SOME STUDIES ON CYTOTOXIC PLANT EXTRACTS

A thesis presented by

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ABSTRACT

The work described in this thesis is concerned with the separation and identification of the cytotoxic constituents of the Chilean plants *Pleocarphus revolutus* and *Ovidia pillo pillo*.

The literature on cytotoxic compounds of plant origin is reviewed.

The isolation and characterisation of two triterpenes, three flavones and five novel sesquiterpenes of the guaiane and isopatchoulane sub-groups is described together with details of two partially characterised sesquiterpenes.

The use of a relatively simple *in vitro* cytotoxicity test as a guide to the fractionation of crude plant materials has been investigated. By means of this test four of the isolated sesquiterpenes, including those partially characterised, and eight incompletely separated fractions are shown to possess some cytotoxic activity.

Some structure-activity studies have been carried out with two of the novel sesquiterpenes.

ACKNOWLEDGEMENTS

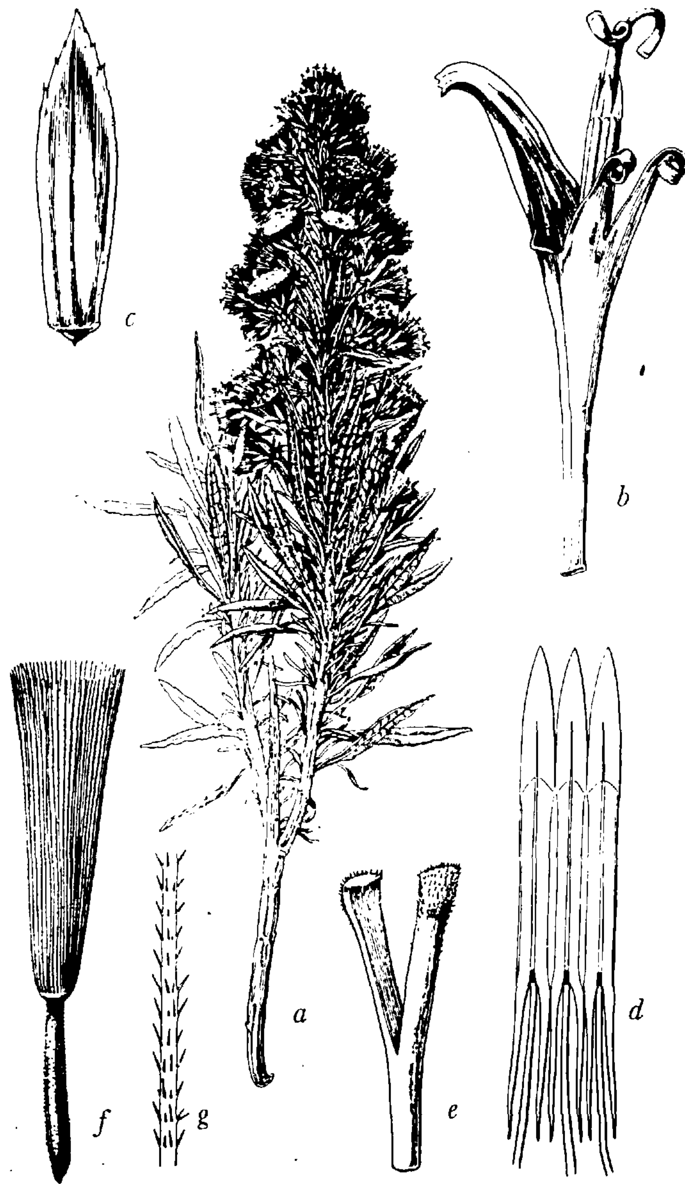
I would like to express my sincere thanks to Dr. Peter Sammes for his guidance and encouragement throughout the course of this work. I thank Professor Sir Derek H. R. Barton F. R. S. for the privilege of working in his department and the Tropical Products Institute for my financial support.

I am indebted to Dr. T. H. Connors and Mr. K. Merai of the Chester Beatty Research Institute for their assistance with the cytotoxicity testing, and to Dr. M. Silva and his staff at the University of Concepcion, Chile, for provision of plant materials. Thanks also to Lynn for her help with the typing of this thesis.

Many thanks go to all my colleagues in the Heilbron laboratory, particularly Drs. Barry Arnold, Frank Ellis, Kayhan Göktürk, Tim Wallace, Peter Machin, and Robert Watt.

Grateful thanks to my parents for their continued support, far beyond the line of duty.

Thanks finally to Jane, now my wife, for the support and encouragement which have really made this work possible.



LXXIII *Compositae. Pleocarphus revolutus.* a, ramita florífera $(x\ ^{2}/_{3})$ b, flor hermafrodita (x) c, brácteas del receptáculo (x) d, estambres (x) e, estilo (x) *l*, aquenio con papo (x) g, detalle de un pelo del papo (x). SGO. Nº 45.049. Original. E. Sierra

Plate 1. Pleocarphus revolutus (Compositae)

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REVIEW

INTRODUCTION

In 1970 Searle reported[1] that, of the 550,000 deaths recorded annually in England and Wales, about 110,000 could be ascribed to the group of diseases known collectively as cancer. The earliest record of an attempted cure for such a malignant disease, dates from about 1550 BC.[2] This early treatment involved unnamed crude plant extracts and was the first of many examples of the use of plant materials in this way.[3] However, it was only in 1938 that the work by Dustin,[4] on the cytotoxicity of colchicine, heralded the start of a world wide search for naturally occurring antitumour agents.

In the last 25 years a large number of antitumour agents have been isolated from the higher plants. It is the purpose of this review to give a brief account of the main screening methods used in the isolation of these compounds and to outline some of the chemically more interesting groups of antitumour agents, particularly the sesquiterpenes, with reference to any known structure-activity relationships and possible clinical uses. In addition, the most widely held theory of the mechanism of antitumour action, the alkylation theory, will be discussed.

SCREENING METHODS

Antitumour assays have been widely used, not only for the testing of pure compounds and for the preliminary screening of plant extracts, but also as a means of identifying the active fractions throughout the separation procedures. This latter use has become very important and Kupchan has commented[5] that this has led to the isolation of important minor plant constituents which would probably have been missed by the classic phytochemical approach. In this, the compounds studied were generally those most easily separated and those most readily crystallised.

Both in vitro and in vivo systems have been used for these types of screening. The in vitro systems are generally cultures of a well defined tumour cell line, the two most used systems being the KB-human epidermoid carcinoma of the nasopharynx and H.Ep. -2 - human epidermoid carcinoma of the larynx.

The testing of compounds against these cell cultures actually provides a measure of the cytotoxicity (i.e. toxicity towards cells) rather than of the in vivo antitumour activity of the substance and, although most antitumour compounds are cytotoxic, not all cytotoxic compounds have antitumour activity! Thus correlation of cytotoxicity with antitumour action is not always possible. This applies even when, as in the work described by Hirschberg,[6] biopsy tissue is used in culture in order to compare the effect of drugs on the patient with the effect on the corresponding culture. In this work the biopsy tissue cultures correctly predicted the improvement of fifteen out of nineteen 'cured' patients, but in addition they incorrectly predicted improvement in nine clinically unresponsive patients.

Hirschberg has also discussed[6] some work in which an examination was made of the effects of some antitumour drugs on normal human cell cultures. Very few preferential effects could be observed, in comparison with the effect of the same drugs on tumour cells.

The result of in vitro testing is normally expressed as an ED_{50} value. This is defined as the dose level, in μg/ml, at which 50% inhibition of tumour cell growth is observed, compared with untreated controls. A value ≤ 1.0 is considered significant.

This ED_{50} activity threshold of 1.0 has been arrived at statistically by the CCNSC (see below), to allow a low enough percentage of passes to be selective and to keep subsequent work within practical bounds.[2] The work of Eagle and Foley[7] and of Foley et al.[8](the latter including the use of microorganisms), has shown that this ED_{50} value of 1.0 is adequate to allow inclusion of virtually all of the active compounds from a large number which had previously proved active to in vivo tests. However, it also included a number of compounds known to be inactive in vivo ("false positives").

A large number of in vivo testing systems are now available most of which involve the use of transplantable tumours in mice or rats. Some of these systems have been developed as models for specific types of human cancer, but these tumours tend to lose their special characteristics very rapidly during serial transplantation and thus are of only limited use.

Most in vivo testing of plant materials is now carried out using the, WM-Walker carcinosarcoma 256 in the rat and LE-Leukaemia L-1210 in the mouse. Results are expressed as simple ratios of the test and control systems (T/C). With solid tumours,such as WM, the assessment of antitumour effect is made by measurement of the change in tumour weight, significant activity being attained at an inhibition of greater than 58% compared with the control (T/C $\leq$0.42). For leukaemias (LE) an increased life span of 25% or more is considered to represent appreciable activity (T/C$\geq$1.25).

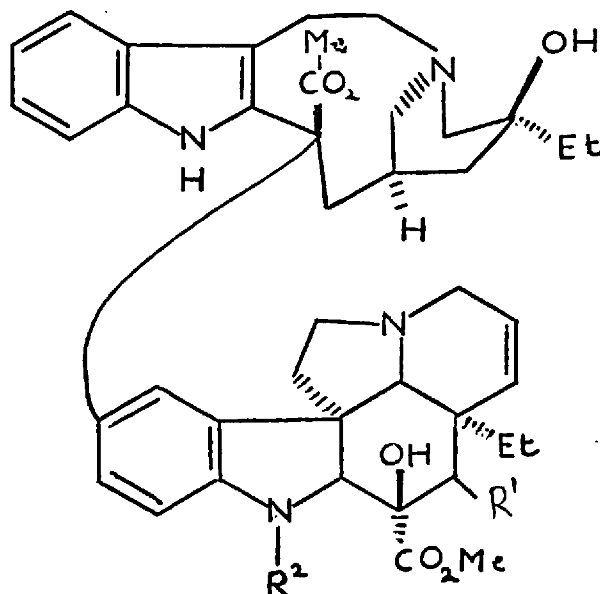
A further factor, taken into consideration before selection of a drug for clinical trials, is the therapeutic index (TI). This is the ratio of the minimum tolerated dose to the minimum effective dose. A compound will not normally be selected for

clinical trials unless it has a TI greater than 2.

The majority of world antitumour testing is now carried out under the auspices of The Cancer Chemotherapy National Service Center, Maryland, U.S.A. (CCNSC). Hartwell and Abbott have reported[2] that in the nine years, 1964-73, in which the CCNSC have been accepting plant products, over 40,000 crude extracts have been tested. Of these about 3.5% have shown reproducible activity in one or more systems and, to date, over 150 of the agents responsible for this activity have been isolated.

CATHARANTHUS ALKALOIDS

The first clinically useful drug of plant origin was the dimeric indole alkaloid vinblastine (1). This was originally isolated from the shrub catharanthus roseus during a search for new drugs to control diabetes mellitus[9]. Subsequently, more than seventy alkaloids have been isolated from plants of the catharanthus genus and of these, approximately a third have been dimeric indoles.[10] Of these only two, vinblastine and vincristine (2) have been of clinical use.



(1) R^1 = OAc; R^2 = Me (2) R^1 = OAc; R^2 = CHO

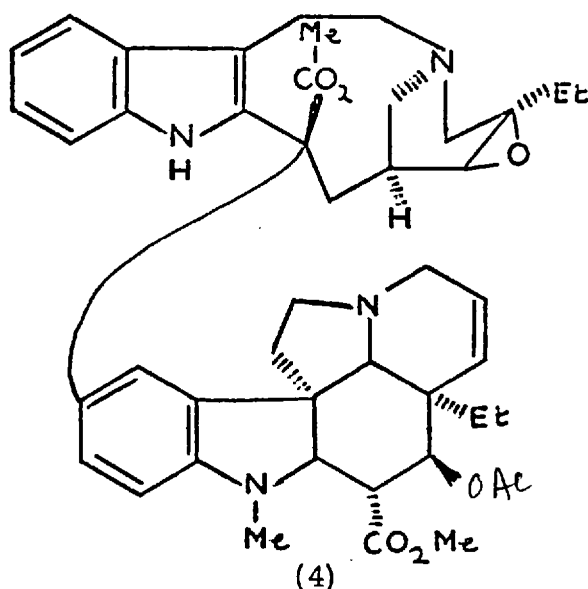
(3) R^1 = OCO-CH_2-NMe_2; R^2 = Me

Vinblastine has been used in the treatment of Hodgkins disease and choriocarcinoma.[11] It is more effective than vincristine for solid tumours but is more injurious to the bone marrow. Vincristine has a broader spectrum of activity and has been used for the treatment of acute leukaemia in children. Both compounds cause a number of side effects, including leukopenia, (a decrease below the normal number of leukocytes),[12] but these are comparatively mild for drugs of this type.

Structure-activity work with vinblastine derivatives has shown that activity is destroyed by removal of the acetoxy group, acetylation of the tertiary hydroxyls, or reduction of the methoxycarbonyl group to the corresponding carbinols.[10] Other chemical modifications including replacement of the acetoxyl group with $O\text{-}CO\text{-}CH_2X$ where X = Cl, CN or $NR'R''$ with R' and R'' alkyl groups or part of a heterocyclic ring, have no effect on the activity.[13]

As a result of these structure-activity studies the compound vinglycinate sulphate (3) has been prepared.[10] It has been clinically useful in certain cases, although the dose required is approximately ten times that of vinblastine.

The alkaloid vinleurosine (4), isolated from catharanthus lanceus, demonstrated a high activity against P-1534 leukaemia in mice[9] but proved disappointing on clinical trials. It has been suggested[14] that this lack of activity may be due to binding by plasma proteins.

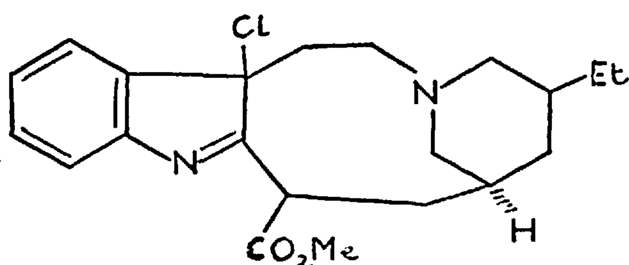


(4)

Three other related alkaloids, vinrosidine, leurosivine and rovidine, have also been shown to have in vivo activity.[15] As yet these compounds have not been fully characterised and have not been applied to the treatment of human tumours.

The mechanism of action of this group of alkaloids is still not fully understood. Biochemical studies have shown, however, that these compounds act as mitotic spindle poisons and inhibit the synthesis of various forms of RNA.[16]

Recently a new synthesis of dimeric alkaloids of the vinblastine type has been described.[17] This involved condensation of the chloroindolenine (5) with vindoline (6) to give the dimer (7).



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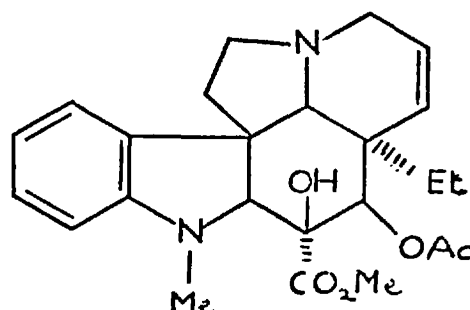

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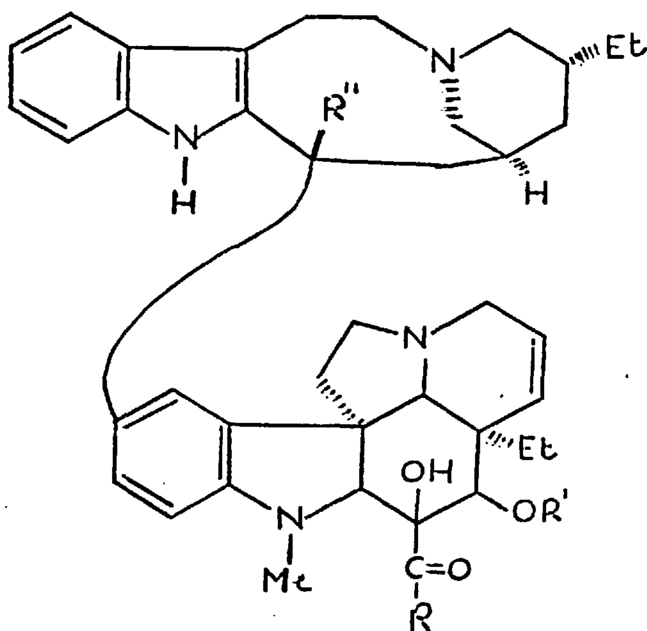


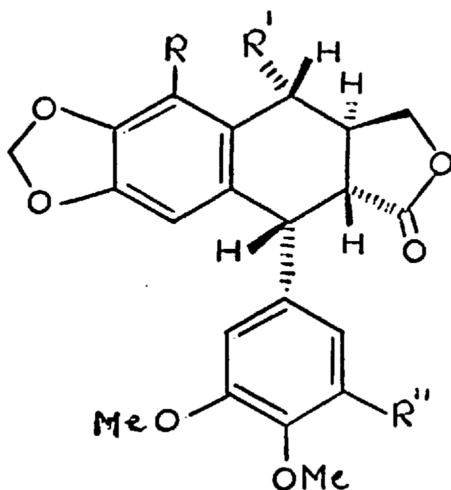
(7) R = OMe; R' = CH_3CO; R'' = $COOCH_3$ (8) R = $NHNH_2$; R' = H; R''=COOCH

(9) R = OMe; R' = CH_3CO; R'' = H (10) R = $NHNH_2$; R' = R'' = H

The generality of this method was demonstrated by its use in the production of the dimeric alkaloids (8), (9) and (10). Preparation of suitably substituted chloroindolenines and vindolines should provide a ready synthesis of this important group of catharanthus alkaloids.

PODOPHYLLUM COMPOUNDS

Extracts of the Himalayan shrub Podophyllum emodi have been used in the treatment of malignant diseases for over 2,000 years.[12] The active constituents of this plant and of the May apple, Podophyllum peltatum, have been identified as the lignan podophyllotoxin (11) and its β-D-glucoside (12). In addition, podophyllotoxin and deoxypodophyllotoxin (13) have been shown to be responsible for the activity of a number of plants in the families Bursuraceae and Pinaceae.[18]

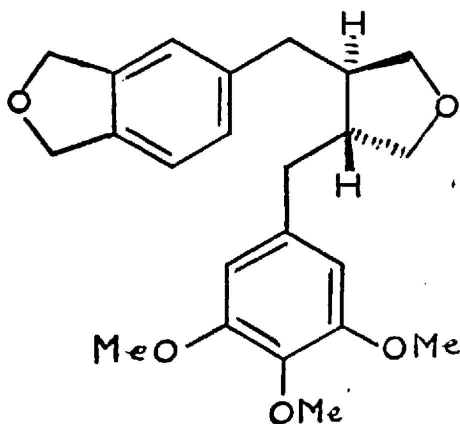


(11) R = H; R' = OH; R'' = OMe
(12) R = H; R' = O-glucosyl; R'' = OMe
(13) R = H; R' = H; R'' = OMe
(14) R = OMe; R' = H; R'' = OMe
(15) R = OMe; R' = H; R'' = H

The benzylidene-D-glucoside of podophyllotoxin has been used in the treatment of a number of human tumours including those of the breast, uterus, and cervix. It can be administered orally and although high doses may induce intestinal disorders, blood disturbances are seldom observed.

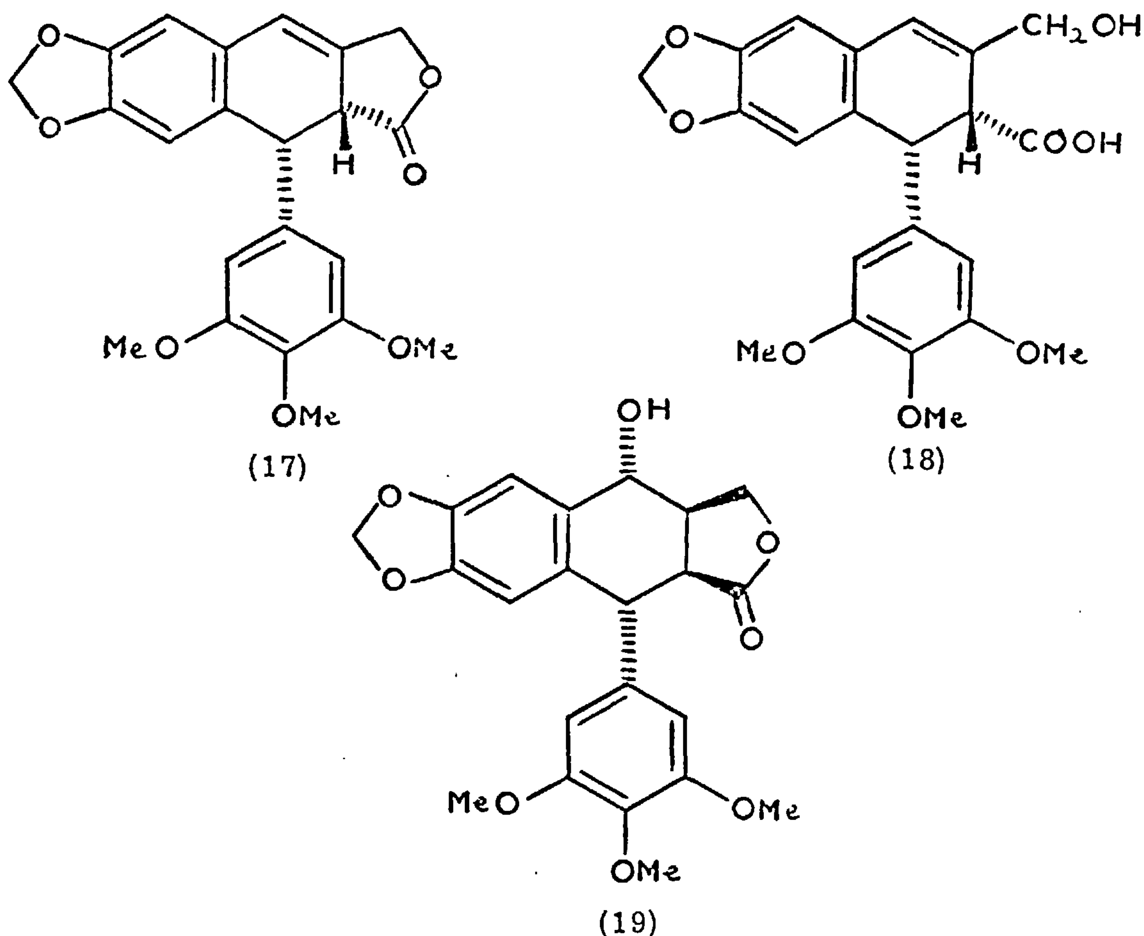
The ethyl hydrazide of podophyllic acid(podophyllotoxin is the γ-lactone of podophyllic acid) has also been used clinically for the treatment of various forms of cancer, including tumours of the ear, nose and throat. Side effects are similar to those of the benzylidene-D-glucoside.

Three other lignans, similar to podophyllotoxin, have been reported as showing experimental antitumour activity. These are β-peltatin-A-methylether (14) and 5'-desmethoxy-β-peltatin-A-methylether (15) both isolated from Bursera fagaroides and active in the WA16 tumour system,[19] and burseran (16). isolated from Burseran microphylla.[20] This latter compound has an ED_{50} of 2.6×10^{-2} against KB (deoxypodophyllotoxin has an ED_{50} of 2.6×10^{-3} and podophyllotoxin of $< 1 \times 10^{-2}$ in the same system), however *in vivo* testing results have not been reported.



(16)

Although synthetic routes to α-apopicropodophyllin (17) and α-apopodophyllic acid (18) have been known for over fifteen years,[21] the first synthesis of podophyllotoxin was not reported until 1966 when Gensler and Gatsonis succeeded in preparing it from the epimer picropodophyllin (19).[22] Gensler et al. had previously synthesised picropodophyllin from methylenedioxybenzene and trimethoxybenzoyl chloride,[23] thus the epimerisation represented a total synthesis of podophyllotoxin.



The clinically useful β-D-glucoside of podophyllotoxin has been prepared by condensation of tetra-O-acetyl-β-D-glucopyranosyl bromide with podophyllotoxin followed by zinc chloride - catalysed methanolysis of the product.[24]

BISBENZYLISOQUINOLINE ALKALOIDS

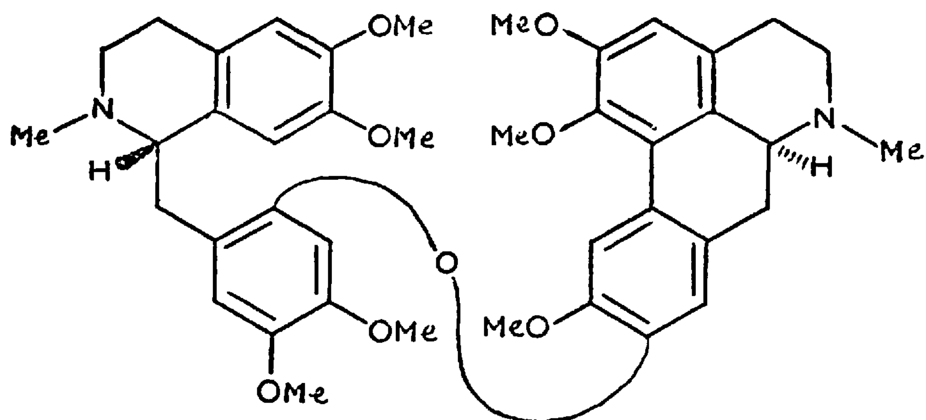
Several members of the bisbenzylisoquinoline group of alkaloids have shown cytoxicity in KB cell culture, however significant in vivo activity has only been shown by thalicarpine (20), dl - tetrandrine (21) and thalidasine (22).[2]

Thalicarpine and thalidasine were isolated from the plant Thalictrum dasycarpum.[25] Thalicarpine induces severe side effects in dogs including cardiac arrest, while thalidasine has a therapeutic index of only 1.5 against W256. Thus it seems unlikely that either of these compounds will be of use clinically, although thalicarpine is undergoing clinical trials.[26]

Both the d-and ℓ-enantiomorphs and the racemic from of tetrandrine have been isolated from plant materials.[27] The racemate has a therapeutic index of greater than 2 in the W256 system and is now undergoing clinical trials.[26]

Kupchan and Altland[26] have prepared and tested a large number of variants of the bisbenzylisoquinoline alkaloids. Their results indicate that the activity, a) is independent of the size of the macrocyclic ring, when present, b) does not depend on methylation of the nitrogen atoms, and c) does not seem to show any preferred stereospecificity. In addition they noted that the corresponding monomeric alkaloids are inactive.

On the basis of these results Kupchan and Altland have proposed that this group of alkaloids exert their antitumour effect 'by means of a stepwise sequence leading to the bis alkylation of the biological macromolecules involved in growth regulation.' One such proposed mechanism required a prior metabolic dehydrogenation to the bis (dihydroisoquinolinium) system shown in (23). This type of structure was expected to be very reactive towards nucleophilic attack at C-1 and C-1' and thus increased activity was predicted.



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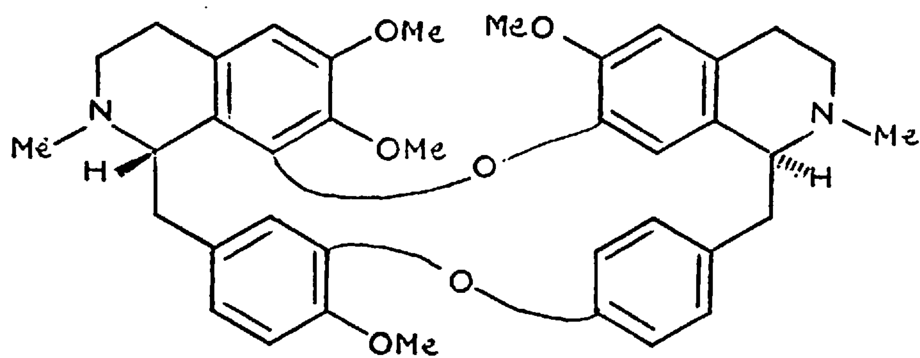

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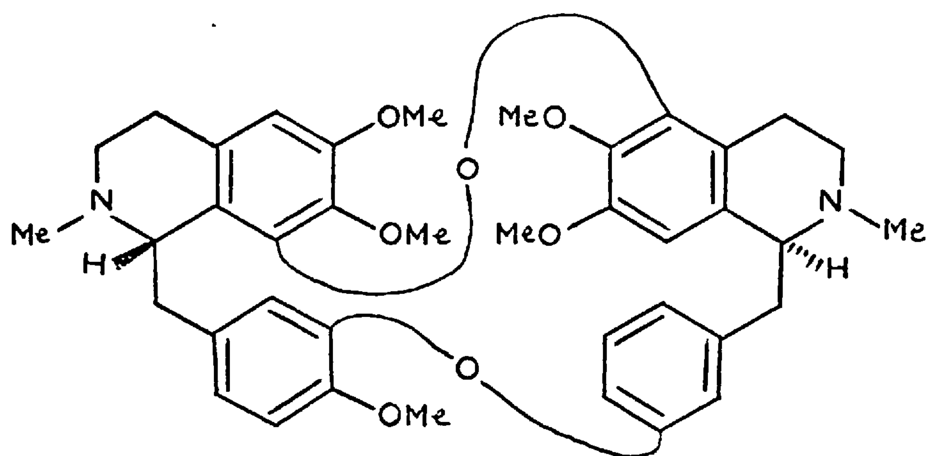

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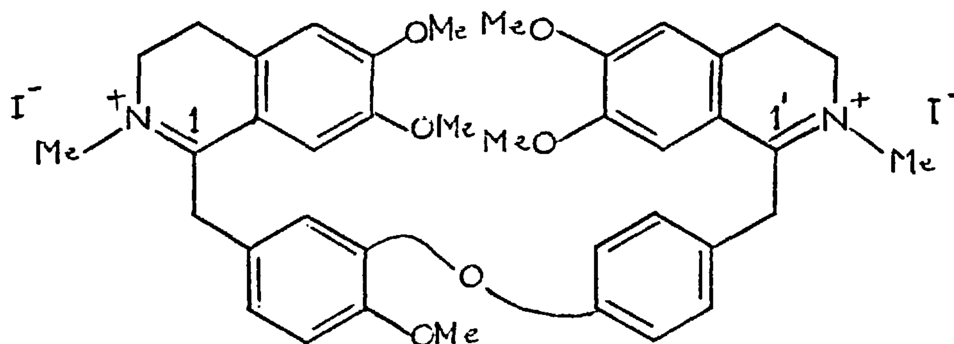
In fact, this compound (23) proved to be inactive while the compound (24) which is unlikely to undergo metabolic transformation to (23), showed considerable activity.



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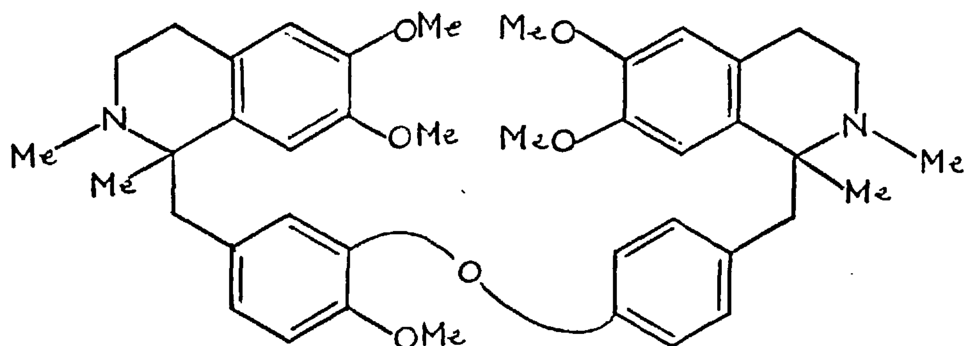
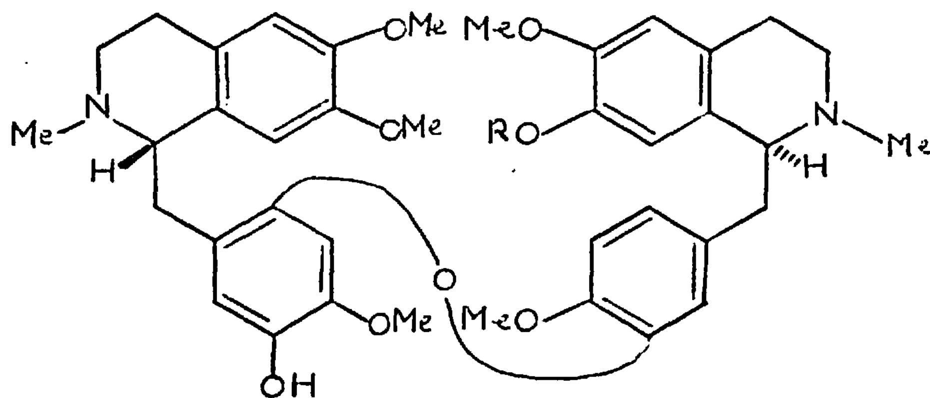
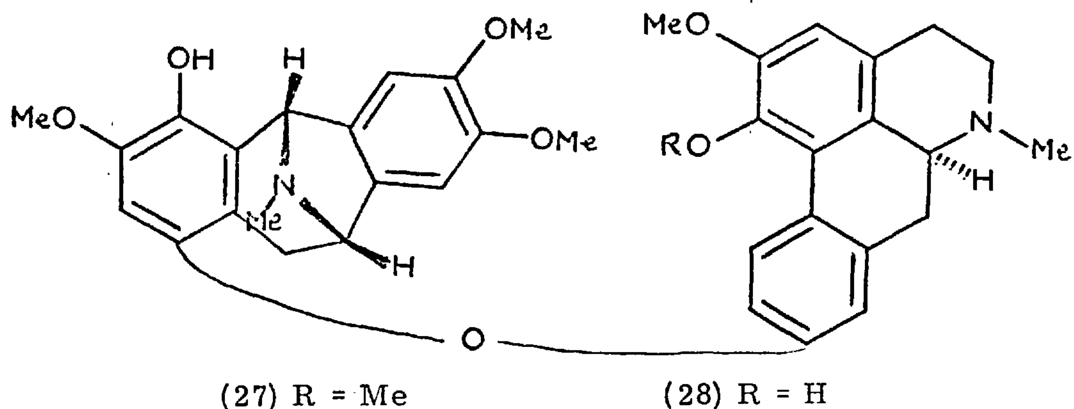


(24)

Recent work by Shamma and Moniot with extracts from the plant Thalictrum polygamum has led to the isolation of four new dimeric alkaloids, pennsylvanine (25), pennsylvanamine (26), pennsylpavine (27) and pennsylpavoline (28).[28] The latter two compounds are the first known examples of aporphine-pavine dimers. No testing data has, as yet, been reported for these compounds.



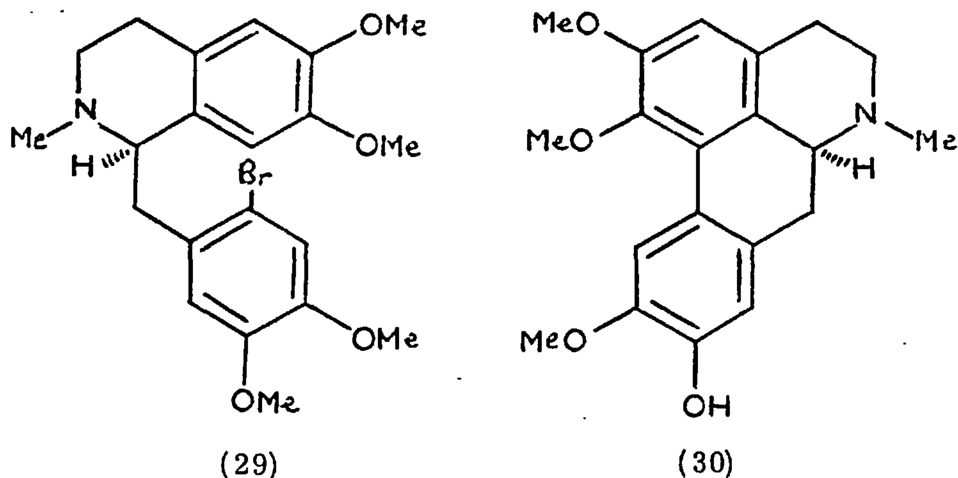
(25) R = Me (26) R = H



(27) R = Me (28) R = H

The synthesis of thalicarpine (20) was first accomplished by means of a modified Ullmann condensation of (S)-(+)-6-bromolaudanosine (29) with isocorydine (30).[29] Kupchan and Liepa have since described a second route to this natural product, starting with the diaryl ether (31) and proceeding via the naturally-occurring alkaloid hernandaline (32).[30]

Inubushi et al. have synthesised dl-tetrandrine (21) by a complex route which includes, an Ullmann condensation between (33) and (34), a second Ullmann reaction to provide the required di-ether linkage and a Bischler-Napieralski cyclisation.[31]



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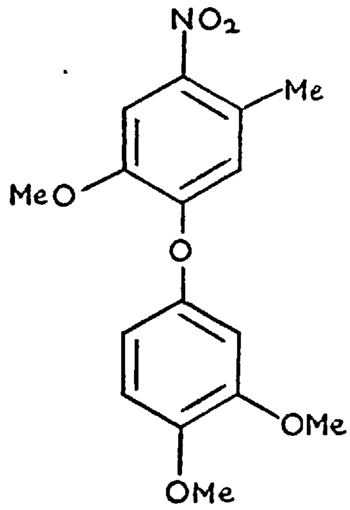

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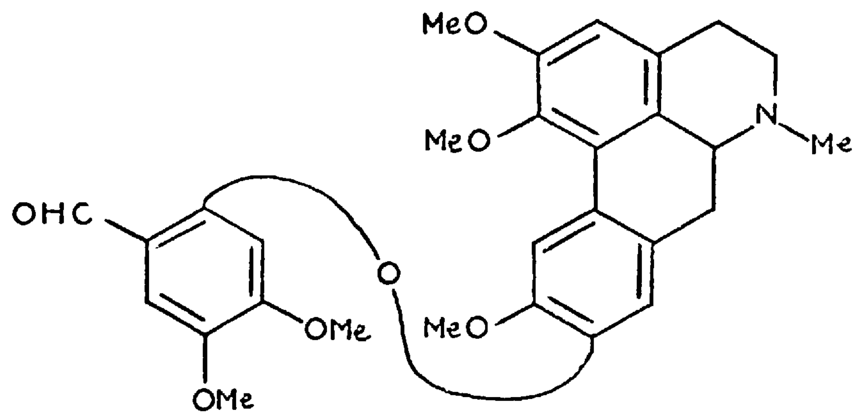

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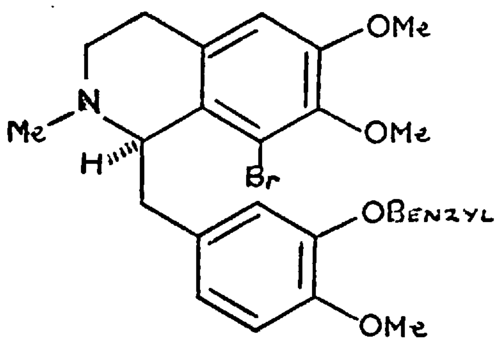

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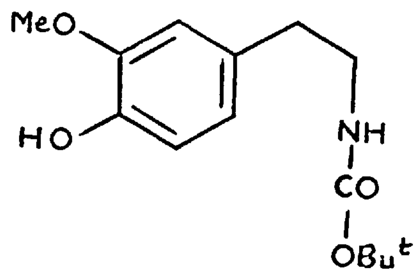

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ANSA MACROLIDES

A new series of cytotoxic ansa-macrolides has recently been isolated from a number of plant species. The first member of this series was maytansine (35) obtained, in a yield of $0.2 \times 10^{-6}\%$ of the dried plant material, from Maytenus ovatus.[32] Maytanprine (36), Maytanbutine (37) and Maytanvaline (38) have been obtained, also in very low yield, from Maytenus buchananii,[33] and colubrinol (39) and colubrinol acetate (40) from colubria taxensis.[34]

(35) R = Me; R' = H; R'' = H.

(36) R = Et; R' = H; R'' = H.

(37) R = $CH(Me)_2$; R' = H; R'' = H.

(38) R = $CH_2.CH.(Me)_2$; R' = H; R'' = H.

(39) R = $CH(Me)_2$; R' = OH; R'' = H.

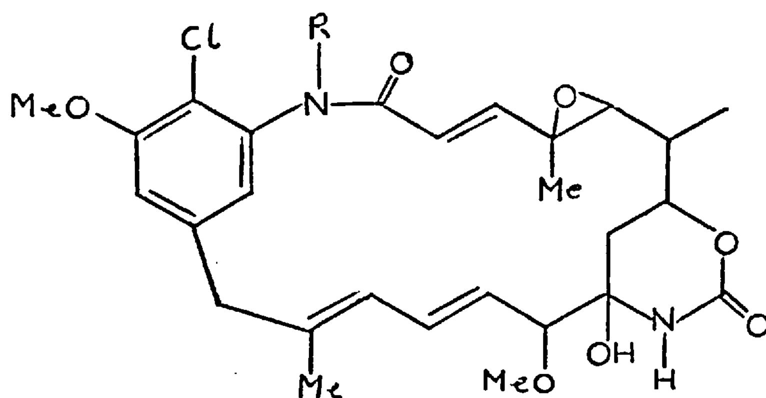
(40) R = $CH(Me)_2$; R' = OAc; R'' = H.

(41) R = Me; R' = H; R'' = Et.

These compounds have typical ED_{50} values against KB of 10^{-4} to 10^{-5} and have shown in vivo activity against a range of tumour systems including the Lewis Lung carcinoma and P388 leukaemia. Maytansine is now undergoing toxicological examination in preparation for clinical trials.

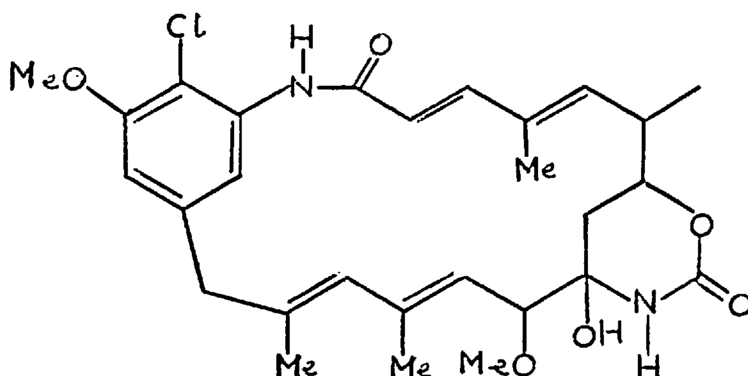
Kupchan has proposed[35] that the important functionalities for the antitumour activity are, the aryl chloride, the epoxide and the carbinolamine, "all constituting possible targets for enzymic nucleophilic attack." Confirmation of the importance of the carbinolamine was obtained by preparation of the ethyl ester (41) and the subsequent discovery that this had no <u>in vivo</u> activity.[33]

The three related compounds maysine (42) normaysine (43) and maysenine (44), isolated from <u>maytenus buchananii</u>,[33] are considerably less cytotoxic (by 10^3) than the maytansine type compounds and in addition have no antileukaemic activity. Their discovery has thus demonstrated the importance of the ester side chain for activity.



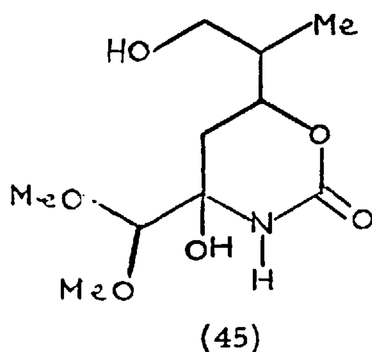
(42) R = Me

(43) R = H



(44)

Their considerable antitumour activity and low natural abundance make this group of ansa-macrolides particularly attractive synthetic targets. To date the only report of a synthetic approach to these compounds is that of Meyers and Shaw, who succeeded in preparing the carbinolamine moiety (45).[36]



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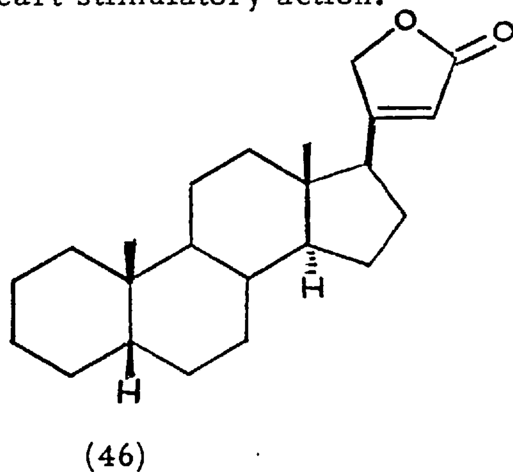
CARDENOLIDES, BUFADIENOLIDES and WITHANOLIDES

Considerable cytotoxicity has been reported for members of three groups of steroid lactones, the cardenolides (46), containing a γ-lactone attached to C-17, the bufadienolides (47), containing a similarly attached δ-lactone, and a small group of compounds related to Withaferin A (48) (Withanolides).[2]

Many of these compounds have long been known for their powerful heart stimulatory action.



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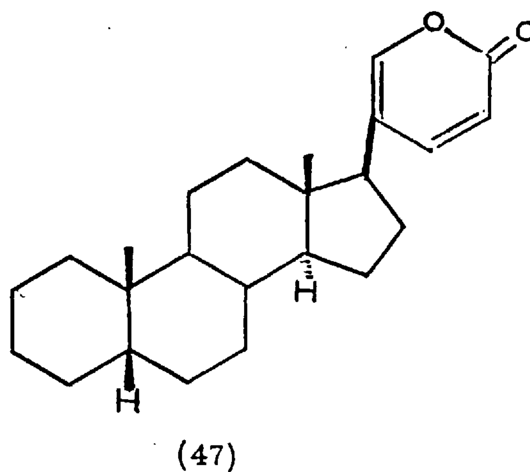

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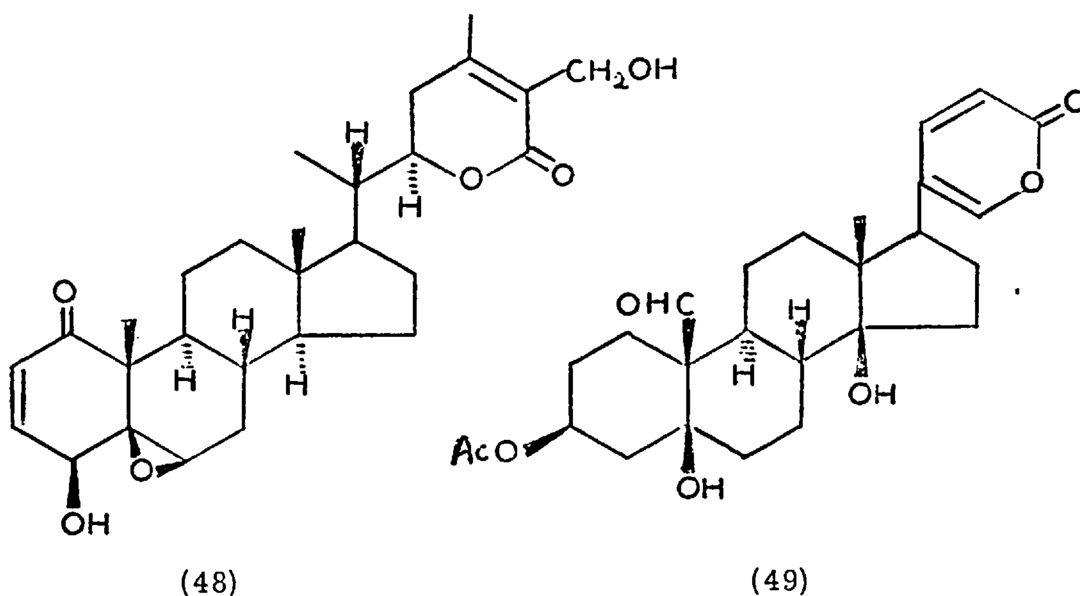
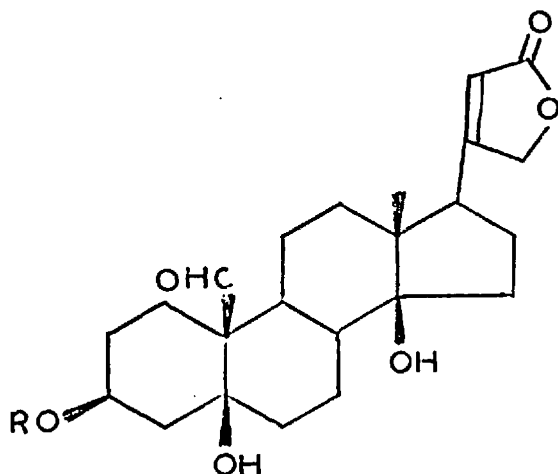
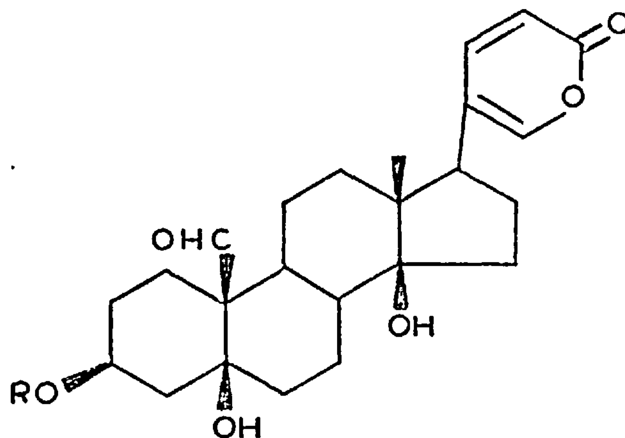


(48) (49)

Over 70% of the compounds tested from these three groups have shown cytotoxicity against the KB cell culture (typical ED_{50} values of 10^{-1} to 10^{-3}), however only hellebrigenin 3-acetate (49) obtained from Bersama abyssinica[37] and Withaferin A (48) from Acnistus arborescens [38] have shown any in vivo activity. Neither of these compounds have therapeutic indices large enough to warrant further study.

Of the 150 steroids tested by Pike et al.,[39] all of the most active contained an α, β-unsaturated lactone. Kelly and co-workers[40] confirmed this observation and demonstrated that, although both glycosides and aglycones were cytotoxic, the former were consistently more active than the latter. This group also concluded that the cardenolide and bufadienolide rings conferred equal activity, the two most active compounds tested, convallatoxin (50) and hellebrin (51), being virtually identical apart from this lactone ring. These compounds had ED_{50} values of 2×10^{-3} and 6×10^{-3} respectively, against the KB cell culture.

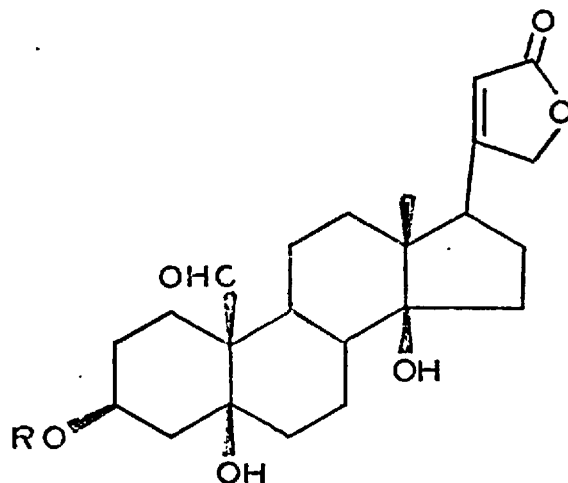




(50) R = L-Rhamnose (51) R = Glucose-L-rhamnose

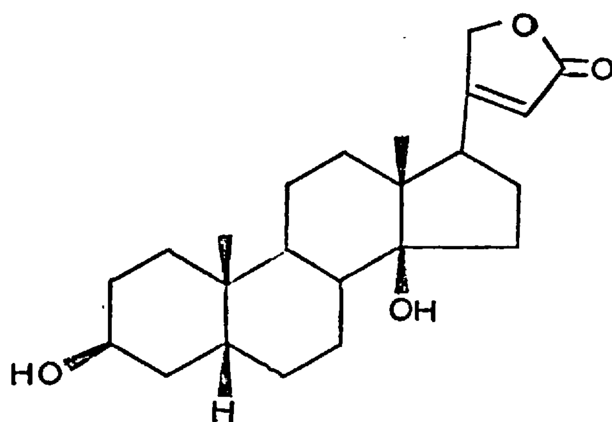
Kupchan's group have prepared derivatives of strophanthidin (52) and have examined the effect of structural variations on both the cytotoxicity and on the inhibition of the adenosine triphosphatases (ATPases) involved in the transport of Na^+ and K^+.[41] The cytotoxicity was unaffected by reduction of the C-19 aldehyde to a carbinol or an oxime, although conversion to the C-19 carboxylic acid, or its methyl ester, resulted in the loss of activity. Alteration of the cis configuration of the A/B or C/D ring junctions also caused loss of activity while saturation of the lactone double bond produced compounds with reduced activity. By variation of the C-3 ester group it was possible to increase the cytotoxicity, structure (53) being the most active compound tested, approximately 100 times more active than strophanthidin itself. The cytotoxicity of these compounds essentially paralleled their inhibition of ATPase, most KB active compounds effecting at least 70% inactivation.

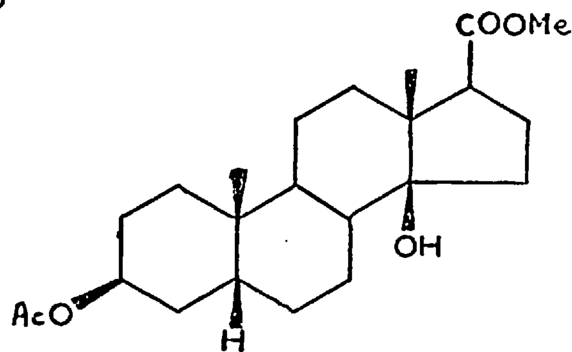
Cardenolides inhibit the active transport of amino acids and Kupchan et al. have postulated[42] that, as tumour cells are very active in accumulating amino acids, the cytotoxicity may be due to the inhibition of amino acid accumulation by the KB tumour cells.



(52) R = H
(53) R = $CO.CH_2.N_3$.

The first synthesis of a cardenolide was that of digitoxigenin (54) in 1962. This was prepared by Sondheimer <u>et al.</u> from methyl 3β - acetoxy - 14β - hydroxy - 5β - etianate (55).[43] Since that time a number of other syntheses have been reported,[44] however in many cases the difficulties of synthesis are such that it has been more economical to obtain the compounds, either directly from plant materials, or by modification of a closely related naturally occurring precursor.



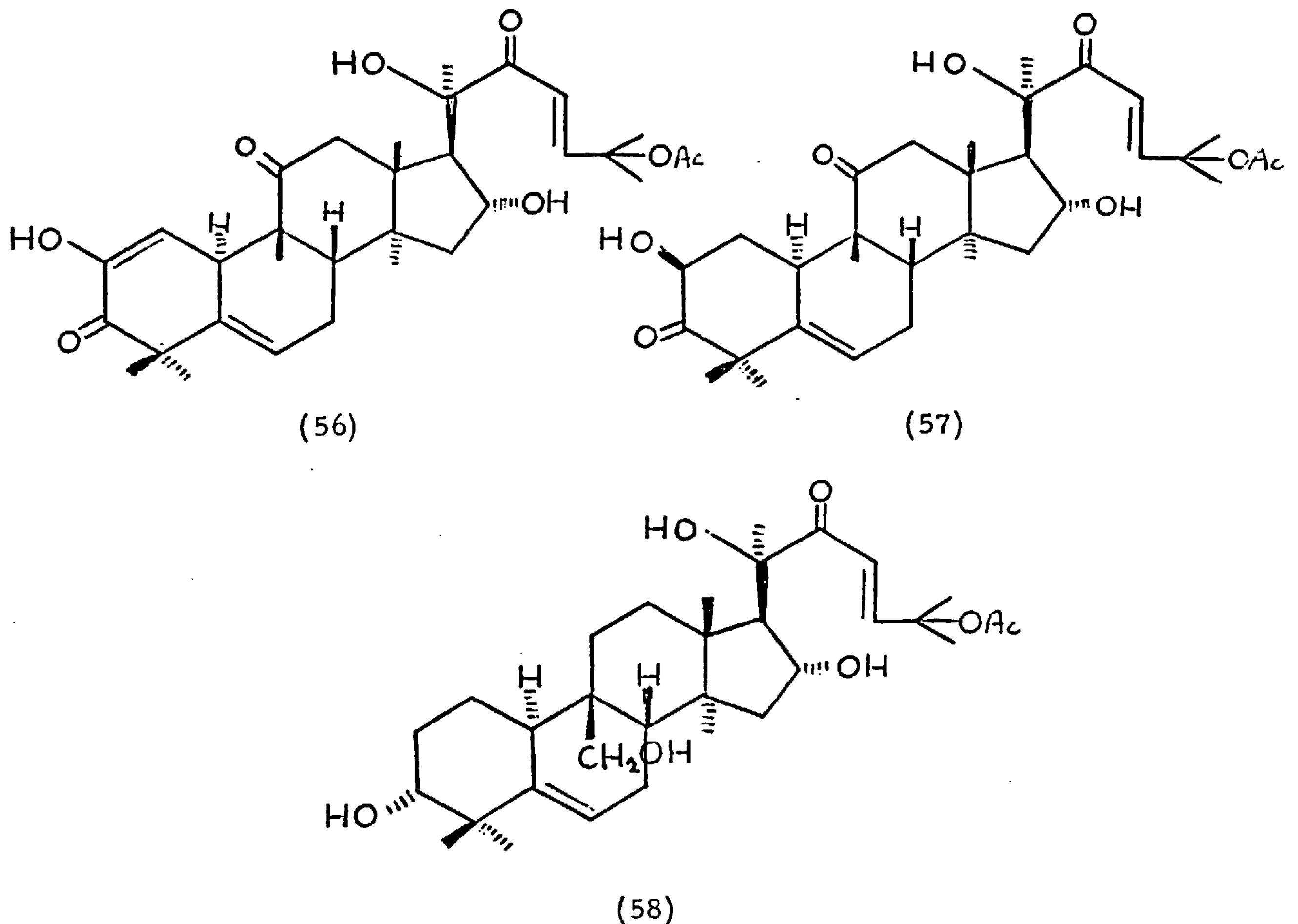


(54)

(55)

CUCURBITACINS

The cucurbitacins are among the most cytotoxic compounds known,[45] cucurbitacin E (56) having an ED_{50} against KB of 4.5×10^{-7} and cucurbitacin B (57) of 2.5×10^{-6}.[2] Despite this high cytotoxicity in vivo activity has only been observed for the cucurbitacins C (58) and E (56).

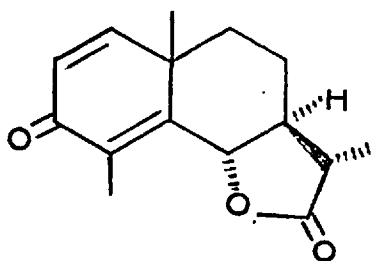


(56) (57) (58)

The structural features responsible for the high toxicity are not understood and although the natural compounds have an, α, β- unsaturated ketone in the side chain, saturation of this, as in dihydrocucurbitacin B, does not completely destroy the activity (dihydrocucurbitacin B has an ED_{50} of 1.7×10^{-3}). It has also been suggested[10] that the 25-acetyl group may have some importance.

SESQUITERPENES

The sesquiterpenes constitute by far the largest group of isoprene derived natural products, there being approximately 1,000 known members of the group.[46] The pharmacological properties of these compounds, which include antibiotic, fungistatic and anthelmintic properties, have long been known e.g. santonin (59), first isolated in 1830, has been used in India for many years to treat intestinal worms.[47] More recently the cytotoxic properties of some sesquiterpenes have been discovered. The structure of these cytotoxic compounds fall mainly into four of the sesquiterpene sub groups,the germacranolides (60), e.g. eupatolide (61)[48] and eupaserrin (62),[49] the guaianolides (63), e.g. euparotin (64)[50] and florienalin (65),[51] the pseudoguaianolides (66), e.g. ambrosin (67) and parthenin (68)[52] and the elemanolides (69), e.g. vernomenin (70) and vernolepin (71).[53] A small number of other structural types such as the seco-eudesmanolides eriolanin (72) and eriolangin (73),[54] are also represented among the fifty or so sesquiterpenes so far reported to show some cytotoxicity.



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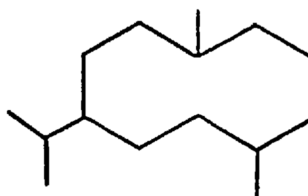

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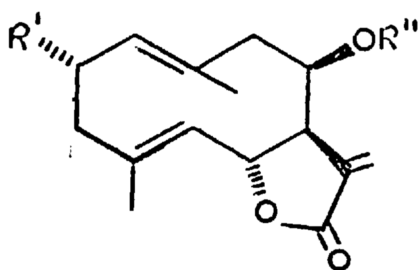
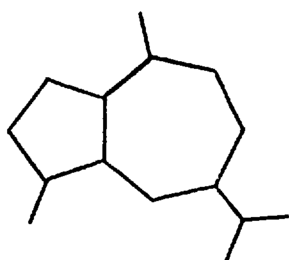


(61) R',R''= H.

(62) R'= OH; R''= CO. C(CH_2OAc)=CH.Me



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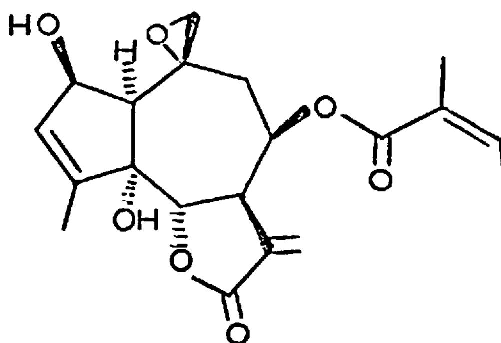

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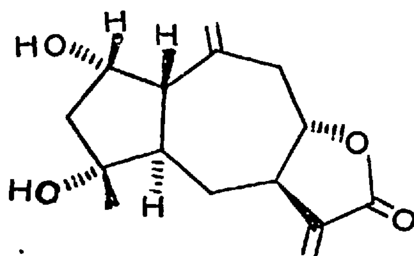

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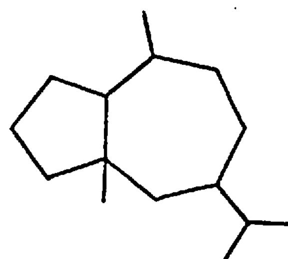

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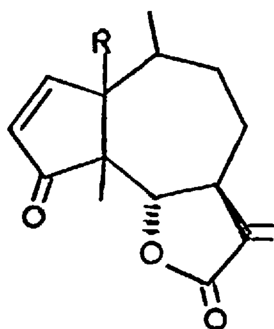
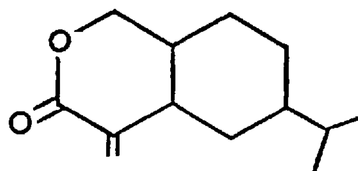


(67) R = H.

(68) R = OH.



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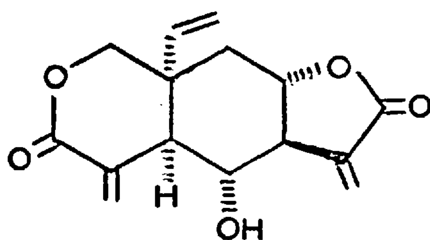

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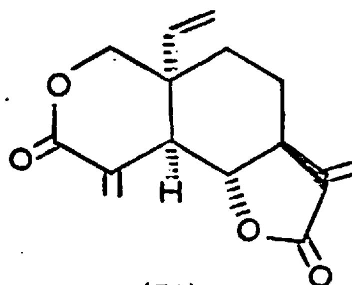

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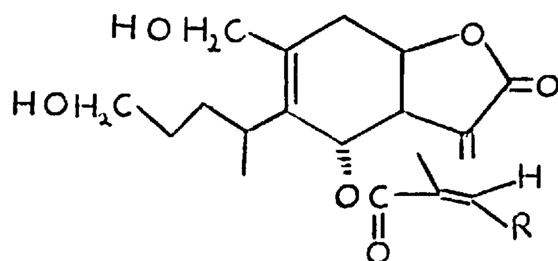
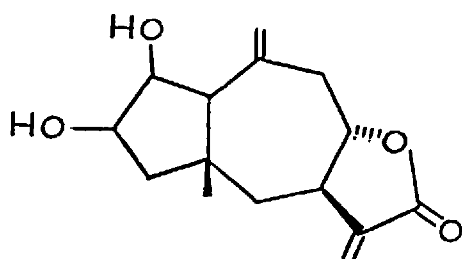


(72) R = H
(73) R = Me

Apart from the basic skeleton, all but two of the cytotoxic sesquiterpenes have one definite structural feature in common, all contain at least one α -methylene - γ -lactone grouping.

This structural feature in itself does not guarantee activity, there being a number of sesquiterpenes containing this moiety which have no appreciable cytotoxicity, e.g. pulchellin C (74) which has an ED_{50} against KB of >100,[2] however virtually all of the compounds known to be active do contain this common factor.



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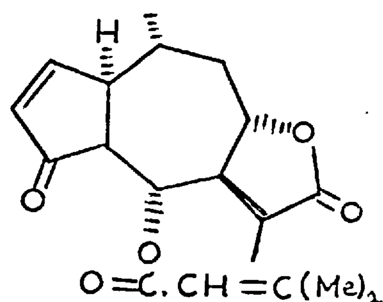

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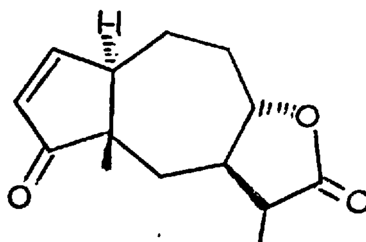
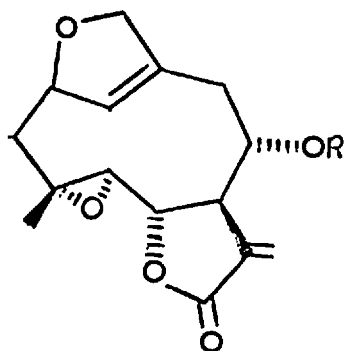


(76)

The two exceptions to this structure-activity rule are fastigilin B (75) and plenolin (76). Fastigilin B was isolated by Herz et al. from Gaillardia fastigiata [55] and has an ED_{50} of 1.0 against KB. Plenolin was recently obtained by Lee and co-workers from the plant Baileya pleneradata.[56] It has an ED_{50} of 3.8 against the H.Ep.-2 cell culture.

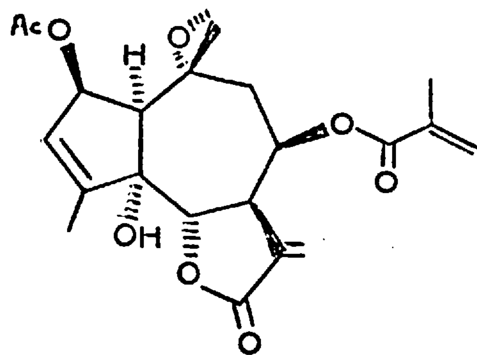
Kupchan, Eakin and Thomas have reported [57] that, of the fifty or so cytotoxic sesquiterpenes only seven have shown in vivo activity. These include elephantopin (77) and elephantin (78) isolated from Elephantopus elatus,[58] euparotin acetate (79) from Eupatorium rotundifolium [50] and vernomenin (70) and vernolepin (71) obtained from Vernonia hymenolepis.[53]

In addition to the α - methylene - γ - lactone all of these in vivo active compounds contain either an α- methylene - δ - lactone or a conjugated side chain ester, and a hydroxyl or acyloxy group adjacent to the methylene function of the γ - lactone. No compound with this structural combination has failed to show in vivo activity.



(77) R = CO. CH. CMe_2.

(78) R = CO. CMe. $=CH_2$.

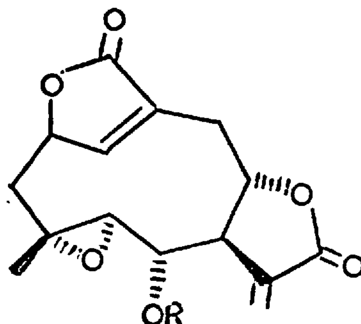


(79)

Elephantopin and Elephantin have the largest number of structural features of these seven in vivo active compounds, two α, β-unsaturated lactones, one with an exocyclic and one with an endocyclic double bond, an α, β-unsaturated ester and an epoxide. Elephantopin has demonstrated activity against WM, LE, PS and KB tumour systems, while elephantin is active in WM and KB, the only two systems in which it has been tested. Despite this, neither of these compounds has a therapeutic index large enough to warrant further examination.[10]

Structure-activity work with the cytotoxic sesquiterpenes has been carried out by two groups of workers, Kupchan's group working with elephantopin (77), elephantin (78) and vernolepin (71) and Lee et al. concentrating on Helenalin (85) (vide infra).

Kupchan et al. prepared[57] dihydrolelephantopin, tetrahydroelephantopin and tetrahydroelephantin in all of which the α-methylene-γ-lactone double bond had been reduced, but which still retained the α, β-unsaturated endocyclic lactone. Although these three compounds were inactive, as were dihydroelephantol and elephantoldihydroisobutyrate, elephantol acetate (80) and elephantol isobutyrate (81) did show some activity.

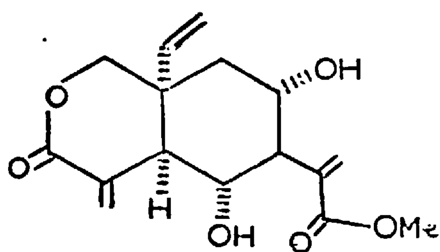


(80) R = Ac.

(81) R = CO. CH(Me)$_2$.

Elephantopin and elephantin reacted readily with cysteine to form both mono - and bis - adducts.[59] The mono-adducts, formed by nucleophilic attack on the double bond of the α -methylene - γ- lactone retained some cytotoxicity, while the bis-compounds were inactive. The rate of cysteine addition was enhanced by the presence of a hydroxy or an acyloxy group adjacent to the lactone ring junction, but no correlation could be found between the rate of cysteine addition and the cytotoxicity. The reaction of α, β-unsaturated endocyclic lactones with cysteine was much less rapid, and resulted in less stable products, than with the corresponding exocyclic compounds.[60]

Reduction of the ethylidene double bond in vernolepin caused no loss of activity but destruction of the α- methylene - γ - lactone, either by reduction or by formation of the methanol adduct (82), resulted in decreased activity.[5] Reduction of both α, β-unsaturated lactones produced an essentially inactive compound. As in the cases of elephantopin and elephantin, vernolepin readily formed an active mono - cysteine adduct (83) and an inactive bis - compound (84).[59]



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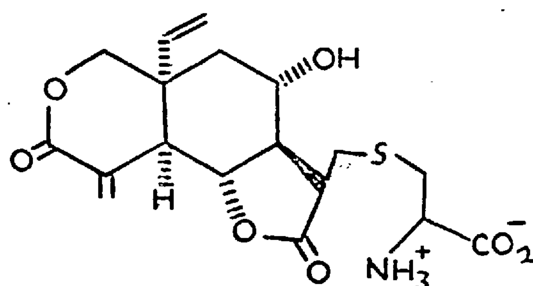

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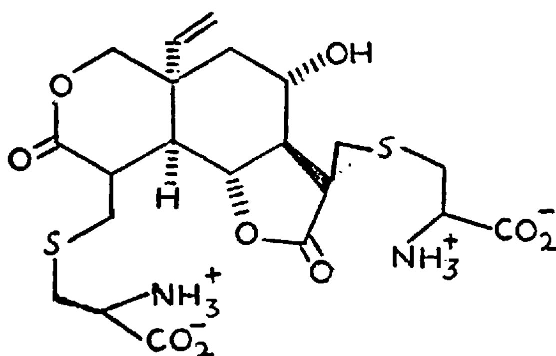


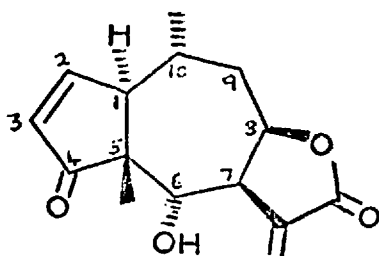
(84)

Elephantopin, elephantin and vernolepin have been shown to inhibit the extension growth of wheat coleoptile sections, an effect which is blocked by addition of sulphydryl compounds such as mercaptoethanol to the medium and which can be reversed by the addition of indole acetic acid.[60] The ease of reversibility of this process has led Kupchan to propose[61] that these compounds interfere with an essential step in cell wall synthesis and do not act merely as toxic principles.

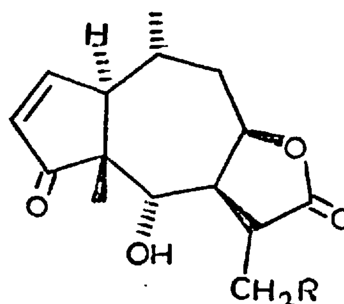
From these results Kupchan et al. have concluded[57] that the most important structural feature for cytotoxicity in this series is the α- methylene - γ - lactone, the stereochemistry of the lactone ring junction being unimportant. A definite relationship has also been observed between lipophilic character and cytotoxicity. Furthermore, the presence of a conjugated ester, a cyclopentenone or an α - methylene - δ - lactone, in addition to the α - methylene - γ -lactone increases the activity by more than can be explained simply by changes in lipophilicity.

As mentioned above α , β -unsaturated - γ - lactones of this type have been shown to react very readily with sulphydryl containing compounds such as cysteine or the enzyme phosphofructokinase.[62] In contrast reactions with amino acids are very slow.[63] This has led to the proposal that, in general the sesquiterpene lactones exert their cytotoxic effect by means of an alkylation reaction with suitable biological nucleophiles.

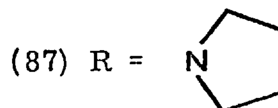
Lee et al. have carried out a considerable amount of work with the pseudoguaianolide helenalin (85). This was originally isolated from Helenium autumnale by Clark[64] and has since been obtained from other Helenium species.[65] This compound has an ED_{50} against KB of 0.22[66] and has been reported as being as effective as 5-fluorouracil in the in vivo Ehrlich ascites carcinoma.[67] Hartwell and Abbott have reported[2] the testing of helenalin in 7 other common in vivo systems from which no indication of activity was obtained.



(85)



(86) R = $N(Me)_2$.

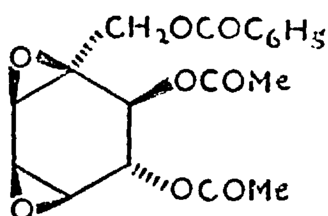
(87) R = 

Lee's group have prepared several hydrogenated helenalin derivatives and a number of michael type secondary amine adducts e.g. (86) and (87).[68] Reduction of the cyclopentenone, to give 2, 3-dihydrohelenalin, resulted in a fourty-fold loss of activity, while similar modification of the exocyclic methylene group, either by hydrogenation or by formation of a michael type amine, produced a five to ten fold loss of activity. Modification of both double bonds resulted in total loss of activity.

As a result of this work Lee et al. have concluded that for the maintenance of a high level of cytotoxicity in helenalin the α- methylene - γ - lactone is less important than the α, β-unsaturated ketone moiety.

An increase in the'alkylating potential'and the lipophilicity of helenalin, by modification of the C6 ester group, were mirrored by an increase in cytotoxicity.[67] The activity increased significantly on introduction of a conjugated ester, especially when an aromatic ring was in conjugation with the carbonyl group. An optimum position appeared to be reached with the cinnamate ester, this being five times more active than helenalin. A similar series of results were obtained by variation of the ester in the helenalin dimethylamine adduct (86).

A number of naturally occurring compounds owe their antitumour activity to an epoxide moiety e.g. crotepoxide (88).[69] Lee's group demonstrated[70] that helenalin - 2, 3 -epoxide had activity equivalent to helenalin while formation of the 2, 3, 11, 13 - diepoxide led to a five fold loss of activity.

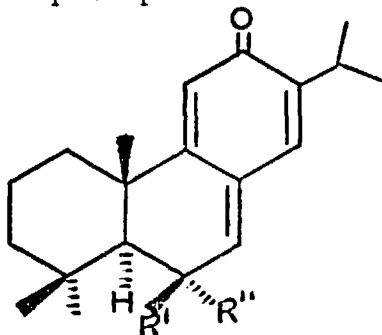


(88)

Little synthetic activity has been reported in the field of cytotoxic sesquiterpenes. This may reflect the fact that, despite their in-vivo activity, none of these compounds has a therapeutic index large enough for it to be considered for use in the treatment of human cancer.

THE ALKYLATION THEORY

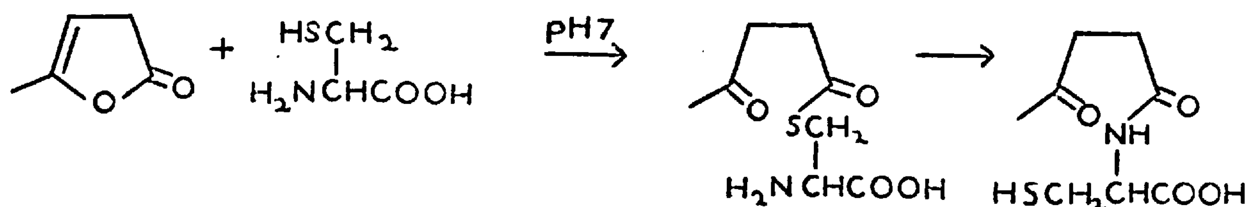
Although the naturally occurring antitumour agents include a wide variety of chemical types, a common mechanism of action, involving alkylation of biological nucleophiles, has been proposed for many of these compounds. Thus, Kupchan and Altland have proposed [26] that the bisbenzylisoquinoline alkaloids act by "a stepwise sequence leading to bis alkylation of the biological macromolecules involved in growth regulation," and Kupchan has reported [35] that the structure and functional groups of Maytansine (35) are " consistent with the selective alkylation theory." An alkylation mechanism has also been proposed to explain the in vivo activity of the quinone methides taxodone (89) and taxodione (90) obtained from Taxodium distichum[71] while, as has been discussed previously in this review, considerable evidence has been accumulated to demonstrate the alkylation reactions of several cytotoxic sesquiterpene lactones.



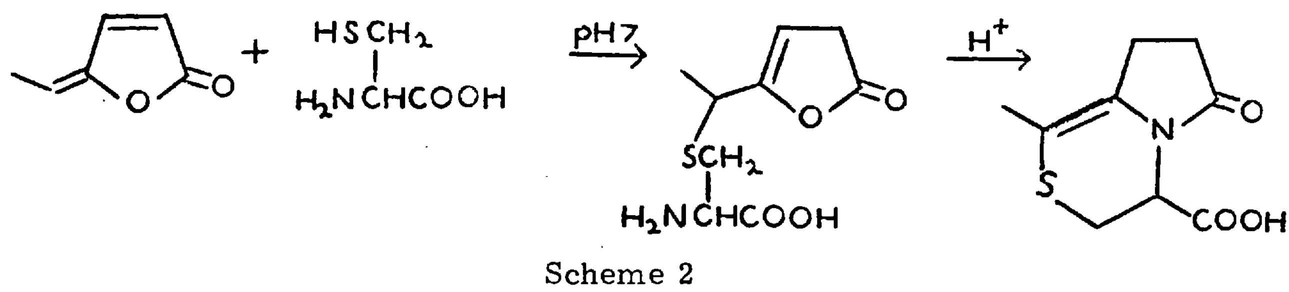
(89) R' = H; R'' = OH (90) R' = R'' = O

Several definite parallels exist between cytotoxicity and carcinogenicity. Dickens and Cooke, in discussing structure-activity relationships for carcinogenicity,[72] reported that five - or six - membered α,β-unsaturated lactones were one of a number of predominantly active chemical types. Dickens had earlier shown[73] that the structural features required for maximum carcinogenicity in γ - lactones were α , β -unsaturation together with an exocyclic double bond attached to position 4.

Whilst engaged in a study of carcinogenicity Jones and Young investigated the reactions of cysteine with both carcinogenic and non-carcinogenic lactones.[74] Two distinct reaction types were observed. The non-carcinogenic lactones reacted, by a sequence such as that shown in scheme 1, to form acylated products, while the carcinogenic compounds reacted, on the whole, more rapidly, to form alkylation products (e.g. scheme 2). Known enzyme catalysed processes are capable of reversing the acylation type reaction, however there are no known biological processes which might reverse the alkylation reaction. These results led Jones and Young to conclude that "all carcinogenic lactones undergo attack (by cysteine) resulting in alkylation of the nucleophile whereas inactive lactones react to give acylated products."



Scheme 1



Scheme 2

The cytotoxic sesquiterpene lactones elephantopin (77), elephantin (78) and vernolepin (71) have been shown to react with cysteine to form alkylated rather than acylated products,[59] however no data is available to show whether or not a non cytotoxic lactone, such as Pulchellin C, (74), reacts via an acylation process.

In general, compounds containing two or more potential alkylating functions have been shown to be more cytotoxic than compounds containing only one such group. This has led to the postulation of a mechanism of action involving cross linking either between two points on the DNA helix or between DNA and protein.[75] The exact site of this reaction is unknown but Dowling _et al._ have reported[75] that the 7 position on guanine and the 3 position on adenine are particularly susceptible to alkylation. These authors believe that this alkylation of DNA is of far greater importance for cytotoxicity than the reaction with cell components such as cysteine. These cell components may be regenerated by the organism, resulting in cell recovery. Injury to the DNA ultimately leads to cell death.

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RESULTS AND DISCUSSION

INTRODUCTION

As part of a world wide screening programme, a systematic search for antitumour activity in extracts of Chilean plants has been organised, during the last five years, by Dr. M. Silva at the University of Concepcion, Chile. Aqueous ethanolic extracts of dried plant materials have been tested against the KB - human epidermoid carcinoma of the nasopharynx and PS-P388 lymphocytic leukaemia systems, by the Cancer Chemotherapy National Service Center, Maryland, USA. Plant extracts showing activity were then subjected to systematic fractionation by solvent extraction using, successively, petroleum-ether, benzene, ethyl acetate, butanol and methyl ethyl ketone, the resulting fractions again being assayed against the experimental tumour systems.

Examination of extracts of the two indigenous Chilean plants, *Pleocarphus revolutus* (Compositae) (Plate 1) and *Ovidia pillo pillo* Meisner (Thymelaeaceae) showed that both exhibited antitumour activity. This was concentrated in the benzene and ethyl acetate soluble fractions of *Pleocarphus revolutus* and in the petroleum-ether and benzene soluble fractions of *Ovidia pillo pillo*.

The primary aim of the research described in this thesis was the isolation and characterisation of the compounds responsible for the antitumour activity of these two plant species, in the hope that these compounds might prove to be useful new agents in the fight against cancer.

Initially a type of classical phytochemical approach was followed, the compounds isolated being those most readily crystallisable. Later, a more systematic approach was adopted, in which the fractionation was followed at every stage by means of a simple cytotoxicity test.

Preliminary Separation of Pleocarphus Revolutus Extracts

The petroleum-ether, benzene and ethyl acetate soluble fractions of Pleocarphus Revolutus were initially seperated by means of column chromatography on silica gel. Crystallisation and, in some cases, further chromatography of the sub-fractions produced, yielded six crystalline compounds, these were; a sesquiterpene diol, a nor-sesquiterpene keto-diol, α-amyrin, α-amyrin acetate, a polyalkane and a flavone (Fig. 1).

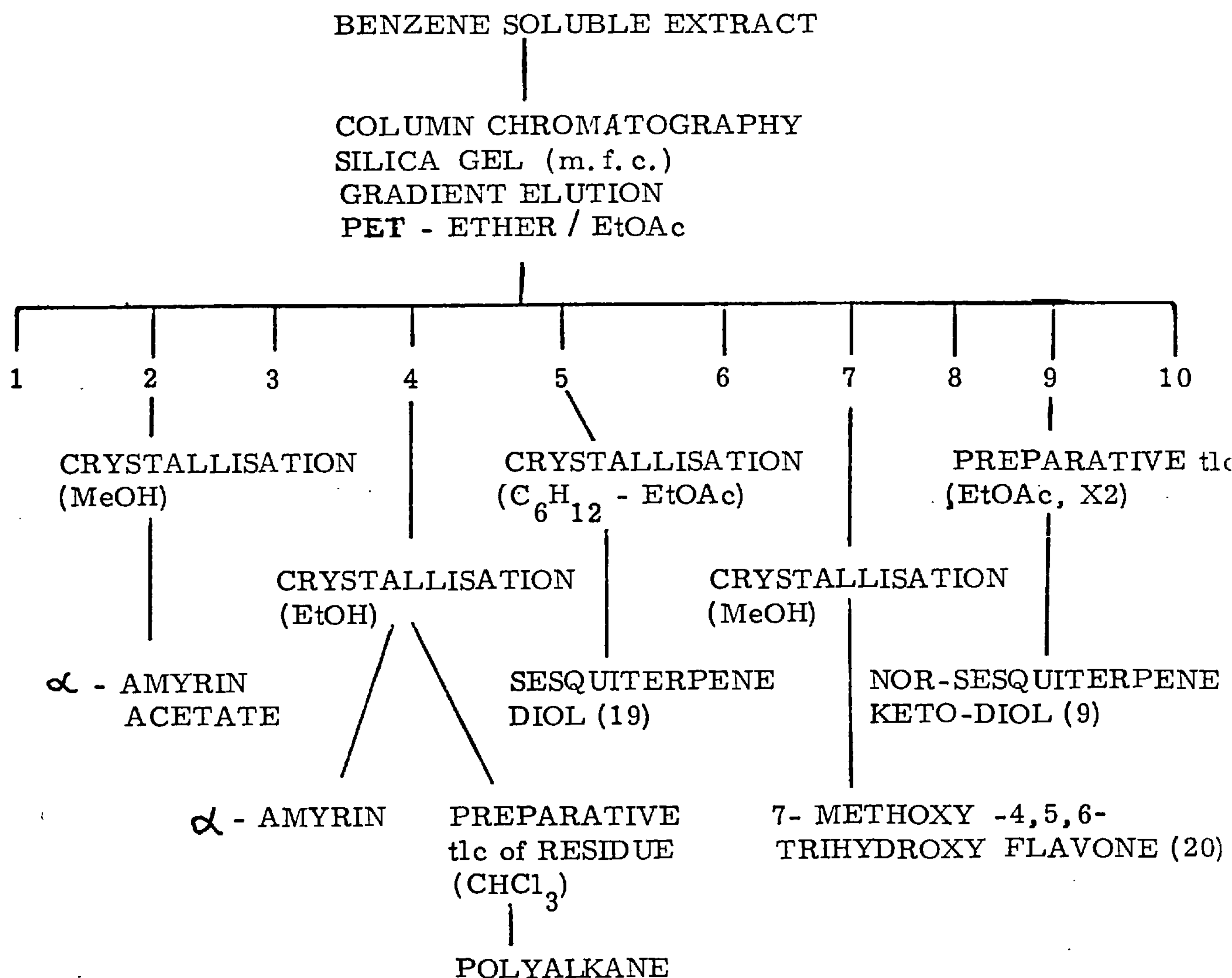


Fig. 1. Initial fractionation diagram of the Pleocarphus revolutus benzene soluble fraction

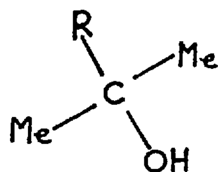
a) <u>The Sesquiterpene Diol</u> (19)

The first of these compounds crystallised directly from one of the benzene sub-fractions as white needles m.p. 133-135°. On treatment with iodine vapour this component gave an intense black colouration which subsequently provided a sensitive method for detection of the compound in tlc fractions. In this way further samples of the material were located in both the petroleum - ether and ethyl acetate soluble fractions of the same plant.

The mass spectrum of this compound showed a highest mass ion at m/e 220 and accurate mass measurement of this, indicated a molecular formula of $C_{15}H_{24}O$. Despite extensive recrystallisation no confirmatory microanalysis could be obtained for this formula. The analytical results, however, were consistent with a composition $C_{15}H_{26}O_2$ andthus indicated a rapid loss of one molecule of water within the mass spectrometer.

Reexamination of the mass spectrum revealed a weak ion at m/e 223, corresponding to the loss of one methyl group from the unobserved parent ion at m/e 238. This was in agreement with the formulation $C_{15}H_{26}O_2$.

In addition to this first, rapid, loss of water, the mass spectrum showed a strong $(M-36)^+$ ion corresponding to the loss of a second molecule of water. This evidence suggested that the compound might be a diol, as ethers do not readily lose water in the mass spectrometer.[1] The presence of the base peak of the mass spectrum at m/e 59 (C_3H_7O) suggested that one of these alcohol functions was present in the form of a 2-hydroxyprop-2-yl group, thus indicating a part structure (1).



(1)

The i.r. spectrum confirmed the presence of at least one hydroxyl group (3350 cm^{-1}) and showed bands at 1640 and 895 cm^{-1} characteristic of an exocyclic methylene group. However, it contained no absorptions attributable to carbonyl functions.

The exocyclic methylene group was also indicated by the n.m.r. spectrum, which included a two-proton resonance at τ 5.3. No other peaks could be observed below τ 8.8 indicating the absence of any protons α to an oxygen function. The spectrum to higher field of this was very complex, but included three, three-proton singlets in the region τ 8.8 - 9.0. These chemical shifts were consistent with the presence of methyl groups attached to hydroxyl bearing carbon atoms and were thus in agreement with the tentative part structure (1).

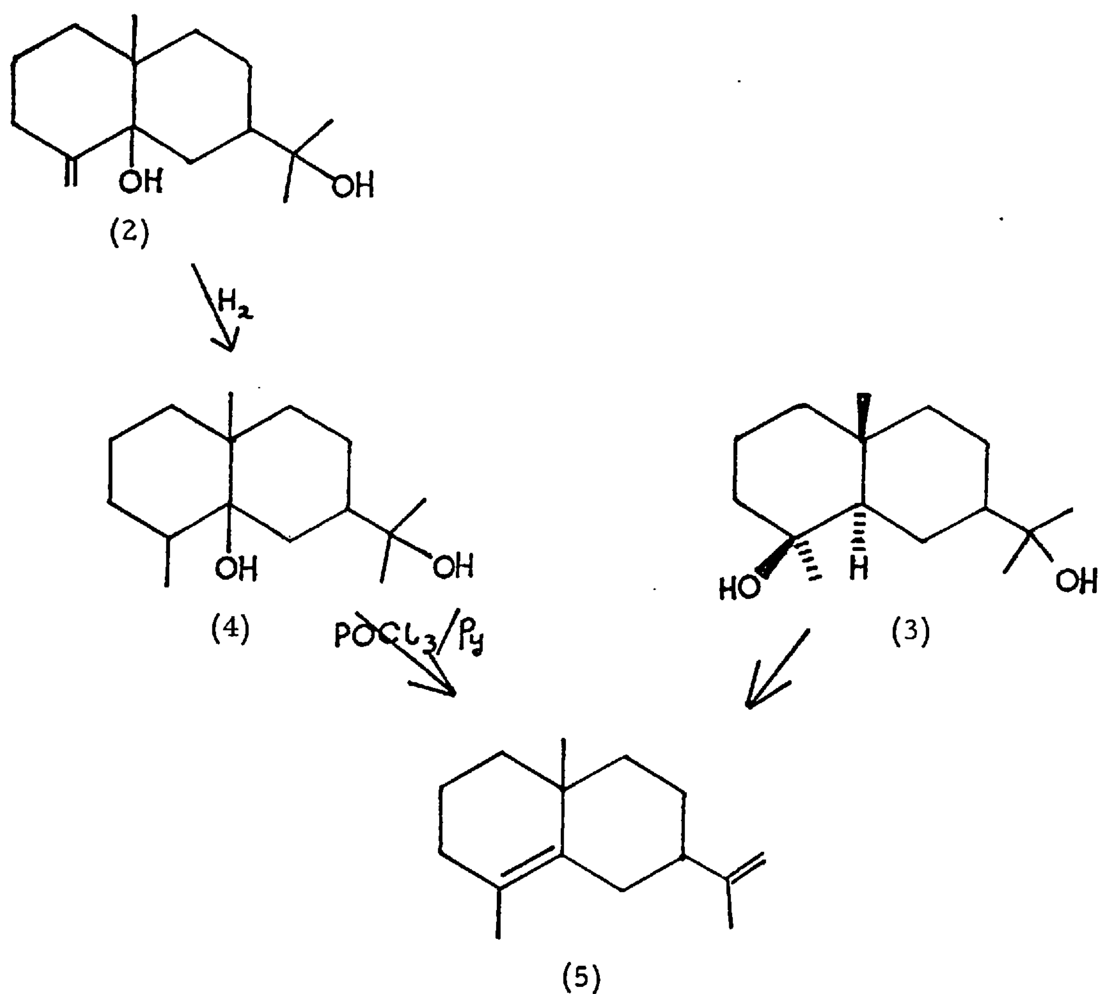
The use of $Eu(fod)_3$ failed to simplify the n.m.r. spectrum to any appreciable extent (Table 1). This may have been a result of the bifunctionality of the molecule, the $Eu(fod)_3$ failing to complex specifically with either one of the hydroxyl groups.

On hydrogenation over platinum, the diol absorbed one equivalent of hydrogen, thus confirming that only one double bond was present in the molecule. The molecular formula, $C_{15}H_{26}O_2$, required three double bond equivalents and therefore, from the above evidence, a bicyclic structure.

CHEMICAL SHIFT (τ) IN CCl_4									
Total Eu $(fod)_3$ ADDED mg	A (3H, s)	B (3H, s)	C (3H, s)	D (1 H, s)	E (1 H, s)	F (1H, m)	G (1H, m)	H (1H, m)	J (1H, m)
0	8.96	8.87	8.87	5.36	5.29	-	-	-	7.00
5.20	8.14	8.10	7.90	4.97	4.97	5.30	6.04	-	6.42
10.24	7.45	7.45	7.09	4.68	4.68	3.09	4.35	5.91	5.75
16.32	6.61	6.69	6.17	4.29	4.38	0.42	2.37	4.67	5.22
22.64	5.74	5.88	5.24	3.93	4.07	-2.04	0.36	3.22	-
28.80	4.87	5.05	4.38	3.60	3.82	-3.95	-1.06	2.05	3.92
36.64	4.34	4.52	3.85	3.43	3.66	-4.85	-1.96	1.23	3.15

Table 1. Eu $(fod)_3$ induced shifts in the n.m.r. spectrum of the sesquiterpene alcohol (19)

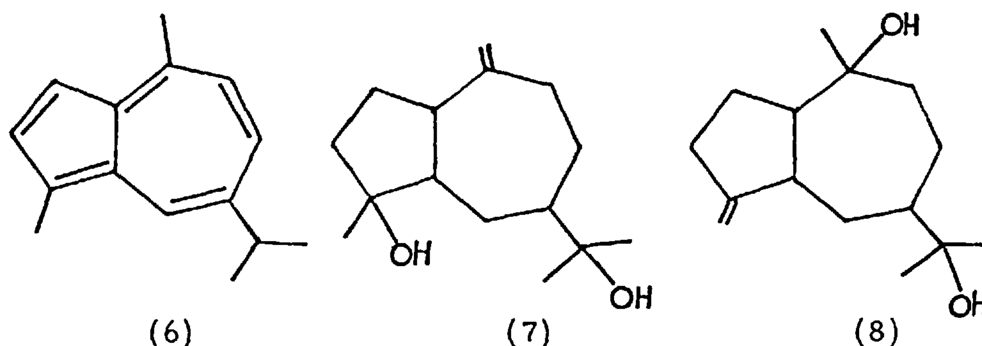
Treatment of the diol with acetic anhydride in pyridine,for twenty four hours, failed to produce any reaction. This fact, coupled with the n.m.r. and mass spectral evidence, indicated that the molecule contained two tertiary alcohol functions. Several structures were considered as being consistent with this information. One of these (2), had obvious similarities to the known natural product cryptomeridiol (3)[2] and the latter provided a rapid means for testing this structure by chemical correlation. Assuming that structure (2) was correct, dehydration of the dihydro alcohol (4) and of cryptomeridiol would be expected to provide at least one common dehydration product (e.g. (5), Scheme 1).



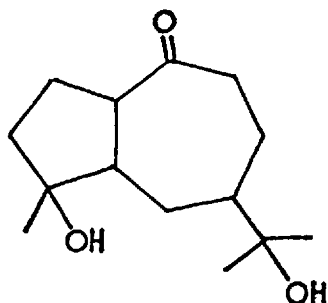
Scheme 1

Although tlc examination of the mixtures obtained from the two dehydration reactions indicated several similarities, treatment of the tlc plate with concentrated sulphuric acid produced entirely different colour reactions for each of the mixtures. Thus structure (2) was incorrect and further, more systematic investigations were continued.

Dehydrogenation of the diol over palladium produced a blue oil which had a u.v. spectrum identical to that reported for S-guaiazulene (6)[3]. This demonstrated that the diol had the guaiane basic skeleton and was not, as had been proposed in structure (2), a eudesmanoloid. Thus, the available evidence indicated either of structures (7) or (8) for this compound.

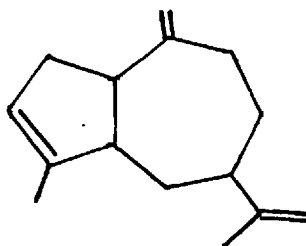


Low temperature (-10°C) ozonolysis of the diol yielded a white crystalline solid with molecular formula, by mass spectroscopy, $C_{14}H_{24}O_3$. The i.r. spectrum of this compound showed a carbonyl stretching frequency at 1695 cm^{-1}, characteristic of a six or higher membered ring ketone, rather than of a five membered ring ketone (1750-1740 cm^{-1}). This firmly indicated structure (9) for the ozonolysis product and thus structure (7) for the diol.



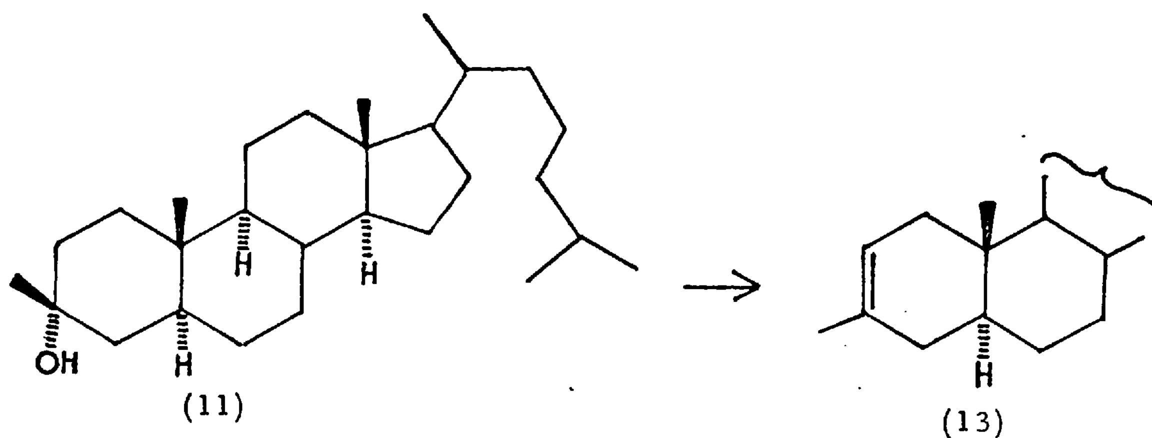
(9)

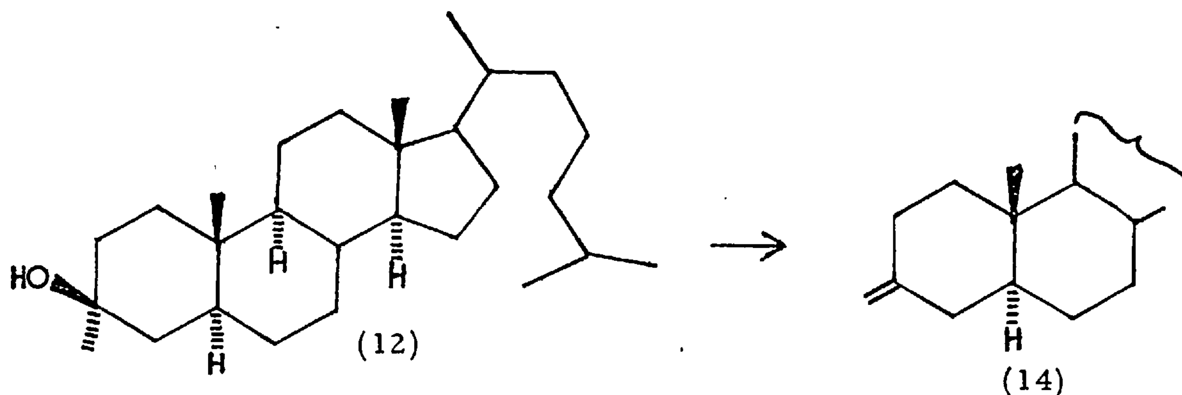
The diol (7) was extremely sensitive to acid, reaction with toluene-p-sulphonic acid at room temperature rapidly producing a black tar. Dehydration was effected more cleanly by means of phosphorus oxychloride in pyridine. The major product of this reaction was shown, by i.r. and mass spectroscopy, to be one of the expected double dehydration products. The n.m.r. spectrum of this compound included bands at τ 4.76 (IH, br s), 5.34 (4H, brm), 8.3 (6H, br s), consistent with structure (10) rather than with any of the other possible double bond isomers. This triene proved to be fairly unstable, decomposing within a period of 48 hours, despite storage at 0°.



(10)

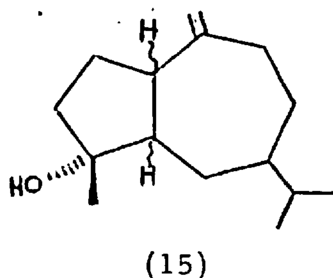
Barton et al.[4] have shown that the stereochemistry, about C-3, of the 3-methylcholestanols (11) and (12), determines the position of the double bond in the subsequent dehydration product. Thus (11), the axial 3α-alcohol was dehydrated to (13) while the equatorial 3β-alcohol (12) provided the corresponding exocyclic isomer (14).



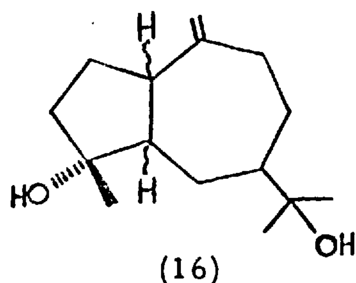


By analogy with these results, Sastry et al.[5] have assigned α stereochemistry to the C-4 hydroxyl group of nardol (15) on the grounds that the only observed dehydration products contained endocyclic double bonds. Dehydration of the Pleocarpus diol (7) produced solely the endocyclic olefin (10) and this, by a simple extension of the above reasoning, appeared to indicate that the compound contained an α-hydroxyl function and was thus represented by structure (16).

However, the validity of this reasoning was not considered to be great, there being three major criticisms; a) the general applicability of Barton's observations to structural systems other than steroids has not been investigated, b) no information was available as to the stereochemistry of the ring junction either in nardol(15) or in the diol(7), and c) 1-methyl-cyclopentanols have generally been observed to dehydrate to give exocyclic olefins.[6] Thus the stereochemistry indicated in structure(16) was considered to be purely speculative in nature.

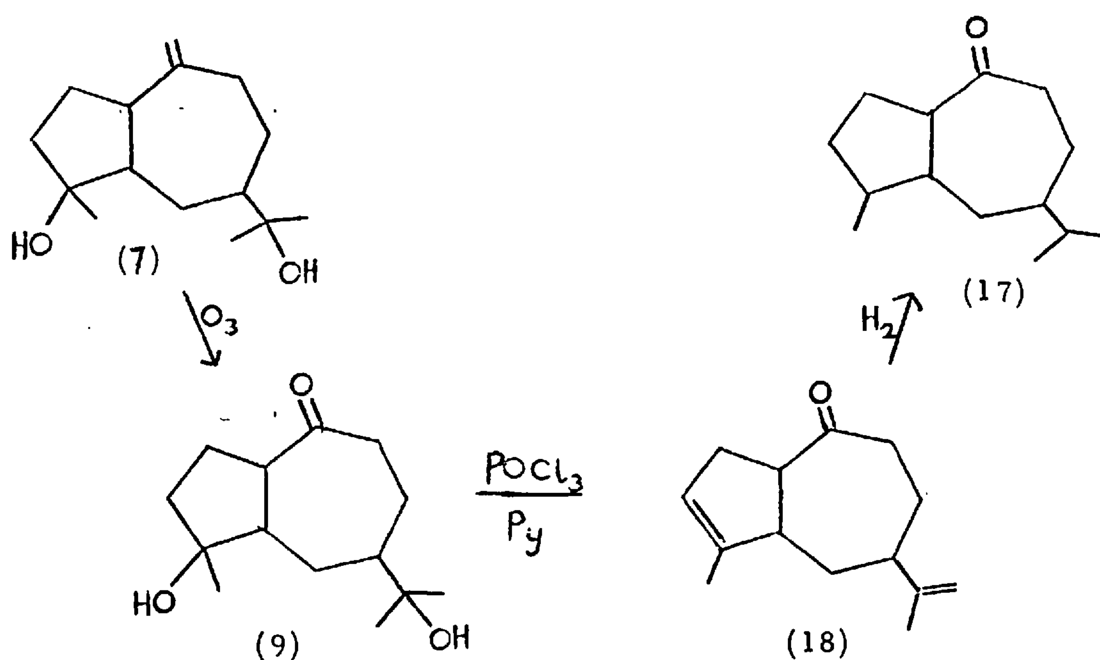


(15)



(16)

A literature search failed to reveal any known compound of structure (7). However, two closely related natural products, nardol (15) and the ketone (17)[7] had previously been reported. This latter compound (17) appeared to provide a suitable target for chemical correlation of the diol(7) by means of the reaction sequence outlined in scheme 2.



Scheme 2

Preparation of the ketone (9) had previously been accomplished by ozonolysis of the diol (7). Dehydration of this keto-diol gave the expected compound, consistent, by n.m.r., i.r. and mass spectroscopy with constitution (18). Hydrogenation of this material over platinum, however, failed to give any product equivalent to the required saturated ketone (17), the reaction mixture showing i.r. absorption bands assigned to both hydroxyl (3620 cm^{-1}) and carbonyl functions (1730 cm^{-1})

^{13}C.m.r. spectroscopy (Figs. 2 and 3) provided a final confirmation of structure (7) for the natural diol. Both the chemical shifts (Table 2) and the off-resonance decoupled splitting patterns were entirely consistent with the proposed structure.

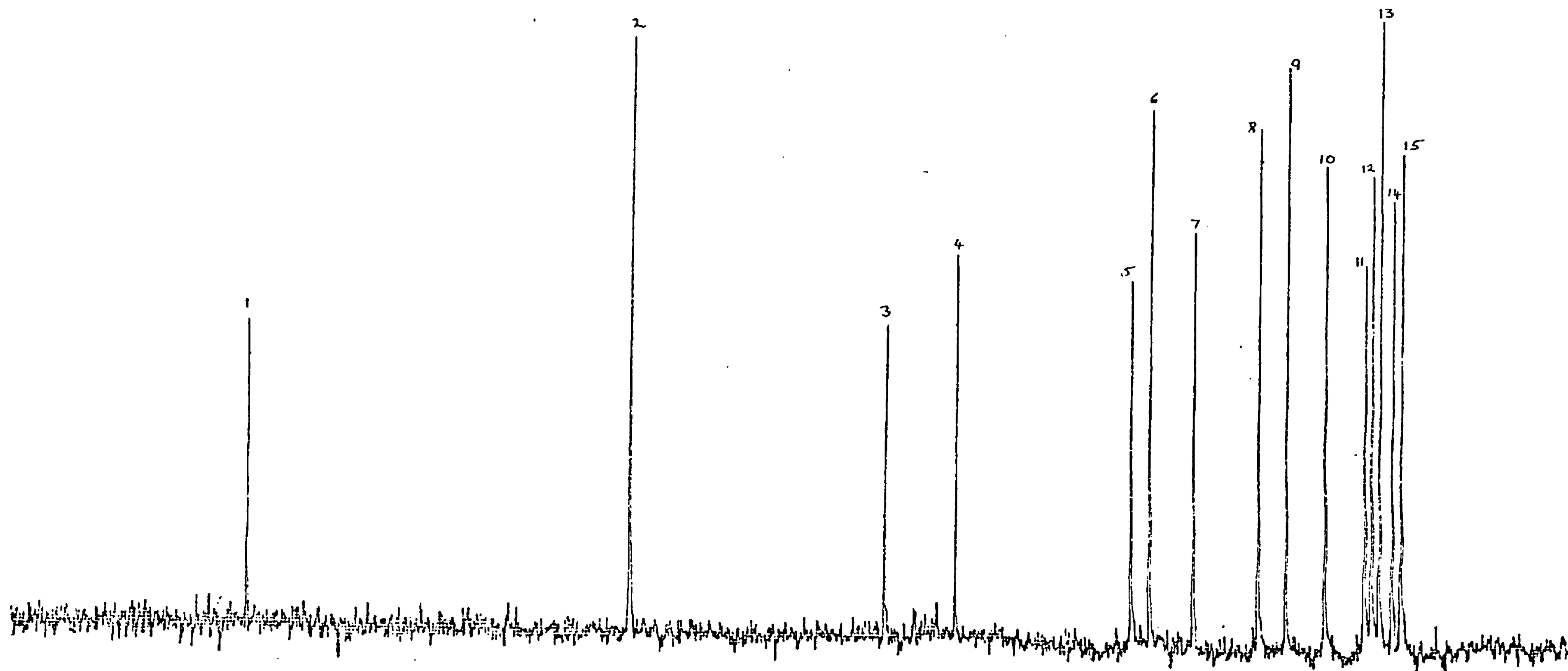


Fig. 2. ^{13}C.m.r. spectrum of sesquiterpene diol (19).

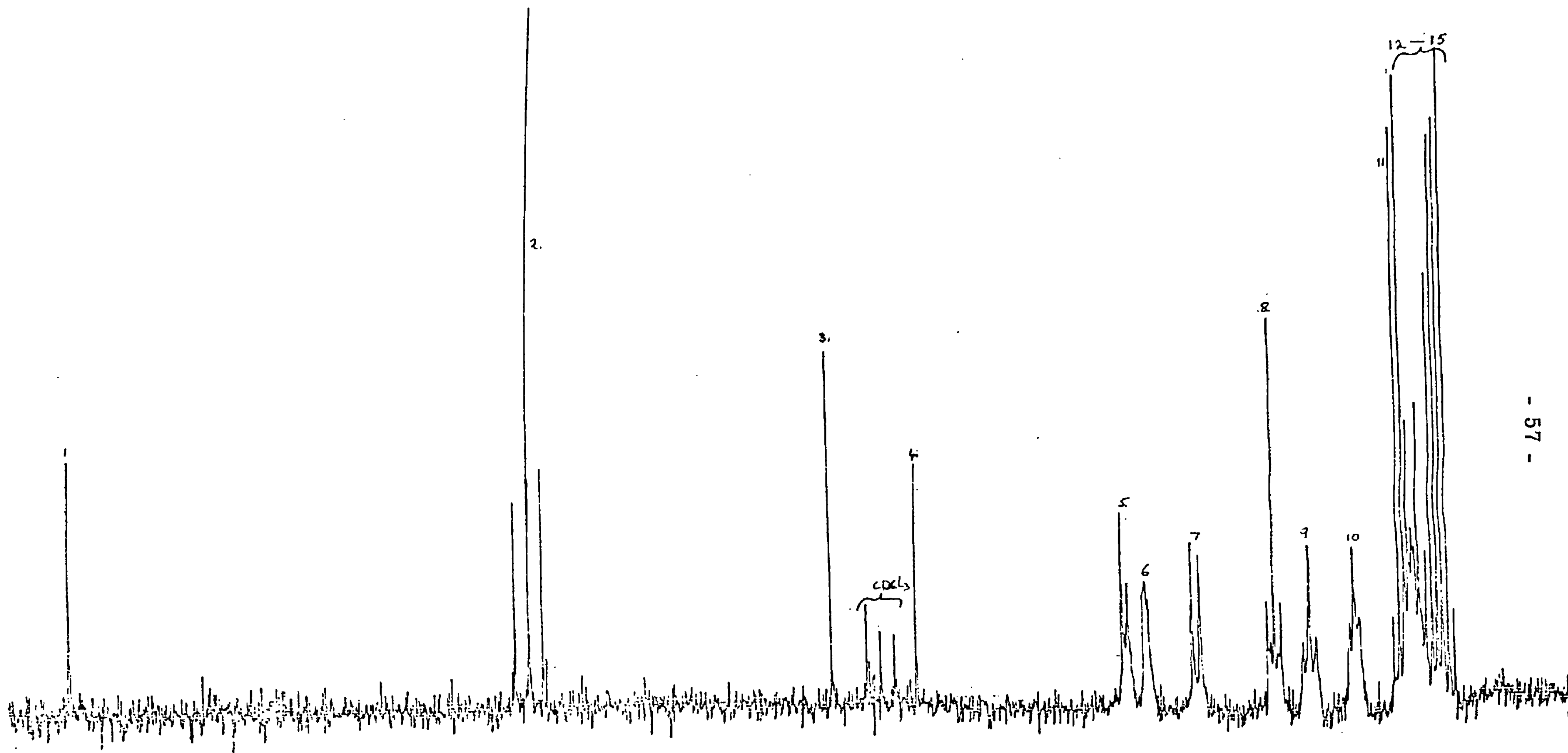
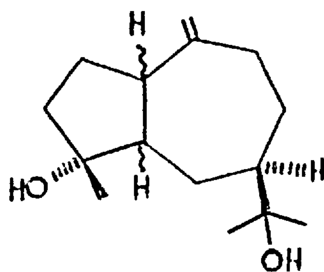


Fig. 3. ^{13}C. m. r. spectrum of sesquiterpene diol (19). Off resonance decoupled.

Peak No.	Chem. shift (ppm from TMS)	Off Resonance Splitting Pattern
1	152.28	s
2	109.90	t
3	81.63	s
4	73.83	s
5	54.04	d
6	52.02	t
7	47.34	d
8	40.24	t
9	37.02	t
10	32.73	t
11	28.33	q
12	27.59	d
13	26.73	t
14	25.34	q
15	24.39	q

Table 2 ^{13}C.m.r. Chemical shifts of sesquiterpene diol (19).

On the basis of the evidence described above, we have proposed structure (7) for this novel diol, isolated from *Pleocarphus revolutus.* Little information has been obtained concerning the stereochemistry of the molecule, however, as a result of the specific course of the dehydration reaction, we have suggested that the C-4 hydroxyl group may have the α orientation. In addition, since virtually all known guaiane derivatives of this type have β-oriented C-7 isopropyl side chains, it is felt that this is also the most likely orientation in the present case. The diol can therefore be tentatively assigned the structure (19).

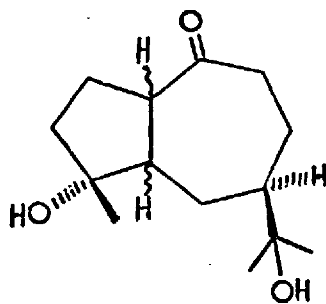


(19)

b) The Nor-sesquiterpene-diol (9)

Further chromatography of the benzene and ethyl acetate soluble fractions of Pleocarphus revolutus showed, that the nor-sesquiterpene keto-diol(9), prepared by ozonolysis of the natural diol(19), was itself a natural product, present in both of these plant fractions.

Microanalysis of the keto-diol, a white crystalline solid m.p. 106-8°, proved difficult, the figures obtained consistently corresponding to the required molecular formula $C_{14} H_{24} O_3$ plus half a mole equivalent of water. However, this molecular formula was confirmed by accurate mass measurement of the molecular ion. N.m.r., i.r. and mass spectra of this compound were as expected for the proposed ketone, which by comparison with the parent diol (19) has structure (20).



(20)

c) The Triterpene components

Crystallisation of sub-fractions of the petroleum-ether and benzene soluble fractions of Pleocarphus revolutus yielded a third white crystalline compound m.p. 224-6°, molecular formula $C_{32} H_{52} O_2$. This was identified as α -amyrin acetate by comparison with the literature values for this compound.[8]

Further chromotography of some slightly more polar plant fractions, later yielded crystalline α -amyrin and also a further white crystalline solid m.p. 76-78°. Apart from a carbonyl stretching frequency at 1705 cm^{-1} and saturated alkane stretching bands, the i.r. spectrum of this compound was virtually featureless. Since the n.m.r. spectrum showed only one peak, a broad singlet at τ 8.7, this material was assumed to be of the polyalkane type and no further work was carried out on this compound.

d) The Flavone (20)

The final compound isolated from the Pleocarphus revolutus extracts by this, non-systematic, approach was a yellow crystalline solid m.p. 272-274°. Mass spectroscopy indicated a molecular formula of $C_{16} H_{12} O_6$ and this, with the yellow colour, suggested a flavanoid type of structure.

The n.m.r. spectrum (d_6-DMSO) immediately indicated a monomethoxy-trihydroxy system with peaks at τ 6.28 (3H, s -OMe), 2.24 and 3.12 (each 2H, d, J = 9Hz. Respectively 3' and 5', 2' and 6'-H), 3.33 and 3.48 (each IH, s, IH attached to C-3 and IH at one of positions 6, 7 or 8), - 0.28 (2H, br.m. - OH), - 3.2 (IH, s. OH).

This last sharp resonance was recognised to be typical of a 5-hydroxy group, rapid proton exchange, and thus signal broadening, being prevented by hydrogen bonding with the 4-carbonyl oxygen.

The u.v. absorption values obtained (Table 3) after addition of sodium methoxide, aluminium trichloride, aluminium trichloride plus hydrochloric acid, sodium acetate and sodium acetate plus boric acid, to methanolic solutions of the flavone, were interpreted according to the methods of Mabry, Markham and Thomas.[9] (Table 4). These confirmed that the compound was a flavone, rather than an isoflavone or flavanol and also indicated the presence of 4' and 5-hydroxy groupsand a 7-methoxy group.

Treatment of the flavone with methyl iodide and potassium carbonate for six hours yielded a tri-methoxy compound identical, by m.p. and u.v., with the known[10] 4', 6, 7 - trimethoxy, 5-hydroxy flavone. Reaction under the same conditions, for twenty four hours yielded 4', 5, 6, 7- tetramethoxy flavone, thus confirming the 4', 5, 6, 7 - oxygenation pattern of the Pleocarphus compound.

Mabry et al. had reported[11] that the benzene induced shifts (relative to carbon tetrachloride) of the methoxy resonances, in the n.m.r. spectra of flavones, or their trimethyl silyl ethers were diagnostic for the position of those methoxy groups in the molecule. Measurement of the shift for the bis (trimethylsilyl ether) of the present flavone gave an observed Δ value ($\delta CCl_4 - \delta C_6D_6$) of 0.43 ppm. This was characteristic for a methoxy group in any of the 2', 3', 4' or 7 positions. As the possibility of 2', 3' or 4' - methoxy groups had already been eliminated by the u.v. and n.m.r. spectra, this confirmed the presence of a 7-methoxy group.

Solution	Band Inm	Band IInm
MeOH	335.5	273.5
NaOMe	394.5, 326.5	276
$AlCl_3$	362	303.5
$AlCl_3/HCl$	358	302
NaOAc	388	275
$NaOAc/H_3BO_3$	341	272

Table 3. U.V. absorptions of 7-methoxy-4', 5, 6 - trihydroxy flavone (20).

Addition	Observed Shift* nm.				Interpretation according to Mabry, Markham and Thomas[9+]
	Pleocarpus flavone		Arisawa *et.al.*[13] flavone		
	I	II	I	II	
NaOMe	+59	+ 2.5			Diagnostic for presence of 4' - hydroxyl group
AlCl3	+26.5	+30	+15	+10)	No B-ring ortho-dihydroxyl group
AlCl3/HCl	+22.5	+28.5		)	
NaOAc	+55.5	+ 1.5	+ 1	+ 1	cannot be a 7-hydroxyl group unless C-6 and C-8 oxygen functions are also present.
NaOAc/ H_3BO_3	+ 5.5	- 1.5			Confirms that there is no B-ring ortho-dihydroxyl group.

* + indicates bathochromic shifts, - hypsochromic shifts.

\+ Band I (usually 300-380 nm) is considered to be associated with absorption due to the B-ring cinnamoyl system and Band II (240-280 nm) with absorption involving the A-ring benzoyl system.

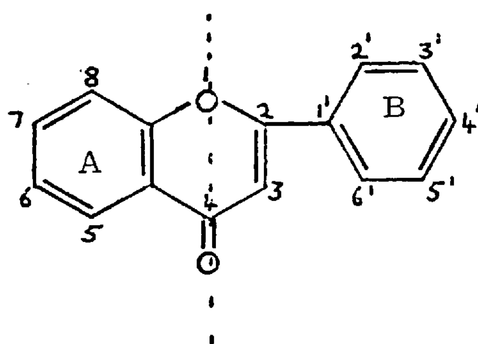
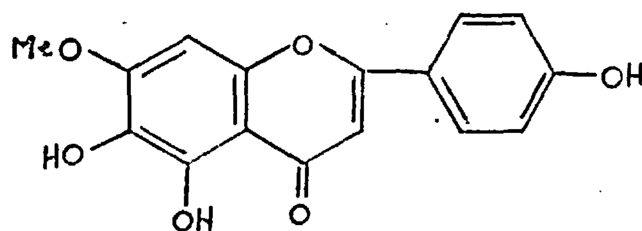


Table 4 Interpretation of u.v. absorptions of 7-methoxy - 4', 5 6 - trihydroxy flavone (20)

This compound must therefore be 7-methoxy - 4', 5, 6-trihydroxy flavone (20).



(20)

A search of the literature revealed that compounds of this structure had previously been reported by two groups of workers. The only information available from the first of these references[12] was a melting point, 190-192°, considerably lower than that obtained in the present work. However, the second of these compounds, isolated by Arisawa et al.[13] had m.p. 290-292° and λ_{max}^{EtOH} 253, 308 nm. The corresponding values for the Pleocarphus flavone were m.p. 272° - 274° and u.v. $\lambda_{max.}^{MeOH}$ 273.5, 335.5 nm. No n.m.r. data were reported for this literature compound, however the very limited u.v. measurements available, when interpreted according to the methods of Mabry, Markham and Thomas, were in agreement with the structure proposed (Table 4). In addition the tri-methoxy compound prepared by Arisawa et al. was identical with that obtained by Fukui et al.[10] and thus with that obtained from the Pleocarphus flavone.

Crystallisation of this latter flavone had proved consistently difficult. Thus the observed discrepancy with the literature melting point may be explained by the existence of minor impurities in our samples. This does not, however, explain the discrepancies between the two sets of u.v. data.

Despite this lack of correlation with reported values, the results described above provide firm evidence for the occurrence of 7 - methoxy - 4', 5, 6 - trihydroxy flavone in extracts of the plant Pleocarphus revolutus.

Preliminary Separation of Ovidia pillo pillo Leaf Extracts

Preparative tlc of the petroleum ether and benzene soluble fractions from the leaves of Ovidia pillo pillo yielded three yellow, crystalline compounds, subsequently shown to be flavones.

The first of these components had m.p. 171-174° and a molecular formula, by mass spectroscopy, of $C_{17}H_{14}O_5$, corresponding to a mono-hydroxy-di-methoxy flavone. The n.m.r. and u.v. spectra, (Table 5) interpreted as for the case of the Pleocarphus flavone, indicated methoxy groups at positions 4' and 7 and a hydroxygroup at position 5.

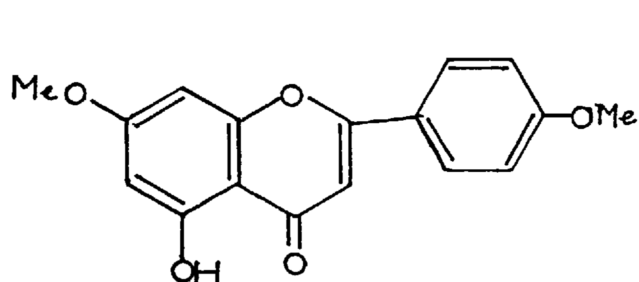
Confirmation of this was obtained from the Eu $(fod)_3$ induced shifts in the n.m.r. spectrum of this compound (Table 6). The "S-values" (proposed by Cockerill and Rackham [14] as the slopes of the straight lines obtained by plotting the shift values against the molar ratio of Eu $(fod)_3$ to the flavone)obtained were in close agreement with those predicted by Okigawa et.al. [15] for 4', 7 - dimethoxy - 5 - hydroxy flavone (21).

Solution	Band I nm.	Band II nm.
Me OH	328.5	268.5
Na OMe	346 (sh)	289
$AlCl_3$	379, 346.5	303, 277
$AlCl_3$/HCl	381, 340.5	301.5, 278
Na OAc	327	289
$NaOAc/H_3BO_3$	330	268

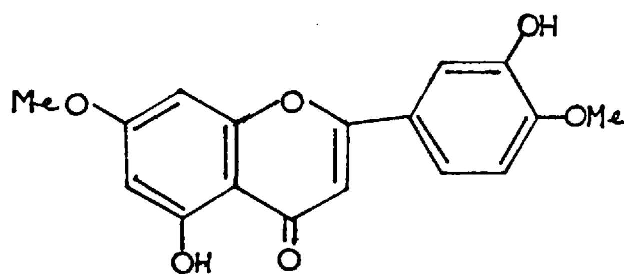
Table 5. U.V. absorptions of 4', 7 - dimethoxy - 5 - hydroxy flavone (21)

Mole ratio: $Eu(fod)_3$ Flavone	Observed Chemical Shifts τ						
	H-2',6'	H-3',5'	H-8	H-6	H-3	OMe-7	OMe-4'
0	2.22	3.06	3.50	3.60	3.71	6.28	6.28
0.056	2.18	3.04	3.42	3.42	3.42	6.09	6.20
0.17	2.12	3.06	3.32	2.29	3.32	6.04	6.19
0.25	2.05	3.06	3.02	1.16	3.02	5.87	6.19
0.45	1.97	3.08	2.68	-0.29	2.54	5.69	6.20
'S-Value	0.60	-0.05	1.80	8.80	1.25	2.55	0.30

Table 6. $Eu(fod)_3$ induced shifts and 'S-value' of 4',7 - dimethoxy - 5 - hydroxy flavone (21)



(21)

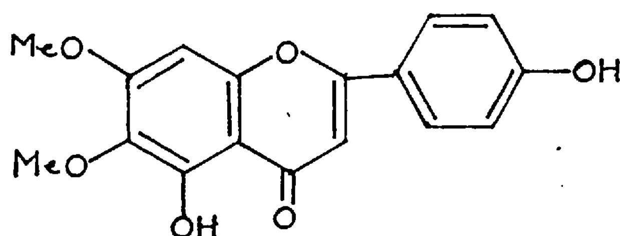


(22)

This compound has previously been isolated by several workers including Rangaswani and Iyer [16] and Bauer and Dietrich [17]. The data obtained in the present work was completely consistent with that reported by these groups for 4', 7 - dimethoxy - 5 - hydroxy flavone.

Slightly more polar on preparative tlc than (21) was the second Ovidia flavone. This was identified as pilloin (4', 7 - dimethoxy - 3', 5 - dihydroxy flavone) (22) first isolated from this same plant species by Nunez-Alarçon [18].

The third of these flavones was only present in relatively minor quantities. It had m.p. 259 - 261° and a molecular formula, by mass spectroscopy, of $C_{17} H_{14} O_{6}$, indicative of a dihydroxy - dimethoxy flavone. U.v .spectroscopy (Table 7) indicated the presence of 4' and 5 - hydroxyl groups and a 7 - methoxy group. A literature search for compounds of this molecular formula and part structure, revealed the compound 6 ,7 - dimethoxy - 4', 5 - dihydroxy flavone (23). The limited data reported [19] for this compound was in fairly close agreement with that obtained in the present work. Thus we propose that this third Ovidia flavone is the compound 6 ,7 - dimethoxy - 4', 5 - dihydroxy flavone (23).



(23)

Solution	Band I nm.	Band II nm.
MeOH	335	267
NaOMe	386	266.5
$AlCl_3$	384, 348	277, 302.5
$AlCl_3/HCl$	381, 344	278.5
NaOAc	387	266, 258.5
$NaOAc/H_3BO_3$	341.5	266

Table 7 U.v. absorptions of 6 ,7 - dimethoxy - 4', 5 - dihydroxy flavone (23)

Literature reports [20] had indicated that extracts of the plant *Ovidia pillo pillo* were also under investigation by Nunez-Alarcon in Chile. In the light of this second investigation, which confirmed the presence of relatively large quantities of flavanoid type compounds in this plant, no further work was carried out on these particular extracts.

Systematic Fractionation of the Benzene Soluble Extract of Pleocarphus revolutus

Introduction

The classical phytochemical approach, described previously in this discussion, had not proved successful as a means of isolating the active constituents of the plants Pleocarphus revolutus and Ovidia pillo pillo. In an attempt to improve on this, a more systematic method of fractionation was adopted. This was based on the use of a simple cytotoxicity test (Appendix I) as a means of identification of the active fractions, thus allowing further work to be carried out selectively on the fractions of interest.

Silica column chromatography of the benzene soluble extract of Pleocarphus revolutus yielded seventeen fractions. Cytotoxicity testing of these, indicated that the activity was concentrated in fractions 3 and 5 and these two fractions were therefore subjected to further fractionation.

a) The Sesquiterpene Keto-Alcohol (37)

Further chromatography of fraction 5, over sephadex LH-20, yielded two active sub-fractions (C and D Fig. 4). However, separation of these to give pure components, resulted in the total loss of activity, possibly as a result of decomposition of the active components during storage.

One of the inactive fractions (B Fig. 4), obtained by sephadex LH-20 chromatography of fraction 5, had proved to be a white crystalline solid m.p. 108 - 109°. Microanalysis and mass spectroscopy of this component indicated a molecular formula of $C_{15} H_{22} O_2$ i.e. 5 double bond equivalents. The i.r. spectrum showed absorption bands characteristic of hydroxyl and carbonyl functions (3480 and 1700 cm^{-1}),

accounting for both oxygen atoms, and of an exocyclic carbon - carbon double bond (1640 and 910 cm^{-1}.). The u. v. spectrum was indicative of an α, β - unsaturated ketone (λ_{max}^{EtOH} 243.5, ϵ 13,400). These structural features accounted for all but two of the required double bond equivalents and thus suggested a bicyclic structure for the compound.

'H.m.r. spectroscopy indicated the presence of two vinylic methyl

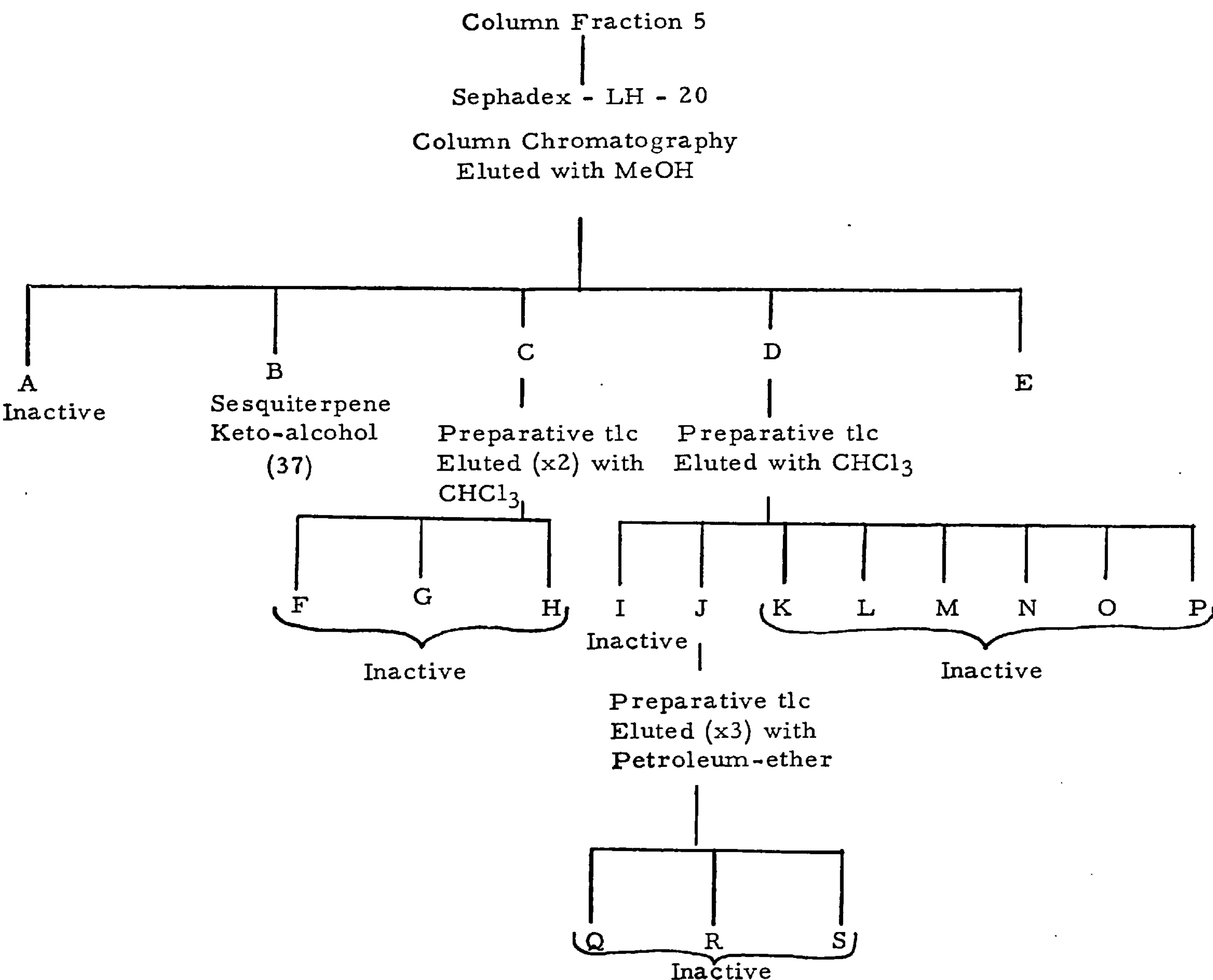
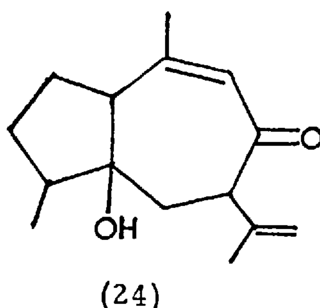


Fig 4. Fractionation diagram of Pleocarpus revolutus benzene extract column fraction 5.

groups τ 8.16 (3H, brs) and 8.37 (3H, d, J = 2 Hz), one secondary methyl group τ 9.04 (3H, d, J = 7 Hz) and three vinylic protons τ 5.2 (3H, brm).

On the basis of this evidence a number of possible structures were proposed for this compound including the keto-alcohol (24), considered as a result of Eu $(fod)_3$ shift experiments (Table 8). These experiments had failed to show any shift typical of a proton adjacent to a hydroxyl function, and thus indicated a tertiary alcohol.



(24)

The spectroscopic properties (i.r. and n.m.r.) of a further component isolated from Pleocarphus revolutus, had indicated that this was a sesquiterpene keto-acetate. Comparison of this spectral data with that obtained from the above keto-alcohol indicated a close similarity between the two compounds. This relationship was readily confirmed by acetylation of the alcohol, with acetic anhydride in pyridine, to give a product, indistinguishable in all respects from the natural keto-acetate.

Neither this acetylation nor the subsequent cinnamoylation (cinnamoyl chloride in pyridine) or formylation (formic-acetic anhydride in pyridine) of this keto-alcohol, were consistent with the presence of the proposed tertiary hydroxyl group. It was therefore necessary to revise the proposed structure to that of either a primary or a secondary alcohol. That the latter was the correct assignment was indicated by both the ^{1}H.m.r. spectrum of the derived keto-acetate and the ^{13}C.m.r. spectrum of the alcohol. The ^{1}H.m.r.

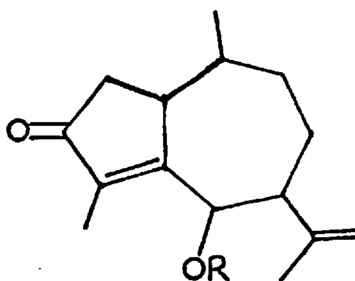
Total Eu (fod)$_3$ Added (mg)	CHEMICAL SHIFTS (τ) in CCl_4												
	C_4-Me (d, J = 2Hz)	C_{10}-Me (d, J = 7Hz)	C_{11}-Me (br s)	$C_{11} = CH_2$ (br s)	(br s)	H_1 (m)	H_2 (dd, J = 18, 5Hz)	(dd, J=18, 1Hz)	H_6 (d, J=1Hz)	H_7 (m)	$H_{8,9}$ (m)	(m)	H_{10} (m)
0	8.37	9.05	8.16	5.18	5.18	6.88	7.83	8.03	5.18	8.08*	8.27*	8.60*	8.08*
2.4	7.86	8.95	8.06	5.10	5.10	6.62	6.96	7.17	4.86	-	-	-	-
5.2	7.16	8.81	7.92	4.96	4.96	-	5.79	6.00	4.43	7.33	7.81	8.19	7.61
7.4	6.70	8.73	7.84	4.86	4.92	5.40	5.01	5.14	4.13	7.00	7.72	8.01	7.42
9.4	6.21	8.64	7.74	4.75	4.85	4.92	4.22	4.43	3.82	6.67	7.56	7.80	7.21
12.3	5.60	8.54	7.61	4.60	4.75	4.37	-	-	3.41	6.17	7.22	7.49	6.93
15.9	4.92	8.39	7.46	4.44	4.65	3.71	2.09	2.29	2.91	5.56	7.10	7.32	6.49
19.7	4.21	8.28	7.31	4.26	4.53	3.01	0.98	1.03	2.37	4.76	6.84	6.94	5.89
28.6	3.17	8.12	7.07	3.96	4.34	1.92	-0.51	-0.46	1.43	-	6.41	6.41	4.56

* Initial chemical shifts estimated by extrapolation.

Table 8 Eu (fod)$_3$ induced shifts in the n.m.r. spectrum of the sesquiterpene keto-alcohol (25)

spectrum of the acetate showed a broad proton singletat τ 4.16 (C$\underline{H}$OAc) while the off resonance decoupled ^{13}C.m.r. spectrum of the alcohol (Fig. 5) contained a sharp doublet at a chemical shift of 70.8 ppm, this being characteristic of a carbon attached to a hydroxyl function and thus of a secondary alcohol.

Assignment of one of the resonances at τ 5.2, in the ^{1}H.m.r. spectrum of the alcohol, as a C$\underline{H}$OH proton also made this spectrum consistent with the proposed secondary alcohol structure. However, this resonance did not show the predicted, large, Eu $(fod)_3$ induced shift, the most rapidly shifting signals being two single proton double doublets both with approximate chemical shifts, in the absence of the Eu $(fod)_3$ of τ 8.0. This indicated that the primary site of complex formation of the keto-alcohol with the Eu $(fod)_3$, was probably on the carbonyl group and not, as had been expected, on the hydroxyl function. Reconsideration of the Eu $(fod)_3$ induced shifts (Table 8) in this light, led to the proposal of (25) as the correct structure for this compound.



(25) R = H

(26) R = Ac

This structure (25) was completely consistent with all the known data for the keto-alcohol, including that obtained from ^{13}C.m.r.

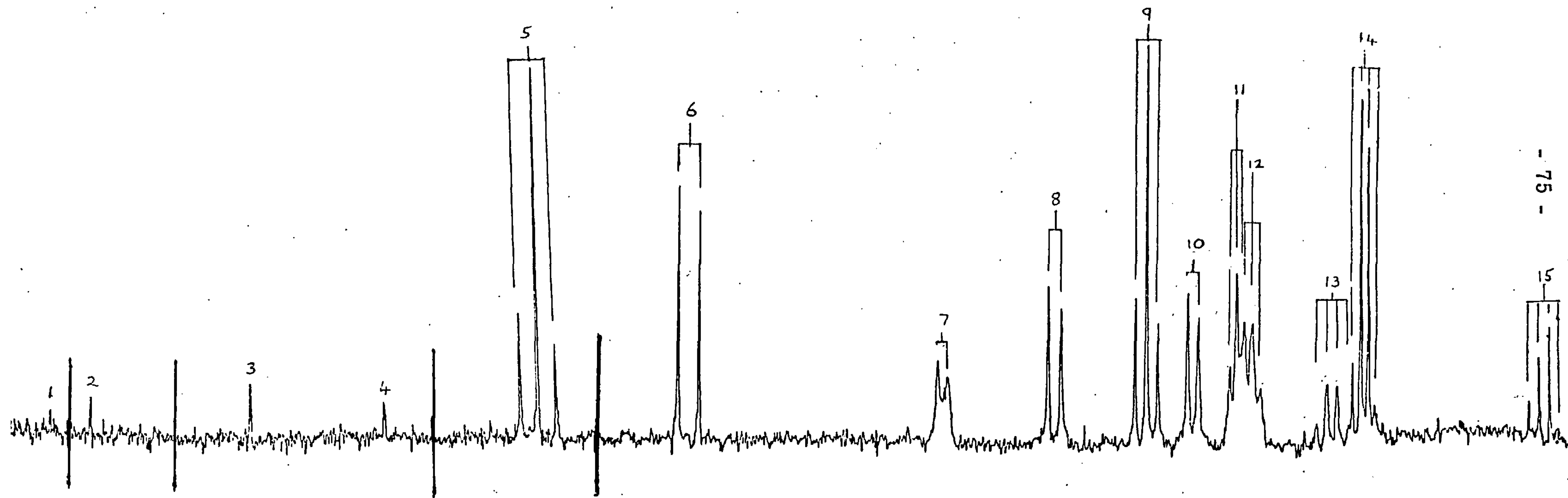


Fig. 5. ^{13}C. m. r. spectrum of the sesquiterpene keto-alcohol (25). Off resonance decoupled.

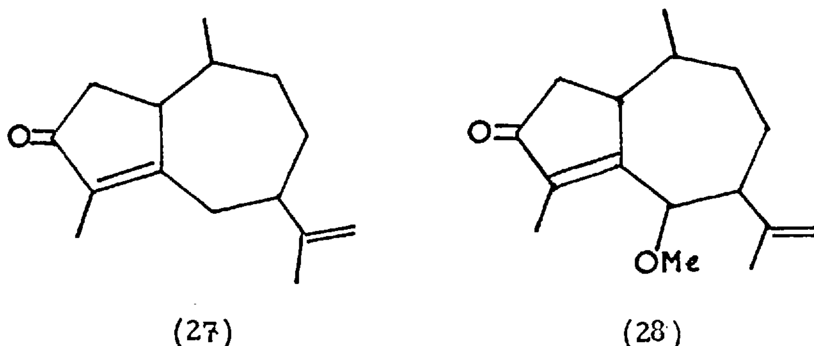
spectroscopy . (Table 9)

Examination of this structure (25) by means of Dreiding models, showed that in the majority of the possible stereoisomers there was considerable steric crowding around the hydroxyl function. This was particularly marked in those structures in which the hydroxyl group was in the cis orientation relative to the C-7 side chain. The proposed structure (25) thus provided a rationalisation for the unusual site of interaction of the keto-alcohol with Eu $(fod)_3$, the steric hindrance (around the hydroxyl oxygen atom) being too great to allow the necessary close approach of the relatively large europium complex.

Peak No.	Chem. Shift (ppm from TMS)	Off Resonance Splitting Pattern
1	180.48	s
2	176.76	s
3	147.00	s
4	134.78	s
5	111.61	t
6	70.80	d
7	51.95	d
8	43.76	d
9	36.95	t
10	33.52	d
11	30.18	t
12	29.05	t
13	23.19	q
14	20.87	q
15	7.79	q

Table 9 ^{13}C.m.r. chemical shifts of the sesquiterpene keto-alcohol (25)

The proposed γ - hydroxy ketone part structure of the keto-alcohol was further confirmed by zinc/acetic acid reduction of this group. The major product of this reaction, apart from the keto-acetate (26), was consistent in all respects with the expected reduction product (27) [21].



(27) (28)

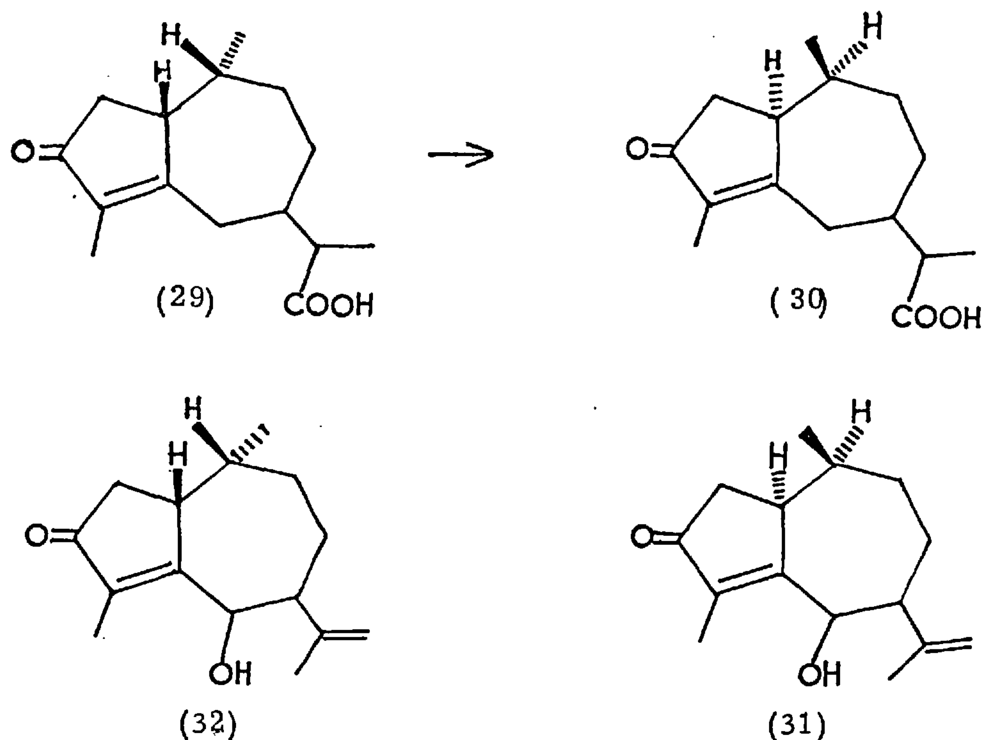
Treatment of the keto-alcohol (25) with pyridine produced no detectable decomposition. However, reaction with toluene-p-sulphonic acid in refluxing methylene chloride resulted in a complex mixture consisting of at least seven components. The major product showed very similar spectroscopic and tlc properties to those of the starting alcohol, excepting that it lacked a hydroxyl function. Mass spectroscopy showed the molecular ion at m/e 248, corresponding to a molecular formula of $C_{16} H_{24} 0_2$, and a fairly intense peak at m/e 216, corresponding to the loss of the elements of methanol from the molecular ion. This evidence suggested that the compound was the methyl ether (28) of the natural alcohol probably formed by reaction with traces of methanol present in the commercial methylene chloride.

Repetition of this reaction with toluene-p-sulphonic acid, using freshly distilled methylene chloride, failed to produce any trace of the methyl ether (28). Preparative tlc of this second reaction mixture allowed isolation of two relatively major products. The i. r., u. v. and mass spectra of the more polar of these, proved to be virtually superimposable on those of the keto-alcohol (25), with which it was isomeric. The two n. m. r. spectra were also very similar, the only observable difference being that the C10-methyl doublet resonance occurred 0.4 ppm

to higher field in the reaction product, than in the natural alcohol.

Buchi, Kauffman and Loewenthal [22] had previously reported a similar shift to higher field on epimerisation of (29) to (30). These workers considered that this unusual shift was the result of a long range diamagnetic shielding of the C-10 methyl group, by the cyclopentenone double bond. Conformational studies had shown that this could only occur in the epimer (30).

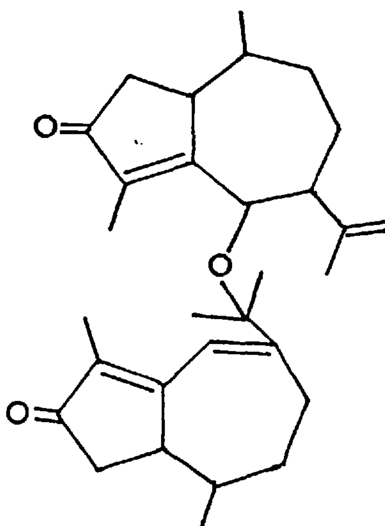
By analogy with this example, the structure of the present toluene-p-sulphonic acid reaction product therefore seemed to be that in which the C-10 methyl group was in the β- orientation (31). Both this compound and the starting alcohol thus contained a cis arrangement of the C-1 and C-10 protons, these being in the β - configuration in the latter (32).



The second, less polar product from the reaction with toluene-p-sulphonic acid, had a molecular weight, by mass spectroscopy, of 450. The high field region (τ 7 - 9.5) of the n.m.r. spectrum of this compound,

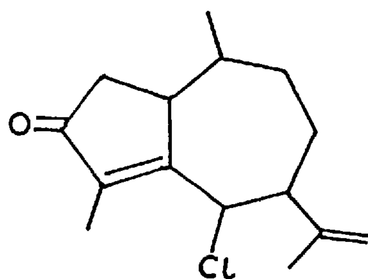
resembled two superimposed keto-alcohol (32) spectra, in which the whole of one spectrum had been shifted by about 2 Hz relative to the other. The n.m.r. spectrum also contained a very broad, four proton resonance centred at τ 5.2. The u.v. spectrum of this compound showed λ_{max}^{EtOH} 243.5, 294 nm consistent with $\alpha, \beta, \gamma, \delta$ and α, β - unsaturated ketones.

Consideration of this evidence led to the proposal that this reaction product was a di-ether of the keto-alcohol (32), having the structure (34).



(34)

Attempted dehydration of the keto-alcohol (32), by treatment with phosphorus oxychloride in pyridine, gave one major product and as expected, the i.r. spectrum of this compound showed no hydroxyl absorption band. Mass spectroscopy, however, demonstrated the presence of chlorine in this molecule and consideration of this and other spectral data indicated that the chlorine had simply displaced the hydroxyl function to give a chloro-compound of structure (35).



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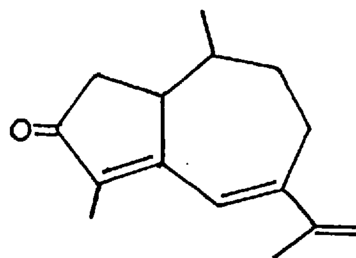


(36)

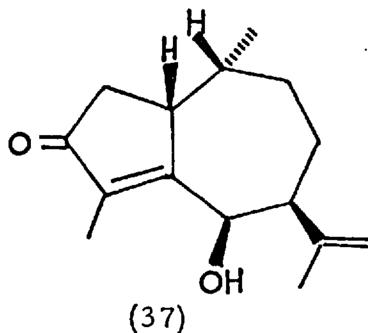
Mass spectroscopy indicated that the molecular weight of a second, minor component isolated from the reaction with phosphorus oxychloride, was the same as that of the expected dehydration product (36). However this second compound had u.v. λ_{max}^{EtOH} 237 and 303 nm while the absorption, calculated from tables,[23] for the conjugated triene-one (36) was, λ max 323 nm. The isolated compound thus appeared to be some double bond isomer of this triene (36).

ORD data for a number of guaiane type compounds with cyclopentenone systems similar to that of the keto-alcohol (32), has been obtained by Piers and Cheng[24]. These authors considered that, since the cyclopentenone ring in compounds of this type was essentially planar, the sign of the Cotton effect was largely determined by the configuration about C-1. A negative Cotton effect, and thus a negative CD, was therefore considered to be diagnostic for compounds with the C-1 proton β-oriented and the C-10 methyl group in the α- configuration.

Application of these results to the keto-alcohol, the CD spectrum of which showed negative absorptions at 240 and 304 nm, confirmed that the stereochemistry shown in (32) was, in fact, correct.

That the C-7 side chain of the keto-alcohol was <u>cis</u>-oriented with respect to the C-6 hydroxyl group, had been suggested as a result of the lack of reaction of this group with Eu $(fod)_3$. This was confirmed by observation of $J_{6,7} = 1$ Hz in the Eu $(fod)_3$ shifted n.m.r. spectrum. Molecular models indicated that the bond angle of close to 90° required by this coupling constant, was only attained with a <u>cis</u>- orientation of the two protons.

The greatest majority of known guaianes have been shown to contain β - oriented C-7 side chains [25]. By analogy with these, we propose that both the C-7 side chain and the C-6 hydroxyl group, of this keto-alcohol, have the β-configuration. This novel sesquiterpene keto-alcohol isolated from the benzene soluble extract of Pleocarphus revolutus thus has the structure shown (37).



(37)

b) The Sesquiterpene Keto-Acetate (38)

Separation of the cytotoxic column fraction 3 by preparative tlc on silica gel, yielded three active sub-fractions (A2, A3, and A4, Fig. 6) Further chromatography, of the second of these active sub-fractions (A3), gave three single components (B4, B5 and B6) two of which (B4 and B5) were shown to have cytotoxic activity.

The second of these active components (B5) was isolated in quantities of less than 5 mg and no further work was carried out with this fraction. The first component (B4) however, was obtained in yields large enough to allow a complete structural elucidation.

This compound (B4) was a colourless oil with a molecular formula, from mass spectroscopy of $C_{17}H_{24}O_3$. Its i. r. spectrum showed two carbonyl absorption bands at 1740 and 1705 cm^{-1}, the former in conjunction with the strong band at 1235 cm^{-1} being indicative of an ester grouping, and exocyclic carbon-carbon double bond absorptions at 1645 and 910 cm^{-1}. U.v. spectroscopy indicated that one of the carbonyl groups was α,β unsaturated (λmax 235 nm ϵ 17,400). The n.m.r. spectrum was complex, particularly in the region

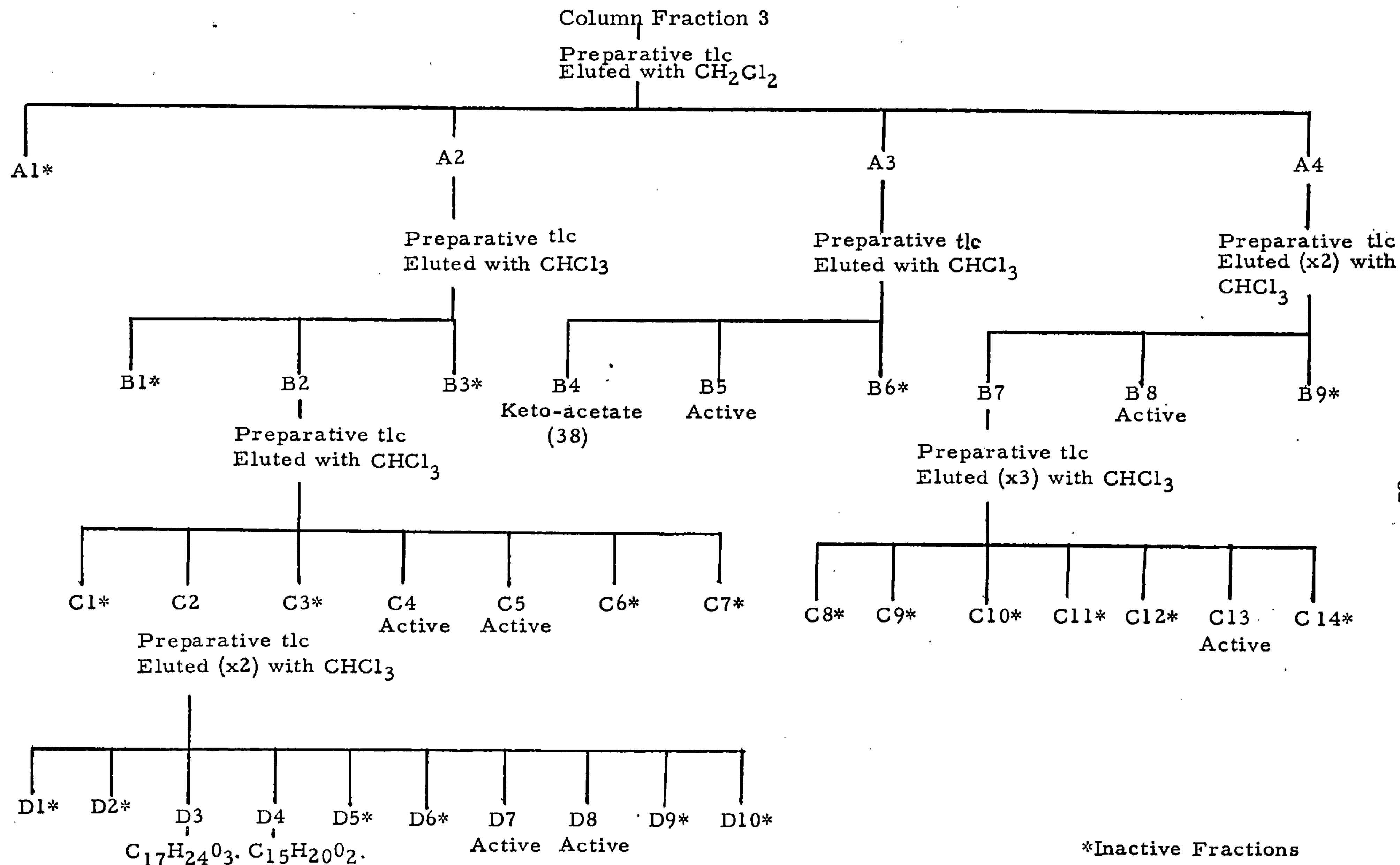
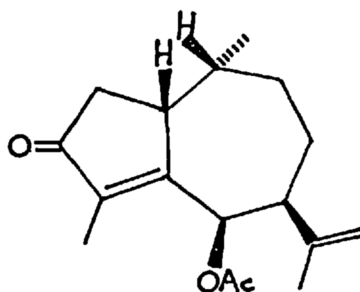


Fig. 6. Fractionation diagram of Pleocarpus revolutus benzene extract column fraction 3

τ 7.4-9.2, but included distinct resonances at τ 4.16 (IH, br s); 5.27 (2H, br m) - vinylic protons; 8.05 (3H, s)-acetate methyl group; 8.22 (3H, s) and 8.29 (3H, d, J = 2Hz) - both vinylic methyl groups; 9.05 (3H, d, J = 7Hz), a secondary methyl group.

This spectral information and also that obtained from Eu $(fod)_3$ induced shift experiments (Table 10) was consistent with this component being a sesquiterpene keto-acetate. Comparison of this data with that obtained for the sesquiterpene keto-alcohol (37) showed considerable similarities, the only significant differences being those attributable to an acetyl rather than to a hydroxyl function. That this active component was the acetate of the keto-alcohol was confirmed both by acetylation of the alcohol with acetic anhydride in pyridine, and by hydrolysis of the acetate with aqueous sodium hydroxide. The products of these reactions being identical with the corresponding natural compound.

On the basis of this correlation structure (38) was proposed for this sesquiterpene keto-acetate. Further evidence for this structure was obtained from zinc-acetic acid reduction of the acetate, the reaction product being identical with the compound (27) previously obtained by similar treatment of the keto-alcohol. Finally, the ORD curves obtained from the keto-acetate and keto-alcohol were virtually superimposable, thus indicating that the two compounds had identical stereochemistry.



(38)

Total Eu(fod)$_3$ added (mg)	CHEMICAL SHIFT (τ) IN CCl_4																	
	C_4-Me (d, J=2Hz)	C_{10}-Me (d, J=7Hz)	C_{11}-Me (br s)	Acetate - Me (s)	H_1 (m)	H_2 (dd, J=19, 5Hz)	H_2 (dd, J=19, 2Hz)	H_6 (br s)	H_7 (m)	$H_{8,9}$ (m)				H_{10} (m)	C_{11}=CH_2 (br s)	C_{11}=CH_2 (br s)
0	8.29	9.05	8.22	8.00	7.16	7.80*	8.00*	4.16	7.85*	8.40*	8.35*	8.70*	8.75*	9.10*	5.27	5.32
5.0	7.16	8.84	8.01	7.74	6.21	-	-	3.49	-	-	-	-	-	-	5.10	5.10
9.8	6.06	8.66	7.81	7.47	5.17	4.42	4.57	2.80	-	-	-	-	-	-	4.96	4.89
15.2	4.68	8.45	7.56	7.07	3.96	2.00	2.27	1.87	6.42	7.22	7.40	-	7.92	6.47	4.76	4.61
20.4	3.84	8.36	7.42	6.80	3.18	0.82	1.03	1.29	6.06	6.97	7.18	7.40	7.73	6.21	4.67	4.46
26.2	2.62	8.21	7.19	6.18	2.00	-0.60	-0.50	0.18	5.42	6.47	6.68	7.02	7.49	-	4.49	4.16
33.9	1.59	8.10	6.95	5.34	0.82	-1.63	-1.63	-1.13	4.76	5.99	6.27	6.77	7.21	5.18	4.32	3.85
38.6	1.36	8.03	6.86	5.14	0.54	-1.91	-1.91	-1.58	4.56	5.85	6.12	6.64	7.12	5.00	4.25	3.73
44.5	0.97	7.98	6.72	4.67	-0.02	-2.47	-2.47	-2.56	4.30	5.65	5.90	-	7.04	4.84	4.19	3.56
53.8	0.52	7.83	6.46	3.95	-0.63	-2.89	-2.89	-3.73	3.86	5.32	5.55	6.20	6.82	4.34	3.95	3.25
78.8	0.00	7.60	6.07	2.21	-1.51	-3.57	-3.57	-5.73	3.26	4.82	5.02	5.96	6.54	3.62	3.79	2.81

* Initial chemical shifts estimated by extrapolation.

Table 10. Eu(Fod)$_3$ induced shifts in the n.m.r. spectrum of the sesquiterpene keto-acetate (38)

Despite extensive purification, a correct microanalysis of the keto-acetate (38) could not be obtained, the results proving consistently high in oxygen. Careful examination of the analytical samples, by multiple elution tlc, showed that these contained a previously undetected impurity, with an Rf value very similar to that of the keto-acetate. This impurity could not be detected in the crude Pleocarphus revolutus plant extracts although a further compound, with identical tlc properties, was obtained (see below). It was observed, however, that purified samples of the keto-acetate became recontaminated with this close running impurity after standing at room temperature for several days.

A further investigation showed that this impurity could consistently be produced by exposing purified samples of the keto-acetate to daylight for at least forty-eight hours. Identical samples of the keto-acetate, stored in darkness, showed no sign of any similar contaminants.

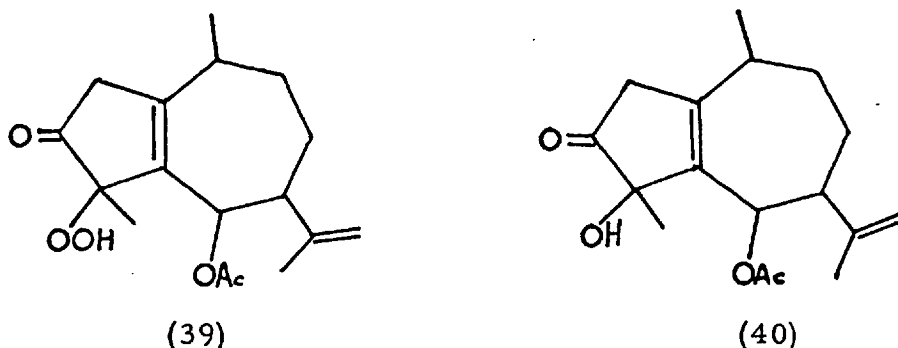
The major product formed in this daylight reaction was isolated, by multiple elution tlc, as a colourless oil. Microanalysis indicated that it was an oxidation product of the keto-acetate, having a molecular formula $C_{17}H_{24}O_5$. Its i.r. spectrum showed, a fairly sharp hydroxyl absorption band (3420 cm^{-1}), a very strong, broad, carbonyl band (1740 cm^{-1}) and carbon-carbon double bond absorptions (1665, 1645 and 960 cm^{-1}). The n.m.r. spectrum was very similar to that of the keto-acetate itself except for the presence of a broad, one proton singlet, exchangeable with D_2O, at τ 2.12.

Treatment of this new compound, either as a solution or as a spot on a tlc plate, with starch-iodide solution produced a dark colour reaction which, with the above spectral evidence, suggested that this oxidation product was a hydroperoxide.

Solvent extraction of the black spots obtained by treatment of the hydroperoxide, on a tlc plate, with starch-iodide solution, yielded a

single product. This component was also obtained by treatment of an ethereal solution of the hydroperoxide with aqueous potassium iodide. Accurate mass measurement of the molecular ion of this compound indicated a molecular formula $C_{17} H_{24} O_4$. Its i. r. spectrum showed a broad hydroxyl absorption band (3460 cm^{-1}), two carbonyl absorption bands (1745 and 1720 cm^{-1}) and carbon-carbon double bond absorptions (1645 and 900 cm^{-1}). This data confirmed that this potassium iodide reduction product was the alcohol obtained by simple reduction of the hydroperoxide.

On the basis of this evidence and also that obtained directly from the hydroperoxide, structures (39) and (40) were proposed as being consistent with the data available for the hydroperoxide and its potassium iodide reduction product, respectively.



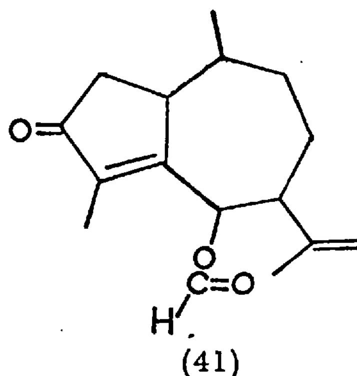
(39) (40)

Although the oxidation of the keto-acetate (38) to the hydroperoxide (39) could readily be carried out in the manner described above (i. e. exposure of the purified, oily, keto-acetate to daylight) attempts to repeat this reaction under more controlled conditions proved unfruitful.

Thus, treatment of the keto-acetate (38) with O. IN hydrochloric acid or with diisopropylethylamine produced no detectable reaction, while the only product from treatment with O. IN aqueous sodium hydroxide solution, was the keto-alcohol (37).

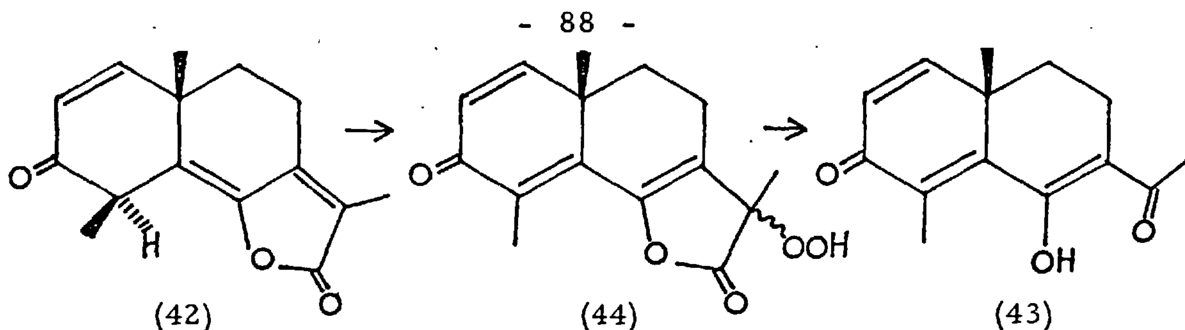
Similarly, irradiation of solutions of the keto-acetate, in benzene or dichloromethane, with a 300W tungsten lamp produced no traces of (39) or (40), even when oxygen was passed through the solution in the presence of methylene blue as sensitiser. Slight traces of the hydroperoxide (39) were obtained, by irradiation of a benzene solution of the keto-acetate with a medium pressure mercury lamp, however, this reaction also provided a considerable mixture of other products. Among these, traces of the hydroperoxide were detected by multiple elution tlc and subsequent treatment with starch-iodide solution.

No similar 'daylight' oxidation reaction was observed in the corresponding keto-formate (41), either as the pure compound or in solution. However, unlike the keto-acetate, which was an oil, the keto-formate was a crystalline solid at room temperature. Thus formation of the hydroperoxide may have been promoted by the presence of dissolved oxygen within the oil of the keto-acetate itself.



(41)

Several examples of photo-sensitised peroxidation reactions of this general type have been reported in the literature [26], although instances of such autoxidations of sesquiterpenes occurring in the absence of any photo-sensitiser are less common. McMurry and Molan, however, have reported [27] one very similar example. They observed that, on standing in ethanol solution, santonene (42) decomposed to the compound (43) via the hydroperoxide (44).



In the case of the present keto-acetate hydroperoxide (39) no trace of any decomposition product corresponding to (43) was observed.

c) Unidentified Sesquiterpenes

Extensive chromatography of column fraction 3 of the Pleocarpus revolutus benzene soluble extract, yielded a total of ten cytotoxic compounds (B4, B5, B8, C4, C5, C8, D3, D4, D7, D8 Fig. 5) of which one (B4) was the sesquiterpene keto-acetate (38), described above. Of the remaining nine fractions only two (D3 and D4) were isolated in sufficient quantities to allow any attempt at structural elucidation.

The less polar of these two compounds (D3) was a colourless oil, the mass spectrum of which showed a highest mass ion at m/e 276, consistent with a molecular formula $C_{17} H_{24} O_3$. Accurate mass measurement of the most intense peaks in this mass spectrum indicated fragment ions of composition, $C_{15} H_{19} O_2$ i.e. $(M-C_2 H_5 O)^+$, $C_{14} H_{18} O$, $C_{12} H_{15}$ and $C_{11} H_{11} O$.

The i.r. spectrum of this compound showed a broad carbonyl absorption band at 1725 cm^{-1} and a strong carbon-carbon double bond absorption at 1635 cm^{-1}. There was no absorption attributable to either a hydroxyl function or to an exocyclic methylene group. The u.v. spectrum indicated the presence of an α, β — unsaturated carbonyl group (λ max 238.5 nm), while the n.m.r. spectrum was complex but included τ 3.23 (IH, br m), 4.90 (IH, m), 6.3 (3H, br m), 8.90 (3H, s) and 8.97 (3H, s).

Insufficient material was available to allow further investigation of this cytotoxic component, the small quantities obtained decomposing slowly even on storage at 0°. From the information obtained the presence or absence of the various functionalities described above were indicated. However, the information was inadequate to allow the postulation of any complete structure for this sesquiterpenoid compound.

The second of these two active components (D4) was a colourless oil which slowly decomposed on standing, even at 0°. Microanalysis of this compound indicated a molecular formula $C_{15}H_{20}O_2$ consistent with the highest mass ion in the mass spectrum at m/e 232.

The i.r. spectrum showed a broad carbonyl absorption band at 1730 cm^{-1} and a carbon-carbon double bond absorption at 1640 cm^{-1}. As in the case of the previous, unidentified compound, absorptions attributable to hydroxyl or exocyclic methylene groups were absent. The u.v. spectrum again indicated the presence of an α, β unsaturated carbonyl group (λmax 238 nm).

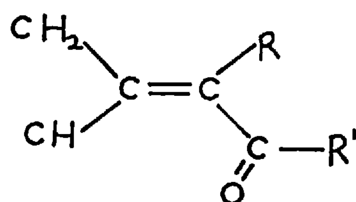
The n.m.r. spectrum of this component included resonances at τ 3.38 (1H, t, J = 3.5 Hz), 6.02 (2H, dd, J= 6, 7Hz), 7.59 (2H, dd, J = 3, 7Hz), 8.95 (3H, d, J = 7Hz), 8.97 (3H, s) and 9.00 (3H, s). The three proton doublet at τ 8.95 was obscured in the normal n.m.r. spectrum and was only observed in the Eu $(fod)_3$ shifted spectrum. The chemical shift of this doublet was obtained by extrapolation, to zero concentration of Eu $(fod)_3$, of the shifts obtained from sequential addition of the shift reagent. By means of these Eu $(fod)_3$ induced shifts (Table 11), resonances attributed to eighteen of the twenty protons of the molecule were observed.

Total Eu $(fod)_3$ added (mg)	CHEMICAL SHIFT (τ) IN CCl_4								
	3H, s	3H, s	3H, d J=7Hz	2H, dd J=14, 8Hz	1H, t J=6Hz	1H, t J=8Hz	2H, dd J=3, 7Hz	2H, dd J=6, 7Hz	1H, t J=3, 5Hz
	9.00	8.97	8.95*	8.15*	8.05*	7.90*	7.59	6.02	3.38
3.4	8.96	8.86	-	-	-	-	7.52	5.89	3.06
6.8	8.94	8.84	-	-	-	-	7.46	5.73	2.86
12.8	8.90	8.78	8.90	-	-	-	7.33	5.59	2.38
18.9	8.86	8.72	-	-	-	-	7.26	5.44	2.04
28.2	8.78	8.63	8.88	-	7.36	-	7.10	5.16	1.37
37.6	8.71	8.53	-	-	7.10	6.82	6.90	4.83	0.63
46.4	8.64	8.43	-	-	6.87	6.54	6.74	4.53	-0.02
58.1	8.53	8.30	8.74	7.65	6.44	6.19	6.53	4.15	-0.88
65.8	8.47	8.23	8.68	7.63	-	5.98	6.40	3.92	-
73.5	8.41	8.16	8.66	7.55	-	5.76	6.27	3.70	-1.82
83.2	8.34	8.06	8.60	7.45	6.01	5.63	6.11	3.41	-
95.4	8.26	7.96	8.52	7.39	5.75	5.21	5.91	3.07	-3.20

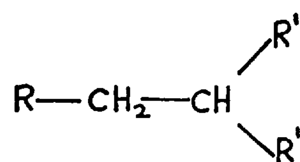
* Initial chemical shifts estimated by extrapolation.

Table 11   Eu $(fod)_3$ induced shifts in the n.m.r. spectrum of the unidentified sesquiterpene $C_{15}H_{20}O_2$

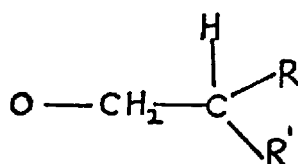
In addition to this information, the use of Eu $(fod)_3$ in the n.m.r. spectrum of this compound also allowed a number of proton decoupling experiments to be carried out. These indicated that the three single proton triplets at τ 3.38, 7.90, and 8.05 were respectively coupled to the two proton double doublets at τ 7.59, 8.15 and 6.02. Consideration of these results in conjunction with the relevant chemical shifts appeared to indicate the three part structures (45), (46) and (47).



(45)



(46)



(47)

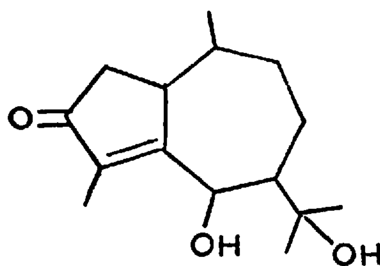
The spectral data obtained for this compound indicated certain similarities both with the previous unidentified compound (D3) and also with the sesquiterpene keto-alcohol (37) and keto-acetate (38). In particular, the three proton doublet at τ 8.95 in the n.m.r. spectrum was characteristic of the C-10 methyl group of the guaiane structural system. However, despite these similarities the evidence was insufficient to allow any definite structure to be proposed for this compound.

d) The Sesquiterpene Keto-Alcohol (56)

Chromatography of column fraction 6 from the Pleocarphus revolutus benzene soluble extract yielded samples of both the keto- alcohol (37) and also of a previously unisolated sesquiterpene component. This component was a pale yellow oil with an Rf value, in chloroform, of slightly less than that of the keto-alcohol (37). Its mass spectrum showed a highest mass ion at m/e 234, corresponding to a molecular formula $C_{15} H_{22} O_2$, microanalysis, however, indicated that the correct molecular formula was $C_{15} H_{24} O_3$. It thus appeared that, during mass spectroscopy, this compound was rapidly losing one molecule of water from the molecular ion, in an analogous manner to the sesquiterpene-diol (19). The loss of a further fragment of 18 mass units, from the ion at m/e 234, suggested that this new compound was also a diol.

The presence of at least one hydroxyl function in this component was confirmed by i. r. spectroscopy (absorption band at 3440 cm^{-1}). This also showed a carbonyl absorption band (1710 cm^{-1}) and a carbon-carbon double bond absorption (1670 cm^{-1}), while the u. v. spectrum indicated that the carbonyl function was α,β- unsaturated (λmax 241 nm).

The above spectral information indicated that this compound was structurally similar to the keto-alcohol (37), the only apparent major differences being the existence of a second alcohol function, and the lack of an exocyclic methylene group. On this basis structure (48) was initially proposed for the new diol.



(48)

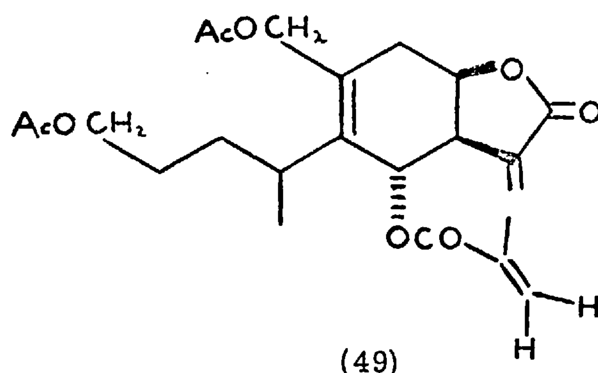
This tentative structure, however, was not consistent with the n.m.r. data. This was complex, particularly in the region τ 7.4 - 9.4 but the spectrum included τ 6.2 (3H, br m), 7.83 (3H, s), 8.28 (3H, s), 9.15 (3H, s), 9.38 (3H, d, J = 6.5 H_z). Although inconsistent with structure (48), this n.m.r. data indicated two definite similarities with the guaiane type skeleton of the keto-alcohol (37 and the keto-acetate (38). These were, the three proton singlet at τ 8.28 very similar to the signal assigned to the C-4 methyl group in the latter two compounds and the three proton doublet at τ 9.38, characteristic of the C-10 methyl group in this structural system. However, the chemical shifts of the two remaining three proton singlets were not consistent with the C-11 methyl groups in structure (48) and the resonance at around τ 5.2, predicted for the C-6, α - hydroxy, proton, was not present.

Treatment of this supposed keto-diol with phosphorus oxychloride in pyridine yielded a complex reaction mixture. Mass spectroscopy of the major reaction product indicated that it had a molecular formula, $C_{15}H_{21}OCl$ consistent with the loss of one of the hydroxyl functions of the keto-diol by dehydration and of the other by displacement with chlorine. This reaction product was thus isomeric with the chloro compound (35) obtained by similar treatment of the keto-alcohol (37). Close examination of the mass spectra of these two compounds however, revealed distinct differences in the fragmentation patterns which thus indicated that the two compounds were not identical.

One of the minor components of the reaction of the diol with phosphorus oxychloride was found to have identical tlc properties, including a colour reaction with p-anisaldehyde : methanol : concentrated sulphuric acid, 1 : 1 : 1 with compound (31). This was an isomer of the keto-alcohol (37) obtained by reaction of the keto-alcohol with p-toluenesulphonic acid. This component was not obtained in sufficient quantities to allow further comparison of the two compounds.

Acetylation of the keto-diol, with acetic anhydride in pyridine, yielded a single major component with molecular formula, by microanalysis, $C_{17} H_{24} 0_3$. The molecular formula of the parent diol thus required that this reaction had involved acetylation of one of the hydroxyl functions and loss of the other by dehydration.

The i. r. spectrum of this mono acetate showed no bands typical of hydroxyl functions but showed two carbonyl absorption bands (1748 and 1710 cm^{-1}) and a carbon-carbon double bond absorption (1670 cm^{-1}). The u. v. spectrum confirmed that the α,β unsaturated carbonyl function was unchanged (λmax 239.5, ε 9675). N. m. r. spectroscopy of this acetate showed absorptions at τ 8.29 (3H, s) and 9.38 (3H, d, J = 6Hz), respectively characteristic of the C-4 and C-10 methyl groups of the keto-alcohol (37) type of structure. The n. m. r. spectrum also included τ 5.7 (2H, ABq, J = 11 Hz), 7.85 (3H, s), 7.98 (3H, s), 8.29 (3H, s) and 9.18 (3H, s). The AB quartet at τ 5.7 indicated the presence of an isolated $C\underline{H}_2$ OAc group, this chemical shift and splitting pattern being consistent with that reported [28] for a similar group in the seco-eudesmanolide, eriolanin-diacetate (49).



(49)

Treatment of this acetate with zinc dust in acetic acid for thirty hours failed to provide any reaction. This indicated that the acetyl function was not in the α or γ position, relative to the α,β unsaturated carbonyl group, acetyl groups in these positions being subject to reduction under such conditions [21].

Attempts to prove, by means of dehydrogenation, that the parent keto-diol had an azulenic structure, were unsuccessful. Despite heating the compound over palladised charcoal in refluxing ethylene glycol (198°,) for six hours, no reaction was observed.

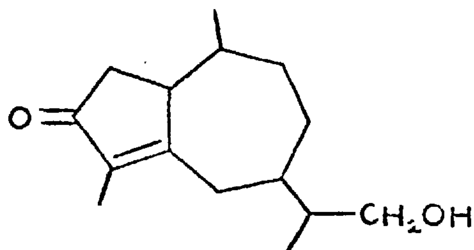
As a result of this lack of reaction and the above, unusual mono dehydration/mono substitution and mono dehydration/mono acetylation reactions of this supposed keto-diol, the evidence for its molecular formula being $C_{15}\ H_{24}\ O_3$ was re-examined.

In the case of the sesquiterpene diol (19), rapid loss of one molecule of water from the molecular ion prevented observation of this ion in the mass spectrum. However, the resultant $(M-18)^+$ ion was not the highest peak in the spectrum, there being a low intensity ion, consistent with the prior loss of one methyl group from the molecular ion, at $(M-15)^+$. In the case of the present keto-diol no such $(C_{15}\ H_{24}\ O_3\ -\ 15)^+$ ion was observed. This evidence, coupled with the unusual dehydration reactions, suggested that the microanalytical result, and thus the molecular formula, for this compound were incorrect. This may have been caused by the retention of 1 mole equivalent of water within the oily diol. Thus the correct molecular formula for this compound, consistent with the mass spectral data was $C_{15}\ H_{22}\ O_2$.

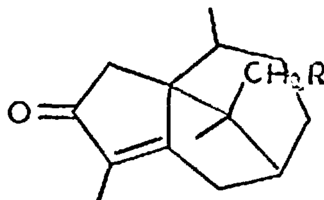
Reconsideration of the structural information for this keto-alcohol, in the light of this revised molecular formula, led to the tentative proposal of structure (50) for this compound. However, this formulation provided only four of the five double bond equivalents required by the molecular formula $C_{15}\ H_{22}\ O_2$.

As none of the spectral evidence indicated the presence of more than one carbon-carbon double bond in this compound, the remaining double bond equivalent was attributed to the existence of an additional ring system. Provision of this third ring, in the manner shown in structure (51), provided a compound which was consistent with all of the observed

data, apart from the three proton singlet at τ 7.85 in the n.m.r. spectrum.

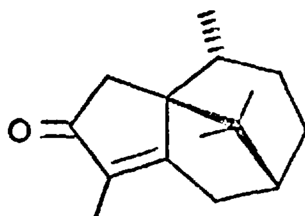


(50)



(51) R = OH

(53) R = OAc

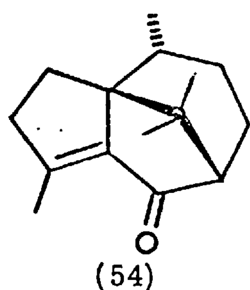


(52)

A search of the literature revealed the natural product isopatchoul - 4(5) -en-3-one (52). The published [29] data for this sesquiterpene ketone, indicated that it was structurally very similar to the present alcohol, the only apparent differences between the two being those attributable to the hydroxyl function. In particular, the n.m.r. spectrum of isopatchoulenone contained a three proton singlet at τ 7.58 very similar to that observed in the spectrum of the Pleocarphus alcohol. This had been attributed by Nigam [30] to the accidental equivalence of three, chemically non-equivalent, protons.

Isopatchoulenone further resembled the Pleocarphus revolutus alcohol in that, Hikino et al. [31] had failed to dehydrogenate this compound by heating over palladised charcoal.

As a result of the close correlation between this literature data and that obtained for the present keto-alchohol, structures (51) and (53) were proposed for the alchohol and its acetate, respectively. No direct evidence was obtained concerning the stereochemistry of these two compounds, however, the virtual superimposability of their spectra, both with those of isopatchoulenone and with those of other similar compounds e.g. patchoulenone (54) [32] and 8α - acetoxyisopatchoulenone (55) [33], suggested that the stereochemistry might be similar. In addition, Hikino et al.[31] working with isopatchoulenone and similar compounds, had suggested that the occurrence of the C-10 methyl group, at a chemical shift of approximately τ 9.40, in the n.m.r. spectrum, was diagnostic of this group being in the α position. On these grounds we tentatively propose structures (56) and (57) for this sesquiterpene keto-alcohol and its acetate.



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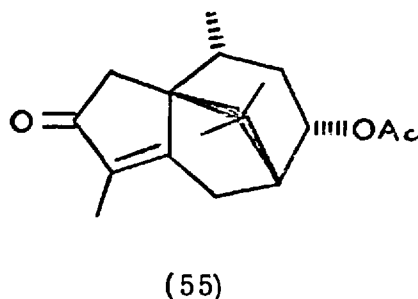

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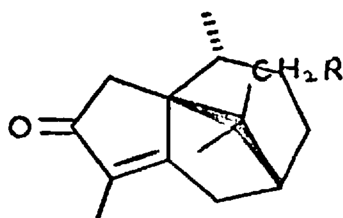
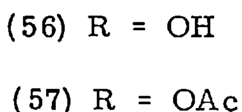



(56) R = OH

(57) R = OAc

e) The Sesquiterpene Keto-Acetate (57)

Extensive chromatography of further samples of the benzene soluble fraction of Pleocarphus revolutus indicated that the sesquiterpene keto-acetate (57), previously prepared by acetylation of the keto-alcohol (56), was present in the plant fraction. This acetate had an Rf value identical with that of the keto-acetate hydroperoxide (39) from which it was inseparable even on multiple elution tlc.

Cytotoxicity Testing

The classical phytochemical approach, adopted in the early part of this work, had not proved successful as a means of isolating the antitumour active constituents of the plants Pleocarpus revolutus and Ovidia pillo pillo. In an attempt to improve upon this a more systematic method of separation was adopted, in which each stage of the fractionation procedure was monitored by means of a simple cytotoxicity test.

A survey of the literature revealed that the majority of known antitumour agents of plant origin had been isolated by the use of such assay-guided fractionation procedures [34]. In particular, much of this reported work had been guided by assay against the in vitro KB-human epidermoid carcinoma of the nasopharynx. Testing against this cell culture system was carried out by the Cancer Chemotherapy National Service Centre, Bethesda, USA. The crude aqueous ethanolic extracts of Pleocarpus revolutus and Ovidia pillo pillo had initially been shown to have activity against this KB cell culture; however, the length of time required before the results of this assay were received (6 months), was too great for it to be of any use during the present separation procedures.

Collaboration with the Chester Beatty Research Institute, however, provided a simple, relatively rapid, in vitro cytotoxicity test, based on cells of the TLX5 mouse mammary lymphoma [35]. It was hoped that this test would provide a screen roughly equivalent to that of the KB system.

The TLX5 assay depends upon a comparison of the uptake of tritiated thymidine, leucine or uridine by normal TLX5 cells, with that of cells previously treated with the test compound (Appendix 1). In the majority of testing carried out for the present work, tritiated thymidine was chosen as this was known to provide a broader screen than either leucine or uridine [36]. The occasional use of labelled leucine or uridine, however,

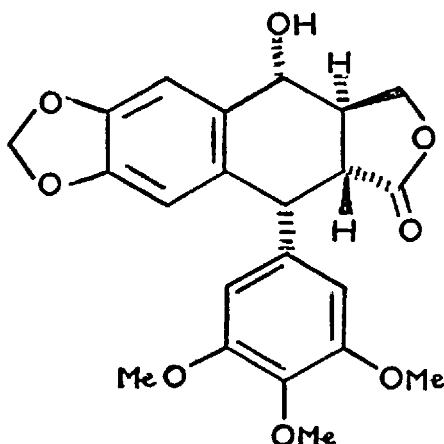
can provide further information on the effect of the test compound on protein and RNA synthesis respectively, the thymidine experiments having principally reflected the effect on DNA synthesis.

The assay results were expressed as a percentage inhibition of the uptake of the radiolabelled precursor, by the treated tumour cells, at a given concentration of the test compound after 75 mins incubation. The concentration was expressed in grammes of test compound per millilitre of cell suspension (g/ml), each ml of cell suspension containing 3×10^6 cells. Thus a 90% inhibition of thymidine uptake at a concentration of 4×10^{-5} g/ml indicated a relatively high level of activity.

In order to obtain some information as to the performance of this TLX5 system, the action on it of a number of various types of known antitumour agents were investigated:-

a) Podophyllin Resin

Podophyllin resin is the alcohol soluble, water insoluble extract of the roots and rhizomes of the wild mandrake, podophyllum. The major component of this extract is the compound podophyllotoxin (58), known to be responsible for the antitumour activity of a number of plant species [37].



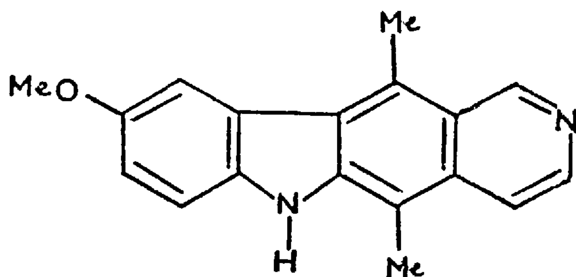
(58)

In the TLX5 system podophyllin resin showed activity at all concentrations at which it was tested. At the lowest concentration (3.5×10^{-5} g/ml) it provided an inhibition of 80% over the control.

b) 9-Methoxyellipticine (59)

This compound has been shown to be active against more than fifteen tumour systems including leukaemias and solid tumours. However, it has been found to be inactive against most ascites systems (tumours formed in the fluid of the peritoneal cavity) [37].

Against the TLX5 tumour, a type of ascites system, 9-methoxyellipticine showed a high level of activity, with a greater than 90% inhibition of thymidine uptake even at a concentration of 3.75×10^{-5}g/ml.

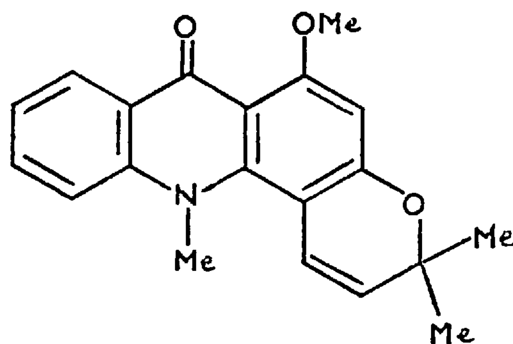


(59)

c) Acronycine (60)

This acridone alkaloid isolated from Acronychia baueri [38] has been reported to have in vivo antitumour activity. It has a wide spectrum of action having shown activity against thirteen of the nineteen systems in which it has been tested. However, it has failed to show activity in four ascites systems. [37]

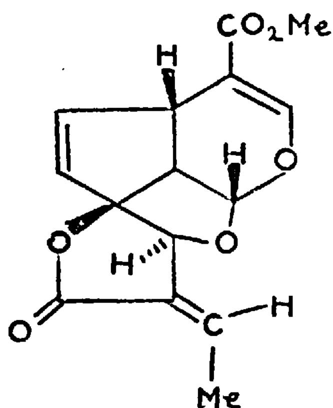
At a concentration of 3.9×10^{-5}g/ml acronycine showed moderate activity against the TLX5 tumour, inhibiting the thymidine uptake by 60%. At higher concentrations the activity increased, a greater than 90% inhibition being obtained. with a concentration of 11.5×10^{-5}g/ml.



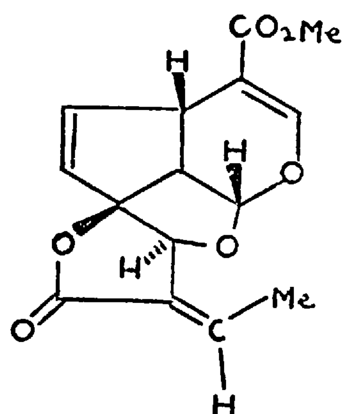
(60)

d) Isoplumericin (61) /Plumericin (62)

A 7/3 mixture of isoplumericin and plumericin produced a 90% inhibition of thymidine uptake by the TLX5 tumour cells, even at a concentration of 4.4×10^{-5}g/ml. This mixture has previously been shown [39] to have antifungal as well as cytotoxic properties, however it has failed to shown any in vivo antitumour effect.



(61)

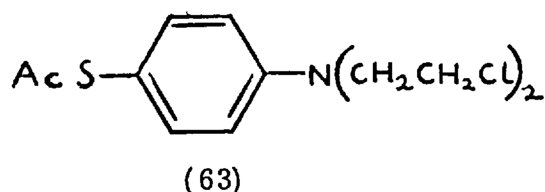


(62)

e) Nitrogen Mustards

The three nitrogen mustards (63), (64) and (65) have been shown to have chemotherapeutic indices, against the Walker 256 rat carcinoma, of 0,17 and 70 respectively[40], compounds (64) and (65) having respective LD_{50} values of 56 and 140 mg/kg in the rat.

These three compounds all showed some activity against the TLX5 system (Table 12) however the relative activities were in a similar order to that observed in the Walker 256 tumour with (64) $\gg$ (65) $>$ (63). Although compound (63) had a very low TLX5 activity at the lowest dose level employed, testing of these three compounds in the TLX5 system, without foreknowledge of the Walker 256 results, would have led to them all being classed as active.



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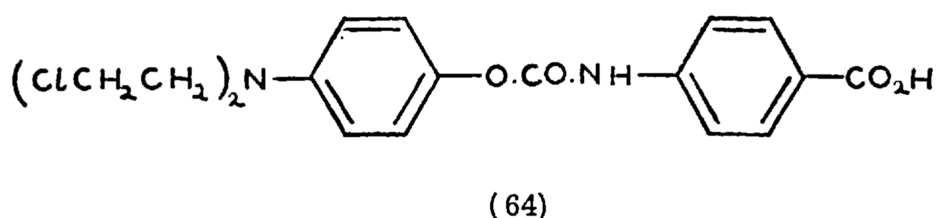
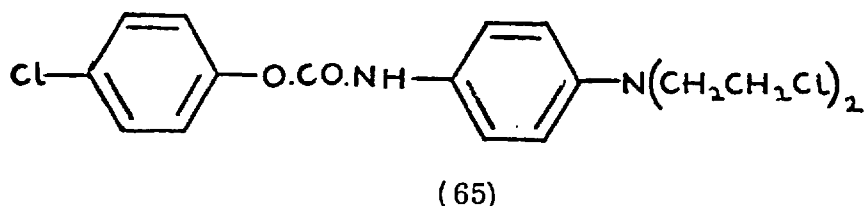


(64)



(65)

f) Erioflorin Methacrylate (66)

Erioflorin methacrylate, a sesquiterpene lactone isolated from the Chilean plant Podanthus ovatifolius [41], was of particular interest as it had been shown to have activity against the P388 mouse leukaemia but not against the KB cell culture.

Against the TLX5 tumour system this compound showed a very high level of cytotoxicity, inhibiting the uptake of thymidine and uridine by 90% and of leucine by 45%, all at a concentration of 2.8×10^{-5} g/ml. That this apparent broad spectrum of activity was due to general toxicity, rather than to any specific antitumour action, was confirmed by testing against the *in vivo* Walker 256 rat carcinoma. Against this system erioflorin methacrylate failed to show any activity. [36]

Compound	Dose mg/ml	Inhibition of Thymidine uptake. %
(63)	9.3×10^{-6}	10
	4.6×10^{-5}	53
	9.3×10^{-5}	85
(64)	9.3×10^{-6}	68
	4.6×10^{-5}	96
	9.3×10^{-5}	97
(65)	9.3×10^{-6}	45
	4.6×10^{-5}	87
	9.3×10^{-5}	95

Table 12 Inhibition of Thymidine uptake by Nitrogen Mustards (63), (64) and (65)

g) Miscellaneous Compounds

A second sesquiterpene lactone of the germacranolide group was examined in the TLX5 system. This compound, isolated from a plant of the Elephantopus spp[42], showed a greater than 90% inhibition of thymidine uptake even at a concentration of 3.4×10^{-5} g/ml.

A lower level of activity was shown by a lignan from a plant of the Bursera spp., previously reported to have activity against the 9KB-adenocarcinoma of the nasopharynx, cell culture[43]. At a concentration of 3.9×10^{-5} g/ml this compound inhibited the thymidine uptake of the TLX5 cells by 30%. This increased to 70% inhibition at 11.7×10^{-5} g/ml and to greater than 90% at 21.9×10^{-5} g/ml.

These testing results confirmed that, although the TLX5 assay did not provide a specific test for in vivo active compounds, it did provide a very broad screening method suitable for use as an aid to the isolation of antitumour agents from Pleocarpus revolutus.

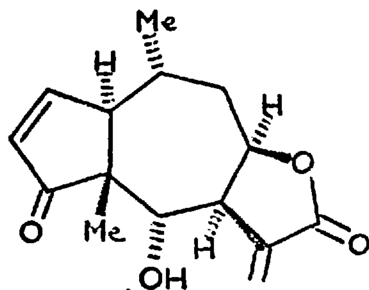
The crude Pleocarpus revolutus benzene soluble extract showed very weak activity against the TLX5 assay. However, after initial separation of this extract, by means of column chromatography, a number of moderately active fractions were obtained. In particular, column fractions 3 and 5 showed considerable activity. As described above, further separation of fraction 5 eventually led to the total loss of activity while the further assay guided separation of fraction 3 is illustrated in Fig. 6.

The assay work can best be described by division into four sections
a) The sesquiterpene keto-alcohol (37) and related compounds,
b) 'Inactive compounds', c) Unidentified active compounds, and
e) the flavones from Ovidia pillo pillo.

a) The Sesquiterpene Keto-Alcohol (37) and related compounds.

Initial testing of the sesquiterpene keto-alcohol (37) indicated that it was inactive at concentrations of up to 1.95×10^{-4}g/ml. The sesquiterpene keto-acetate (38) however, showed moderate activity, providing a 60% inhibition of thymidine uptake at a concentration of 1.2×10^{-4}g/ml. Variation of the C-6 ester group, from alcohol to acetate, had thus produced a considerable change in cytotoxicity.

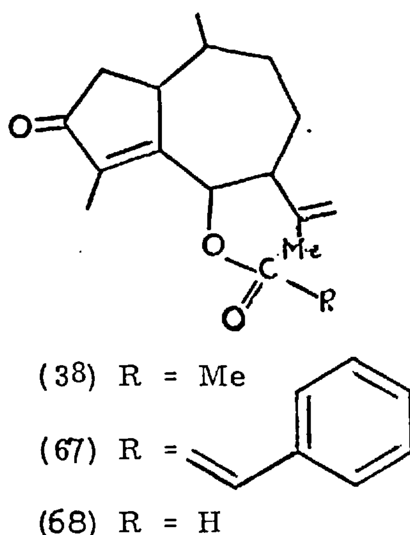
An effect similar to this had previously been reported by Lee et al.[44] working with the sesquiterpene lactone helenalin (66). In this work the cytotoxicity of a large number of C-6 esters of helenalin had been examined and had been found to vary over a wide range. The most active helenalin ester examined by Lee and co-workers, was the cinnamate and it was thus of interest to prepare, and test, the cinnamate of the sesquiterpene keto-alcohol (37).



(66)

This preparation was carried out by stirring the keto-alcohol with cinnamoyl chloride in pyridine. As predicted from Lee's results, the sesquiterpene keto-cinnamate (67) produced showed an increased

activity over the corresponding keto-acetate (38),producing an inhibition of thymidine uptake of greater than 90% at a concentration of 9.3 x 10^{-5}g/ml. At a similar concentration the keto-acetate had provided a 25% inhibition of thymidine uptake.



(38) R = Me

(67) R = [styryl]

(68) R = H

Close examination of the structures of the keto-acetate (38) and of the keto-cinnamate (67), showed that these compounds both contained a part structure which resembled a ring opened α,β-unsaturated-γ-lactone.

As α,β- unsaturated-γ-lactones had frequently been associated with high cytotoxicity, it was felt that this resemblance might be important to the activity of this series of compounds. According to this hypothesis, the keto-formate (68) was predicted to have an activity greater than that of either the acetate or the cinammate, the steric hindrance to the attainment of a lactone type arrangement being at a minimum in this compound.

In fact, this keto-formate, prepared by treatment of the keto-alcohol (37) with formic-acetic anhydride in pyridine, proved to be less active than the cinnamate, having a level of activity very similar to that of the keto-acetate (38). Thus the proposed structure analogy with the γ-lactone system did not appear to be important to the cytotoxicity of this series of compounds.

In the case of Helenalin, Lee et al. had attributed the higher cytotoxicity of the cinnamate to the increased alkylating potential of this molecule as compared with that of the parent alcohol. This hypothesis may thus also explain the observed variation in the cytotoxicity of the esters of the sesquiterpene keto-alcohol (37).

A similar structure-activity relationship to that described above was also observed with the sesquiterpene keto-alcohol (56) and its acetate (57). This keto-alcohol was inactive at a concentration of 19.5×10^{-5}g/ml, while the acetate showed a weak, though definite activity (30% inhibition of thymidine uptake) at half this concentration. Neither, the cinnamate nor the formate of this alcohol, were investigated.

A number of cytotoxic chlorine containing natural products had previously been reported in the literature [45]. For this reason the chloro compound (35), derived from the sesquiterpene keto-alcohol (37) was tested against the TLX5 tumour where it was shown to have a very similar level of cytotoxicity to that of the keto-acetate (38).

b) 'Inactive' Compounds

As described above, both the sesquiterpene keto-alcohols (37) and (56) and the sesquiterpene diol (19) were inactive at concentrations of 19.5×10^{-5}g/ml.

At a concentration of 4.8×10^{-4}g/ml however, all three of these compounds produced a greater than 90% inhibition of thymidine uptake. The high dosage level required to provide this level of cytotoxicity almost certainly rendered these compounds useless as potential therapeutic agents. However, this could only be confirmed by means of further testing.

c) Unidentified Active Compounds

The incompletely characterised sesquiterpenes $C_{17} H_{24} O_3$ and $C_{15} H_{20} O_2$, isolated from the benzene extract of Pleocarphus revolutus, showed a cytotoxicity slightly greater than that of the sesquiterpene keto-acetate (38). These two compounds were thought to possess a guaiane type skeleton similar to that of the sesquiterpene keto-alcohol (37) and keto-acetate (38). Final elucidation of these two unknown structures may thus provide further information of the structure-activity relationships in this series of compounds.

In addition to these two active compounds, eight other fractions derived from Pleocarphus revolutus showed a greater than 60% inhibition of thymidine uptake at concentrations of approximately 9×10^{-5}g/ml. These active fractions, none of which were obtained in quantities of greater than 5 mg., included both mixtures and virtually pure compounds. On further separation these active fractions would certainly yield further natural products with a considerable level of activity.

d) The Flavones

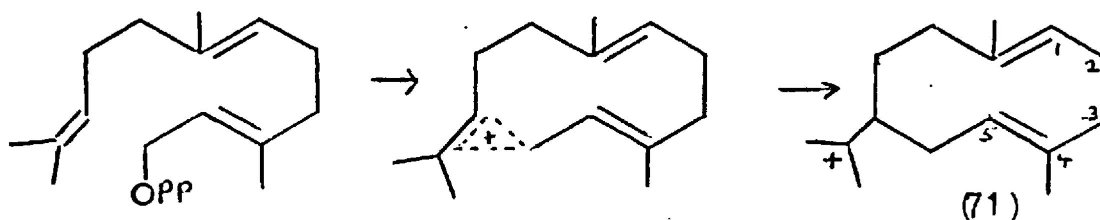
The flavones 4',7-dimethoxy - 3',5-dihydroxy flavone (22) and 4',7-dimethoxy - 5-hydroxy flavone (21) isolated from extracts of the plant Ovidia pillo pillo, were also tested in the TLX5 assay, certain flavones and flavonols having previously been shown [46] to have moderate activity against the KB cell culture. In the TLX5 system both of the Ovidia flavones showed a weak activity, producing a 30% inhibition of thymidine uptake at a concentration of 9×10^{-5}g/ml.

Biosynthesis

Examination of the structures of the five fully characterised novel sesquiterpenoids isolated from extracts of the plant Pleocarpus revolutus showed these compounds to be members of two, closely related sesquiterpene sub-groups. The sesquiterpene diol (19), keto-alcohol (37) and keto-acetate (38) possess a guaiane skeleton while the keto-alcohol (56) and keto-acetate (57) are part of the smaller groups of isopatchoulanes..

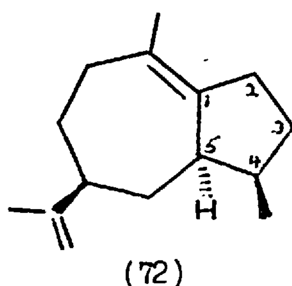
Most workers [47] agree that the biogenetic origin of the guaianes lies in 2-trans-6-trans-farnesyl pyrophosphate formed from acetate via acetyl coenzyme A, mevalonic acid, isopentenyl pyrophosphate and geranyl pyrophosphate.

Further enzymic processes catalyse the loss of the pyrophosphate group from the farnesyl pyrophosphate and the accompanying participation of the terminal double bond. This results in formation of the cation (71) in a sequence represented by Parker et al.[48] in scheme 3.

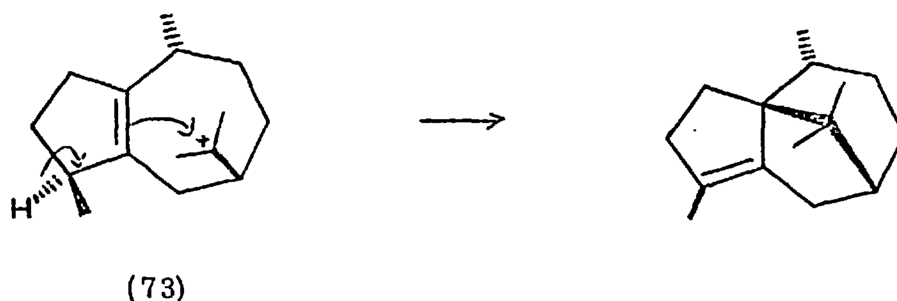


Scheme 3

An anti - Markownikoff cyclisation between carbon atoms 1 and 5 of the cation (71) then yields the basic guaine skeleton (72).

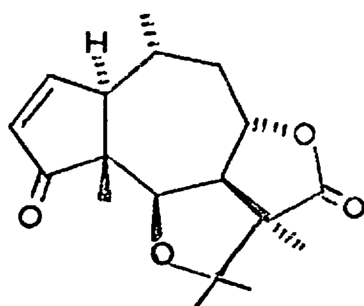


The same biosynthetic pathway is considered to be responsible for the isopatchoulanes, it being postulated [48] that these may be formed by nucleophilic attack of the double bond on the cation derivable from epiguaiol (73) - scheme 4.

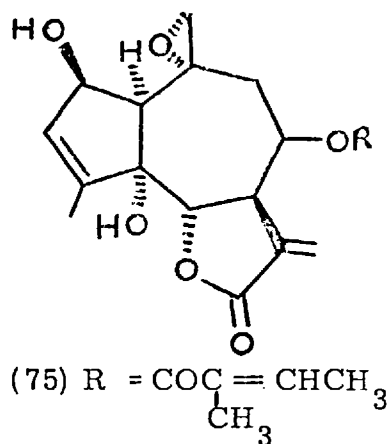


Scheme 4

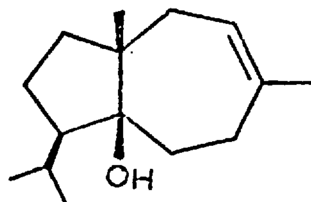
Thus the five Pleocarphus revolutus sesquiterpenes may be considered to be the products of a single biogenetic pathway. This same basic pathway, with minor modifications, is also known to be responsible for the biosynthesis of a number of other groups of sesquiterpenes including the pseudoguaianes e.g. (74) the guaiane lactones (guaianolides) e.g. (75) the carotanes e.g. (76) and the zieranes e.g. (77) [49]. A considerable number of these compounds, particularly of the pseudoguaiane and guaianolide sub-groups, have previously been isolated from plants of the Compositae species [49]. Thus, the occurrence of this particular biogenetic pathway in Pleocarphus revolutus, a member of the Compositae species, could not be considered unusual. Neither would it seem unlikely that compounds representative of one or more of these other biogenetically related sub-groups, some members of which have been shown to have antitumour activity, might also be present in this plant. Indeed, compounds of this type may well be responsible for some or all of the unexplained cytotoxicity of the Pleocarphus revolutus extracts.



(74)



(75) R = COC = CHCH$_3$
$\quad$ CH$_3$



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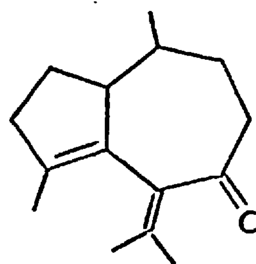


(77)

Conclusion

As a result of the work described in this thesis five novel sesquiterpenes of the guaiane and isopatchoulane types and three previously reported flavones have been isolated and fully identified. Two other sesquiterpenes have been partially characterised.

In addition a relatively simple, but broad, cytotoxicity screening procedure has been examined. By means of this, four of the isolated sesquiterpenes (including those partially characterised) and seven incompletely separated fractions were shown to possess some cytotoxic activity.

Further work, utilising this TLX5 assay guided separation procedure, would, with considerable certainty, be expected to yield further, possibly novel, cytotoxic compounds from the extracts of the Chilean plant Pleocarphus revolutus. However, the likelihood that any of these compounds might be of any therapeutic value cannot, unfortunately, be predicted with any such certainty.

EXPERIMENTAL

All melting points were determined on a Kofler hot stage apparatus and are uncorrected.

Infra-red (i. r.) spectra were recorded on either a Pye-Unicam SP200 or a Perkin-Elmer SP1000 spectrophotometer, for solutions in carbon tetrachloride unless otherwise stated.

Ultra-violet (u. v.) spectra were recorded on a Pye-Unicam SP800 spectrophotometer.

N.m.r. spectra were obtained, apart from certain stated exceptions, for solutions in deuteriochloroform ($CDCl_3$), with tetramethylsilane (TMS) as internal reference, using either a Varian T60 or a Varian HA100 spectrometer. The following abbreviations are used in connection with the n.m.r. spectra:

s = singlet
d = doublet
dd = double doublet
t = triplet
q = quartet
m = multiplet
br = broadened

Mass spectra were recorded on either an AEI MS9 or a Perkin-Elmer PE270 mass spectrometer.

Radioactivity counting was carried out using a Nuclear Enterprises NE 8310 scintillation counter.

Unless otherwise stated thin layer chromatography (tlc) was carried out on glass plates coated with kieselgel GF 254 (ex Merck) and activated for one hour at 110°.

All organic solvent extracts were dried over anhydrous sodium sulphate before evaporation.

All yields of natural products were calculated as percentages of the dried plant material.

Extraction of Pleocarphus revolutus

The powdered, dried leaves and stems of Pleocarphus revolutus (5 kg) were extracted to completion by percolation with EtOH - H_2O (1 : 1). The solvent was removed in vacuo , the residue (550 g) macerated with water and the small amount of precipitate obtained discarded. The aqueous solution was then extracted succesively with petroleum-ether, C_6H_6, EtOAc, n-butanol, and methyl ethyl ketone. On evaporation these extracts respectively yielded approximately 36g, 60g, 35g, 28g and 23g of black tarry material.

Column chromatography of the Pleocarphus revolutus benzene soluble, extract (5 g) on silica gel (m.f.c.) (200 g) yielded ten fractions.

The sesquiterpene diol (19) (Rf 0.53, EtOAc)

On standing, column fraction 5 (1.2g) slowly crystallised to give white crystals of the sesquiterpene diol (19) (238 mg 0.057%) m.p. 133-5° (purified by sublimation under reduced pressure at 45-50°). λ_{max}^{EtOH} 203 nm, ν max (nujol) 3350, 2950, 2875, 1640, 1470, 1380, 1190, 1135, 1080, 950, 895 cm^{-1}; τ 5.28 (1H, br s), 5.36 (1H, br s) (both C-14 protons), 8.88 (3H, s), 8.92 (3H, s), 8.96 (3H, s). m/e 223 (M^+- methyl weak), 220 (M^+- water), 202 (M^+ - 2 molecules water), 59 (base peak). $[\alpha]_D^{24}$ - 4.77^{o} (c 0.41, $CHCl_3$). (Found C, 75.50; H, 11.04; $C_{15}H_{26}O_2$ requires C, 75.63; H, 10.92%).

Further samples of this compound were obtained from preparative tlc of other column fractions, a total of 542 mg (0.13%) being obtained. It was also obtained, by preparative tlc, from the crude petroleum-ether soluble fraction; 143 mg (0.12%) being obtained from 1g of this crude extract.

Hydrogenation of the sesquiterpene diol (19)

Adams catalyst (Pt O_2) (10 mg) was added to a solution of the sesquiterpene diol (50 mg) in ethanol (10 ml) and the solution obtained was shaken under H_2 for $2\frac{1}{2}$ hours. Filtration through celite and evaporation of the solvent yielded white crystals (48.2 mg, 97%) of the reduction product m.p. 156-7° (hexane - EtOAc, 3 : 1).νmax (nujol) 3350, 2950, 2880, 1470, 1385, 1325, 1255, 1170, 1145, 1085, 1075, 940 cm^{-1} τ 8.83 (6H, s), 8.87 (3H, s). m/e 225 (M $^+$- methyl, weak), 222 (M $^+$- water), 204 (M $^+$- 2 molecules water). $[\alpha]_D^{25}$ - 56.52^o (c 1.08, $CHCl_3$). (Found C, 75.25, H, 11.39; $C_{15} H_{28} O_2$ requires C, 75.00; H, 11.67%).

Acetylation of the sesquiterpene diol (19)

The sesquiterpene diol (25 mg) was dissolved in pyridine (3 ml) and a few drops of acetic anhydride were added. Tlc (EtOAc) after 24 hours showed only starting material.

Dehydration of the hydrogenated sesquiterpene diol and of Cryptomeridiol (3)

Small samples (5 mg) of the hydrogenated sesquiterpene diol and of cryptomeridiol were each dissolved in pyridine (3 ml) and a few drops $POCl_3$ were added to each solution. After $1\frac{1}{2}$ hours the solutions were neutralised with HCl extracted into EtOAc and evaporated. Tlc, ($CHCl_3$) of the oils obtained showed that each consisted of three components, the Rf values of the corresponding components in the two mixtures being very similar. On spraying the developed tlc plates with conc. $H_2 SO_4$ the component from the cryptomeridiol reaction mixture gave a blue/ black colour reaction while those derived from the hydrogenated sesquiterpene diol provided a red colouration.

Dehydrogenation of the sesquiterpene diol (19)

The sesquiterpene diol (25 mg) was sealed, under vacuum, into a glass tube with 5% palladium on charcoal (25 mg). The tube was suspended in

refluxing ethylene glycol (b.p. 198°) for 4 hours. Preparative tlc (EtOAc) of the green oil obtained,yielded the blue oily s-guaiazulene (6) (8.3 mg, 40%). λ_{max}^{EtOH} 244, 284, 289.5, 304, 249, 367 nm.

Ozonolysis of the sesquiterpene diol (19)

The diol (500 mg) was dissolved in CH_2Cl_2 (50 ml) and this solution was cooled in an ice-salt freezing mixture (<-10°). The temperature was maintained at <-10° for 2 hours while a current of ozone was passed through the solution. It was then allowed to stand at room temperature for 4 hours before being evaporated to dryness. Preparative tlc (EtOAc, 4 elutions) yielded the ketone (9) (246.3 mg, 49%) as a white crystalline hemi-hydrate m.p. 96-100° (hexane-EtOAC 3:1)or m.p. 106-8° (after heating under vacuum at 70° for 4 hours). ν max (nujol) 3460, 2930, 2870, 1695, 1465, 1405, 1390, 1370, 1315, 1285, 1245, 1225, 1200, 1180, 1150, 1125, 1100, 1075, 1045, 960, 925, 880, 850 cm^{-1}. m/e 240.1722. $C_{14}H_{24}O_3$ requires 240.1725, 222, (M^+- water), 204 (M^+- 2 molecules water),59 (base peak).(Found C, 67.49; H, 10.05; $C_{14}H_{24}O_3 + \frac{1}{2} H_2O$ requires C,67.47; H, 10.04%).

Acid treatment of the sesquiterpene diol (19)

The sesquiterpene diol (50 mg) and toluene-p-sulphonic acid (20 mg) were heated to reflux in dry benzene (50 ml), under a current of dry nitrogen. Work up after 1 hour, by washing with aqueous sodium bicarbonate solution and with water, yielded a dark brown viscous oil (42 mg).

Dehydration of the sesquiterpene diol (19)

A few drops $POCl_3$ were added to a solution of the diol (65 mg) in pyridine (5 ml). After stirring for 1 hour at room temperature this solution was neutralised with HCl, extracted with CH_2Cl_2 and evaporated to yield a yellow oil. Preparative tlc ($CHCl_3$) of this

gave the double dehydration product (10) (45 mg,81%). νmax 3080, 2950, 2880, 1640, 1455, 1385, 1330, 1275, 1180, 1030, 940, 905 cm^{-1}. τ 4.76 (1H, br s), 5.34 (4H, br m), 8.30 (6H, br s). m/e 202 (M^+).

Dehydration of the nor-sesquiterpene keto-diol (9)

The keto-diol (9) (50 mg) was dissolved in pyridine (5 ml) and a few drops of $POCl_3$ were added. The solution was stirred for 1 hour before being neutralised with HCl and extracted with CH_2Cl_2. Preparative tlc($CHCl_3$) yielded the ketone (18) (22.1 mg, 52%). ν max 3070, 3040, 2970, 2930, 2860, 1710, 1645, 1455, 1445, 1405, 1380, 1360, 1275, 1185, 1160, 1120, 895 cm^{-1}. τ4.60 (1H,m), 5.23 (2H, m), 8.32 (6H, br s). m/e 204 (M^+).

Hydrogenation of the nor-sesquiterpene ketone (18)

The ketone (18) (22 mg) and Adams catalyst (25 mg) in EtOH (10 ml) were shaken under hydrogen for $2\frac{1}{2}$ hours. Filtration and evaporation gave a pale yellow oil which, on preparative tlc ($CHCl_3$ - Petroleum ether 2 : 1, 3 elutions), gave two compounds.

a) (9.3 mg) ν max 3620, 2960, 2930, 2870, 1730, 1465, 1385, 1380, 1370, 1285, 1130, 1095, 1070, 1040 cm^{-1}.

b) (5.7 mg) ν max 3620, 2960, 2930, 2870, 1730, 1465, 1385, 1380 1370, 1290, 1125, 1075, 1020 cm^{-1}.

The nor-sesquiterpene keto-diol(9) (Rf 0.28, EtOAc).

Preparative tlc (EtOAc, 2 elutions) of fraction 10 from column chromatography of the benzene soluble extract of Pleocarphus revolutus yielded white crystals (72 mg 0.017%) identical in all respects with the nor-sesquiterpene keto-diol (9).

α-Amyrin acetate

Fraction 2 (0.71g) from column chromatography of the benzene soluble extract of Pleocarphus revolutus crystallised to an oily white solid. (73.2 mg, 0.018%). Recrystallisation (MeOH) yielded white crystals of α-amyrin acetate m.p. 224-6° (lit[8] 224°). ν max (nujol) 3450, 2950, 1730, 1470, 1385, 1255, 1040, 995 cm^{-1} m/e 468 (M^+).

Crystallisation of fractions from the Pleocarphus revolutus petroleum-ether soluble fraction yielded further quantities (17.1 mg 0.012%) of α-amyrin acetate.

α-Amyrin

Crystallisation (EtOH) of fraction 4 (0.37g) from column chromatography of the benzene soluble extract of Pleocarphus revolutus yielded α-amyrin (242 mg, 0.058%) m.p. 181-3° (lit.[8] 186°).

The polyalkane

Preparative tlc of the residue from column fraction 4 of the benzene soluble extract of Pleocarphus revolutus, after removal of the α-amyrin by crystallisation, yielded a second white, crystalline solid m.p. 76-8° (C_6H_{12} - EtOAc, 2:1). ν max (nujol) 1705, 1305, 735, 725 cm^{-1}. τ 7.65 (weak m), 8.70 (strong s), 9.12 (weak m).

7-Methoxy - 4',5,6 - trihydroxy flavone (20) Rf 0.66, $CHCl_3$ + 5% MeOH)

On standing a green/yellow solid precipated from column fraction 7 (0.12g) of the Pleocarphus revolutus benzene soluble extract. Purification by tlc ($CHCl_3$ + 5% MeOH) yielded yellow crystals (41 mg, 0.0098%) of the flavone (20) m.p. 272-4°. ν max (nujol) 3400 (br) 2950, 2880, 1710, 1655, 1610, 1580, 1470, 1385, 1285, 1255, 1170, 1100 cm^{-1} τ(d_6-DMSO) - 3.2 (1H, s, C_5-OH), -0.28 (2H, br m, -OH), 2.24 (2H, d, J = 9Hz , $H_{2'}$ and $H_{6'}$), 3.12 (2H, d, J = 9Hz $H_{3'}$ and $H_{5'}$) , 3.33

(1H, s, H_8), 3, 48 (1H, s , H_3) 6.28 (3H, s, - OMe). m/e 300.0625 $C_{16}H_{12}O_6$ requires 300.0634 285 (M^+ - ethyl) 282 (M^+ - water)
λ_{max}^{MeOH} 273.5, 335.5 nm, $\lambda_{max}^{MeOH-NaOMe}$ 276, 326.5, 394.5 nm.
$\lambda_{max}^{MeOH-AlCl_3}$ 303.5, 362 nm, $\lambda_{max}^{MeOH-AlCl_3}$ 302, 358 nm .
$\lambda_{max}^{MeOH-NaOAc}$ 275, 388 nm. $\lambda_{max}^{MeOH-NaOAc/H_3BO_3}$ 272, 341 nm.

<u>Methylation of 7-methoxy- 4', 5,6 - trihydroxy flavone (20)</u>

a) A solution of the flavone (30 mg), potassium carbonate (4 mg) and methyl iodide (1 ml) in acetone (20 ml) was heated at reflux for 6 hours. Filtration and evaporation yielded a pale yellow solid (80 mg) which, on preparative tlc ($CHCl_3$) gave yellow crystals of 4', 6, 7- trimethoxy - 5 - hydroxy flavone (21.4 mg, 65%) m.p. 192-4° (MeOH). (Lit.[10] 187-8°), λ_{max}^{EtOH} 277, 330 nm. τ -2.20 (1H, s, C_5-OH), 2.18 (2H, d, J = 9Hz, H_2' and H_6'), 3.00 (2H, d, J = 9Hz H_3' and H_5'), 3.45 (1H, s), 3.50 (1H, s), 6.05 (3H, s - OMe), 6.10 (3H, s, - OMe), 6.15 (3H, s, - OMe).

A minor product of the above reaction was consistent with 4', 5, 6, 7-tetramethoxy flavone (3.2 mg, 9.4%), λ_{max}^{EtOH} 267.5, 319 nm.

b) A solution of the flavone (20) (25 mg), potassium carbonate (40 mg) and methyl iodide (1 ml) in acetone (20 ml) was heated at reflux for 24 hours. Preparative tlc ($CHCl_3$ + 3% MeOH) of the solution, after filtration yielded pale yellow crystals of 4', 5, 6, 7 - tetramethoxy flavone (16.9 mg 59%) m.p. 158-9° (MeOH).(Lit[10] 161-2°). λ_{max}^{EtOH} 267, 320 nm. τ 2.11 (2H, d, J= 9Hz H_2' and H_6'), 2.97 (2H, d J= 9Hz, H_3' and H_5'), 3.17 (1H, s), 3.38 (1H, s), 6.00 (6H, s, - OMe) 6.07 (3H, s-OMe), 6.10 (3H, s, - OMe).

Formation of the trimethylsilyl ether of 7 - methoxy - 4',5,6 - trihydroxy flavone (20)

The flavone (20) (30 mg) was dissolved in pyridine (3 ml) and hexamethyldisilazane (0.5 ml) and trimethylchlorosilane (0.5 ml) were added. The solution was stirred for 16 hours and was then evaporated to dryness. The residue was dissolved in CCl_4(10 ml), filtered, and the filtrate evaporated to dryness to leave the required trimethylsilyl ether. τ(CCl_4) 2.16 (2H, d, J= 9Hz), 3.15 (2H, d J = 9Hz), 3.50 (1H, s), 3.70 (1H, s), 6.34 (3H, s - OMe); τ (C_6D_6) 3.12 (2H, d, J = 9Hz), 3.82 (2H, d, J = 9Hz), 4.03 (1H, s), 4.10 (1H, s), 6.77 (3H, s - OMe).

Further samples of the flavone (20) (124 mg, 0.0087%) were obtained by crystallisation of a fraction obtained by column chromatography of the ethyl acetate soluble fraction of Pleocarphus revolutus.

Extraction of Ovidia pillo pillo

The powdered, dried leaves of Ovidia pillo pillo (5kg) were extracted to completion by percolation with EtOH-H_2O (1 : 1). The solvent was removed under reduced pressure and the residue (600 g) was redissolved in H_2O (1l). This aqueous solution was extracted successively with petroleum-ether (3l) and benzene (3l). Evaporation of these solvents yielded the respective extracts (44g) and (67g).

Tlc ($CHCl_3$) of these extracts indicated that both contained the same three major components. Preparative tlc ($CHCl_3$) of the benzene soluble extract (5g) allowed isolation of three major components; 4',7 - dimethoxy-5-hydroxy flavone (21) (200 mg, 0.053%), 4', 7 - dimethoxy - 3',5 - dihydroxy flavone (22) (98 mg, 0.026%) and 6,7 - dimethoxy - 4',5 - dihydroxy flavone (23) (28 mg, 0.0075%).

4', 7 - Dimethoxy - 5 - hydroxy flavone (21)

This was obtained as a yellow crystalline solid m.p. 171-4° (acetone), λ_{max}^{MeOH} 268.5, 328.5 nm (ε 2.96 x 10^{4}, 3.4 x 10^{4}), $\lambda_{max}^{MeOH-NaOMe}$ 289, 346 (sh)nm, $\lambda_{max}^{MeOH-AlCl_3}$ 277, 303, 346.5, 379 nm, $\lambda_{max}^{MeOH-NaOAc}$ 278, 301.5, 340.5, 381 nm, $\lambda_{max}^{MeOH-NaOAc}$ 289, 327 nm, $\lambda_{max}^{MeOH-NaOAc/H_3BO_3}$ 268, 330 nm. ν max (nujol) 2950, 2880, 1670, 1615, 1520, 1470,1455, 1395, 1350, 1325, 1280, 1250, 1225, 1205, 1195, 1170, 1130,1105, 1050, 1030, 1020, 965, 925, 855, 850, 835 cm^{-1}. τ-2. 72 (1H, s C_5- OH), 2.22 (2H, d, J = 9Hz $H_{2'}$ and $H_{6'}$), 3.05 (2H, d, J = 9Hz, $H_{3'}$ and $H_{5'}$), 3.49 (1H, s),3.58 (1H, d, J = 2Hz), 3.70 (1H, d , J = 2Hz), 6.17 (6H, s, - OMe). m/e 298.0850. $C_{17}H_{14}O_5$ requires 298.0841.

4', 7 - Dimethoxy - 3',5 - dihydroxy flavone (22)

This was a yellow crystalline solid m.p. 236-8° (acetone), λ_{max}^{EtOH} 253, 268, 345 nm (ε 2.14 x 10^{4}, 1,91 x 10^{4}, 2.34 x 10^{4}). ν max (nujol) 3320, 2950, 2880, 1655, 1610, 1563, 1520, 1470, 1450, 1365, 1335, 1305, 1290, 1270, 1210, 1190, 1160, 1150, 1125, 1105, 1050, 1020, 990, 945, 885, 875, 855, 830 cm^{-1}. τ (pyridine-d_5) - 3.49 (1H,s, C_5-OH), - 1.40. (1H, br m, C_3 - OH), 2.22 (1H, d, J = 2Hz $H_{2'}$), 2.54 (1H, partially obscured by pyridine $H_{6'}$), 2.95 (1H, d, J = 8Hz, $H_{5'}$), 3.12 (1H, s, H_3), 3.48 (2H, br s, H_6 and H_8), 6.26 (3H, s, - OMe), 6.29 (3H, s, - OMe). m/e 314.0806 $C_{17}H\ _4O_6$ requires 314.0790 (Found C, 64.91:H, 4.68; $C_{17}H_{14}O_6$ requires C, 64.96; H, 4.49%).

6 , 7 - Dimethoxy - 4', 5 - dihydroxy flavone (23)

This compound was purified by preparative tlc ($CHCl_3$) as yellow crystals m.p. 259-61°. λ_{max}^{MeOH} 267, 335nm (ε 1.34 x 10^{4},2.24 x 10^{4}), $\lambda_{max}^{MeOH-NaOMe}$ 266.5, 386 nm, $\lambda_{max}^{MeOH-AlCl_3}$ 277, 302,5,348, 248 nm, $\lambda_{max}^{MeOH-AlCl_3/HCl}$ 278.5, 344, 381 nm, $\lambda_{max}^{MeOH-NaOAc}$ 258.5, 266, 387 nm, $\lambda_{max}^{MeOH-NaOAc/H_3BO_3}$ 266, 341.5 nm. m/e 314.0797 $C_{17}H_{14}O_6$ requires 314.0790.

Systematic fractionation of the Pleocarphus revolutus benzene soluble extract.

The benzene soluble extract of Pleocarphus revolutus (10g) was subjected to column chromatography on silica gel (m.f.c.) (300g). Initial elution with CH_2Cl_2, followed by a stepwise gradation to pure EtOAc yielded a total of 17 fractions.

Sesquiterpene keto-alcohol (37)(Rf. 0.3, $CHCl_3$)

Further chromatography of column fraction 5 (224.5mg) on sephadex LH-20 (25g) in MeOH yielded the white crystalline keto-alcohol (37) (125 mg 0.015%) m.p. 108-9° (crystallised from C_6H_{12} - EtOAc 3 : 1). λ^{EtOH}_{max} 243.5 nm ε 13,400. νmax 3480, 2950, 1700, 1640, 1450, 1415, 1385, 1340, 1320, 1285, 1190, 1155, 1145, 1115, 1090, 1070, 1045, 1030, 1000, 960, 950, 910 cm^{-1}. τ5.18 (3H, br m), 6.88 (1H, m), 8.16 (3H, s,),8.37 (3H, d, J = 2Hz), 9.04 (3H, d, J = 7Hz). m/e 234 (M^+), 216 (M^+- H_2O), 109 (base peak). $[\alpha]^{22}_{D}$ - 144.9^{o} (c 1.65, $CHCl_3$). (Found: C, 77.10; H, 9.32. $C_{15}H_{22}O_2$ requires : C, 76.92; H, 9.40%) $\Delta\varepsilon_{304}$-2.35, $\Delta\varepsilon_{240}$-10.3, $\Delta\varepsilon_{203}$5.4.

Additional samples (630 mg 0.076%) of this keto-alcohol were obtained on chromatography of column fractions 4, 6 and 7.

Acetylation of the sesquiterpene keto-alcohol (37)

The keto-alcohol (37) (25 mg) was dissolved in pyridine (3 ml) and a few drops acetic anhydride were added. The solution was stirred for 24 h. before being acidified with HCl and extracted into $CHCl_3$. Preparative tlc ($CHCl_3$) of the organic fraction yielded a colourless oil, (18.8 mg, 63%) consistent in all respects with the expected mono-acetate (26). ν max 2950, 2880, 1740, 1705, 1645, 1450, 1415, 1380, 1345, 1285, 1235, 1175, 1115, 1090, 1065, 1050, 1035, 1005, 995, 970, 965, 940, 910 cm^{-1}. τ 4.16 (1H, s),5.27 (2H, br m),7.12 (1H, m), 8.05 (3H, s), 8.22 (3H, s), 8.29 (3H, d, J = 2Hz), 9.04 (3H, d, J = 7Hz). m/e 276 (M^+), 216 ($M^{\pm}$ acetic acid). This acetate proved to be identical to the naturally occurring keto-acetate (38) subsequently isolated

from column fraction 3. (See below)

Cinnamoylation of the sesquiterpene keto-alcohol (37)

A few drops of cinnamoyl chloride were added to a solution of the keto alcohol (37) (25 mg) in pyridine (3 ml). This solution was stirred for 6 hours before being made acidic with HCl and extracted into CH_2Cl_2. After evaporation, preparative tlc ($CHCl_3$) yielded the cinnamate (67) (33.1 mg, 90%) as a colourless oil λ_{max}^{EtOH} 280 240, 223 nm (ε 26,000, 17,500, 21,500). τ 2.20 (1H, d, J = 16Hz), 2.50 (5H, m), 3.48 (1H, d, J = 16Hz), 3.90 (1H, br s), 5.10 (2H, br s), 8.13 (6H, br s), 9.0 (3H, d, J = 7Hz). m/e 364 (M^+), 216 (M^+- cinnamic acid). $[\alpha]_D^{20}$ - 174.95° (c 1.05, $CHCl_3$). (Found, C, 78.85; H, 7.67 $C_{24}H_{28}O_3$ requires C, 79.12, H, 7.69%).

Formylation of the sesquiterpene keto-alcohol (37)

The keto-alcohol (37) (25 mg) was stirred at room temperature in pyridine (2 ml) containing a few drops of formic-acetic anhydride. After 24 hours the mixture was worked up by acidification with HCl and extraction with CH_2Cl_2. Preparative tlc ($CHCl_3$ + 5% MeOH) yielded, starting material (8.8 mg, 35%) and the white crystalline keto-formate (41) (12.7 mg, 48%) m.p. 104-5° (C_6H_{12} - EtOAc 3 : 1). λ_{max}^{EtOH} 238 nm (ε 17,628). νmax 3090, 2960, 2930, 2880, 1735, 1710, 1645, 1445, 1410, 1380, 1335, 1280, 1200, 1160, 1110, 1085, 1055, 955, 925, 905 cm^{-1}. τ 1.83 (1H, s), 3.90 (1H, brs), 5.13 (2H, m), 8.16 (6H, m), 9.05 (3H, d, J = 7Hz). m/e 262 (M^+), 216 (M^+- formic acid). $[\alpha]_D^{23}$ - 127.70° (c 0.621, $CHCl_3$). (Found C, 73.16, H, 8.62; $C_{16}H_{22}O_3$ requires C, 73.28, H, 8.40%).

Reduction of the sesquiterpene keto-alcohol (37)

Zn dust (25 mg) was added to the keto-alcohol (37) (50 mg) in acetic acid (10 ml). The solution was heated at reflux for 18 hours, fresh portions of Zn dust being added after 13 and 15 hours. Work up, by neutralisation

with NaOH, extraction into CH_2Cl_2 and evaporation gave an oil (45.3 mg). Separation of this by preparative tlc ($CHCl_3$) yielded the starting alcohol(8.7 mg, 17.5%) and the reduction product (27) (24.5 mg, 63%) as a colourless oil. λ_{max}^{EtOH} 239 nm (ϵ 15,000). ν max 3070, 2960, 2930, 2880, 1705, 1648, 1460, 1445, 1412, 1385, 1345, 1285, 1180, 1155, 1075, 1045, 945, 910, 895 cm^{-1}. τ 5.2 (2H, m), 8.9 (3H, s), 8.95 (3H, d, J = 2Hz), 9.0 (3H, d, J = 7Hz), m/e 218 (M^+). $[\alpha]_D^{21}$ 29.33° (c 0.5, $CHCl_3$). (Found C, 82.51; H, 10.15; $C_{15}H_{22}O$ requires C, 82.57; H, 10.09%).

Reaction of the sesquiterpene keto-alcohol (37) with pyridine

The keto- alcohol (37) (5mg) was stirred for 24 hours in pyridine (3 ml.). After this time tlc ($CHCl_3$) showed only starting material.

Reaction of the sesquiterpene keto-alcohol (37) with toluene-p-sulphonic acid.

I The keto-alcohol (37) (20 mg) and toluene-p- sulphonic acid (20 mg) were heated to reflux in CH_2Cl_2 (10 ml). After 6 hours the reaction mixture was washed with aqueous $NaHCO_3$, and with water, before evaporation of the organic fraction to give a yellow oil (24.1 mg). Tlc ($CHCl_3$, 2 elutions) of this indicated seven distinct components, the major one of which was separated by preparative tlc ($CHCl_3$) as the oily keto-alcohol methyl ether (28) (6.3 mg, 25%). ν max 2960, 2930, 1710, 1640, 1445, 1410, 1380, 1360, 1335, 1280, 1170, 1125, 1095, 950, 925, 895 cm^{-1}. m/e 248 (M^+), 216 (M^+-MeOH).

II The keto-alcohol (37) (100 mg) was dissolved in CH_2Cl_2 (20 ml) which had been freshly distilled from P_2O_5. Dried toluene-p-sulphonic acid (100 mg) was added to this solution and the whole was heated at reflux for 14 hours. The solution was then poured into water, and extracted with further CH_2Cl_2. After evaporation of the organic phase, preparative tlc ($CHCl_3$, 2 elutions) yielded, in addition to starting material (18.4 mg, 18.4%) two major products. The more polar of these proved to be an isomer of the starting alcohol (31) (11.2 mg, 11.2%).

λmax. 3540, 2980, 2910, 1710, 1645, 1445, 1410, 1385, 1312, 908 cm^{-1}. τ 5.1 (3H,m), 8.1 (3H, br s), 8.2 (3H, br s), 9.4 (3H, d, J = 7Hz). m/e 234 (M^{+}- H_2O), 109 (base peak).

The less polar product was the compound (34) (19.6 mg, 39.2%) the di-ether of the keto-alcohol (37). λ_{max}^{EtOH} 243.5, 294 nm (ϵ 11,250, 27,900). ν max 2960, 2925, 2870, 1710, 1648, 1448, 1410, 1385, 1320, 1280, 1200, 1175, 1160, 1100, 1070, 940, 900 cm^{-1}. m/e 450 (M^{+}), 234 (M^{+}- $C_{15}H_{20}O$), 216 (base peak, M^{+}- $C_{15}H_{22}O_2$). $[\alpha]_D^{24}$ 186.2° (c 0.51, $CHCl_3$). (Found C, 79.96; H, 9.23; $C_{30}H_{42}O_3$ requires C, 80.00; H, 9.33%).

Dehydration of the sesquiterpene keto-alcohol (37)

A few drops of $POCl_3$ were added to a solution of the keto-alcohol (37) (25 mg) in pyridine (3 ml). The whole was stirred for one hour before being poured into water, acidified with HCl and extracted into CH_2Cl_2. After evaporation of the CH_2Cl_2 preparative tlc ($CHCl_3$) yielded two major components. (1) The less polar chloro-compound (35) (9.2 mg, 35%). λ_{max}^{EtOH} 243 nm. ν max 3075, 2920, 2870, 1710, 1640, 1450, 1410, 1390, 1380, 1310, 1260, 1235, 1175, 1100, 1065, 915, 900 cm^{-1}. τ 8.1 (3H, d, J = 3Hz), 8.2 (3H, br s), 9.05 (3H, d, J = 7Hz). m/e 25.4 (M^{+}+ 2), 252 (M^{+}), 217 (M^{+}-Cl), 216 (M^{+}-HCl). Ratio of intensities 254 : 252, 1 : 3.

(11) The more polar product a tri-ene (3.0 mg, 13%). λ_{max}^{EtOH} 237, 303 nm. m/e 216 (M^{+}), 249 (base peak).

Sesquiterpene keto-acetate (38) (Rf. 0.6, $CHCl_3$)

Preparative tlc ($CHCl_3$) of column fraction 3(410 mg) from the *Pleocarpus revolutus* benzene extract, eventually yielded the keto-acetate (38) (148 mg, 0.018%) as a colourless oil. λ_{max}^{EtOH} 235 nm (ϵ 17,400). ν max 2950, 2880, 1740, 1705, 1645, 1450, 1415, 1380, 1345, 1285, 1235, 1175, 1115, 1090, 1065, 1050, 1035, 1005, 995, 970, 965, 940, 910 cm^{-1}. τ 4.16 (1H, s), 5.27 (2H, br m), 7.12 (1H, br m), 8.05 (3H, s), 8.22 (3H, s), 8.29 (3H, d, J = 2Hz), 9.05

(3H, d, J = 7Hz). m/e 276 (M^+), 216 (M^+- acetic acid). $\Delta\varepsilon_{307}$ -0.55, $\Delta\varepsilon_{238}$ -3.1, $\Delta\varepsilon_{196}$ 3.1.

Further samples of this keto-acetate (38) (350 mg, 0.042%) were obtained from chromatography of column fractions 2, 4 and, 6.

Hydrolysis of the sesquiterpene keto-acetate (38)

The keto-acetate (38) (25 mg) was stirred for 20 hours in THF (5 ml) to which 0.1N aqu. NaOH solution (3 ml) had been added. The reaction mixture was poured into water (10 ml) and extracted with CH_2Cl_2. After evaporation of the organic fraction preparative tlc ($CHCl_3$) yielded starting material (4 mg, 16%) and a compound identical with the sesquiterpene keto-alcohol (37).

Reduction of the sesquiterpene keto-acetate (38)

The keto-acetate (38) (25 mg) and Zn dust (15 mg) were refluxed for 20 hours in acetic acid (10 ml) fresh Zn dust being added after 17 and 19 hours. The solution was neutralised with NaOH and extracted into CH_2Cl_2. Preparative tlc ($CHCl_3$) of the evaporated organic phase yielded starting acetate (6.3 mg, 25%), and a compound identical with the reduction product (27) (8.3 mg, 42%) obtained from similar treatment of the keto-alcohol (37) (see above).

Daylight photolysis of the sesquiterpene keto-acetate (38)

Two samples of the sesquiterpene keto-acetate (38) (5 mg), freshly purified by preparative tlc(petroleum-ether : $CHCl_3$ 1 : 2,3 elutions), were sealed under nitrogen in separate glass flasks. One of the samples was wrapped in tin foil and stored in complete darkness, the other was allowed to stand in daylight.

After 48 hours tlc of the 'dark' sample showed keto-acetate only. The 'light' sample, however, was shown to contain a second very close running, component. Preparative tlc (petroleum-ether : $CHCl_3$ 1 : 2, 3 elutions) allowed isolation of this second, slightly more polar, component (39) as a colourless oil. λ_{max}^{EtOH} 233, 284 nm

(ε 6,400, 250). ν max 3420, 2960, 2930, 2850, 1740, 1665, 1645, 1460, 1440, 1380, 1330, 1295, 1235, 1155, 1115, 1080, 1055, 1020, 975, 960 cm^{-1}. τ 2.12 (1H, m, exchangeable D_2O), 4.49 (1H, br s), 5.18 (2H, br s), 7.89 (3H, s), 8.18 (3H, s), 8.20 (3H, d, J = 3Hz), 8.98 (3H, d, J = 7Hz).m/e 308 (M^+) $[\alpha]_D^{24}$ - 26.30° (c 0.635, $CHCl_3$). (Found : C, 66.32; H, 7.89; $C_{17}H_{24}O_5$ requires, C, 66.23, H, 7.79%).

Decomposition of the hydroperoxide (39)

The hydroperoxide (39) (10 mg) dissolved in Et_2O (3 ml) was stirred with aqueous KI solution for 2 hours. The Et_2O layer was removed and washed with sodium thiosulphate solution and with water. Evaporation of the organic layer provided an orange oil which, on preparative tlc ($CHCl_3$), yielded the alcohol (40) (3.7. mg 43%) as a colourless oil. ν max 3460, 2930, 2860, 1745, 1720, 1645, 1460, 1375, 1330, 1230, 1120, 960, 900 cm^{-1}. m/e 292.1673 (M^+) $C_{17}H_{24}O_4$ requires 292.1674, 232 (M^+ - acetic acid), 214 (M^+- acetic acid - water).

Acid treatment of the sesquiterpene keto-acetate (38)

The sesquiterpene keto-acetate (38) (3 mg) dissolved in THF (1 ml) was stirred at room temperature with 0.1N HCl (1 ml). Tlc(petroleum-ether : $CHCl_3$ 1 : 2,3 elutions) of this solution after 24 hours showed only keto-acetate.

Base treatment of the sesquiterpene keto-acetate (38)

a) The keto-acetate (38) (7.5 mg) was dissolved in CH_2Cl_2 (3 ml) and a few drops of diisopropylethylamine were added. The solution was stirred at room temperature for 24 hours after which tlc (petroleum-ether : $CHCl_3$ 1 : 2, 3 elutions) showed keto-acetate only.

b) The keto-acetate (3 mg) was dissolved in THF (1 ml) and stirred with 0.1N NaOH (1 ml) for 24 hours. TLC (petroleum-ether : $CHCl_3$ 1 : 2, 3 elutions) showed only one spot other than that ascribed to the keto-acetate. This was shown to be due to the formation of the keto-alcohol (37).

Photolysis of the keto-acetate (38)

a) A freshly purified sample of the keto- acetate (38) (6 mg) was dissolved in $CH_2 Cl_2$ (5 ml) and a current of oxygen was bubbled through the solution. At the same time this was irradiated with light from a 300 W tungsten lamp. Tlc (petroleum-ether : $CHCl_3$ 1 : 2, 3 elutions) after $2\frac{1}{2}$ hours showed only keto-acetate.

A small amount of methylene blue was added to the above solution and it was irradiated for a further $2\frac{1}{2}$ hours. Tlc (petroleum-ether : $CHCl_3$ 1:2, 3 elutions) again failed to show any trace of the hydroperoxide (39).

b) A current of oxygen was bubbled through a solution of freshly purified keto-acetate (38) (5 mg) in benzene (5 ml) and the whole irradiated with a medium pressure mercury lamp. Tlc (Petroleum-ether : $CHCl_3$ 1 : 2, 3 elutions) of the solution after two hours showed a considerable mixture of compounds, one of which had identical tlc properties, including spraying with starch-iodide solution, with those of the hydroperoxide (39).

Daylight photolysis of the sesquiterpene keto- formate (41)

The keto-formate (41) (5 mg) was sealed, under nitrogen into a glass container. This was allowed to stand in daylight for 72 hours after which tlc ($CHCl_3$), showed only starting material.

A solution of the keto-formate (5 mg) dissolved in $CH_2 Cl_2$ (5 ml) was exposed to the light for a further 72 hours. Tlc ($CHCl_3$) again showed no reaction.

Unidentified sesquiterpenes

Extensive preparative tlc of column fraction 3 (410 mg) from the *Pleocarphus revolutus* benzene extract yielded a total of ten cytotoxic fractions;

(1) The sesquiterpene keto-acetate (38)(see above)

(2) A colourless oil (D3) (7.8 mg, 0.0095%) Rf. 0.51, $CHCl_3$. λ_{max}^{EtOH} 238.5 nm. ν max 2950, 1725, 1635, 1475, 1465, 1435, 1395, 1375, 1360, 1325, 1290, 1280, 1235, 1225, 1200, 1165, 1150, 1130, 1115, 1090. 1075, 1050, 1040, 970, 960 cm^{-1}. τ 3.23 (1H, br m), 4.90 (1H, m), 6.30 (3H, br m), 8.90 (3H, s), 8.97 (3H, s). m/e 276 (M^+). Accurate mass measurements;

m/e	276.172	$C_{17}H_{24}0_3$	requires	276.1725
	231.1381	$C_{15}H_{19}0_2$	requires	231.1384
	202.1347	$C_{14}H_{18}0$	requires	202.1357
	159.1182	$C_{12}H_{15}$	requires	159.1174
	159.0811	$C_{11}H_{11}0$	requires	159.0810

(3) A colourless oil (D4) (42.7 mg, 0.0051%) Rf. 0.33 $CHCl_3$. λ_{max}^{EtOH} 238, 293 nm. ν max 2980, 2880, 1730, 1640, 1470, 1425, 1390, 1370, 1350, 1320, 1310, 1270, 1240, 1225, 1160, 1120, 1110, 1080, 1065, 1030, 960, 955, 945 cm^{-1}. τ 3.38 (1H, t, J = 3.5Hz), 6.02 (2H, dd, J = 6, 7 Hz), 7.59 (2H, dd, J = 3, 7 Hz), 8.95 (3H, d, J = 7Hz), 8.97 (3H, s), 9.00 (3H, s). m/e 232 (M^+), 202 (base peak). $[\alpha]_D^{21}$ - 77.67° (c 1.3, $CHCl_3$). (Found C, 77.61; H, 8.79; $C_{15}H_{20}0_2$ requires, C, 77.59; H, 8.62%).

The remaining seven active compounds were all obtained as oils in the following very low yields;

(4) 3.6 mg, 0.00043% (B5)

(5) 4.2 mg, 0.0005% (B8)

(6) 3.7 mg, 0.00044% (C4)

(7) 2.8 mg, 0.00034% (C5)

(8) 2.4 mg, 0.00029% (C13)

(9) 14.8 mg, 0.0018% (D7)

(10) 1.4 mg, 0.00017% (D8)

The sesquiterpene keto-alcohol (56) (Rf. 0.16 $CHCl_3$)

Preparative tlc of column fraction 6 (709 mg) from the Pleocarphus revolutus benzene extract yielded, in addition to the sesquiterpene alcohol (37) (112 mg, 0.013%) a pale yellow oil (181.2 mg, 0.022%) Rf 0.16 ($CHCl_3$), eventually identified as the sesquiterpene keto-alcohol (56). λ_{max}^{EtOH} 241, 277 (sh) nm. ν max 3440, 2930, 2880, 1710, 1670, 1465, 1430, 1415, 1380, 1330, 1305, 1250, 1165, 1135, 1025, 910 cm^{-1}. τ 6.2 (3H, br m), 7.83 (3H, s), 8.28 (3H, s), 9.15 (3H, s), 9.38 (3H, d, J = 6.5Hz). $[\alpha]_D^{19}$ 22.96° (c 1.52, $CHCl_3$). m/e 234 (M^+), 216 ($M^+ - H_2O$). (Found C, 71.48; H, 9.47; $C_{15}H_{22}O_2$ + H_2O requires C, 71.43; H. 9.52%).

Treatment of the sesquiterpene keto-alcohol (56) with phosphorus oxychloride

The sesquiterpene keto-alcohol (56) (25 mg) was dissolved in pyridine (2 ml) and a few drops of $POCl_3$ were added. The solution was stirred for 15 mins. before being neutralised with HCl extracted into $CHCl_3$ and evaporated to leave a yellow oil. Tlc ($CHCl_3$) of this oil showed ten components one of which had the same Rf and gave the same blue/black colour reaction on spraying with p-anisaldehyde : H_2SO_4 : MeOH 1 : 1 : 1 as compound (35).

Preparative tlc ($CHCl_3$) of the reaction mixture yielded a yellow oil (2.8 mg, 10.4%). m/e 254 ($M^+ + 2$), 252 (M^+). Ratio of intensities 254 : 252, 1 : 3 consistent with $C_{15}H_{21}OCl$.

Acetylation of the sesquiterpene keto-alcohol (56)

A few drops of acetic anhydride were added to a solution of the keto-alcohol (56) (30 mg) in pyridine (3 ml) and the whole was stirred for 12 hours. Work up, by neutralisation with HCl and extraction into CH_2Cl_2 yielded a yellow oil which, on preparative tlc ($CHCl_3$, 2 elutions), yielded the colourless, oily, acetate (57) (10.2 mg, 31%). λ_{max}^{EtOH} 239.5 nm (ϵ = 9,675). ν max 1960, 1748, 1710, 1670, 1465, 1430, 1420, 1390, 1380, 1365, 1330, 1302, 1275, 1235, 1180, 1035 cm^{-1}. τ 5.7 (2H, ABq, J = 11 Hz), 7.85 (3H, s), 7.98 (3H, s), 8.29 (3H, s), 9.18 (3H, s), 9.38 (3H, d, J = 6Hz). m/e 276 (M^+), 216 (M^+- acetic acid). $[\alpha]_D^{23}$ - 43.9° (c 0.5, $CHCl_3$). (Found C, 73.75; H, 8.62; $C_{17}H_{24}O_3$ requires C, 73.91; H, 8.70%).

Reduction of the sesquiterpene keto-acetate (57)

The sesquiterpene keto-acetate (57) (5 mg) was dissolved in acetic acid (5 ml) and Zn dust was added. The solution was heated at reflux for 20 hours, fresh Zn dust being added after 15 hours and 17 hours. Tlc ($CHCl_3$) of the reaction mixture after this time showed only starting material.

Dehydrogenation of the sesquiterpene keto-alcohol (56)

The keto-alcohol (56) (25 mg) and Pd (5%) on charcoal (25 mg) were sealed, under vacuum, into a glass tube and this was heated in refluxing ethylene glycol (198°). After 6 hours there was no indication of any blue colouration and tlc ($CHCl_3$) showed only the starting alcohol.

APPENDIX 1.

Appendix 1

TLX5 Cytotoxicity Test

A small number of cells of the TLX5 tumour were implanted into the peritoneal cavity of a mouse, where they were incubated for seven days. The mouse was then sacrificed and the TLX5 tumour cells, contained in the intraperitoneal fluid were removed, washed with saline and suspended in TC199/horse serum (60 : 40) at a concentration of 3×10^6 cells/ml.

After equilibration for one hour at 37°, 8 ml aliquots of this cell suspension were added to each of the test compounds, previously dissolved in 0.3 ml dimethylsulphoxide. These mixtures of tumour cells and test compound were then incubated for a further 4 hours at 37°. To provide a control experiment an 8 ml aliquot of the cell suspension was also added to 0.3 ml dimethylsulphoxide only (i.e. containing no test compound). This control solution was subsequently treated in an identical manner to those solutions containing compounds under test.

After incubation for four hours aliquots (6 ml) from each of the above cell solutions were added to aqueous solutions of the tritiated thymidine, uridine or leucine(3 Ci/ml of cells) and the resulting radioactive solutions were reincubated at 37°. Samples (1 ml) of these latter solutions were removed at fifteen minute intervals between 0 and 75 minutes after addition of the cells to the radio labelled precursor. Each sample was filtered through a Whatman glass fibre filter (GF/C) which was then washed succesively with 0.9% saline solution, 0.2N perchloric acid and further 0.9% saline solution. The filter was then placed in a glass counting vial in which it was allowed to air dry overnight before addition of liquid scintillator solution and counting:

Each sample was counted until three separate values, each of 10,000 counts, had been obtained which, after correction for quenching, were all within two standard deviations (i.e. 2%) of their mean value.

From the counts obtained a least squares plot (see below) of cpm vs the time of sampling was drawn for each test solution. By comparison of the curves obtained from the treated cells with that obtained from the corresponding control experiment, an estimation of the inhibition of uptake of radiolabelled precusor and thus of the cytotoxicity of each compound was obtained.

The liquid scintillator solution used in counting was prepared by addition of 2, 5-diphenyloxazole (5g) and 1,4-di (2-(5-phenyloxazolyl)) - benzene (0.25g) to toluene (1l). The toluene was purified by distillation from phosphourus pentoxide.

Calculation of the least squares plots

e.g. The control solution for one particular assay which gave the following results:-

Sampling Time (mins)	Counts cpm			
	I	II	III	Ave.
0	636	614	624	625
15	5,063	5,047	5,016	5,042
30	11,871	11,542	11,587	11,667
45	15,048	15,040	15,182	15,090
60	21,651	21,875	21,538	21,688
75	25,892	25,607	25,407	25,635

Applying the method of least squares.

The above points may be represented by

x	0	1	2	3	4	5
y	625	5,042	11,667	15,090	21,688	25,635

The sum of the squares (s) of the residual distances relative to the line $y = mx + c$ is

$$s = (625 - c)^2 + (5,042 - c-m)^2 + (11,667 - c-2m)^2 = --- + (25,635 - c-5m)^2$$

In order that s may be a minimum for variations of c and m (i.e. for variations of the line) it is necessary that:

$$\frac{ds}{dc} = 0$$
$$= -2\ ((625-c) + (5,042-c-m) + (11,667-c-2m) + --- + (25,635-c-5m))$$

and $$\frac{ds}{dm} = 0$$
$$= -2\ ((5,042 - c-m) + 2\ (11,667-c-2m) + --- +5\ (25,635-c-5m))$$

This gives

$$6c + 15m = 625 + 5,042 + 11,667 + --- + (25,635)$$

i.e. $6c + 15m = 79,747$

and $15c + 55m = 5,042 + 2\ (11,667) + --- +5\ (25,635)$

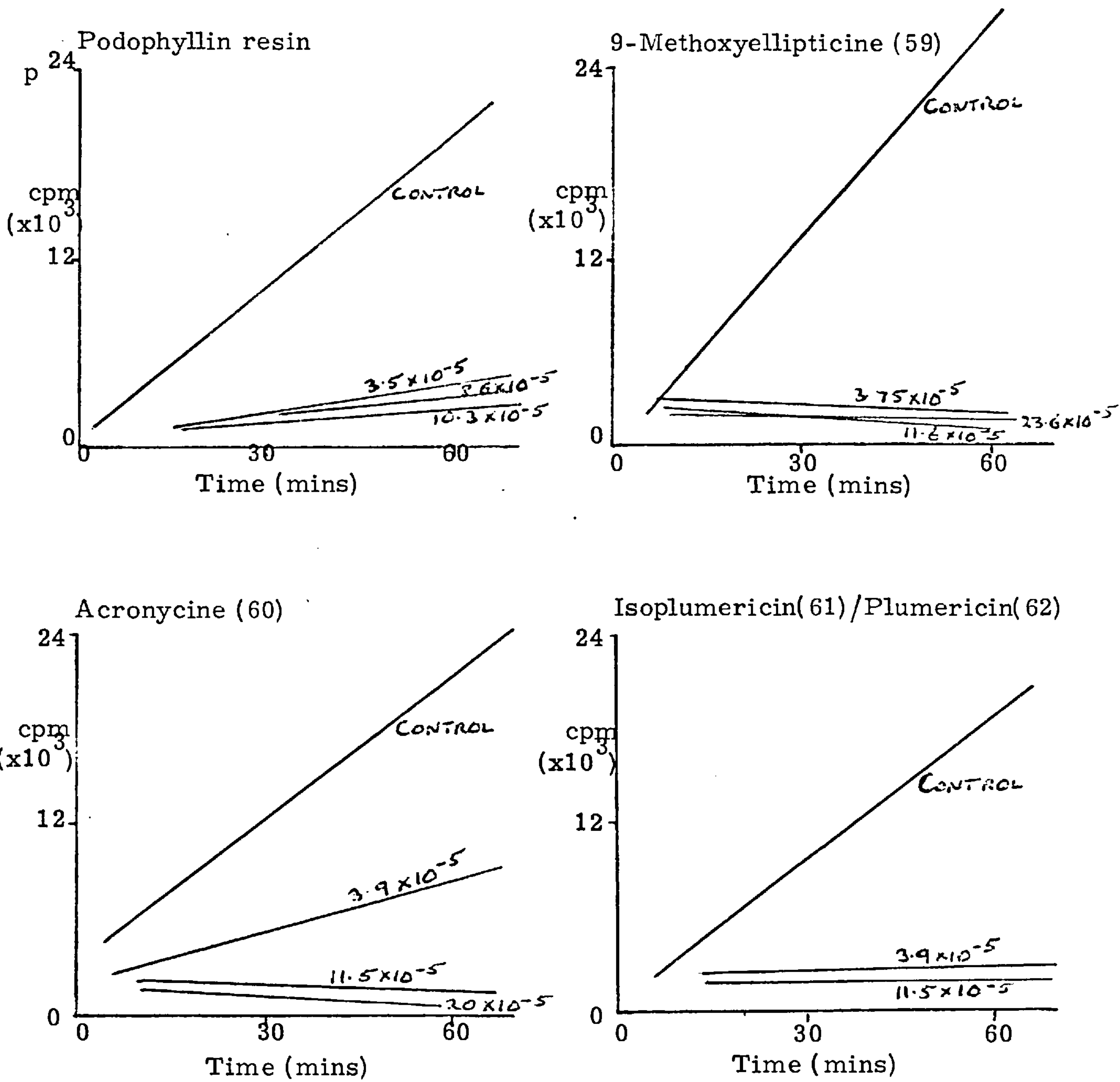
i.e. $15c + 55m = 288,573$

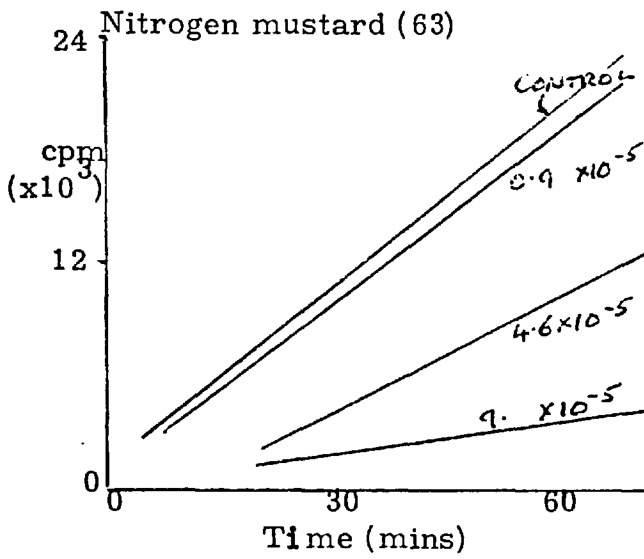
Solving these 2 simultaneous equations gives the equation of the line of best fit as:-

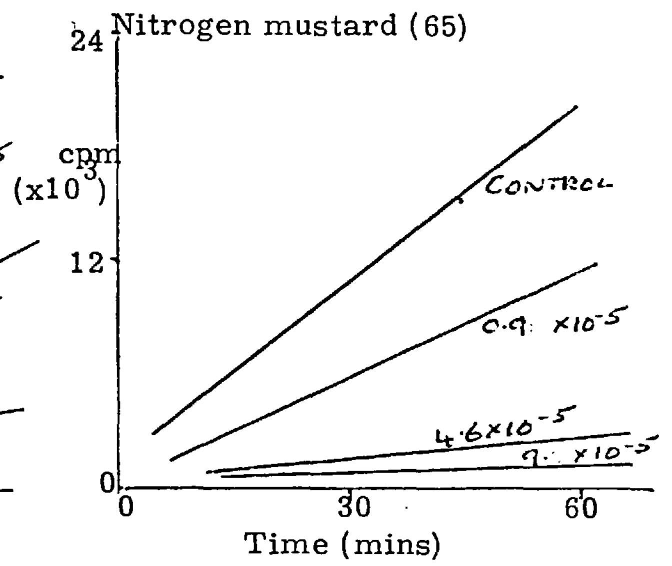
$$y = 5,097.5x + 547.5$$

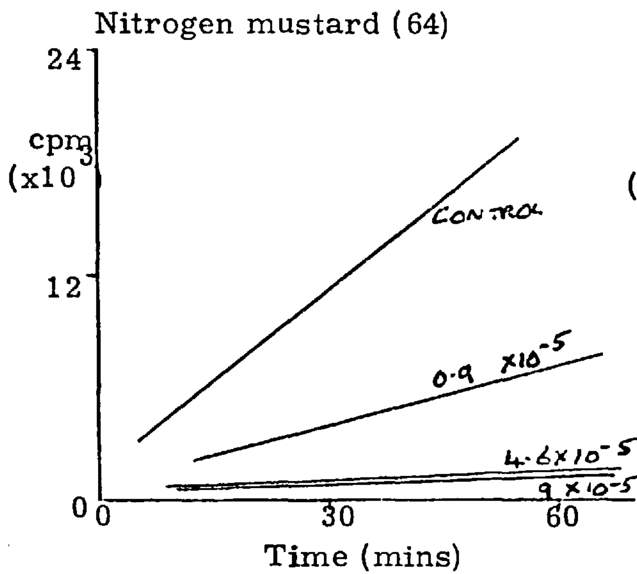
CYTOTOXICITY TESTING RESULTS

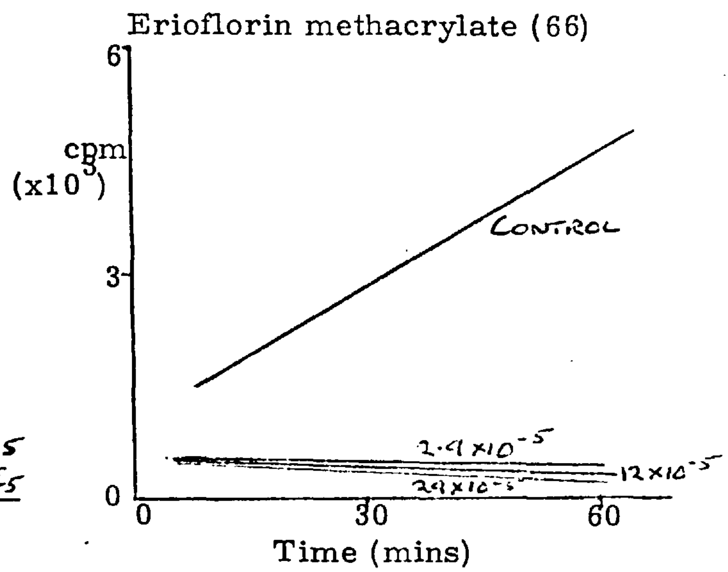
The results shown all represent inhibition of thymidine uptake unless otherwise stated.
Concentrations are in terms of g/ml of cell suspension i. e. g/3x10^6 cells.

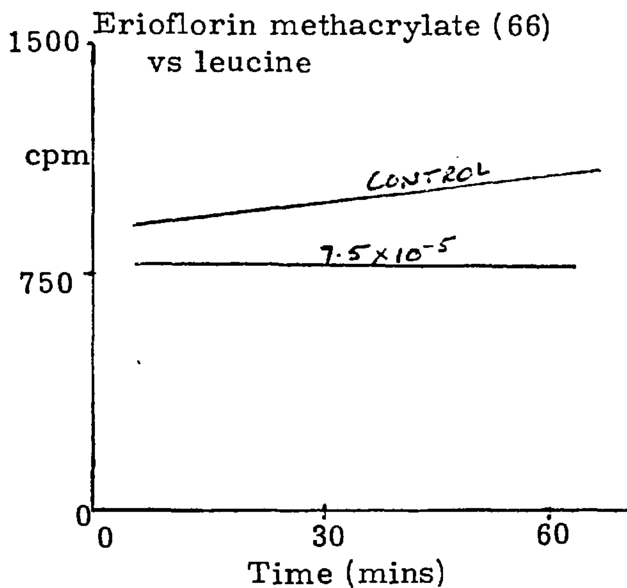


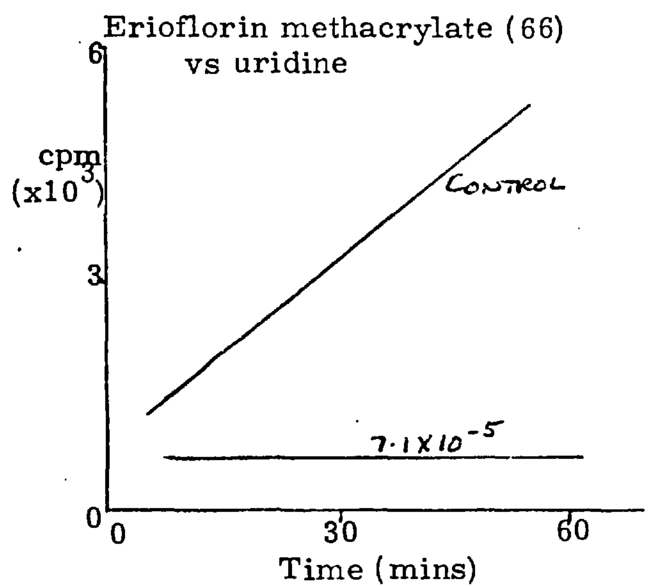

Nitrogen mustard (63)
24
cpm
(x10^3)
12
0
0
30
60
Time (mins)

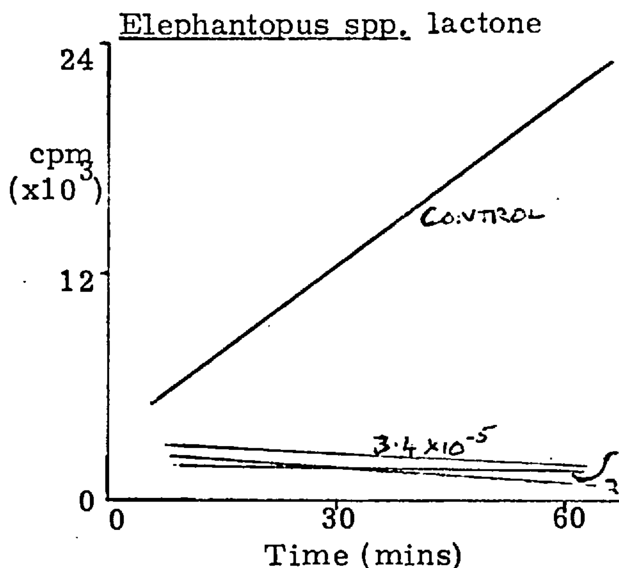

Nitrogen mustard (65)
24
cpm
(x10^3)
12
0
0
30
60
Time (mins)

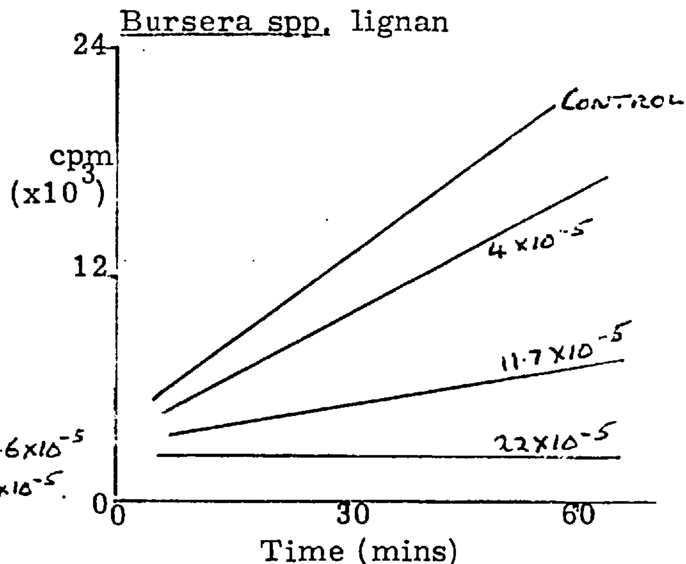

Nitrogen mustard (64)
24
cpm
(x10^3)
12
0
0
30
60
Time (mins)

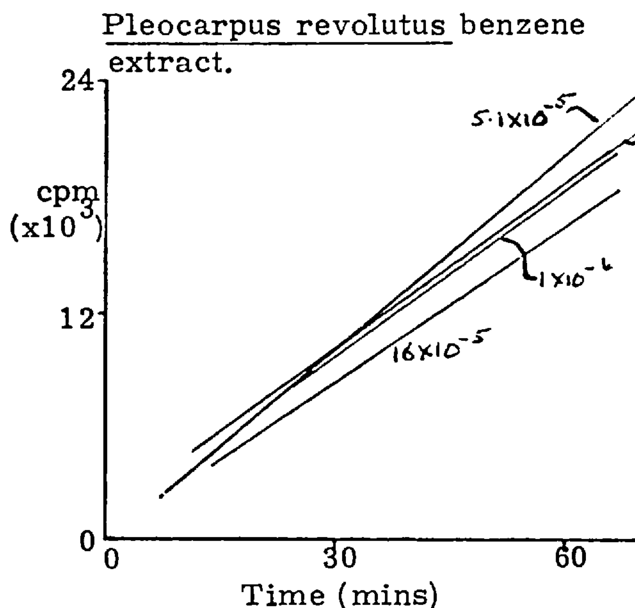

Erioflorin methacrylate (66)
6
cpm
(x10^3)
3
0
0
30
60
Time (mins)

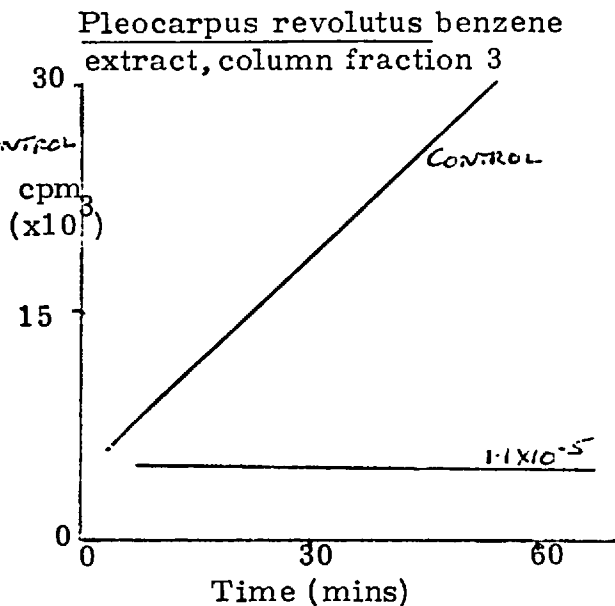

Erioflorin methacrylate (66)
vs leucine
1500
cpm
750
0
0
30
60
Time (mins)

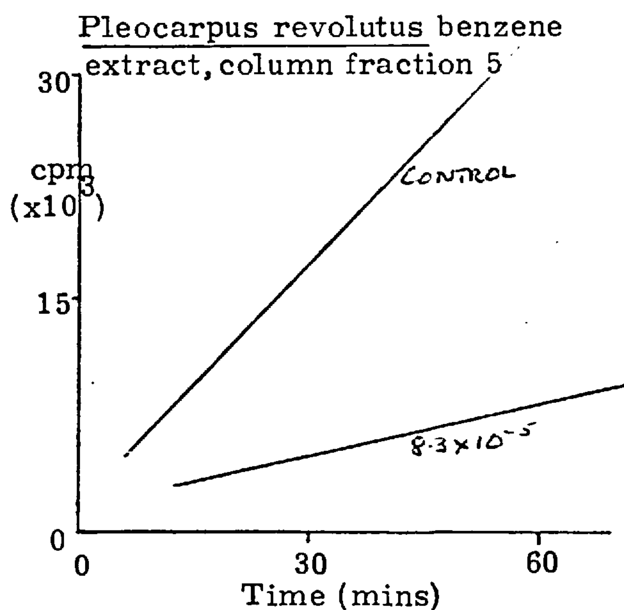

Erioflorin methacrylate (66)
vs uridine
6
cpm
(x10^3)
3
0
0
30
60
Time (mins)

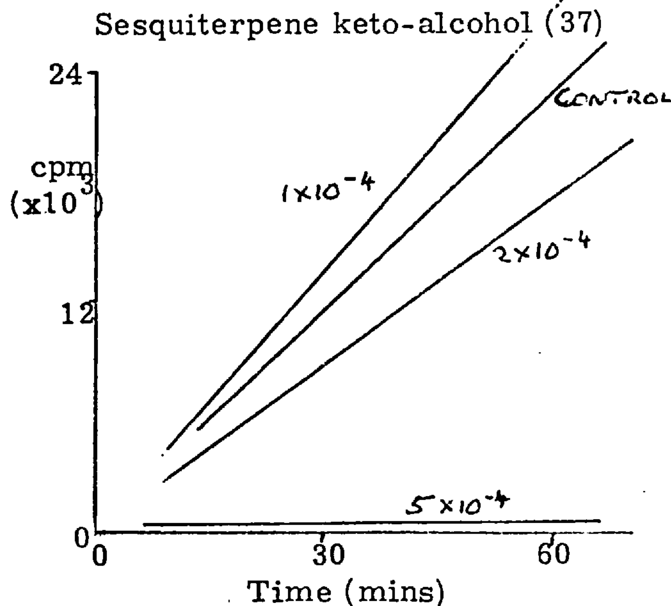

Elephantopus spp. lactone
cpm ($x10^3$)
24
12
0
CONTROL
3·4 x10^{-5}
11·6x10^{-5}
21x10^{-5}
0
30
60
Time (mins)

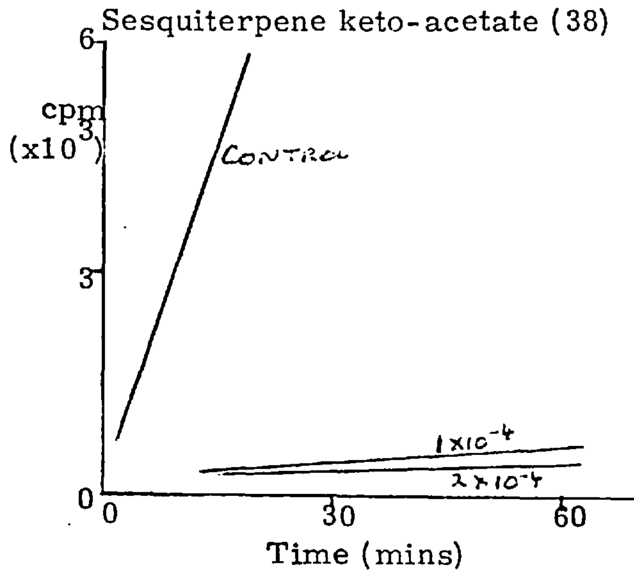

Bursera spp. lignan
cpm ($x10^3$)
24
12
0
CONTROL
4 x10^{-5}
11·7x10^{-5}
22x10^{-5}
0
30
60
Time (mins)

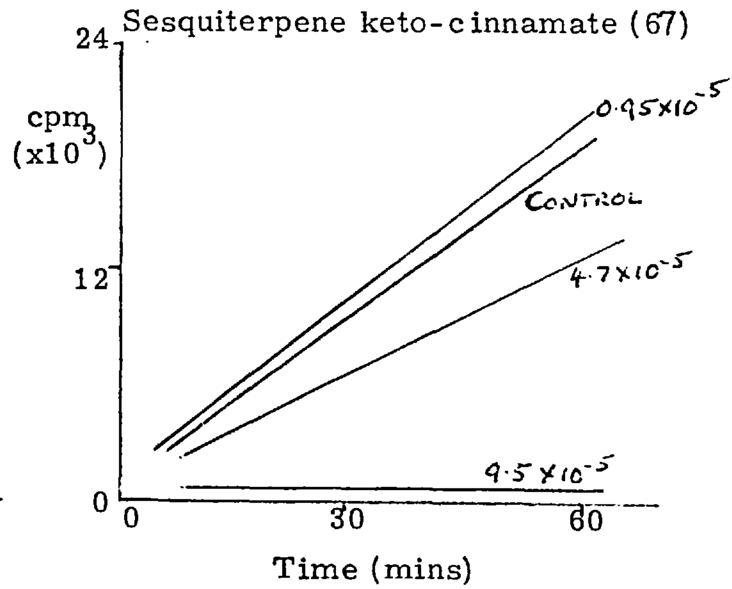

Pleocarpus revolutus benzene extract.
cpm ($x10^3$)
24
12
0
5·1x10^{-5}
CONTROL
1x10^{-4}
16x10^{-5}
0
30
60
Time (mins)

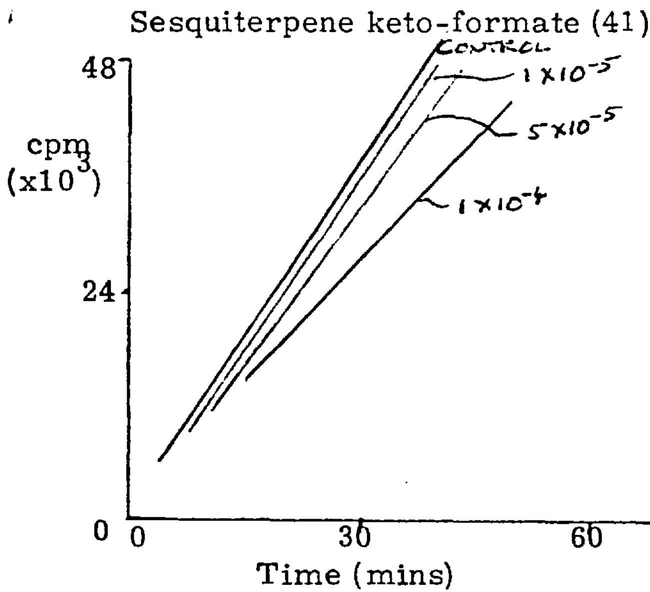

Pleocarpus revolutus benzene extract, column fraction 3
cpm ($x10^3$)
30
15
0
CONTROL
1·1x10^{-5}
0
30
60
Time (mins)

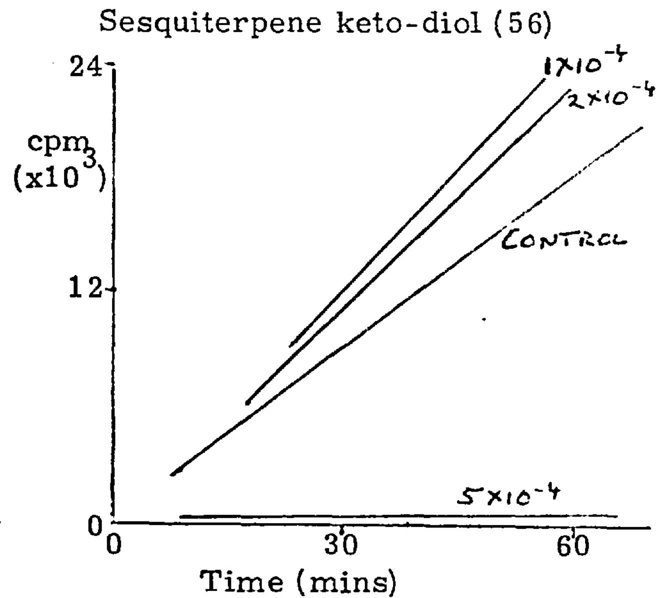

Pleocarpus revolutus benzene extract, column fraction 5
cpm ($x10^3$)
30
15
0
CONTROL
8·3x10^{-5}
0
30
60
Time (mins)

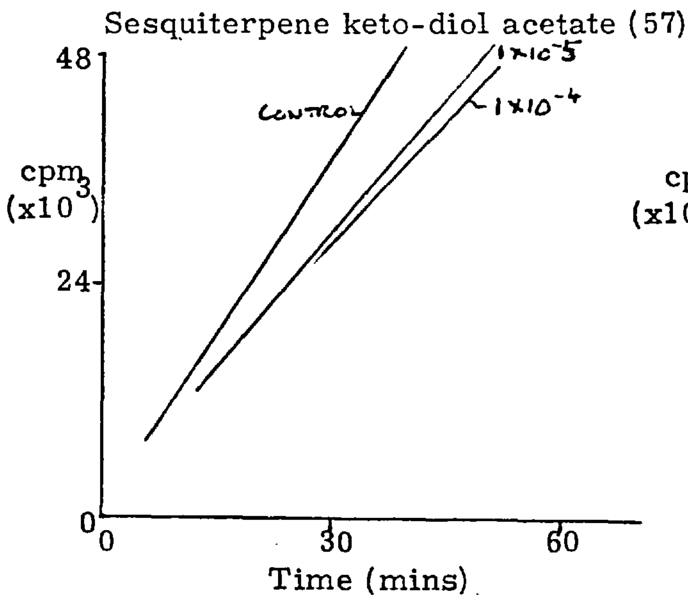

Sesquiterpene keto-alcohol (37)
cpm ($x10^3$)
24
12
0
1x10^{-4}
CONTROL
2x10^{-4}
5x10^{-4}
0
30
60
Time (mins)

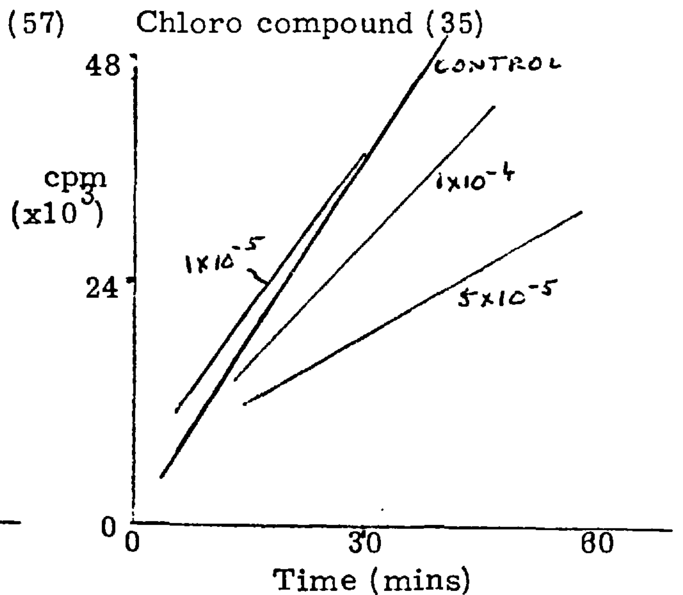

Sesquiterpene keto-acetate (38)
cpm
(x10^3)
6
3
0
Control
1x10^-4
2x10^-4
0
30
60
Time (mins)

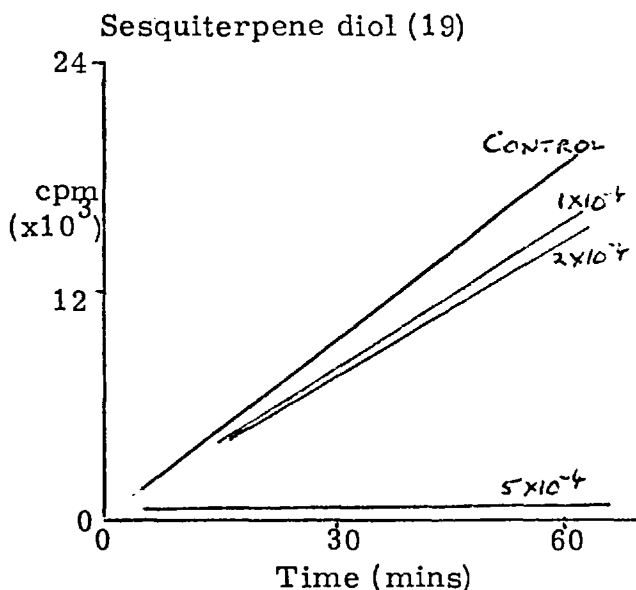

Sesquiterpene keto-cinnamate (67)
cpm
(x10^3)
24
12
0
0.95x10^-5
Control
4.7x10^-5
9.5x10^-5
0
30
60
Time (mins)

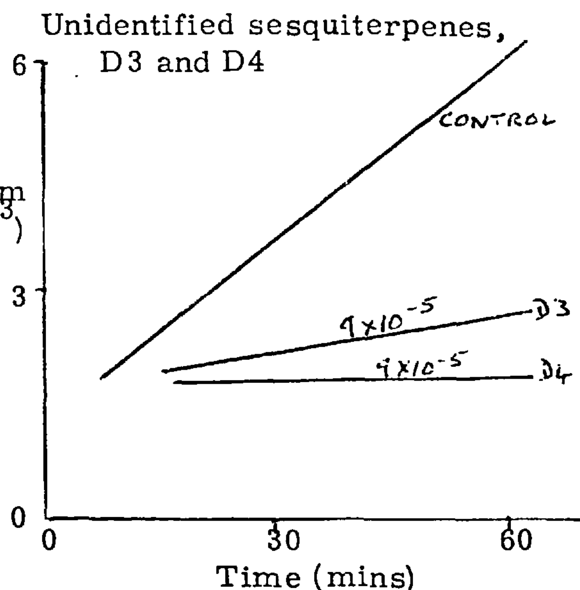

Sesquiterpene keto-formate (41)
cpm
(x10^3)
48
24
0
Control
1x10^-5
5x10^-5
1x10^-4
0
30
60
Time (mins)

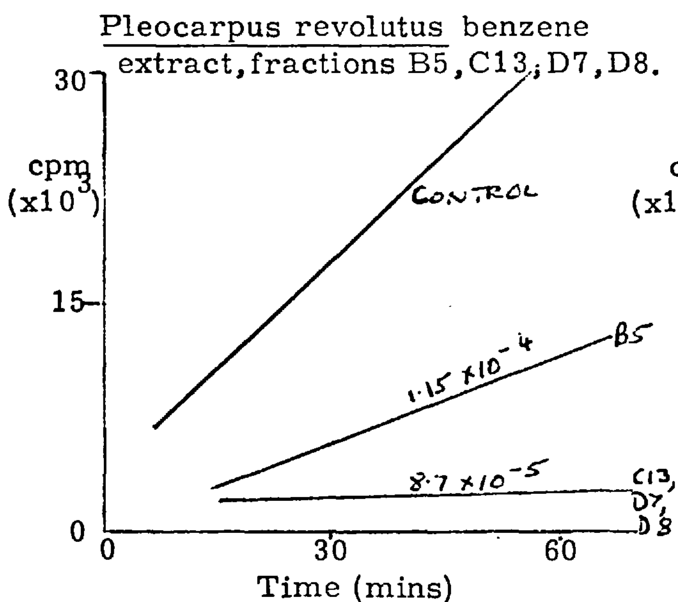

Sesquiterpene keto-diol (56)
cpm
(x10^3)
24
12
0
1x10^-4
2x10^-4
Control
5x10^-4
0
30
60
Time (mins)

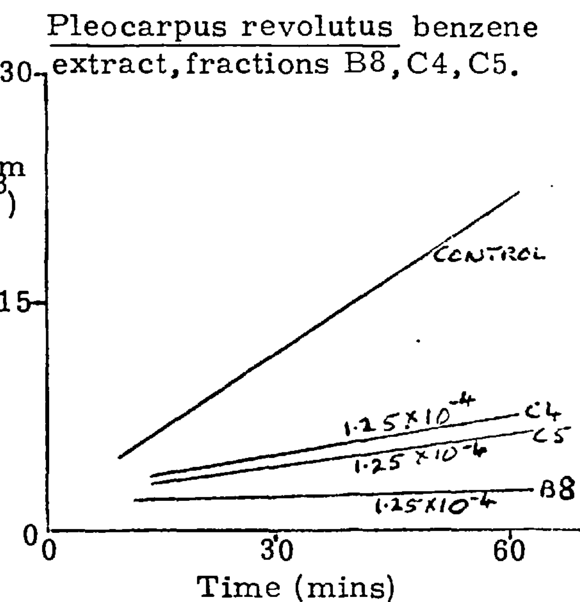

Sesquiterpene keto-diol acetate (57)
cpm
(x10^3)
48
24
0
Control
1x10^-5
1x10^-4
0
30
60
Time (mins)

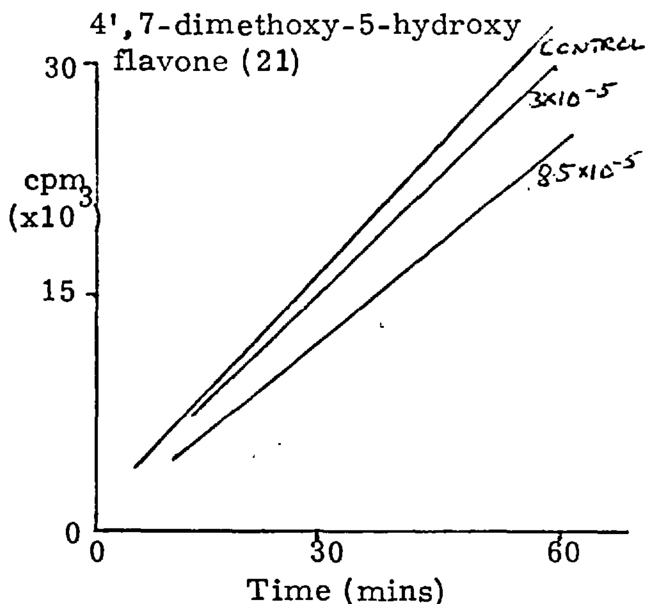

Chloro compound (35)
cpm
(x10^3)
48
24
0
Control
1x10^-5
1x10^-4
5x10^-5
0
30
60
Time (mins)

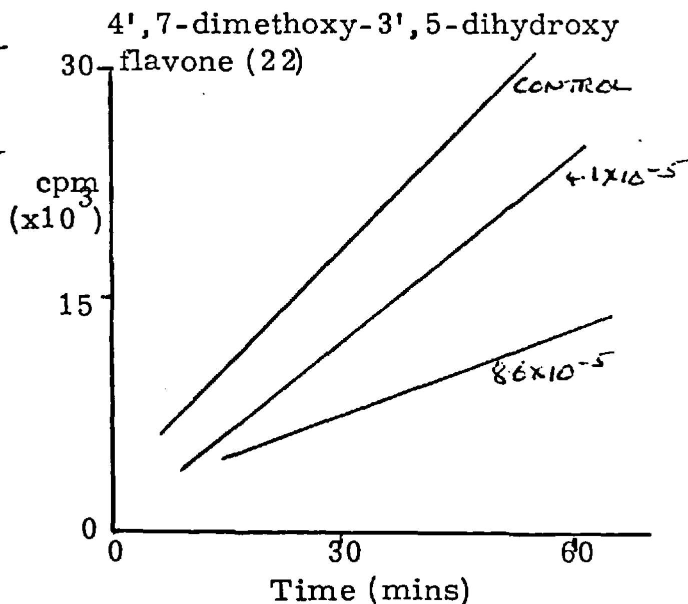

Sesquiterpene diol (19)
24
12
0
cpm
(x10^3)
CONTROL
1x10^-4
2x10^-4
5x10^-4
0
30
60
Time (mins)


Unidentified sesquiterpenes,
D3 and D4
6
3
0
cpm
(x10^3)
CONTROL
9x10^-5
D3
9x10^-5
D4
0
30
60
Time (mins)


Pleocarpus revolutus benzene
extract, fractions B5, C13, D7, D8.
30
15
0
cpm
(x10^3)
CONTROL
1.15 x10^-4
B5
8.7 x10^-5
C13,
D7,
D8
0
30
60
Time (mins)


Pleocarpus revolutus benzene
extract, fractions B8, C4, C5.
30
15
0
cpm
(x10^3)
CONTROL
1.25x10^-4
C4
1.25x10^-4
C5
1.25x10^-4
B8
0
30
60
Time (mins)


4',7-dimethoxy-5-hydroxy
flavone (21)
30
15
0
cpm
(x10^3)
CONTROL
3x10^-5
8.5x10^-5
0
30
60
Time (mins)


4',7-dimethoxy-3',5-dihydroxy
flavone (22)
30
15
0
cpm
(x10^3)
CONTROL
4.1x10^-5
8.6x10^-5
0
30
60
Time (mins)

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