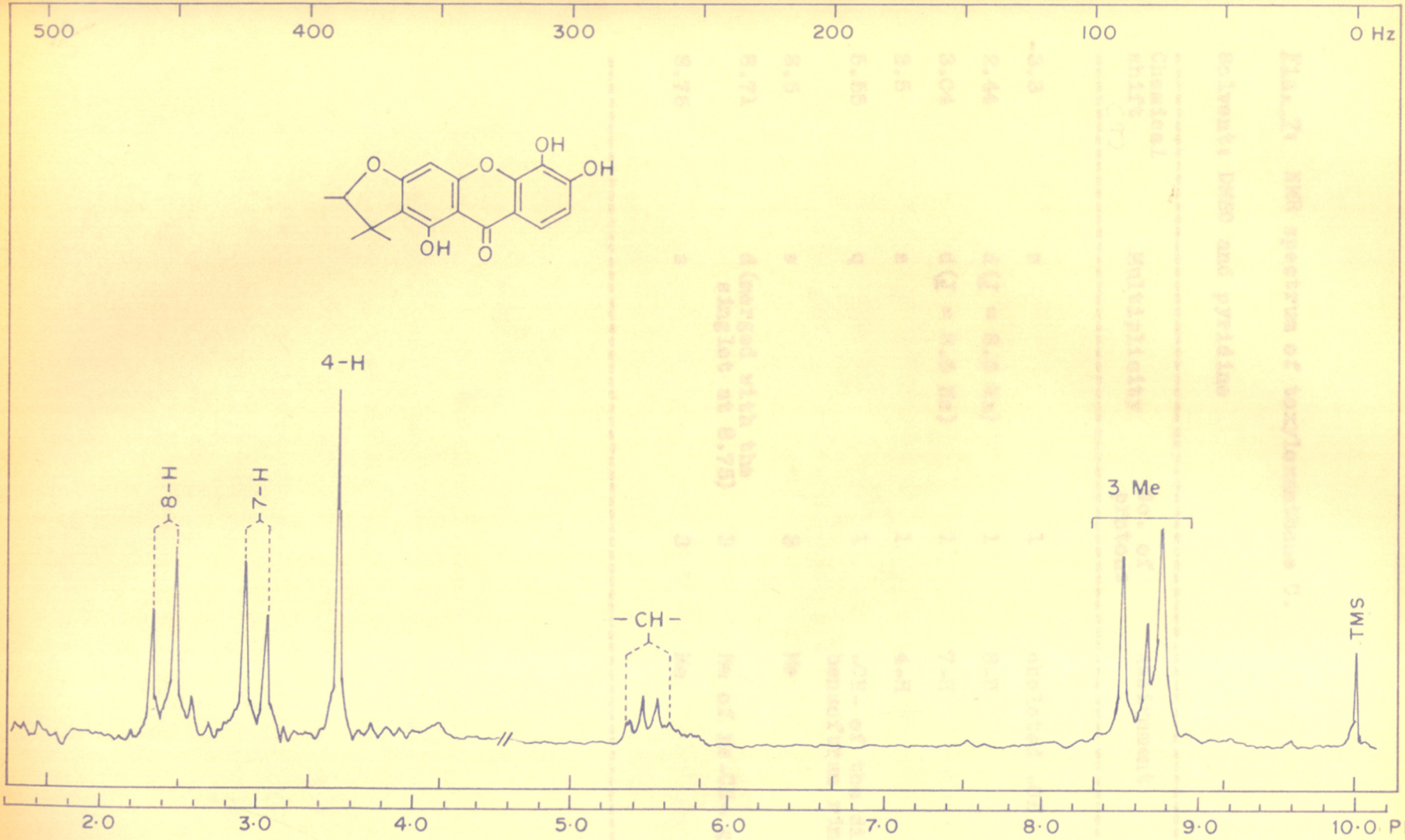
(XIV)

a methyl group resulting in a stable pyrylium cation.[27]

Xanthone (6) (Toxyloxanthone C), m.p. 290-291°, $C_{18}H_{16}O_6$ (M^+ 328). Unlike the other xanthones isolated from Toxylon pomiferum, toxyloxanthone C is optically active: $(\alpha)_D^{ethanol}$ + 59°. The UV spectrum is characteristic of 1,3,5,6-tetraoxygenated xanthones (Table 3) (248, 286 and 336 nm; log ε 4.54, 4.26 and 4.17 respectively). As in other products the IR spectrum shows characteristic bands for hydroxyls and xanthone carbonyl group (3450 and 1650 cm^{-1}).

Toxyloxanthone C formed a diacetate (M^+ 412) m.p.150° on treatment with acetic anhydride and pyridine, but a trimethyl ether, m.p. 97-100° on prolonged refluxing with dimethyl sulphate and potassium carbonate in acetone. No hydrogen was absorbed in presence of Adams catalyst in acetic acid.

The NMR spectrum (solvent: DMSO, pyridine; Fig. 7) shows the absence of methoxyl. In the downfield region a single-proton singlet at -3.3, disappearing by deuteration can be assigned to a hydroxyl bonded with a xanthone carbonyl group. In the aromatic region two doublets centered at 2.44 and ≙ 3.04 (J = 8.5 Hz) represent protons in the 8- and 7-positions respectively in a xanthone; a third signal at 3.50 can be assigned to proton at C-2 or C-4, indicating the phloroglucinol substitution pattern in ring A. Two methyl



FIG·7 NMR SPECTRUM OF TOXYLOXANTHONE C IN DMSO AND PYRIDINE

Fig. 7: NMR spectrum of toxyloxanthone C.

Solvent: DMSO and pyridine

Chemical shift (τ)	Multiplicity	No. of protons	Assignment
-3.3	s	1	chelated -OH
2.44	d (J = 8.5 Hz)	1	8-H
3.04	d (J = 8.5 Hz)	1	7-H
3.5	s	1	4-H
5.55	q	1	-CH- of the dihydro-benzofuran ring
8.5	s	3	Me
8.71	d (merged with the singlet at 8.75)	3	Me of Me-CH- group
8.75	s	3	Me

groups appear as singlets at 8.5 and 8.75, and a third methyl group appears as a doublet at 8.71 (partly overlapping with the singlet at 8.75). A single-proton quartet at 5.55 shows vicinal coupling with the methyl at 8.71. The data suggest the presence of a trimethyl dihydrofuran ring fused to a phloroglucinol nucleus, toxyloxanthone C having one of two possible structures (XV and XVI). The third alternative involving cyclisation with the 1-OH group is ruled out because of the presence of a hydrogen-bonded hydroxyl group. Differentiation between the two alternative structures (XV) and (XVI) was possible by observing the benzene-induced solvent shifts of the methoxyl resonances. All the three methoxyls in Toxyloxanthone C trimethylether appear as a single signal at 5.95 in $CDCl_3$, but in benzene they are seen at 6.02, 6.19 and 6.64, indicating that one OMe group has suffered a significant upfield shift. This can only happen if two of the OMe groups are sandwiched between two substituents, supporting structure (XV) for toxyloxanthone C. Structure (XV) is also favoured by the preferential formation of a diacetate, because the 1-hydroxyl in (XV) may be expected to exhibit steric hindrance to acetylation. A similar steric effect was noticed in artocarpin, which forms a diacetate under mild conditions of acetylation although there are three hydroxyl groups and a triacetate can be produced by more vigorous acetylation.

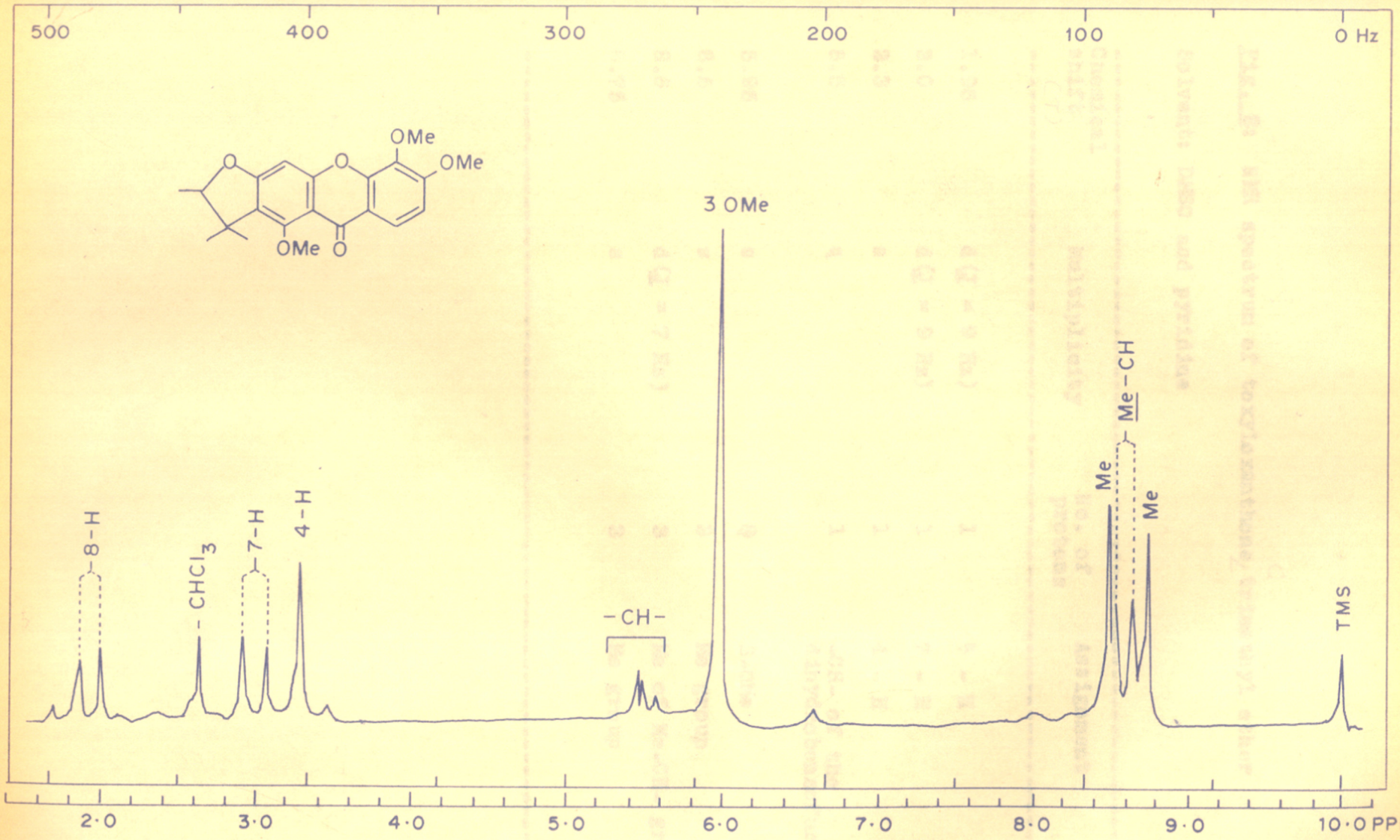
The NMR spectra of the trimethyl ether (Fig. 8) and the diacetate (Fig. 9) are also in complete agreement with structure (XV).

So far no natural trimethyldihydrofuranoxanthone has been reported, although some of them have been synthesised.[26] One furanoxanthone (XVII) from the extracts of the heartwood of Allanblankia floribunde[28] (Guttiferae) proved to be an artefact produced from the uncyclised isomer (XVIII) during its isolation by preparative layer chromatography involving the use of glacial acetic acid. It is known that under acetic acid conditions a 1,1-dimethylallyl group adjacent to a hydroxyl can cyclise to a trimethyldihydrofuran.[21]

Xanthone (6) has been shown to be not an artefact by comparing its R_f value with the total extract on TLC (silica gel: acetone-benzene as solvent).

Although a trimethyldihydrofuran not been encountered among natural flavonoids and xanthones so far, two mould metabolites, one naphthaquinone, two coumarins and two alkaloids have been encountered from different natural sources (Table 4).

Xanthone (7) (Toxyloxanthone D), m.p. 250-252°, $C_{23}H_{24}O_6$ (M^+ 396). The UV spectrum closely resembles 1,3,5,6-tetraoxygenated xanthones (see Table 3), λ_{max} 248, 284, 352 nm (log ε 4.46, 4.23 and 4.20), ν_{max} 1650, 3350 cm^{-1}. It forms a tetramethyl ether, m.p. 132°, on methylation with DMS.

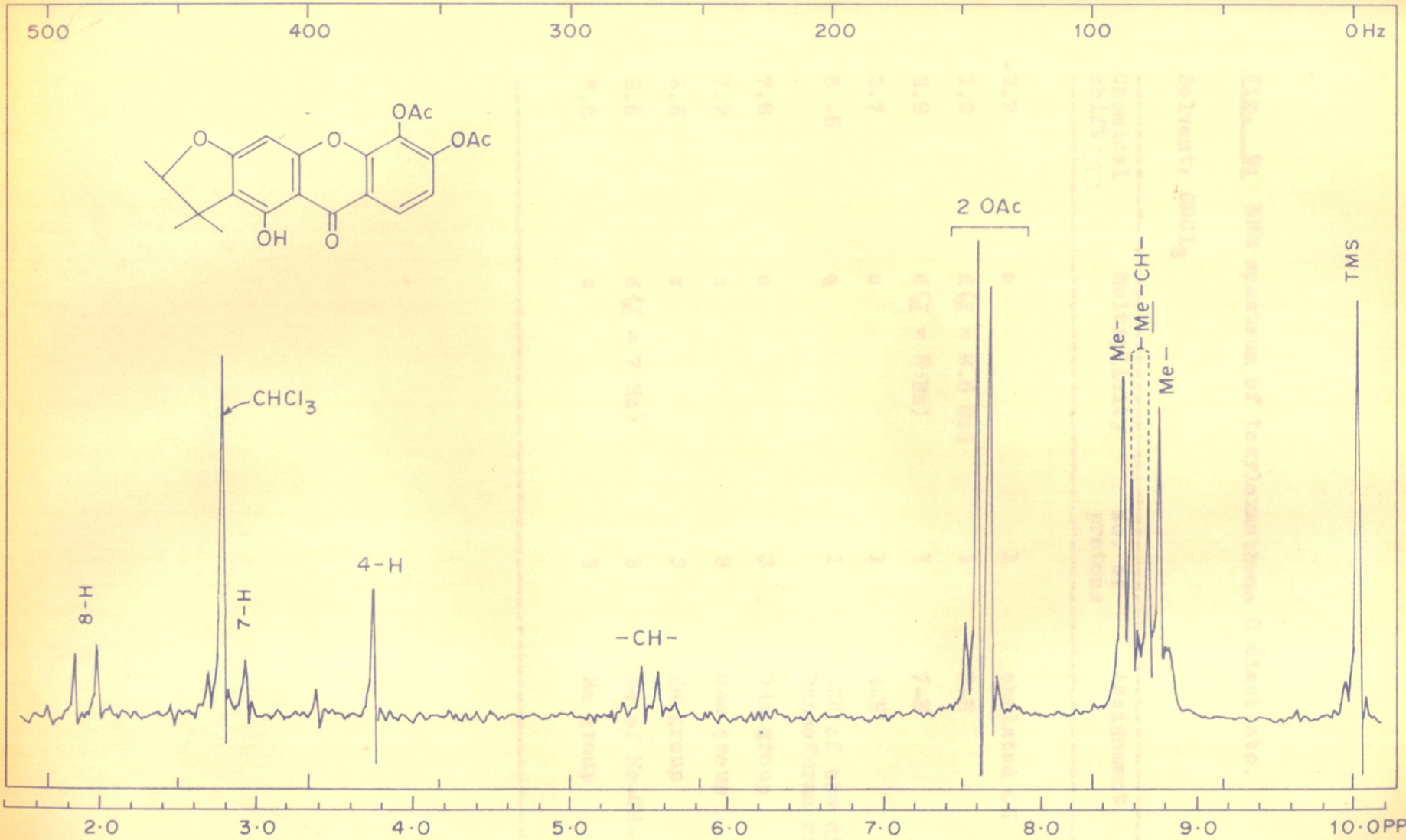


FIG·8 NMR SPECTRUM OF TOXYLOXANTHONE C TRIMETHYL ETHER IN $CDCl_3$

Fig. 8: NMR spectrum of toxyloxanthone C trimethyl ether

Solvent: DMSO and pyridine

Chemical shift (τ)	Multiplicity	No. of protons	Assignment
1.95	d (J = 9 Hz)	1	8 - H
3.0	d (J = 9 Hz)	1	7 - H
3.3	s	1	4 - H
5.5	q	1	-CH- of the dihydrobenzofuran ring.
5.95	s	9	3-OMe
8.5	s	3	Me group
8.6	d (J = 7 Hz)	3	Me of Me-CH- group
8.75	s	3	Me group

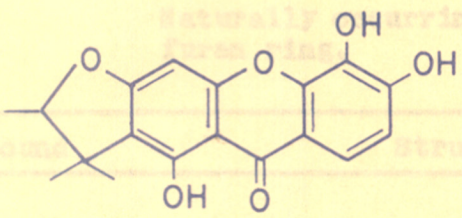


FIG·9 NMR SPECTRUM OF TOXYLOXANTHONE C DIACETATE IN $CDCl_3$

Fig. 9: NMR spectrum of toxyloxanthone C diacetate.

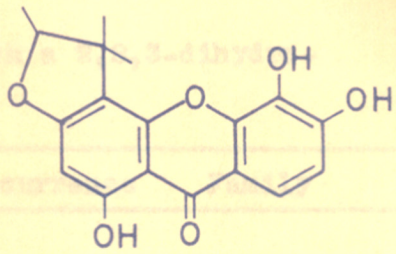
Solvent: $CDCl_3$

Chemical shift (τ)	Multiplicity	No. of protons	Assignment
-2.7	s	1	chelated OH
1.9	d (J = 8.5 Hz)	1	8-H
2.9	d (J = 8.5Hz)	1	7-H
3.7	s	1	4-H
5 .5	q	1	-CH of the dihydro-benzofuran ring.
7.6	s	3	OAc group
7.7	s	3	OAc group
8.5	s	3	Me group
8.6	d (J = 7 Hz)	3	Me of Me-CH- group
8.8	s	3	Me group

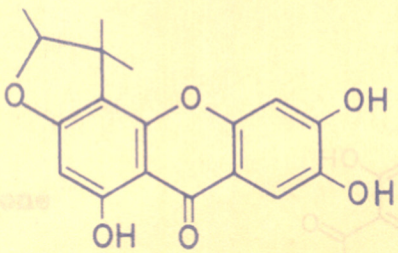


(XV)

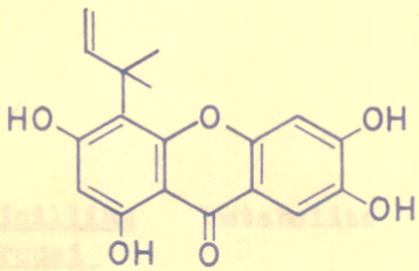
TOXYLOXANTHONE C



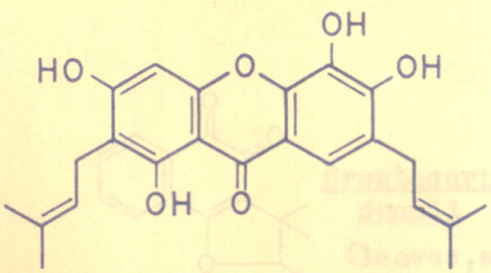
(XVI)



(XVII)



(XVIII)

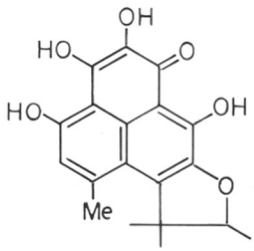
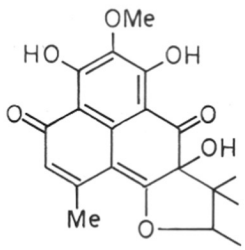
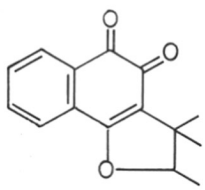


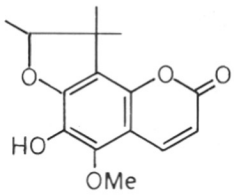
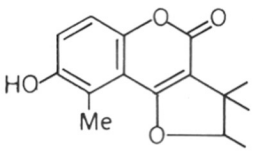
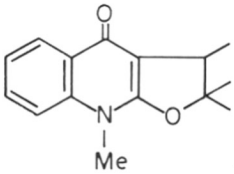
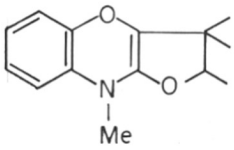
(XIX)

TOXYLOXANTHONE D

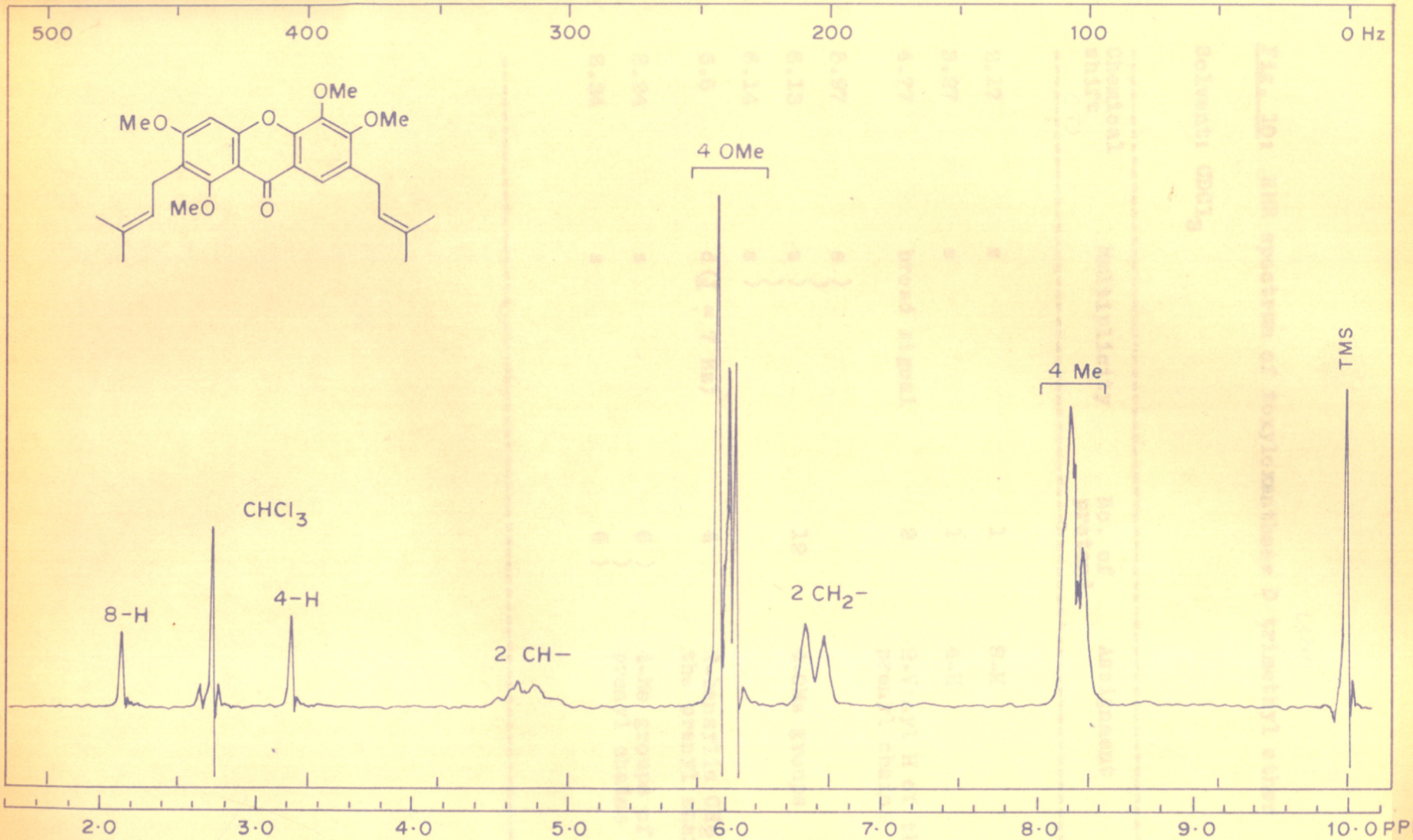
TABLE 4

Naturally occurring compounds with a 2,3,3-dihydrofuran ring.

Compound	Structure	Occurrence	Family	Ref.
Atrovenetin		Penicillium atrovenetum	Mould metabolite	29
Herqueinone		Penicillium herquei	Metabolite	30
Dunnione		Streptocarpus dunnii (leaves, stem flowers)	Gesneraceae	31

Nieshoutol		<u>Ptaeroxylon utile</u> (heartwood)	Meliaceae	32
Glaupalol		<u>Glaucidium palmatum</u> (rhizome)	Ranun-culaceae	33
Glucoside of glaupalol		-do- (rhizome)	-do-	34
Spectabiline		<u>Lemonia spectabilis</u> (leaves)	Rutaceae	35
Ifflaiamine		<u>Flindersia ifflaiana</u> (heartwood)	Rutaceae	36

The NMR spectrum in acetone-d_6 shows the absence of methoxyl groups in the molecule. There are characteristic signals for the presence of two 3,3-dimethylallyl groups attached to aromatic rings. The NMR spectrum of the tetramethyl ether in $CDCl_3$ (Fig. 10) shows two broad signals at 8.24 and 8.34 integrating for 12H; together with a four-proton doublet at 6.6 and a broad two-proton signal at 4.77, they can be assigned to the two 3,3-dimethylallyl groups. Four methoxyl groups are seen at 5.97 (6H), 6.13(3H) and 6.14(3H). In the aromatic region there are only two singlets at 2.17 and 3.27. The latter can be assigned to a proton at C-2 or C-4 of a 1,3-dihydroxyxanthone with an alkyl or alkenyl substituent at C-4 or C-2. The singlet at 2.17 has to be assigned to the proton at C-8. ~~The singlet at 2.17~~ Thus the A and B rings of the xanthone are substituted by four hydroxyls and two 3,3-dimethylallyl groups. Considering that the A-ring carries hydroxyls at C-1 and C-3 positions, structure (XIX) is suggested in preference to other possibilities on the basis of benzene-induced solvent shifts of the OMe groups. The OMe groups at 5.97, 6.13 and 6.14 in $CDCl_3$ appear at 6.07, 6.17(6H) and 6.57 after the addition of benzene; only one methoxyl signal undergoes an upfield shift and therefore has an ortho-position unsubstituted.



FIG·10 NMR SPECTRUM OF TOXYLOXANTHONE D TETRAMETHYL ETHER IN $CDCl_3$

Fig. 10: NMR spectrum of toxyloxanthone D tetra trimethyl ether

Solvent: $CDCl_3$

Chemical shift (τ)	Multiplicity	No. of protons	Assignment
2.17	s	1	8-H
3.27	s	1	4-H
4.77	broad signal	2	2-Vinyl H of the prenyl chains
5.97	s	12	4-OMe groups
6.13	s		
6.14	s		
6.6	d (J = 7 Hz)	4	2-benzylic CH_2 of the prenyl chains
8.24	s	6	4-Me groups of the prenyl chains
8.34	s	6	

The phenolic constituents of the heartwood of T. pomiferum

Reinvestigation of the heartwood of T. pomiferum revealed the presence of resorcinol kaempferol and quercetin which are not reported previously, besides morin,[4] dihydromorin,[5] dihydrokaempferol[5] and oxyresveratrol.[4] Recently the presence of resorcinol has been reported in the wood of many Morus and Artocarpus species.[37] The isolation of resorcinol from the genus Maclura, also belonging to the family, Moraceae, is of significant importance in the biogenesis of many phenolic compounds and also in chemotaxonomy of Morus species.

A deliberate attempt was made to detect the presence of 1,3,6,7-tetrahydroxyxanthone reported earlier from the heartwood of T. pomiferum. We were unable to detect the presence of 1,3,6,7-tetrahydroxyxanthone in the present sample of heartwood confirmed by a direct comparison on a paper chromatogram (phenol saturated with water as solvent) with an authentic sample of xanthone, kindly supplied by Dr. H.B. Bhat.

The powdered heartwood of T. pomiferum was soxhlet extracted with acetone and then with methanol. Both the extracts behaved identically on a TLC plate and hence were mixed. The extract was concentrated to a small volume,

mixed with the exhausted wood powder and extracted with benzene, ether and acetone.

The benzene soluble contained mostly of waxes together with some triterpenes and was not investigated further.

The ether soluble and acetone soluble behaved identically on a TLC silica gel plate and hence were mixed. The mixture was chromatographed on a silica gel column using benzene and benzene-acetone for elution. Further purification was carried out by chromatographing the mixture on a column of cellulose using water and water-acetic acid for elution. Resorcinol, kaempferol, dihydro-kaempferol, oxyresveratrol, dihydromorin and quercetin were obtained in the yields of 0.007, 0.01, 0.3, 2.0, 0.4, 0.005 and 0.35 per cent respectively.

Biogenesis of xanthones

In a recent review, Carpenter, Locksley and Scheinmann[25] have assessed the various proposals concerning xanthone biogenesis. The present knowledge favours the suggestion that xanthones, particularly in higher plants, are formed by intramolecular oxidative coupling of hydroxybenzophenones (Chart 1).

Some xanthones, earlier encountered in molds, have been derived entirely from a polyacetyl chain by multiple cyclisation, e.g. griscoxanthone[43,44] (Chart 2).

The oxygenation patterns of all xanthones isolated from higher plants suggest that they are formed from hydroxybenzophenones, which in turn might have resulted by the condensation of shikimate- and acetate- derived moieties. The acetate-derived part is an acyclic β-triketide, which may condense with a hydroxybenzoic acid (derived from shikimate unit) to give the corresponding benzophenone. Although various mechanisms have been postulated for the formation of xanthones from benzophenones, involving direct phenol oxidative coupling,[45] quinone addition,[46] dehydration between two hydroxyl groups,[47] or spirodienone formation and subsequent rearrangement to form a xanthone,[48] Carpenter *et al.*[25] have shown convincingly that xanthones in higher plants are formed from benzophenones by direct oxidative coupling.

Very recently Seshadri and his co-workers[49] have proposed a new scheme of biogenesis, particularly for the xanthones isolated from the family Guttiferae, to explain their frequent co-occurrence with 4-phenylcoumarins and other neoflavonoids. They have suggested that a 4-phenylcoumarin can undergo oxidative or dehydrative cyclisation to give an intermediate oxido structure (see chart 3), which on further oxidation loses the ∝-pyrone ring to give rise to a xanthone. The validity of this postulation can be questioned, although they have accomplished a chemical conversion of several 4-phenylcoumarins into the corresponding xanthones, because the mere co-occurrence of compounds of different chemical types does not necessarily indicate a common biosynthetic pathway. Further, there is no reason to believe that the "intermediate oxido" type compound will oxidise so rapidly to give xanthones that their presence cannot be detected. Thus there are biflavonoids which accompany 4-phenylcoumarins and xanthones in the heartwood of some plants.

All the xanthones of Toxylon pomiferum contains prenyl groups, partly in modified forms. Prenylation probably occurs after xanthone formation. Cyclisation of the side chain with an adjacent hydroxyl group, which may be followed by dehydrogenation, leads to the various xanthones

CHART 1

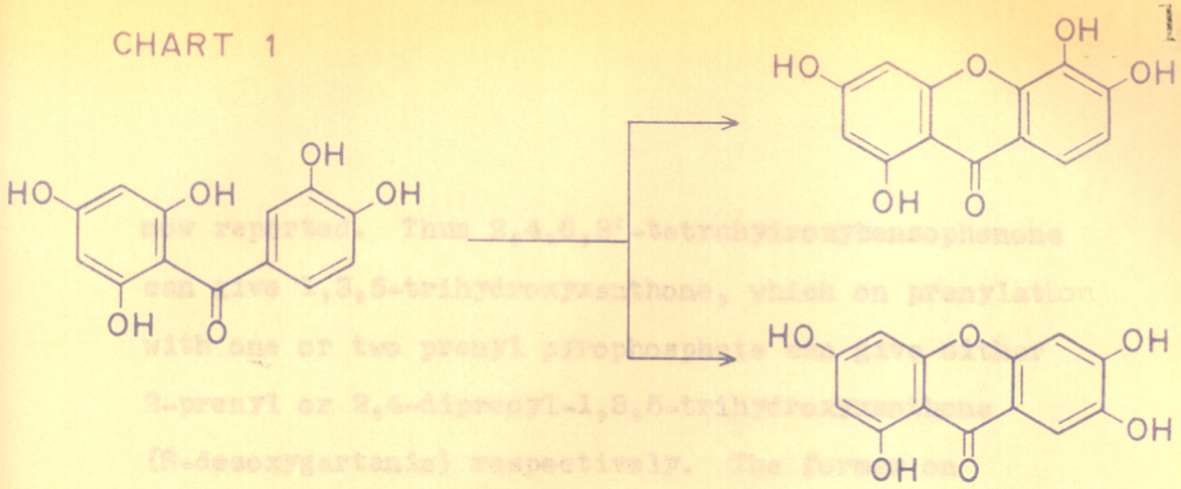


CHART 2

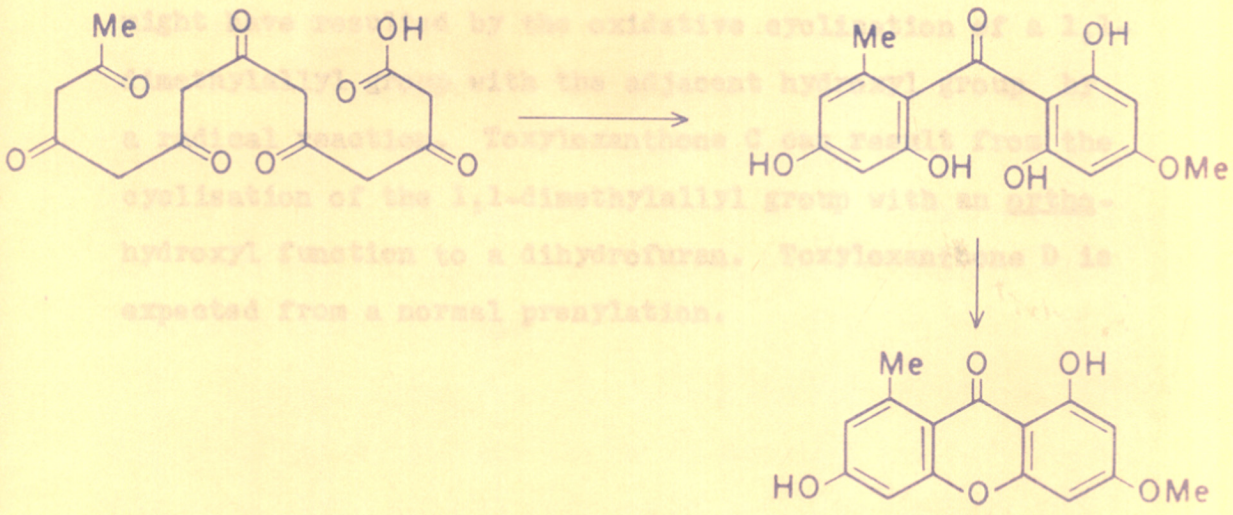
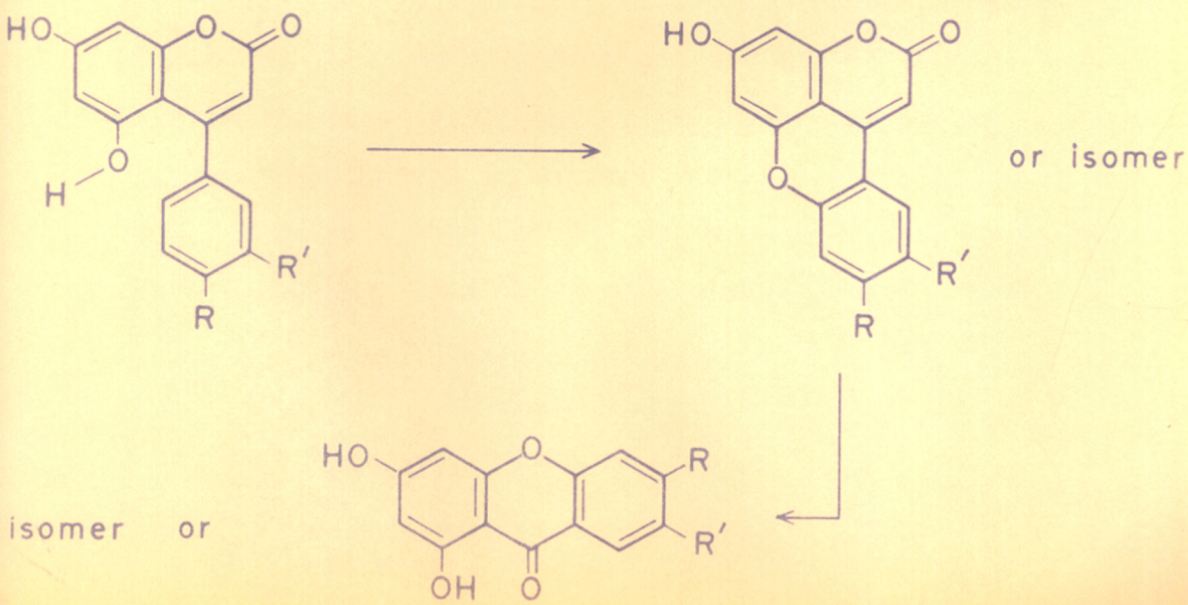


CHART 3



now reported. Thus 2,4,6,3'-tetrahydroxybenzophenone can give 1,3,5-trihydroxyxanthone, which on prenylation with one or two prenyl pyrophosphate can give either 2-prenyl or 2,4-diprenyl-1,3,5-trihydroxyxanthone (8-desoxygartanin) respectively. The former on oxidative cyclisation can lead to 6-desoxyjacareubin and the latter to toxyloxanthone A. Toxyloxanthone B might have resulted by the oxidative cyclisation of a 1,1-dimethylallyl group with the adjacent hydroxyl group by a radical reaction. Toxyloxanthone C can result from the cyclisation of the 1,1-dimethylallyl group with an ortho-hydroxyl function to a dihydrofuran. Toxyloxanthone D is expected from a normal prenylation.

EXPERIMENTAL

Extraction of T. pomiferum bark

The powdered bark of T. pomiferum (600 g) was extracted in a soxhlet with acetone. The extract was concentrated to a small volume, mixed with the exhausted bark powder and successively extracted with hexane, benzene, ether and acetone.

The hexane (5 g) and benzene (18 g) extracts showed identical behaviour on TLC plates (solvent: acetone-benzene 1:4). The ether extract (8 g) contained the slower moving compounds present in benzene. The acetone extract (8 g) contained mostly tannins.

The residue from the hexane extract (5 g) was dissolved in the minimum amount of methanol and left aside. The white solid (1.8 g) that separated was a mixture of triterpenes. The mother liquor gave a sticky product (3 g) on removal of the solvent. It was dissolved in benzene and loaded on a column of silica gel (60 g). The column was initially eluted with benzene and then with benzene-acetone. The acetone content in benzene was gradually increased to 40%. Fractions, each of 150 ml, were collected and examined on TLC plates. Similar fractions were pooled together.

Fractions 1 to 5 (1.0 g) contained mostly waxes. Fractions 6 and 7 (0.04 g) were found to be a mixture of two products with close R_f values on silica gel TLC plates developed with 0.5% acetone in benzene. The two fractions were collected by PLC. The fast moving fraction (xanthone 1) crystallised in yellow needles (0.15 g) from hexane-benzene, m.p. 165°, and was found to be identical with the known xanthone, 8-desoxygartanin,[20] m.p. 166.5°. (Found: C, 72.3; H, 6.2. $C_{23}H_{24}O_5$ requires C, 72.6; H, 6.1%). The slower moving compound (xanthone 2), toxyloxanthone A, crystallised in yellow needles (0.15 g), m.p. 165-66°, from hexane-benzene (Found: C, 72.6; H, 5.5. $C_{23}H_{22}O_5$ requires C, 73.0; H, 5.6%).

Fraction 8 (0.02 g) was a mixture of xanthones 2 and 3.

Fractions 9 to 11 (0.015 g) contained exclusively xanthone 3. It crystallised from hexane-benzene in yellow plates, m.p. 232°. (Found: C, 69.2; H, 4.5. $C_{18}H_{14}O_5$ requires C, 69.4; H, 4.5%). Its physical and chemical properties are identical with those of 6-desoxyjacareubin,[38] m.p. 211-13°.

Fractions 12 to 18 (0.1 g) were a mixture of xanthones 3 and 4, while fraction 19 contained exclusively xanthone 4 (0.04 g). The latter compound crystallised from benzene in yellow needles, m.p. 241° (lit.[38] m.p. 249-52°). (Found: C, 69.4; H, 4.6. $C_{18}H_{14}O_5$ requires C, 69.4; H, 4.5%). Its physical and

chemical properties are identical with those of osajaxanthone, (lit.[6] m.p. 264-65°), first isolated from the root bark of this plant.

Fractions 20 to 24 (0.4 g) contained a mixture of xanthones 4 and 5.

Fractions 25 to 27 contained xanthone 5 (toxyloxanthone B). It crystallised from acetone-benzene in yellow plates (0.02 g), m.p. 300°. (Found: C, 66.2; H, 4.3. $C_{18}H_{14}O_6$ requires C, 66.2; H, 4.7%).

Fractions 28 to 32 (0.1 g) contained mostly a mixture of xanthones 5 and 6.

Fractions 33 to 35 (0.2 g) contained a homogeneous compound, xanthone 6 (toxyloxanthone C). It crystallised from acetone-benzene in yellow plates (0.15 g), m.p. 290-291° (Found: C, 65.5; H, 4.7. $C_{18}H_{14}O_6$ requires C, 65.8; H, 4.8%).

Fraction 36 (0.01 g) contained xanthone 7 (toxyloxanthone D). It crystallised from acetone-benzene in pale yellow needles, m.p. 250-252°. (Found: C, 69.1; H, 5.9. $C_{23}H_{24}O_6$ requires C, 69.6; H, 6.0%).

Separation of the mixture into the individual compounds was effected by PLC on silica gel plates.

Benzene extract

The benzene extract of T. pomiferum (18 g) was adsorbed on silica gel (40 g) and packed over a column of silica gel (400 g) and eluted with benzene and benzene-

acetone mixture as in previous experiments. Like fractions were mixed. All the seven xanthones have been collected by fractional crystallisation from the respective fractions, except the second fraction which was a mixture of xanthones 1 and 2 together with waxes. The separation of these compounds was effected on PLC plates. The Table 5 indicates the compounds collected and the compounds present in the mother liquor of the respective fractions.

Table 5

Fr. No.	Xanthone	Quantity obtained (g)	Wt. of the product left in the mother liquor. (g)	Xanthone present in the mother liquor.
1	-	-	2 g.	waxes
2	1	0.01	0.54	waxes
3	2	0.01	-	2
4	3	0.06	0.6	2 & 3
5	4	0.16	1.5	3 & 4
6	5	0.2	1.2	4, 5 & 6
7	6	0.7	1.0	6 & 7
8	7	0.1	2.0	7 and a slow moving fraction.

6-Desoxyjacareubin dimethyl ether

A solution of 6-desoxyjacareubin (0.05 g) and dimethyl sulphate (0.07 ml) in acetone (20 ml) was refluxed with anhydrous potassium carbonate (0.4 g) for 24 hours. Distillation of acetone and treatment of the residue with water yielded a solid free from ferric colour. It crystallised from hexane-benzene in colourless needles (0.027 g), m.p. 201-202° (lit.[38] m.p. 198-199°) (Found: C, 70.9; H, 5.5. $C_{20}H_{18}O_5$ requires C, 71.0; H, 5.3%).

Osajaxanthone diacetate

A mixture of osajaxanthone (0.06 g), acetic anhydride (2 ml) and pyridine (1 ml) was heated on a steam bath for 2 hr. The product crystallised from ethanol (0.04 g), m.p. 185° (lit.[11] m.p. 203-204°) (Found: C, 67.2; H, 4.4. $C_{22}H_{18}O_7$ requires C, 67.0; H, 4.5%).

Toxyloxanthone B trimethylether

A mixture of toxyloxanthone B (0.08 g), dimethyl sulphate (0.12 ml) and potassium carbonate (0.8 g) was refluxed in acetone (20 ml) for 24 hours. The product crystallised from hexane-benzene in pale yellow needles (0.035 g), m.p. 192-193° (Found: C, 68.4; H, 5.6. $C_{21}H_{20}O_6$ requires C, 68.5; H, 5.4%).

Toxyloxanthone B dimethyl ether

To a solution of toxyloxanthone B (0.05 g) in absolute methanol, a solution of ethereal diazomethane was added and the mixture left in a refrigerator for 15 hr. The excess diazomethane was decomposed by the addition of acetic acid. The product showed two spots on TLC plates (solvent: benzene-acetone, 4:1). The two fractions were separated by PLC on silica gel plates using the same solvent system. The two fractions were collected and crystallised from hexane-benzene. The fast moving fraction was identified as the dimethyl ether (0.01 g), m.p. 210-212°. The slow-moving fraction was identified as the trimethyl ether (0.012 g), m.p. 192°.

Dihydrotoxyloxanthone B

Toxyloxanthone B (0.025 g) in ethanol (15 ml) was hydrogenated with platinum oxide. The compound took one mole of hydrogen in 12 hr. The catalyst was filtered off, and

the solvent removed under reduced pressure; the yellow solid crystallised from ethanol in pale yellow needles (0.015 g), m.p. 249-51° (Found: C, 65.6; H, 4.9. $C_{18}H_{16}O_6$ requires C, 65.8; H, 4.8%).

Toxyloxanthone C trimethylether

A solution of toxyloxanthone C (0.11 g) and dimethyl sulphate (1 ml) in acetone (50 ml) was refluxed with anhydrous potassium carbonate (3 g) for 12 hr. The product showed a mixture of three spots on a TLC plate (solvent benzene-acetone 9:1). It was separated by PLC using the same solvent system. The major fraction crystallised from hexane-benzene in colourless needles, m.p. 97-100° (Found: C, 67.6; H, 5.8. $C_{21}H_{22}O_6$ requires C, 68.0; H, 5.4%).

Toxyloxanthone C diacetate

A mixture of toxyloxanthone C (0.05 g), acetic anhydride (1 ml) and pyridine (1 ml) was heated for 2 hr. The product crystallised from ethanol in yellow needles, m.p. 150-151°, which gave a green colour with alcoholic ferric chloride (Found: C, 64.3; H, 4.9. $C_{22}H_{20}O_8$ requires C, 64.1; H, 4.9%).

Toxyloxanthone D tetramethyl ether

A solution of toxyloxanthone D (0.045 g) and dimethyl sulphate (0.5 ml) in acetone (20 ml) was refluxed

with anhydrous potassium carbonate (1 g) for 12 hr. The product was purified by passing a chloroform solution through a short column of silica gel. The fast moving fraction crystallised from hexane-benzene in colourless needles (0.02 g), m.p. 132° (Found: C, 71.1; H, 7.0. $C_{27}H_{32}O_6$ requires C, 71.6; H, 7.1%).

Extraction of T. pomiferum heartwood

The powdered heartwood (700 g) was soxhlet extracted with acetone for 48 hours and then with methanol for 48 hours. The acetone extract (21 g) and methanol extract (30 g) were examined on a TLC silica gel plate (30% acetone in benzene) and by paper chromatography (15% acetic acid in water). Both the extracts behaved identically and hence were mixed. The extract was concentrated to a small volume, mixed with the exhausted wood powder, and extracted with benzene, ether and acetone. The benzene soluble (1 g) contained mostly of waxes together with some quantity of triterpenes and was not investigated further. The ether soluble and ether insoluble showed same behaviour on a TLC silica gel plate and paper and hence were mixed.

The mixture (15 g) was adsorbed on silica gel (30 g) and packed on a column of silica gel (170 g). The column was first eluted with benzene and then with benzene-acetone mixture. The acetone content in benzene was gradually

increased to 30%. Fractions (200 ml) were collected and examined by TLC silica gel and paper chromatography. Similar fractions were mixed and in this way ten different fractions were obtained.

Fraction (1) contained mainly of some faster moving waxes (0.5 g).

Fraction (2) on crystallisation from benzene gave colourless needles, m.p. 110° (0.015 g), lit.[39] m.p. 110°, which gave a brown colour with alcoholic ferfic chloride. This was identified as resorcinol by its TLC behaviour and mixed m.p. with an authentic sample.[39] IR spectra were superimposable.

Fraction (3) on crystallisation from acetone-benzene mixture gave pale yellow needles, m.p. 280°. This gave green colour with alcoholic ferric chloride and a red colour in the Shinoda test. This was identified as kaempferol (0.02 g), lit.[40] m.p. 276-78°. The mixed m.p. with an authentic sample was undepressed and IR spectra were superimposable.

Fraction (4) was a mixture of kaempferol and dihydrokaempferol.

Fraction (5) crystallised from acetone-benzene mixture as white needles, m.p. 230°. This gave a green ferric colour and a red colour in the Shinoda test. This was identified as dihydrokaempferol (0.6 g); lit.[5] m.p.228°. The mixed m.p. with the authentic sample was undepressed.

Fraction (6) was a mixture of dihydrokaempferol and oxyresveratrol. Fraction (7) crystallised from water, m.p. 201°, gave green ferric colour and was identified as oxyresveratrol (4.0 g), lit.[5] m.p. 201°.

Fraction (8) showed a single spot on a TLC silica gel plate (3:7 acetone-benzene), but was found to be a mixture of dihydromorin and oxyresveratrol when examined by paper chromatography (15% acetic acid in water). These two compounds were separated over a cellulose column using water and increasing the percentage of acetic acid (10%) as eluant. Dihydromorin (0.9 g), m.p. 224°, lit.[5] m.p.228°, was crystallised from water and oxyresveratrol (0.1 g) was also isolated.

Fraction (9) was found to be a mixture of oxyresveratrol, dihydromorin and quercetin. After removal of solvent this fraction was left in water. Mixture of oxyresveratrol and dihydromorin separated as a yellow solid and was filtered off. The mother-liquor when examined on a TLC plate showed the presence of dihydromorin and quercetin. This mixture was loaded on a PLC plate and faster moving major band was isolated. This crystallised from methanol as yellow needles, m.p.302° (0.01 g), lit.[41] m.p. 315°. This was characterised as quercetin. Mixed m.p. with the authentic sample was undepressed.

Fraction (10) crystallised from water, m.p. 290° (0.7 g), lit.[42] m.p. 300°, and was identified as morin(0.7 g).

All the above compounds have also been isolated from many Morus species[39] in our laboratory and were used for mixed m.p.

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CHAPTER III

CHEMICAL INVESTIGATION OF THE LEAVES OF *Calycopteris floribunda*

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INTRODUCTION

Calycopteris floribunda Lamk. belonging to the family Combretaceae is a species found in Malabar and other parts of South India. It is a diffuse shrub with drooping branches.[1]

The copper coloured leaves of C. floribunda are reputed to have laxative and anthelmintic properties,[2] Leaves ground into paste and made into fine-grain pills, they are administered for the expulsion of round worms. Leaves ground and boiled in oil are applied to ulcers. Leaves also ground and given with butter cure dysentery and malarial fever. Roots of C. floribunda ground to paste with that of Croton oblongifolium is applied to the bites of phoosa snake. Kurz[3] divides this shrub into two species: C. nutans and C. floribunda.

From the acetone extract of the leaves of C. floribunda calycopterin was isolated, m.p. 225-226°. This was found to be a flavone of molecular formula $C_{19}H_{18}O_8$ containing two hydroxyl groups and four methoxyl groups.[4] Calycopterin has also been reported from the leaves of Digitatis thapsi L.[5] (Spanish digitalis) belonging to family Schrophulariaceae.

Further work carried on calycopterin proved that it was a 4',5-dihydroxy 3,6,7,8-tetramethoxyflavone[6] (I).

Calycopterin has been synthesised[7] by condensing 2-hydroxy-ω-3,4,5,6-pentamethoxyacetophenone with the anhydride of potassium salt of p-benzoyloxybenzoic acid according to the method of Allan and Robinson. The product obtained was 4'-hydroxy, 3,5,6,7,8-pentamethoxyflavone which when subjected to partial demethylation using hydrogen-bromide yielded calycopterin.

On the strength of a report[6] from Pharmacology Research Officer from Madras and the statement of Nadkarni[2] anthelmintic properties were ascribed to calycopterin[4]. But Mahal[8] studied the anthelmintic action of calycopterin in vitro by directly immersing round worms, tape worms and leeches in solution of varying concentration. He found that calycopterin does not possess any anthelmintic or germicidal properties.

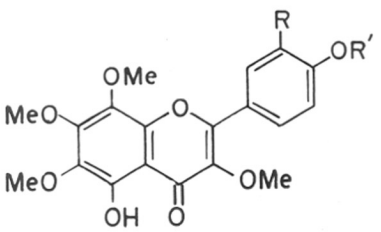
Very recently Rodriguez et al.[9] have sought UV, NMR and mass spectral data for confirming the structure (I) of calycopterin, although conclusive chemical and synthetic evidence was available.[6,7] The main purpose of Rodriguez et al., which was to distinguish between (I) and the isomer (II), was served by the NMR signal at -2.30 for a hydrogen bonded hydroxyl group. Citing unpublished data of P. V. Radhakrishnan and A.V. Rama Rao, Venkataraman[10] showed that two NMR spectra were adequate for determining the structure of calycopterin. In the spectrum of calycopterin

there are four methoxyl groups, a chelated hydroxyl at -2.58 (5-OH) and a second hydroxyl at -0.36 (7 or 4') and four aromatic protons as two doublets at 1.96 and 2.98 (J = 9.0 Hz) which appeared at 1.84 and 2.58 in the spectrum of the diacetate. The A_2B_2 pattern of the aromatic proton signals could only arise from the B-ring, and the downfield shift of 0.4 ppm in the signal for the 3',5'-protons located the second hydroxyl group in the 4'-position.

PRESENT WORK

A reexamination of the leaves of Calycopteris floribunda, obtained from Kerala State has revealed the presence of two minor constituents accompanying calycopterin,[4] the major pigment. The two minor constituents have been identified as 4'-methylether of calycopterin and 3'-methoxy-calycopterin.

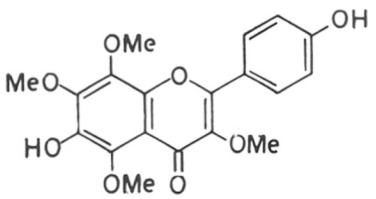
The dried leaves of C. floribunda were extracted in a soxhlet with acetone. The residue from the acetone extract was washed with hexane to remove chlorophyll and resinous matter. Hexane insoluble residue was taken up in ether and was extracted with 5% aqueous sodium hydroxide. The phenolic pigments were recovered by acidification and extraction with ether. The alkali-insoluble portion was found to contain only one compound, which crystallised from methanol



I ; R = H , R′ = H

III ; R = H , R′ = Me

IV ; R = OMe , R′ = H



II

in pale yellow needles (0.03% on weight of leaves), m.p.120°; $C_{20}H_{20}O_8$ (M⁺ 388); λ_{max} 276 (4.38), 333 (4.44) nm; deep orange colour with Mg + HCl and green with $FeCl_3$. The base peak in the mass spectrum corresponds to the loss of a methyl radical from the molecular ion, and as in calycopterin; there are peaks at M-43 (loss of $-COCH_3$ characteristic of a 3-methoxylflavone) and at m/e 211 (retro Diels-Alder cleavage).

The NMR spectrum in $CDCl_3$ (Fig. 1) shows the presence of five methoxyl groups in the region of 5.89 to 6.10 (chemical shifts on the τ scale), a chelated hydroxyl at -2.38, and four aromatic protons in a typical A_2B_2 pattern as two doublets (J = 9 Hz) centered at 1.88 and 2.67, corresponding to 2',6'- and 3',5'-hydrogens respectively in a flavone. A ring is fully substituted and the 3-position is also occupied as further confirmed by the absence of 3-proton signal around 3.2. From this evidence, it is clear that the compound is the 4'-methyl ether of calycopterin (III), confirmed by a direct comparison with the product of the diazomethane methylation of calycopterin. The product (5-hydroxyauranetin) was isolated earlier from the peel of Citrus aurantium.[11]

The alkali-soluble part consisted mainly of calycopterin and was crystallised from methanol in yellow prismatic needles (0.1% on weight of leaves), m.p. 225-226° (lit.[4] m.p. 225-226°). After removal of calycopterin, repeated PLC of the mother-liquor (silica gel) yielded a very minor amount of a new pigment (0.0005% on weight of leaves), which still contained traces of calycopterin. The mass spectrum shows the molecular ion at m/e 404, corresponding to a methoxy-calycopterin. The base peak is formed by the loss of methyl radical (m/e 389) from the molecular ion to form the cation (XIV). Peaks at 361 (M-43) and 211 indicate identical substitution of the "A" ring as in calycopterin. The main paths of fragmentation are outlined in Chart (1).

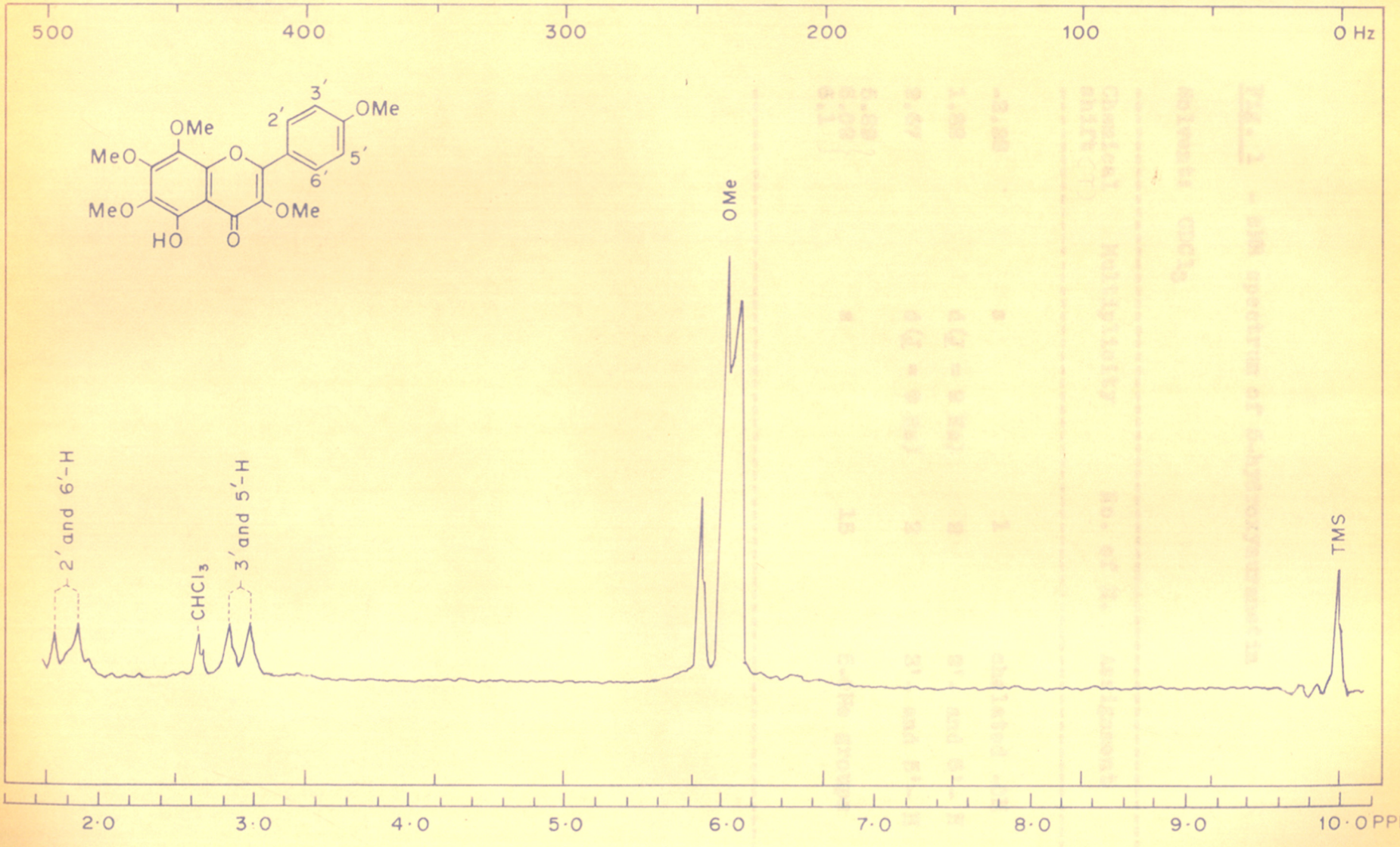


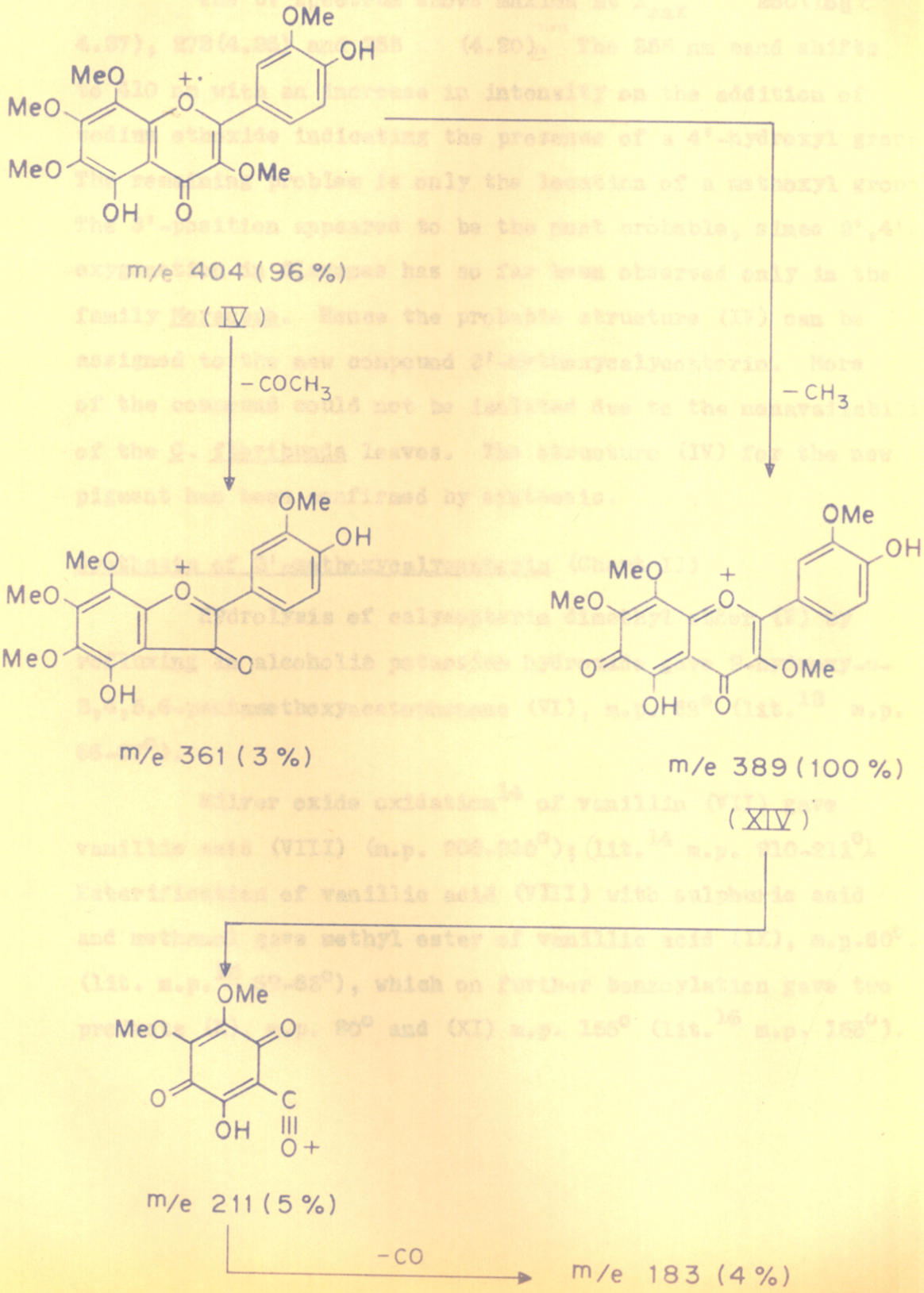
FIG. 1 NMR SPECTRUM OF 5-HYDROXYAURANETIN IN $CDCl_3$

Fig. 1 - NMR spectrum of 5-hydroxyauranetin

Solvent: $CDCl_3$

Chemical shift (τ)	Multiplicity	No. of H.	Assignment
-2.38	s	1	chelated -OH
1.88	d (J = 9 Hz)	2	2'- and 6'- H
2.67	d (J = 9 Hz)	2	3'- and 5'- H
5.89 6.02 6.1	s	15	5-OMe groups

CHART 1


OMe
OH
MeO
MeO
MeO
OMe
OH
O
+·
m/e 404 (96 %)
(IV)
−COCH3
−CH3
m/e 361 (3 %)
m/e 389 (100 %)
(XIV)
m/e 211 (5 %)
−CO
m/e 183 (4 %)

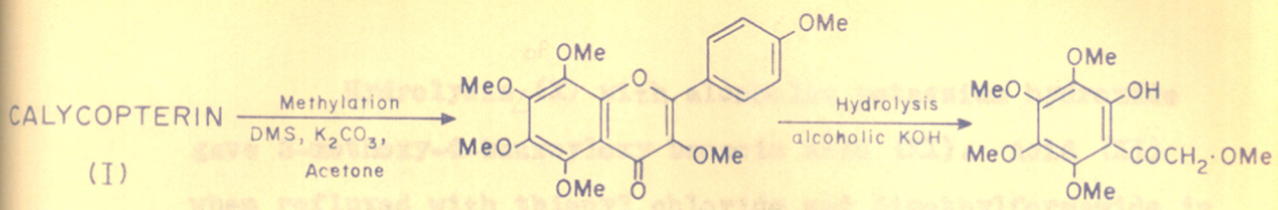
The UV spectrum shows maxima at $\lambda_{max}^{ethanol}$ 260(log ε 4.37), 278(4.36) and 355 nm (4.20). The 355 nm band shifts to 410 nm with an increase in intensity on the addition of sodium ethoxide indicating the presence of a 4'-hydroxyl group. The remaining problem is only the location of a methoxyl group. The 3'-position appeared to be the most probable, since 2',4'-oxygenation in flavones has so far been observed only in the family Moraceae. Hence the probable structure (IV) can be assigned to the new compound 3'-methoxycalycopterin. More of the compound could not be isolated due to the nonavailability of the C. floribunda leaves. The structure (IV) for the new pigment has been confirmed by synthesis.

Synthesis of 3'-methoxycalycopterin (Chart II)

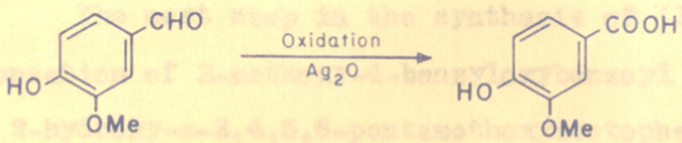
Hydrolysis of calycopterin dimethyl ether (V) by refluxing in alcoholic potassium hydroxide gave 2-hydroxy-ω-3,4,5,6-pentamethoxyacetophenone (VI), m.p. 63° (lit.[13] m.p. 66-67°).

Silver oxide oxidation[14] of vanillin (VII) gave vanillic acid (VIII) (m.p. 208-210°); (lit.[14] m.p. 210-211°). Esterification of vanillic acid (VIII) with sulphuric acid and methanol gave methyl ester of vanillic acid (IX), m.p.60°, (lit. m.p.[15] 62-63°), which on further benzoylation gave two products (X), m.p. 80° and (XI) m.p. 155° (lit.[16] m.p. 155°).

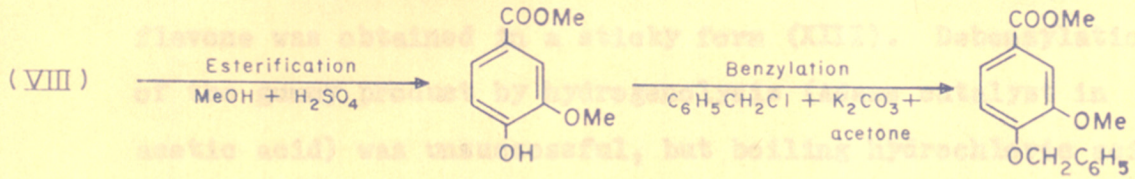
CHART II



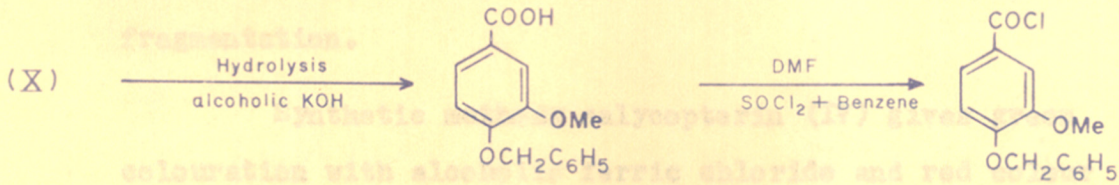
(V) (VI)



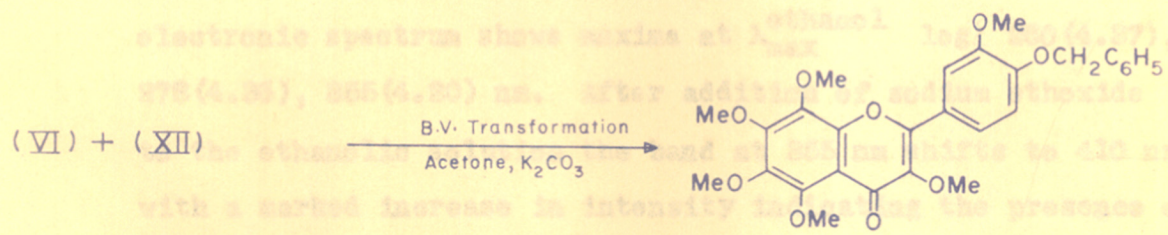
(VII) (VIII)



(IX) (X)



(XI) (XII)



(XIII)

Acetic acid + HCl

3'- METHOXYCALYCOPTERIN (IV)

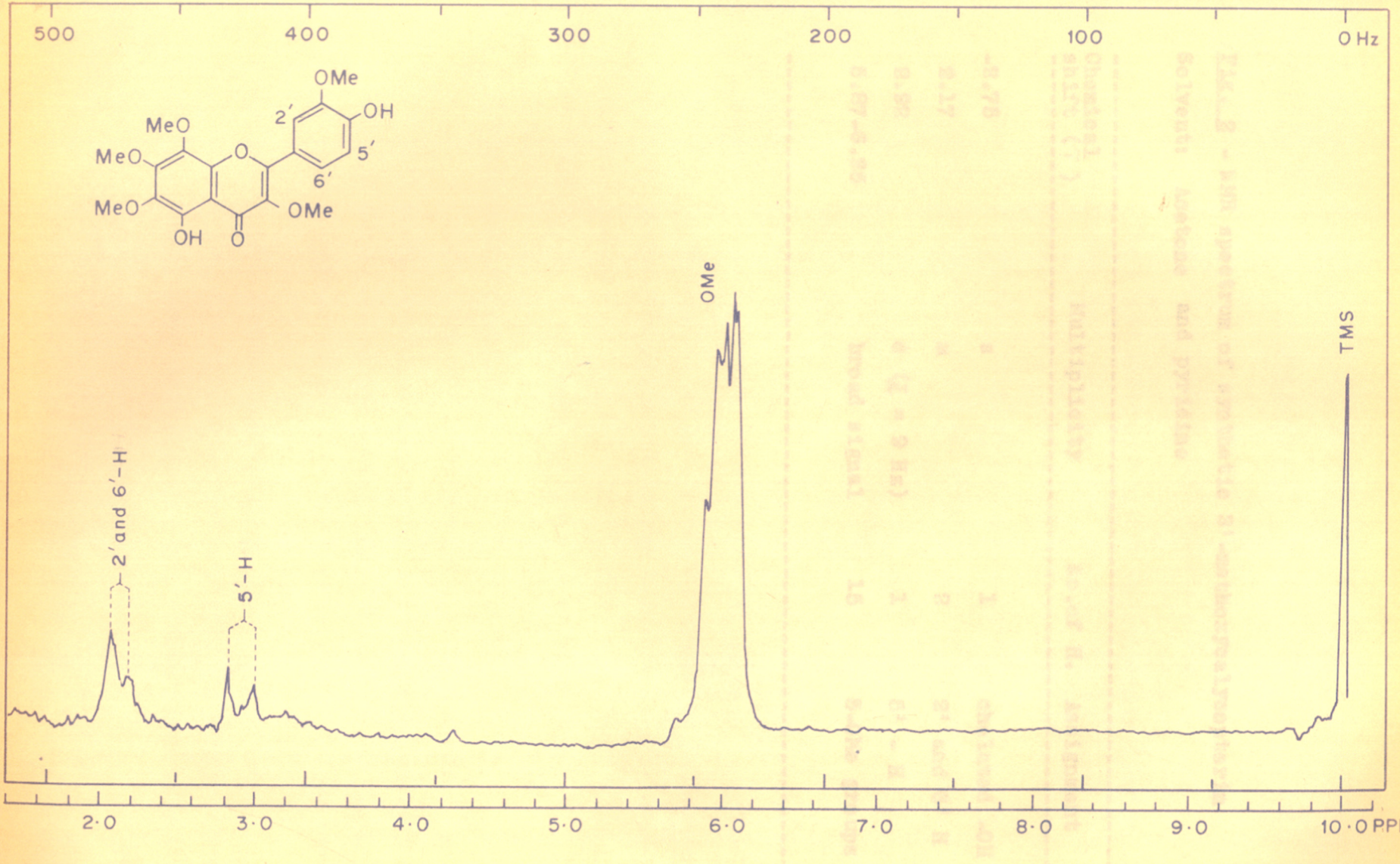
Hydrolysis of (X) with alcoholic potassium hydroxide gave 3-methoxy-4-benzoyloxy benzoic acid (XI). Acid (XI) when refluxed with thionyl chloride and dimethylformamide in dry benzene gave the corresponding acid chloride (XII), m.p. 78° (lit.[17] m.p. 80°).

The next step in the synthesis of (IV) is the condensation of 3-methoxy-4-benzyloxybenzoyl chloride (XII) with 2-hydroxy-ω-3,4,5,6-pentamethoxyacetophenone (VI) in acetone and potassium carbonate (Baker-Venkataraman transformation)[18] when 4'-benzyloxy 3',3,5,6,7,8-hexamethoxyflavone was obtained in a sticky form (XIII). Debenzylation of the gummy product by hydrogenolysis (Adams catalyst in acetic acid) was unsuccessful, but boiling hydrochloric acid in acetic acid yielded (IV), brownish yellow needles, m.p. 160-62° from methanol, identical with natural methoxycalycopterin in chromatographic behaviour and mass spectral fragmentation.

Synthetic methoxy calycopterin (IV) gives green colouration with alcoholic ferric chloride and red colour in Shinoda test. Mass spectrum shows the molecular weight to be 404 and the compound analysis for $C_{20}H_{20}O_9$. The electronic spectrum shows maxima at $\lambda_{max}^{ethanol}$ logε 260(4.37), 278(4.36), 355(4.20) nm. After addition of sodium ethoxide to the ethanolic solution the band at 355 nm shifts to 410 nm with a marked increase in intensity indicating the presence of 4'-hydroxyl group.

NMR spectrum (Fig. 2) recorded in acetone shows 5-methoxyl groups at 5.87 to 6.25, a chelated hydroxyl at -2.75 which disappears on addition of D_2O. The aromatic region of the spectrum shows a doublet at 2.92 (Hz = 9.5 cps), indicating the presence of 5' proton and a multiplet at 2.17 showing the presence of 2' and 6' protons. The A ring of flavone is fully substituted and the 3-position is also occupied as confirmed by the absence of 3 proton signal at about 3. Since the 5-hydroxyl is located, the other hydroxyl could be placed at 4'-position from the electronic spectrum.

From the above evidence structure (IV) could be assigned for the synthetic product which has the same R_f value as the natural 3'-methoxycalycopterin.



FIG·2 NMR SPECTRUM OF 3′-METHOXYCALYCOPTRIN IN ACETONE AND PYRIDINE

Fig. 2 - NMR spectrum of synthetic 3'-methoxycalycopterin

Solvent: Acetone and pyridine

Chemical shift (τ)	Multiplicity	No.of H.	Assignment
-2.75	s	1	chelated -OH
2.17	m	2	2' and 6' H
2.92	d (J = 9 Hz)	1	5' - H
5.87-6.25	broad signal	15	5-OMe groups

EXPERIMENTAL

Extraction of Calycopteris floribunda leaves

The dried leaves (2 kg) of Calycopteris floribunda were extracted in a soxhlet with acetone for 48 hours. The acetone extract (60 g) was washed with hexane. The hexane insoluble residue (18 g) was taken up in ether. The ether soluble (6 g) was extracted with 5% sodium hydroxide.

Isolation of 5-hydroxyauranetin

The sodium hydroxide insoluble portion (0.8 g) when spotted on a TLC (silica gel plate (solvent system 3:7 acetone-benzene) showed a single spot moving faster than calycopterin and was corresponding to the monomethyl ether of calycopterin. 5-Hydroxyauranetin was crystallised from methanol in brownish yellow needles (0.7 g), m.p. 120° (lit.[9] m.p. 126°) (Found: C, 62.6; H, 5.2. $C_{20}H_{20}O_8$ requires C, 61.9; H, 5.2%).

Isolation of calycopterin

Sodium hydroxide soluble (2.3 g) when spotted on a TLC silica gel plate showed a single spot corresponding to calycopterin. Calycopterin was crystallised from methanol in yellow prismatic needles (2 g), m.p. 225-226° (lit.[4] m.p. 225-226°).

Isolation of 3'-methoxycalycopterin

After the removal of calycopterin the mother liquor (0.25 g) was loaded on PLC silica gel plates (solvent system:

acetone-benzene 1:9). After repeated developments, the minor slower moving band was isolated (0.010 g). Methoxycalycopterin was contaminated with traces of calycopterin.

Preparation of monomethylether of calycopterin

Calycopterin (0.1 g) in methanol (30 ml) was treated with excess of ethereal diazomethane and the product was worked out as usual. Monomethylether of calycopterin crystallised from methanol in yellow needles (0.05 g), m.p. 122°; lit.[4] m.p. 124°.

Synthesis of methoxycalycopterin

Methylation of calycopterin

Calycopterin (2 g) was refluxed with excess of dimethyl sulphate in acetone (100 ml) with potassium carbonate (15 g) under anhydrous conditions for 12 hours. The product was worked out as usual and dimethyl ether of calycopterin crystallised from methanol in pale yellow needles (1.4 g) m.p. 128°; lit.[4] m.p. 131°.

Hydrolysis of dimethyl ether of calycopterin

Dimethyl ether of calycopterin (1.4 g) was refluxed with 10% alcoholic potassium hydroxide (30 ml) for 2 hours. After removal of alcohol the residue was treated with water and the mixture was acidified with hydrochloric acid and

extracted with ether. The ether layer was extracted with 5% sodium bicarbonate and 5% sodium hydroxide solution. Sodium hydroxide soluble was acidified with hydrochloric acid and extracted with ether. The ketone (VI) obtained shows a single spot on TLC silica gel plate (1:9 acetone-benzene). It was crystallised from methanol in yellow needles (0.9 g), m.p. 63°; lit.[13] m.p. 66-67°.

Synthesis of vanillic acid

Vanillic acid[14] (VIII) was prepared by silver oxide oxidation of vanillin (VII) (30 g). Vanillic acid was crystallised from water (26 g), m.p. 208-210°; lit.[14] m.p. 210-211°.

Esterification of vanillic acid

Vanillic acid (15 g) was refluxed with methanol (160 ml) and sulphuric acid (2 drops) for 15 hours. After removal of methanol the residue was treated with water and extracted with ether. The methyl ester of vanillic acid (IX) was crystallised from methanol (14 g), m.p. 60°; lit.[15] m.p. 62-63°.

Benzylation of methyl ester of vanillic acid

A mixture of methyl ester of vanillic acid (14 g), benzyl chloride (22 ml) and potassium carbonate (80 g) was refluxed in dry acetone (250 ml) for 24 hours. After removal

of acetone, water was added to the reaction mixture and then extracted with ether. Traces of benzyl chloride from the aqueous layer was removed by steam distillation. The product separated was 3-methoxy-4-benzyloxy benzoic acid (XI). This was crystallised from water in white needles (5 g), m.p. 155°; lit.[16] m.p. 155°.

After removal of ether from the ether layer, water was added to the residue and steam distilled to remove benzyl chloride. The methyl ester of 3-methoxy-4-benzyloxy-benzoic acid (X) separated was crystallised from water in white needles (5 g), m.p. 80°.

Hydrolysis of methyl ester of 3-methoxy-4-benyloxy-benzoic acid (X)

The acid (X) (5 g) was refluxed with 10% alcoholic potassium hydroxide (30 ml) for 2 hours. The reaction mixture was worked out as usual and 3-methoxy-4-benzyloxybenzoic acid (XI) was crystallised from water (4 g), m.p. 155°.

Preparation of acid chloride

The acid (XI) (3 g) was refluxed with thionyl-chloride (5 ml) and dimethylformamide (2 drops) in dry benzene (15 ml) for 2 hours. Thionylchloride, dimethyl-formamide and benzene were distilled off under reduced pressure and the white solid separated (1.9 g) was dried, m.p. 78°; lit.[17] m.p. 80°.

Synthesis of 3'-methoxycalycopterin

A mixture of 3-methoxy-4-benzyloxybenzoyl chloride (XII) (1.9 g), 2-hydroxy-ω-3,4,5,6-pentamethoxyacetophenone (VI) (0.8 g) and potassium carbonate (10 g) in dry acetone (100 ml) was refluxed for 16 hours. The reaction mixture was worked out as usual to give a sticky product (XIII)(0.5 g).

Demethylation of XIII

A mixture of the sticky product (0.5 g) (XIII), concentrated hydrochloric acid (5 ml) and acetic acid (5 ml) was heated on a water bath for 3 hours. This was poured in cold water and the product separated gave green colour with alcoholic ferric chloride. When the product was examined by TLC (silica gel) (1:4 acetone-benzene) it showed a mixture of three compounds. The major compound was isolated and purified by PLC (silica gel). This was crystallised from methanol in brownish yellow needles (0.04 g), m.p. 160-62°. The product was found to be having the same R_f value as the natural 3'-methoxycalycopterin on a TLC silica gel plate (solvent system 1:9 acetone-benzene) (Found: C, 60.0; H, 5.2. $C_{20}H_{20}O_9$ requires C, 59.4; H, 4.9%).

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CHAPTER IV

Tissue Culture of Populus nigra

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INTRODUCTION

The aseptic culture of plant cells and tissues as a technique known as tissue culture presents an opportunity for studying the metabolic potential of isolated plant cells grown under carefully controlled conditions. In recent years tissue cultures from a number of plants have been shown to contain secondary metabolites, such as alkaloids,[1] amino acids,[2] antibiotics,[3] carbohydrates,[4] enzymes,[5] flavonoids,[6] sterols,[7] triterpenoids,[8] etc. In some cases the biosynthesis of certain compounds has also been demonstrated.[9-11] This work has been extensively reviewed,[12-15] and will not be dealt with in detail in this chapter. Particular reference however will be paid to literature on tissue cultures obtained from different *Populus* species and the compounds isolated from them.

The genus *Populus* commonly known as poplar, aspen and cottonwood, belongs to the family *Saliaceae*.[16] About forty species of this genus are known, mainly from the extra-tropical regions of the world, and include shrubs and trees. In India the poplars are chiefly distributed in the forests of the Western Himalayas where the altitude reaches upto 7000 ft. In other regions where ecological conditions are favourable poplars are cultivated as ornamental plants.[17]

Poplars have very soft wood which finds application in making cricket bats, packing cases and in other useful industries where soft woods are utilised.[18]

From the bark and leaves of Populus nigra L. phenolic glycosides salicin, salicortin and nigracin have been isolated.[19] The buds of P. nigra have been shown to contain galangin, pinocembrine, chyrsin, izalpinine,[20] kaempferol-3-o-rhanodiglucoside,[21] 3-O-methyl kaempferol, 3-O-methyl galangin and pinostrobin.[22] The compounds isolated from the other species of Populus are listed in Table 1.

Tissue cultures have also been reported from different species of Populus. Hildebrandt and Riker[41] established callus cultures of P. deltoides on a mineral salt medium supplemented with ∝-naphthalene acetic acid, 2,4-dichlorophenoxy acetic acid, pantothenic acid, glycine and 15% coconut milk. The culture did not survive on prolonged subculture. Callus cultures of P. tremula were obtained by Jacquiot[42] on knops half strength solution[43] with sugars, vitamins and other growth factors. Wolter[44] established continuous cultures of P. tremuloides on a modified Reinert and White's medium. These cultures were later induced to differentiate.[45] P. tremuloides cultures were also obtained by Mathes[46] using media containing

TABLE 1

Source	Compounds isolated	Ref.
P. tremuloidin (bark)	tremuloidin	23
	salireposide, salicin	24
	p-coumaric acid, p-hydroxybenzoic acid	25
	pyrocatechol, vanillic acid, ferulic acid, coniferin, syrigin, populine glycerol, linoleic acid, oleic acid	26
(heartwood)	α- and β-amyrin, lupeol, butyrospermol, α-amyrenonol, 24-methylenecycloartanol	30
(leaves)	tremuloiden, populin, salireposide, succinic acid, pyrocatechol, ferulic acid, p-coumaric acid, p-hydroxybenzoic acid, vanillic acid, salicyclic acid, quercetin-3-glucoside, quercetin-3-galactoside	34
P. grandidentata (bark)	salicin, salireposide, tremuloidine, salicyl alc., p-hydroxybenzoic acid, p-coumaric acid, vanillic acid, syringic acid, vanillin	28
	grandidentain	29
	pyrocatechol, salicatin, populin, grandidentoxide, populoxide	38
P. deltoides	Salicortin, salicin, salicyl alc. pyrocatechol, trichocarposide, ω-salicyclonylsalicin, grandidentatin, grandidentoside, populoside, trichocarposide, 6-methyldihydroquercetin	40
(leaves)	salicortin, salicin, salicyl alc. pyrocatechol, 1-O-P-coumaronyl-β-D-glucoside, populoside, chyrsin-7-glucoside, deltoidin	

Source	Compounds isolated	Ref.
P. candicans (leaves)	quercetin-3-β-D-glucoside, myricetin-3-β-D-galactoside, luteolin-7-β-D-glucoside and quercetin	39
P. trichocarpa (bark)	trichocarpin	31
	salicin, salicyl alc. themuloidin, pyrocatechol, p-coumaric acid, trichocarpaside	35
	trichoside, salireposide	32
P. balsamifera (bark)	salicin, trichocarpin, salireposide salicyol alcohol, gentisyl alcohol gentisic acid	33
P. tremula (wood)	myoinositol, glucose, fructose, sucrose, raffinose, stachyose, L-rhamnose	27
(bark and leaves)	tremuloidin, salicin, sucrose, populin	32
(leaves and catbins)	cyanidin-3-glucoside, cyanidin-3-xylosyl-glucoside, cyanidine-3-rhamnosylglucoside	36

mineral salts, glycine, thiamine, naphthalene acetic acid, sucrose and coconut milk. Citric acid (0.5%) stimulated root formation, whereas supplements of ∝-naphthalene acetic acid (1mg/l) and kinetin (0.8 mg/l) occasionally produced leafy shoots which failed to elongnate. Winton[47] also obtained plantlets from callus tissues of P. tremuloides on a modified Wolter and Skoog's[48] medium.

Vanverloo[49] detected the presence of lignans from P. nigra callus cultures. Matsumo et al.[50] isolated an anthocyanin pigment from callus cultures of Populus, (P. nigra X P. maximiwiczii) grown in light on an agar medium containing 2,4-dichlorophenoxy acetic acid. The anthocyanin was characterised as chrysanthemin.

PRESENT WORK

In this laboratory viable tissue cultures were raised from Tectona grandis, Artocarpus heterophyllus, Morus alba and Populus nigra on different media.[51] From the M. alba cultures Kulkarni et al.[52] reported the presence of β-sitosterol. Ghugale et al.[53] observed that in the presence of indole acetic acid, indole butyric acid and indole propionic acid, root differentiation was consistently present with the cultures of M. alba. In the case of P. nigra, root formation was noticed occasionally in the presence of α-naphthalene acetic acid, whereas shoot formation occured in only one instance in the presence of gibberellic acid.

The present work deals with the effect of a few amino acids and carbon sources on growth of callus cultures of P. nigra and the isolation of a new triterpene from this tissue in the yields of 0.08%. Earlier Allison[54] has reported the isolation of three new sesquiterpene lactones, paniculide A, B, C from callus cultures of Andrographis paniculata N. Tomita[55] et al. have isolated sesquiterpenoids lindenenol, lindenenol acetate, linderalactone, lindestrene and caryophyllane from the callus of Lindera strychnifolia. They also detected the presence of β-sitesterol, campesterol and stigmasterol by gas chromatography. Heble[56] et al. have

isolated diosgenin and β-sitosterol from tissue cultures of Solanum xanthocarpum. Tomita et al.[57] have reported the presence of diosgenin, yonogenin and tokarogenin in the tissue cultures of Dioscorea tokoro. Williams[58] et al. have isolated from cultures of Paul's scarlet rose β-amyrin, β-sitosterol, r-sterol, lanosterol and squalene. From tobacco tissue cultures campesterol, β-sitosterol, stigmasterol, cycloartenol, 24-methylenecycloartanol, citrostadienol, and 28-norcitrostadienol have been isolated.[7]

Figures 1 and 2 give the effect of a few amino acids and carbon sources respectively on P. nigra tissues. These compounds were tested over three subcultures to eliminate effects on growth due to carryover of nutrient with the inoculum.

Effect of amino acids

The amino acids were added to the basal medium (Table 2) at 2 ppm, and the effect on the overall growth was compared in all cases with the control medium to which glycine was not added. In the first passage growth in all cases was more or less identical to the control, whereas in the second and third passages wet and dry weight increases were observed with phenylalanine, glycine, aspartic acid and tyrosine. Good growth took place even in the

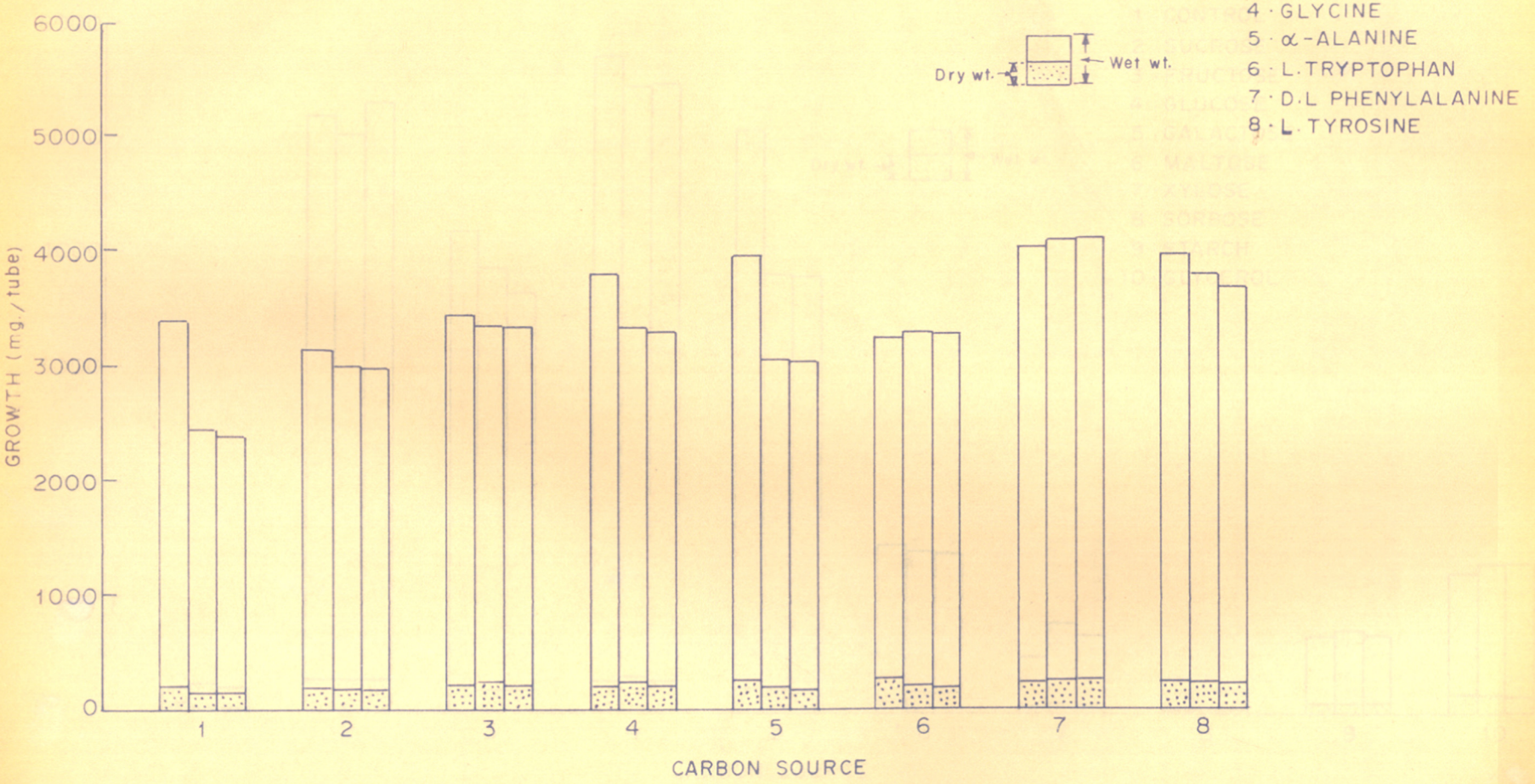


FIG. 1 EFFECT OF DIFFERENT AMINO ACIDS ON GROWTH OF P. NIGRA. THE 3 COLUMNS REPRESENT MEAN WET WEIGHTS AND DRY WEIGHTS AT THE END OF THREE CONSECUTIVE PASSAGES

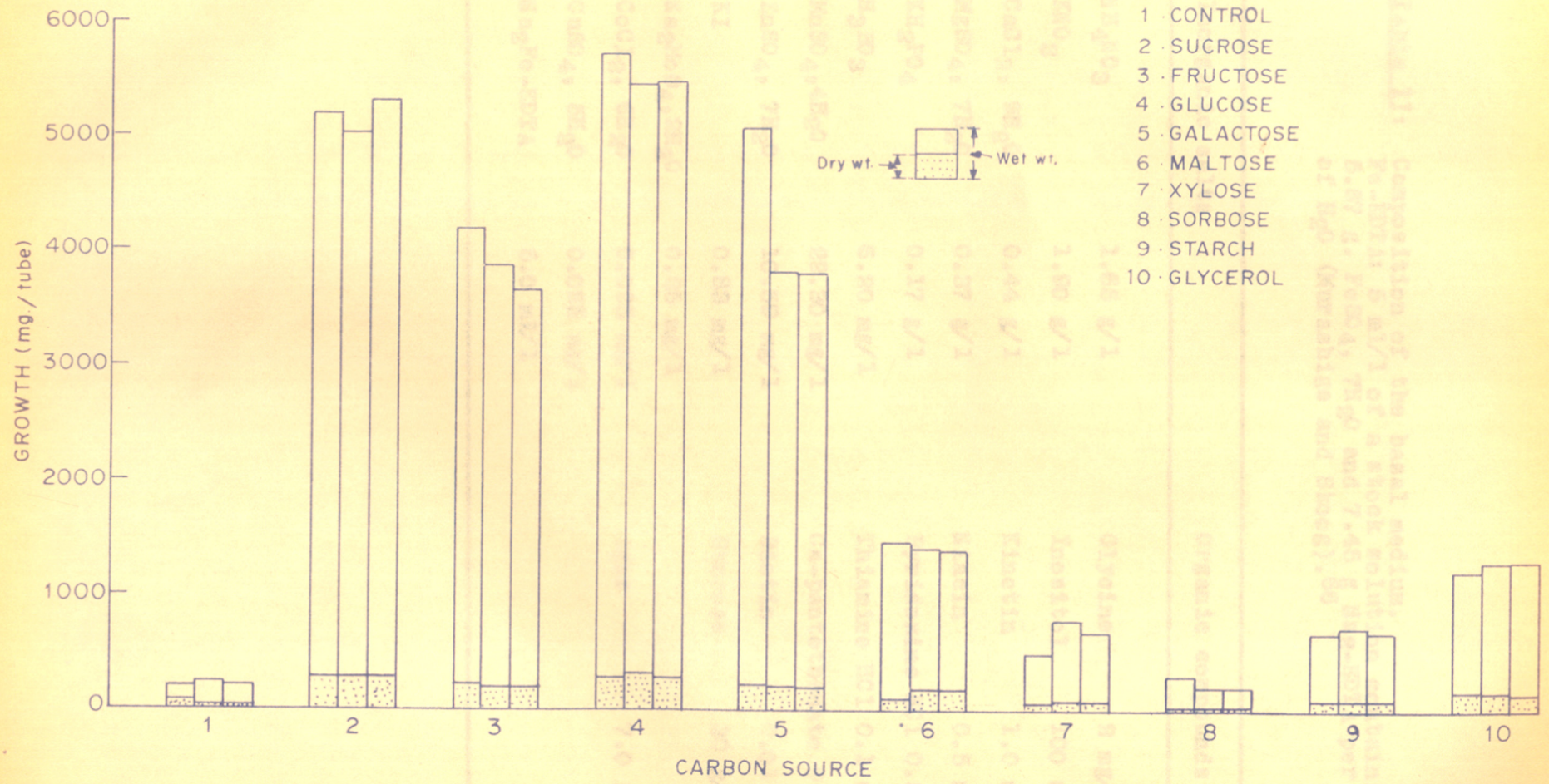


FIG. 2 EFFECT OF DIFFERENT CARBON SOURCES ON GROWTH OF P. NIGRA. THE 3 COLUMNS REPRESENT MEAN WET WEIGHTS AND DRY WEIGHTS AT THE END OF THREE CONSECUTIVE PASSAGES

Table II: Composition of the basal medium.
Fe-EDTA: 5 ml/l of a stock solution containing 5.57 g. $FeSO_4$, $7H_2O$ and 7.45 g Na_2-EDTA per litre of H_2O (Murashige and Skoog).[66]

Inorganic salts		Organic compounds	
NH_4NO_3	1.65 g/l	Glycine	2 mg/l
KNO_3	1.90 g/l	Inositol	100 mg/l
$CaCl_2$, $2H_2O$	0.44 g/l	Kinetin	1.0 mg/l
$MgSO_4$, $7H_2O$	0.37 g/l	Niacin	0.5 mg/l
KH_2PO_4	0.17 g/l	Pyridoxine HCl	0.5 mg/l
H_3BO_3	6.20 mg/l	Thiamine HCl	0.1 mg/l
$MnSO_4$,$4H_2O$	22.30 mg/l	Ca-pantothenate	0.1 mg/l
$ZnSO_4$, $7H_2O$	10.59 mg/l	Biotin	0.01 mg/l
KI	0.83 mg/l	Sucrose	30 g/l
Na_2MoO_4,$2H_2O$	0.25 mg/l		
$CoCl_2$, $6H_2O$	0.025 mg/l	Agar	7.0 g/l
$CuSO_4$, $5H_2O$	0.025 mg/l		
Na_2Fe-EDTA	5.0 ml/l		

absence of an amino acid. Tulecke[59] found that for tissue originating from pollen of Ginkgo biloba arginine was necessary for growth whereas Risser and White[60] considered glutamine essential for spruce tumor cells. Though poplar cultures did not show an essential requirement for any of the amino acids tested, a slight growth stimulation was noticed after the second and third passages in their presence.

Effect of different carbon sources

Fig. 2 indicates the effect of different carbon sources which include sugar starch and glycerol. The carbon sources were added at 2% concentration. There was no growth in the absence of a carbon source (Fig. 2; medium 1). Good growth was obtained with glucose, sucrose, galactose, and fructose. All tissue culture media contain either sucrose or glucose,[41,61,62] although in some cases galactose has also been used as a carbon source.[63,64] With starch xylose and glycerol very little growth was observed whereas sorbose was found to be toxic. Of the different carbon sources tested only glucose, fructose or galactose provided a satisfactory substitute for sucrose.

In these growth studies only the effect of a few amino acid and carbon sources were tested at a single

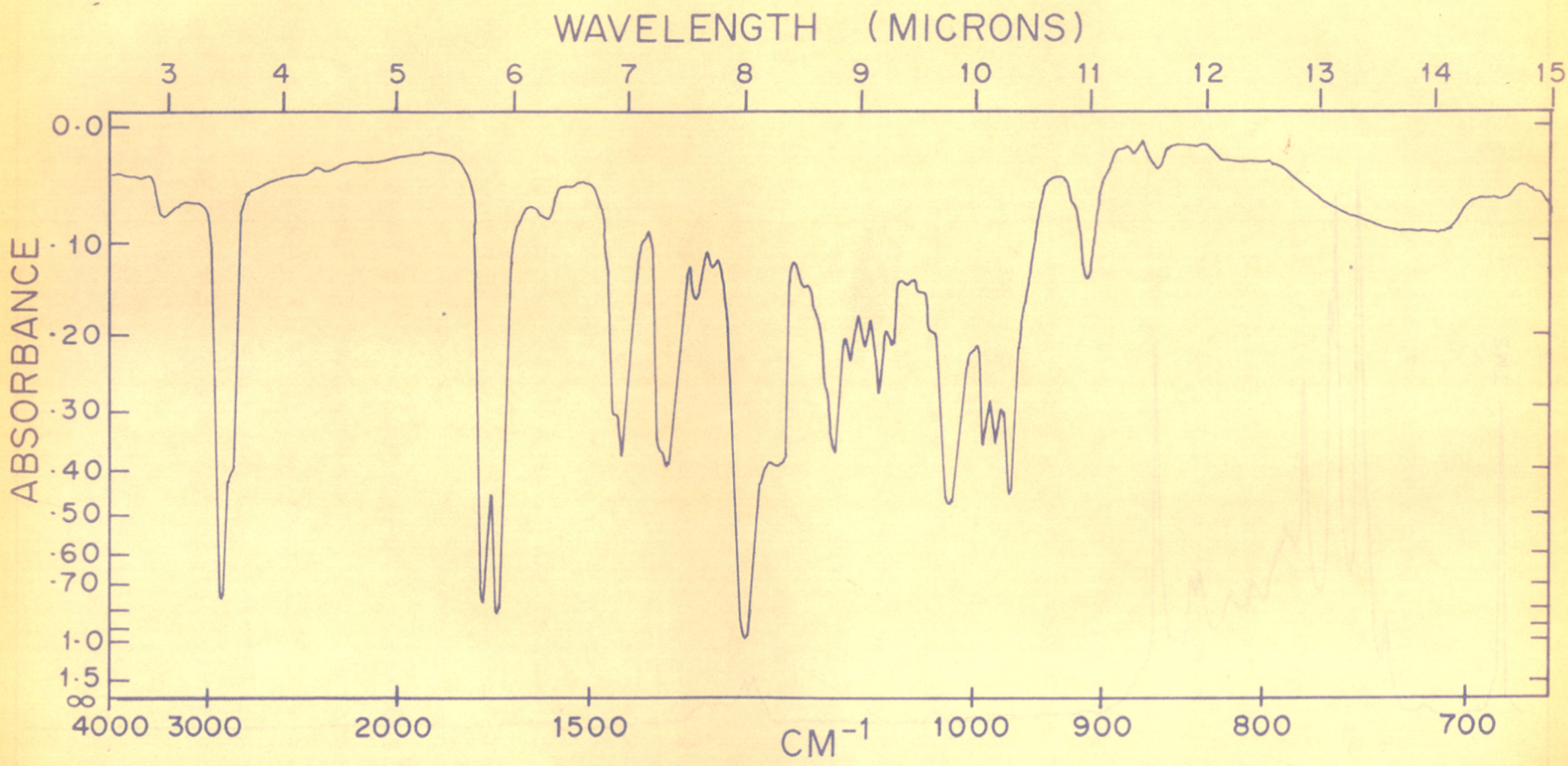
concentration. Further studies will however be necessary to confirm the optimum requirements of Populus nigra tissue with respect to different auxins, cytoleinéns, gibberellin, etc. tested over a wide concentration range.

Isolation of Populus terpene

The dried tissues of P. nigra were extracted in a soxhlet with acetone. After removal of acetone the residue was extracted with ether. The ether layer was dried over anhydrous sodium sulphate. After removal of ether a light brown semicrystalline residue was obtained. This was washed twice with hexane, and the cream-coloured residue, on repeated crystallisation from methanol, gave glistening colourless needles, m.p. 325° (sintering at 310°) $(\alpha)_D$ - 25° (C = 1 g/ 100 ml of $CHCl_3$). The compound was found to be homogeneous on a TLC silica gel plate, using benzene-acetone as solvent and detecting the spot by spraying with vanillin-sulphuric acid. A pink colour in the Liebermann-Burchard test indicated a steroid or triterpenoid. There was no colouration with tetranitromethane, indicating the absence of unsaturation, confirmed by the failure to add bromine. The mass spectral molecular weight (M+ 498) and elemental analysis led to the molecular formula $C_{32}H_{50}O_4$. The UV spectrum shows only a weak end absorption (λ_{max}^{EtOH} 214 nm; ε 4680). The IR spectrum in

chloroform (Fig. 3) shows two intense bands in the carbonyl region at 1733 and 1753 cm^{-1}. A band at 1250 cm^{-1} coupled with the band at 1733 cm^{-1} can be assigned to an acetate group. The band at 1753 cm^{-1} can be due to a five-membered ketone or a five-membered saturated lactone. The bands at 1360 and 1380 cm^{-1} can be assigned to gem-dimethyl groups. The presence of a hydroxyl group is not clearly evident from the IR spectrum, and treatment with acetic anhydride and pyridine left the compound unchanged. Treatment with dinitrophenylhydrazone gave a negative result. A clear colour reaction in the hydroxamic test for lactones was not obtained. Mild hydrolysis of the terpene with methanolic sodium hydroxide gave a crystalline alcohol, m.p. 310°; $C_{30}H_{48}O_3$, M^+ 456 $(\alpha)_D$ - 9.8 (c = 1.0 g/100 ml. $CHCl_3$). The IR spectrum showed band at 3200 cm^{-1} and 1758 cm^{-1} indicating the presence of a hydroxyl group and probably a saturated 5-membered lactone ring. However, the deacetylated compound was stable to boiling 20% ethanolic potassium hydroxide.

The NMR spectrum of the parent compound (Fig. 4) in $CDCl_3$ (chemical shifts on the τ scale) shows signals assignable to seven quaternary methyl groups (8.87-9.2). A singlet signal at 8τ is characteristic of an acetoxyl group. A broad signal at 5.3 to 6.0 can be assigned to



FIG·3 IR SPECTRUM OF POPULUS TERPENE IN $CHCl_3$

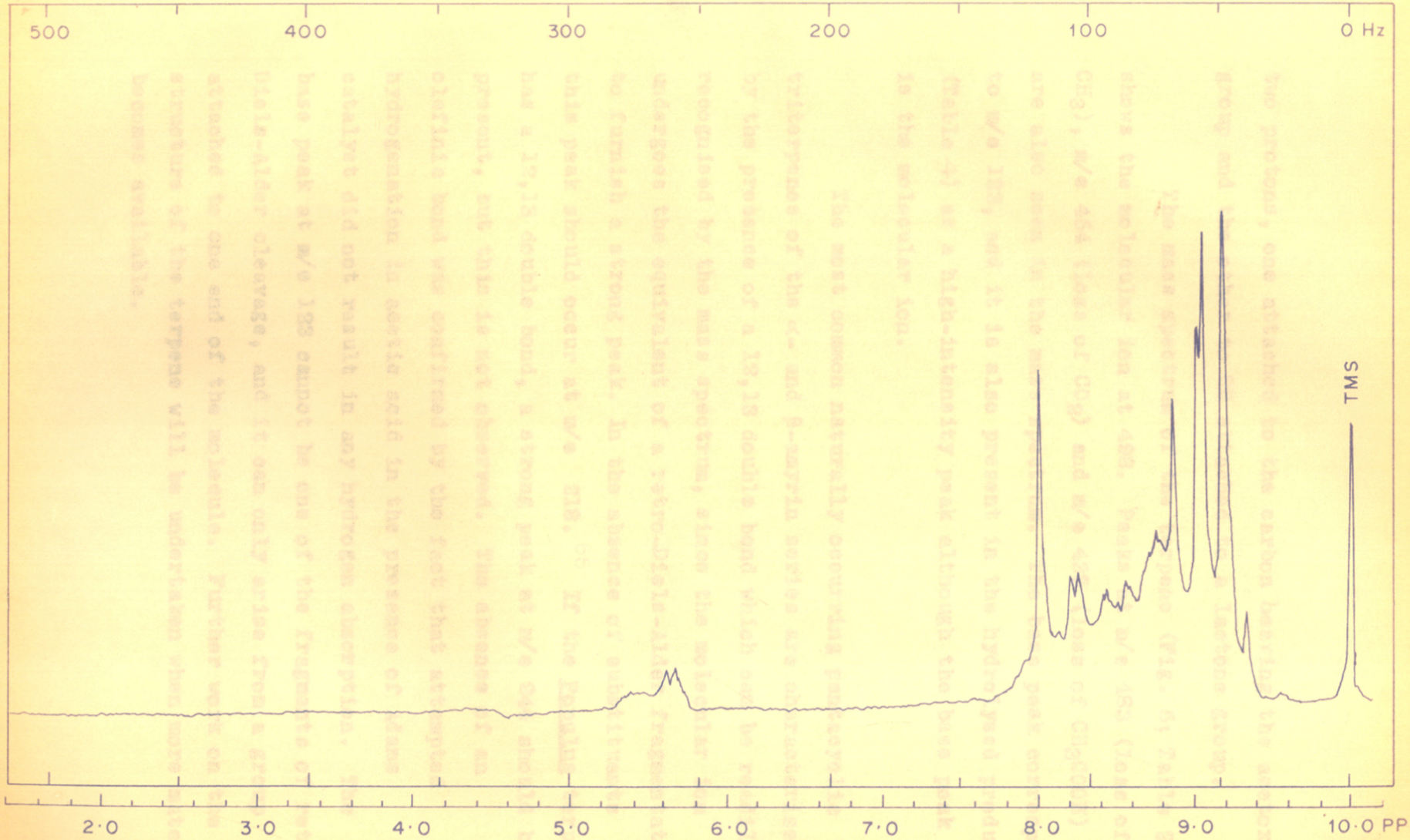
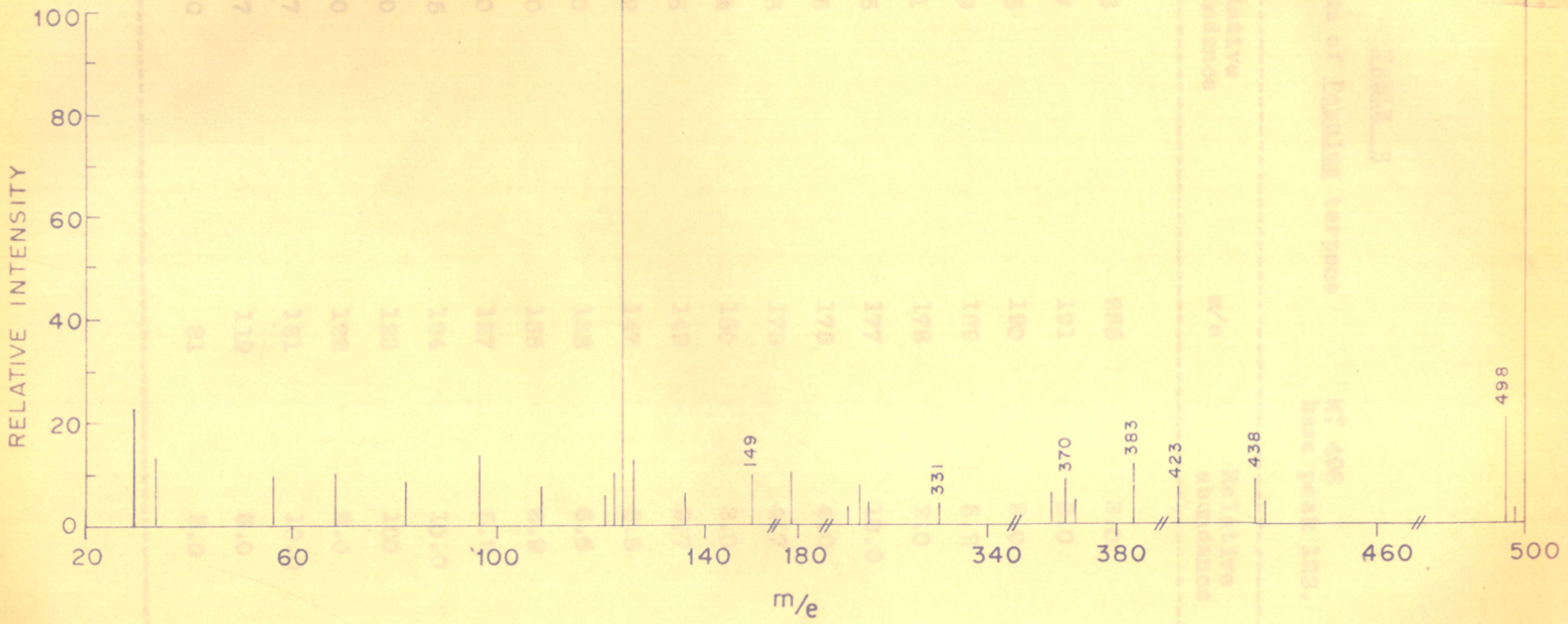


FIG. 4 NMR SPECTRUM OF POPULUS TERPENE IN $CDCl_3$

two protons, one attached to the carbon bearing the acetoxyl group and the other to CH attached to a lactone group.

The mass spectrum of the terpene (Fig. 5; Table 3) shows the molecular ion at 498. Peaks at m/e 483 (loss of CH_3), m/e 454 (loss of CO_2) and m/e 438 (loss of CH_3COOH) are also seen in the mass spectrum. The base peak corresponds to m/e 123, and it is also present in the hydrolysed product (Table 4) as a high-intensity peak although the base peak is the molecular ion.

The most common naturally occurring pentacyclic triterpenes of the α- and β-amyrin series are characterised by the presence of a 12,13 double bond which can be readily recognised by the mass spectrum, since the molecular ion undergoes the equivalent of a retro-Diels-Alder fragmentation to furnish a strong peak. In the absence of substituents this peak should occur at m/e 218.[65] If the Populus terpene has a 12,13 double bond, a strong peak at m/e 244 should be present, but this is not observed. The absence of an olefinic bond was confirmed by the fact that attempted hydrogenation in acetic acid in the presence of Adams catalyst did not result in any hydrogen absorption. The base peak at m/e 123 cannot be one of the fragments of retro-Diels-Alder cleavage, and it can only arise from a group attached to one end of the molecule. Further work on the structure of the terpene will be undertaken when more material becomes available.


RELATIVE INTENSITY
100
80
60
40
20
0
20
60
100
140
180
340
380
460
500
m/e
123
149
331
370
383
423
438
498

FIG·5

TABLE 3

Mass spectrum of *Populus* terpene $M^{\dagger}$ 498
base peak 123.

m/e	Relative abundance	m/e	Relative abundance
499	8.3	255	3.0
498	22.7	191	3.0
483	0.5	190	2.0
454	1.9	189	5.2
439	6.1	178	2.0
438	15.5	177	13.0
423	10.5	175	4.0
395	1.8	173	4.7
384	4.1	150	3.0
383	13.6	149	9.7
382	5.2	147	5.5
371	7.0	133	6.6
370	10.0	135	6.9
369	7.0	137	5.2
355	2.5	124	10.0
331	5.0	123	100
315	8.0	122	8.0
314	2.7	121	10.0
271	2.7	119	6.0
269	3.0	81	8.0

TABLE 4

Mass spectrum of deacetylated terpene M^{+} 456
base peak 456

m/e	Relative abundance	m/e	Relative abundance
457	36	189	44
456	100	187	29
		178	37
441	8	177	85
439	13	176	24
		175	42
438	33	174	
423	33	165	26
		163	40
385	13	161	26
384	33.3	159	22
383	88	150	37
370	21	149	53
		148	21
369	28	147	44
316	18	145	25
		137	41
315	33	136	22
290	10	135	56
289	37	134	24
		133	53
271	17	125	18
269	40	124	56
255	21	123	94
		122	60
205	16	121	61
204	17	125	18
		120	24
203	18	119	45
201	20	111	22
191	24	110	20
		109	46
		108	33
		107	57
		106	14
		105	46

EXPERIMENTAL

Culture conditions

For the large scale production of tissues the callus derived from young twigs of *Populus nigra*,[51] subcultured for over two years, was grown in the test tubes (6"x1") containing 20 ml of Murashige and Skoogs medium[66] supplemented with growth factors as shown in Table 2. The inorganic salts used for the preparation of media and the carbon sources were of analytical grade (British Drug House or E. Merck). The amino acids and growth factors were either Sigma or Nutritional Biochemicals. Agar was from DIFCO. The pH of the medium was adjusted to 5.8 ± 0.1 on a Philips pH meter, and then autoclaved at 15 lbs. pressure for twenty minutes, followed by steaming for thirty minutes on the subsequent day. Heat sensitive compounds were sterilised by passing through a Seitz bacterial filter and were added aseptically to the medium at the required levels after the autoclaved medium had been steamed on the second day and had come down to a temperature of 40°. The contents of the tube were then mixed thoroughly.

The wet weight of the inoculum was between 80-100 mg. After inoculation the cultures were incubated in a room at 25 ± 1° and illuminated with diffuse artificial light from fluorescent tubes (5 foot candles) for about four hours per day.

Wet and dry weights were taken as a measure of the growth of the tissues after incubation for forty days when the tissue was still in its logarthomic phase of growth. Dry weights were obtained by drying the tissue at 95 to 100°. The results are the average of five replicate cultures .

For isolation studies callus tissues which had undergone subcultures was harvested after forty days growth. Any adhering medium was removed carefully and the tissues were dried in lyophilizer.

Isolation of Populus terpene

The tissues (2500 g) (dry weight 200 g) were extracted in a soxhlet with acetone for 72 hours. After removal of acetone the residue (2 g) was extracted with ether. The ether extract (1.2 g) was washed with hexane. After removal of the hexane-soluble portion, a cream-coloured solid was obtained (0.3 g). This on repeated crystallisation from methanol gave colourless needles (0.17 g), m.p. 325° (sintering at 310°) (Found: C, 77.1; H, 10.3. $C_{32}H_{50}O_4$ requires C, 77.1; H, 10.1%). The compound showed a single spot on a TLC silica gel plate (solvent system 0.5:9.5 acetone-benzene) on spraying with 2% vanillin in sulphuric acid (1:1).

Hydrolysis of the terpene

A suspension of the terpene (0.1 g) in 10% methanolic sodium hydroxide was refluxed for two hours. After removal of methanol, the mixture was cooled and extracted with chloroform. The product shows a single spot on TLC silica gel plate (solvent system 1:9; acetone-benzene) on spraying with 2% vanillin sulphuric acid (1:1). It crystallised from methanol in colourless needles (0.05 g), m.p. 310° (Found: C, 78.88; H, 10.4. $C_{30}H_{48}O_3$ requires C, 78.9; H, 10.5%).

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SUMMARY

SUMMARY

Chapter I: Chemical investigation of the trunk bark of Artocarpus heterophyllus

From the trunk bark of *A. heterophyllus* three new flavones occurring in minute quantities have been isolated. The structures of the two relatively major pigments have been demonstrated as (I) and (III), based on UV, IR, NMR and mass spectral data.

Hexane and benzene extracts of the trunk bark of *A. heterophyllus* from Kerala, on removal of solvent and shaking with cold methanol, gave a colourless crystalline compound, m.p. 316°, identified as betulinic acid. After the isolation of betulinic acid the filtrate was concentrated, adsorbed on a polyamide column, and eluted with hexane, benzene and acetone. The benzene extract was further subjected to column chromatography on silica gel. Elution with benzene and benzene-acetone gave cycloheterophyllin and heterophyllin in yields of less than 0.01 and 0.001% respectively.

Cycloheterophyllin (I), m.p. 205-206°, $C_{30}H_{30}O_7$ (M^+ 502) responds to the colour reactions of flavones and gives a green ferric colour. It forms a trimethyl ether (M^+ 544) with dimethyl sulphate and potassium carbonate in

boiling acetone. Hydrogenation of the trimethyl ether yields a hexahydro derivative (M^+ 550), showing the presence of three olefinic bonds. In its UV spectrum cycloheterophyllin closely resembles cycloartocarpin, a flavone isolated from the heartwood.

The NMR spectrum of cycloheterophyllin indicates the presence of a 2,2-dimethylchromene system in addition to a γ,γ-dimethylallyl group attached to an aromatic nucleus. It also suggests that the 2'-hydroxyl of the B-ring has oxidatively cyclised with the doubly allylic methylene group as in cycloartocarpin. There are only two aromatic protons and they appear as singlets, indicating 2,4,5-substitution in the B-ring. Based on the benzene-induced solvent shifts of the methoxyl groups and the effect of the 5-acetoxyl group on the chromene doublets the positions of the different substituents have been assigned.

The mass spectral fragmentation fully supports structure (I).

Biogenetic aspects of Morus and Artocarpus flavonoids are discussed in relation to cycloheterophyllin.

The second pigment, heterophyllin has not yet been obtained in the pure state. On methylation with diazomethane it forms a trimethyl ether which shows a green colouration

with ferric chloride. Its mass spectrum shows the molecular ion at 546, indicating that cyclisation to one of the rings D or E of cycloheterophyllin has not taken place. From the mass spectrum of the trimethyl ether, structure (II) is tentatively suggested.

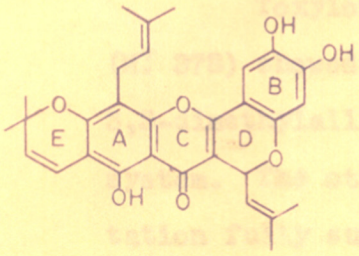
The third pigment, isocycloheterophyllin (m.p. 231°) was isolated from the trunk bark of A. heterophyllin, obtained from Poona, by extracting the benzene-soluble part of the acetone extract with aqueous sodium hydroxide, recovering the phenolic pigments by acidification and chromatographing a benzene solution on a silica gel column. The elemental analysis and molecular weight showed that it is an isomer of cycloheterophyllin. Methylation gave a trimethyl ether (M^{+} 544). The NMR spectrum showed all the expected signals for the methyl groups and olefinic protons of the chromene ring, the protons of the dimethylallyl group, the three methoxyl groups and the aromatic protons of the ring B. The position of the methoxyl groups in ring B was proved by the benzene-induced solvent shifts. Structure (III) was assigned to isocycloheterophyllin on the basis of the following considerations. Evidence for the 7-member character of the D-ring was provided by the broad two-proton signal representing the methylene group by the absence of the characteristic CH proton of the D-ring in cycloheterophyllin, and by the

abnormally high chemical shift of the methyl group which is probably shielded by the dihydro-oxepine ring system. The mass spectral fragmentation of the compound fully supports the structure (III).

Chapter II: The phenolic constituents of the heartwood and bark of Toxylon pomiferum.

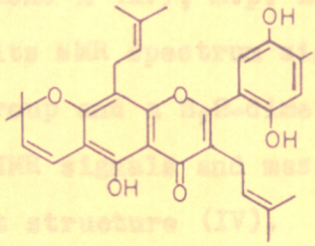
From the heartwood of T. pomiferum an American species, quercetin, morin, kaempferol, dihydromorin, dihydrokaempferol and oxyresveratrol have been isolated. The presence of resorcinol has been detected.

From the trunk bark, seven xanthones have been isolated, three of which, 8-hydroxygartanin, 6-desoxyjacareubin and osajaxanthone, were reported earlier from other sources. The four new xanthones have been designated as toxyloxanthones A, B, C, D and structures (IV), (V), (VI) (VII) respectively have been assigned to them, based on UV, IR, NMR and mass spectral data. All the seven pigments exhibited colour reactions and UV absorptions characteristic of xanthones. The four new xanthones carry C-prenyl groups, and in three they have undergone oxidative cyclisation. Toxyloxanthones A, C & D have a 1,3,5 or 1,3,5,6 oxygenation pattern, and toxyloxanthone B has a 1,3,6,7 oxygenation pattern.



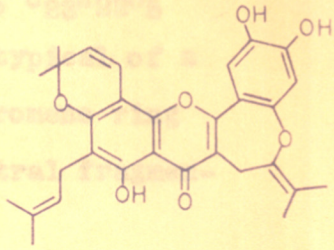
(I)

CYCLOHETEROPHYLLIN



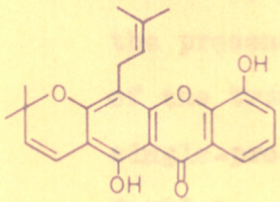
(II)

HETEROPHYLLIN



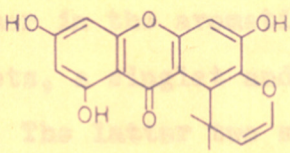
(III)

ISOCYCLOHETEROPHYLLIN



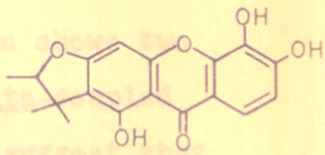
(IV)

TOXYLOXANTHONE A



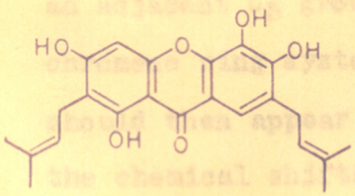
(V)

TOXYLOXANTHONE B



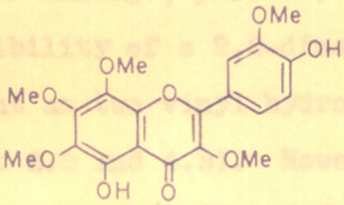
(VI)

TOXYLOXANTHONE C



(VII)

TOXYLOXANTHONE D



(VIII)

3′-METHOXYCALYCOPTERIN

Toxyloxanthone A (IV), m.p. 165-66°, $C_{23}H_{22}O_5$ (M^+ 378) showed in its NMR spectrum signals typical of a 3,3-dimethylallyl group and a 2,2-dimethylchromene ring system. The other NMR signals and mass spectral fragmentation fully support structure (IV).

Toxyloxanthone B (V), m.p. 300°, $C_{18}H_{14}O_6$ (M^+ 326) is the first natural product having a 4,4-dimethylchromene ring system. It forms a trimethyl ether with dimethyl sulphate, potassium carbonate in boiling acetone indicating the presence of three hydroxyl groups. The NMR spectrum of the trimethyl ether in the aromatic region shows two single-proton doublets, a singlet and two meta-coupled doublets, ~~x xingkck~~ The latter two signals suggest that one of the benzene rings is substituted at 1,3-positions by methoxyl groups. Of the four oxygens, other than the two which form part of the xanthone ring system, three are present as methoxyl groups; the remaining oxygen must therefore be involved in an ether linkage, probably with an adjacent C_5 group. The possibility of a 2,2-dimethylchromene ring system is ruled out as two vinyl hydrogens should then appear higher (about 3.3 and 4.3). However, the chemical shifts of the two doublets (1.87 and 4.27), together with the six-proton singlet at 8.5, suggest the other alternative of a 4,4-dimethylchromene ring system.

This might have resulted by the oxidative cyclisation of a 1,1-dimethylallyl group with an adjacent hydroxyl group by a radical mechanism. The benzene-induced solvent shifts of the methoxyl groups, together with other data, supports the structure (V).

Toxyloxanthone C (VI), m.p. 290-291°, $C_{18}H_{16}O_6$ (M^+ 328), is the first natural xanthone having a trimethyldihydrofuran ring system. The NMR spectrum in the downfield region shows a single-proton singlet indicating a chelated hydroxyl group. In the aromatic region two doublets represent protons in the 8- and 7-positions respectively in a xanthone; a third signal can be assigned to the C-4 proton. Two methyl groups appear as singlets and a third methyl group appears as a doublet. A single-proton quartet at 5.5 shows vicinal coupling with the methyl group. The data suggest the presence of a trimethyldihydrofuran ring fused to a phloroglucinol nucleus. The NMR spectra of the trimethyl ether and the diacetate are also in complete agreement with the structure (VI).

Toxyloxanthone D (VII), m.p. 250-252°, $C_{23}H_{24}O_6$ (M^+ 396), forms a tetramethyl ether indicating the presence of four phenolic hydroxyls. The NMR spectrum of the methyl ether shows signals characteristic of two 3,3-dimethylallyl

groups, and the aromatic region shows two singlet signals which can be assigned to 4 and 8-H of the xanthone nucleus. The position of the methoxyl groups are assigned on the basis of the benzene-induced solvent shifts.

Chapter III: Chemical investigation of the leaves of Calycopteris floribunda

A reexamination of the leaves of Calycopteris floribunda obtained from Kerala State has revealed the presence of two minor constituents accompanying calycopterin, the major pigment. They have been characterised as 3'-methoxy-calycopterin (VIII), a new compound, and 5-hydroxyauranetin isolated earlier from Citrus aurantium.

The total acetone extract of the leaves was washed with pet.ether to remove chlorophyll and resinous matter. The residue was extracted with ether and the ether solution extracted with 5% aqueous sodium hydroxide. The alkali-insoluble portion contained only one compound, identified as 5-hydroxyauranetin. The alkali-soluble part contained mainly calycopterin. Removal of the major amount of calycopterin by crystallisation from methanol and repeated PLC of the mother-liquor yielded 0.0005% of the new pigment, 3'-methoxycalycopterin, which still contained traces of calycopterin (M^+ 404). Mass spectral fragmentation pattern

indicates identical substitution of the A-ring as in calycopterin. The UV spectrum shows maxima at 278 and 355 nm, the latter shifting to 410 nm with increase in intensity on the addition of sodium ethoxide. A 4'-hydroxyl group is therefore indicated. The remaining methoxyl group can be placed in the 3'-position, since 2',4'-oxygenation in flavones has so far been observed only in the family Moraceae. The structure for the new pigment has been confirmed by synthesis. 2-Hydroxy-ω,3,4,5,6-pentamethoxyacetophenone, obtained by alkaline hydrolysis of calycopterin dimethyl ether, was condensed with 3-methoxy-4-benzyloxybenzoyl chloride and potassium carbonate in boiling acetone. Debenzylation of the gummy product in boiling hydrochloric acid and acetic acid, which also effected demethylation in the 5-position, yielded 3'-methoxycalycopterin, brownish yellow needles, m.p. 160-62°, from methanol, identical with natural methoxycalycopterin in chromatographic behaviour and mass spectral fragmentation.

Chapter IV: Tissue culture of Populus nigra

Tissues of P. nigra were grown on Murashige and Skoogs medium. The effect of a few amino acids and carbon sources on the growth of callus cultures of P. nigra was studied. It was observed that amino acids were not essential

for the growth of the tissues, but phenylalanine, glycine, aspartic acid and tyrosine were found to stimulate the growth. The effect of different carbon sources including sugar, starch and glycerol was studied. There was no growth in the absence of a carbon source. Good growth was obtained with glucose, sucrose, galactose and fructose.

From the callus cultures of P. nigra a colourless crystalline triterpenoid compound was isolated in very low yield (0.08% on dry weight of tissue), m.p. 325° (sintering at 310°). The elemental analysis and molecular weight (M^+ 498) are in agreement with the molecular formula, $C_{32}H_{50}O_4$. The UV spectrum shows only an end absorption. The IR spectrum shows bands characteristic of an acetate (1250, 1733 cm^{-1}). A band at 1753 cm^{-1} suggests that a five-membered ketone or a five-membered saturated lactone. Mild hydrolysis gives a crystalline alcohol, m.p. 310°, $C_{30}H_{48}O_3$, (M^+ 456). The NMR spectrum of the parent compound shows the presence of seven methyl groups all of which appear to be tertiary, and a singlet signal at 8τ, characteristic of an acetoxyl group. A broad signal at 5.3 to 6.0 can be assigned to two protons, one attached to the carbon bearing the acetoxyl group and the other to CH attached to a lactone group. The terpene does not respond to chemical tests for a ketone or a lactone. Further work on its structure will be undertaken when more material becomes available.

ACKNOWLEDGMENT

I take this opportunity to express my deep sense of gratitude to Professor K. Venkataraman, National Chemical Laboratory, Poona, for suggesting the problem and able guidance throughout the course of this work. I am also indebted to Dr. A. V. Rama Rao, NCL, for his constant support and guidance in the course of this investigation.

I greatly acknowledge the help and encouragement I received from Dr. P.C. Parthasarathy and Dr. T.G. Manjrekar during the early stage of this work.

I also wish to record my grateful thanks to Dr. V.H. Deshpande, Dr. A.F. Mascarenhas and Miss D.D. Ghugale for their help.

Assistance from Spectroscopic and Microanalytical Sections of the laboratory is gratefully acknowledged.

The award of a fellowship by the U.S. Department of Agriculture under PL-480 during the entire course of this investigation is gratefully acknowledged.

I am thankful to the Director, NCL, for allowing me to submit this work in the form of a thesis.

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