PHYTOCHEMICAL STUDIES ON CERTAIN SOUTH AFRICAN SPECIES OF THE RUTACEOUS GENERA *AGATHOSMA* WILLD. AND *ZANTHOXYLUM* L.

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by

NATHAN FINKELSTEIN M.Sc., M.P.S.

School of Pharmaceutical Sciences,
Rhodes University,
Grahamstown,
Republic of South Africa.

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With esteem and gratitude this work is dedicated

to

my friend and former mentor, John M. Calderwood.

His interest afforded me the opportunity
for academic advancement, while his inspiration evoked
in me a love for natural product chemistry.

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SUMMARY

Selected species of two South African rutaceous genera, *Agathosma* Willd. and *Zanthoxylum* L., were phytochemically studied. Two species of the former genus, *A. puberula* (Steud.) Fourc. and *A. clavisepala* R. A. Dyer, yielded a novel *O*-prenylcoumarin, puberulin, the structure of which was fully elucidated as 6,8-dimethoxy-7-prenyloxycoumarin. This represents the first report of a coumarin from that endemic genus.

In another study the alkaloids in three species of *Zanthoxylum*, *Z. davyi* (Verdoorn) Waterm., *Z. thorncroftii* (Verdoorn) Waterm. and *Z. humile* (E.A. Bruce) Waterm., were investigated. Chelerythrine, nitidine, (-)-α-*N*-methylcanadine, (+)-laurifoline and (+)-magnoflorine were isolated and characterized in the stem and root bark of *Z. davyi*, while the root bark of *Z. thorncroftii* contained skimmianine and decarine in addition to the alkaloids present in *Z. davyi*. Skimmianine, decarine, *N*-norchelerythrine, chelerythrine, (-)-α-*N*-methylcanadine, candicine and tembetarine were identified in the root bark of *Z. humile*. The chemotaxonomic significance of these alkaloids occurring in the South African taxa in relation to other African *Zanthoxylum* taxa, is discussed. Several non-alkaloids (lupeol, β-sitosterol and (-)-sesamin) were also isolated and characterized in *Z. davyi* and *Z. humile*. Horizontal cellulose column chromatography and low pressure liquid chromatography have been applied to the separation of quaternary alkaloids.

1. INTRODUCTION

In his closing address at the 1976 International Symposium on the Chemistry of Natural Products, Lord Todd quoted a suggestion made sixteen years earlier. He stated that the type of structural study applied to isolated plant products is no longer a spearhead in the advancement of the science of natural product chemistry, but this does not mean that such studies have lost their practical importance.[1] Although his prediction of a changing pattern of natural product chemistry is generally justified by current events, the field not only continues to develop, but has also retained its importance.

In recent years we have witnessed both the medical value and industrial stimulus provided by a natural alkaloid such as reserpine, isolated from plant material, which for long was used in oriental folk-medicine. It is more than likely that other compounds with meaningful and perhaps invaluable pharmacological properties will be obtained from plant materials, and that, incidentally, clues to some of them may still be found in the folk-medicine of primitive people.

Since several alkaloids of the Rutaceae exhibit promising activity against experimental neoplasms[2], the family has been, and still is, enjoying considerable attention. To phytochemists, the rutaceous plants are fascinating due to the structural diversity of their secondary products. Although the chemosystematic potential of secondary metabolites has been mentioned in several reviews[3-6], the emphasis in this regard has been somewhat modified by developments in the field of plant-animal interactions. There is now the growing realization that secondary compounds are of prime importance in relation to ecology. They are particularly significant in defence, provide the chemical signals of pheromonal interactions, contribute towards disease resistance and are involved in allelopathy[7].

A phytochemical study on certain endemic species obtained from two rutaceous genera was therefore prompted by the belief that such an investigation might yield yet further novel compounds of potential medicinal interest. In the present work, the isolation and characterization of many secondary metabolites from *Agathosma* Willd. and *Zanthoxylum* L. are described. The chemical data obtained from the South African species of *Zanthoxylum* are a further contribution to the chemosystematics of the genus in Africa.

1.1 The plant family Rutaceae

According to the system of Engler and Prantl[8] the family Rutaceae is included in the order Geraniales and in the sub-order Geraniineae. The Rutaceae are generally trees, shrubs or shrublets sometimes armed with prickles or spines and have simple or pinnate leaves which are dotted with odoriferous, pellucid glands[9,10].

The family numbers about 150 genera and approximately 900 species which are widely distributed in warm and temperate regions particularly in Africa and Australia. In Southern Africa the family is represented by some 21 genera and approximately 270 species[10]. The Rutaceae also contain genera having economic importance in horticulture (*Citrus* L.), silviculture (*Chloroxylon* Scop., *Flindersia* R.Br., *Zanthoxylum* L.) and medicine (*Pilocarpus* Vahl, *Agathosma* Willd.)[5].

From a phytochemical point of view, the Rutaceae abound with biogenetically fascinating secondary metabolites often presenting impressive structural versatility. At least twelve structural classes of alkaloids are recognized[11] though biogenetically these can probably be ascribed to tyrosine, phenylalanine, anthranilic acid,

tryptophan or histidine precursors[12]. Of these arbitrary classes at least four are found exclusively in this family. The coumarins, including furano- and pyranocoumarins are predominant among the metabolites of cinnamic acid, while essential oils of commercial value are found in at least four sub-families. A plethora of characteristic flavonoids (hesperidin and diosmin), phenolic compounds (chromones, chromenes, phloroglucin derivatives), tetra- and pentacyclic triterpenes and limonoids have also been isolated from many rutaceous genera[13].

In view of the interesting diversity of rutaceous secondary plant metabolites, plant extractives from two genera *viz.* *Agathosma* Willd. and *Zanthoxylum* L. have now been investigated for the presence of coumarins and alkaloids respectively.

1.2 The genus *Agathosma* Willd.

The genus, consisting of some 135 species, belongs to the sub-family Rutoideae and tribe Diosmeae. They usually occur as shrubs or undershrubs having simple gland-dotted leaves. The oil-glands in the leaf contain an essential oil which is so characteristically pungent in some species, that detection of the odoriferous shrub by the collector is facilitated. The genus is endemic to South Africa and species are found in the south-western Cape extending into Natal and Lesotho[10]. The preponderance of species occurs in the south-western Cape where their distribution extends from the coast to the summits of the highest mountains. Many species are restricted to certain soils, altitudes, aspects and conditions of dryness and moisture[14]. There are 12 species confined to the eastern Cape and a botanical key to these was recently devised on the basis of leaf characters[15].

Pillans, in a review of the genus, united the genera *Barosma* Willd. and *Agathosma* Willd. into one genus, preference being given to *Agathosma* since the characters which are used to separate these genera, (arrangement of leaves and position of flowers) are not constantly associated. By assigning preference to the larger genus *i.e. Agathosma*, changes in nomenclature will be largely minimized[14].

However, as a result of taxonomic uncertainty, some commercially important *Agathosma* species were again revised. This resulted in *A. crenulata* (L.) Pillans (consisting of *Barosma crenulata* (L.) Hook. and *Barosma serratifolia* (Curt.) Willd.) being sub-divided into *A. crenulata*(L.) Pillans *pro parte* and *A. serratifolia* (Curt.) Spreeth *comb. nov.*, while *A. betulina* (Berg.) Pillans is retained in accordance with the revision of the genus by Pillans[16].

Buchu leaf, obtained from certain commercial *Agathosma* species, has been widely used ethnobotanically as a brandy tincture or vinegar for curing diseases of the kidney and urinary tract, in the local treatment of bruises and for the relief of rheumatic pains. Many ethnic uses have been reported for other *Agathosma* species[17]. An investigation of this genus for compounds of medicinal interest therefore seemed feasible.

1.2.1 Phytochemical Review

1.2.1.1 Volatile oil

Most of the phytochemical research on this genus has been confined to investigations of the essential oil components, because the oil enjoys economic importance in the manufacture of synthetic flavourings and in medicine.

1.2.1.2 Terpenoids

The early investigations[18,19] of buchu leaf oil revealed the presence of (+)-limonene, (-)-menthone, diosphenol ("buchu camphor") and (-)-pulegone.

An investigation of a commercial oil published some ten years ago[20], reported the presence of the following terpenoids, which were isolated by a combination of fractional distillation and preparative gas chromatography and identified both spectroscopically and by gas chromatographic comparison with authentic materials: α-pinene (0,5%), myrcene (1%), (+)-limonene (10%), *p*-cymene (0,5%), (+)-menthone (9%), (-)-isomenthone (35%), (+)-isopulegone (3%), (-)-iso-isopulegone (3%), terpinenol-4 (0,5%), (-)-pulegone (11%), (±)-piperitone epoxide (9,5%), diosphenol (12%) and traces of camphene, β-pinene, α-terpinene, and γ-terpinene. The monocyclic terpenes were particularly intriguing as they belonged to the stereochemical series of (+)limonene compounds which, at that stage, were rare. This work repudiated Kondakow's results[18] in that the oil does not contain (-)-menthone, and further questioned the status of diosphenol as a genuine constituent of the leaves, since it was known that piperitone epoxide can rearrange into diosphenol on acidification —— conditions readily fulfilled during steam distillation of plant material.

Kaiser *et al.*[21] subsequently verified the occurrence of six major and ten minor constituents cited in the earlier literature, but could not detect the presence of piperitone epoxide. These workers demonstrated convincingly that a diethyl ether extract of the leaves contained ψ-diosphenol as well as diosphenol in the same ratio as found in steam-distilled oil, thereby discounting the suspicion that diosphenols might be artefacts formed from piperitone epoxide during oil production.

By means of a sophisticated GLC-MS coupling system, they additionally identified[21] in buchu leaf oil a total of 123 compounds consisting of monoterpene hydrocarbons (17), monoterpene oxides (7), monoterpene alcohols (24), monoterpene esters and lactones (12), monoterpene aldehydes (2), monoterpene ketones (10), bifunctional monoterpene ketones (30) consisting of sulphurated and oxygenated derivatives of *p*-menthan-3-one, sesquiterpenes (6), aliphatic compounds (4), cycloaliphatic compounds (3), aromatic compounds (6) and heterocyclic compounds (2).

The oils obtained from four eastern Cape *Agathosma* species were analysed by gas chromatography using polar and non-polar phases[22]. The following major terpenoids were tentatively identified in *A. apiculata* G.F.W.Mey, *A. ovata* (Thunb.) Pillans, *A. clavisepala* R.A.Dyer and *A. puberula* (Steud.) Fourc. by their gas chromatographic retention times *viz.* α-pinene, β-pinene, myrcene, limonene, ocimene, linaloöl, caryophyllene, isopulegol and terpineol. With the exception of β-pinene in the case of *A. ovata* oil, the percentage compositions are in reasonable agreement for the two columns used.

Recently a preliminary gas chromatographic analysis[23] of the oils of *A. barosmaefolia* Eckl. & Zeyh., *A. bisulca* (Thunb.) Bartl. & Wendl.*f*., *A. capensis* (L.) Duemmer, *A. ciliaris* (L.) Druce, *A. imbricata* (L.) Willd. and *A. cerefolium* (Vent.) Bartl. & Wendl.*f*. was carried out on a G.E.S.E. 30 column. Peaks were tentatively identified by relative retention times using camphene and anethole as internal standards. Some of the major components were then separated on a S.E. 30 preparative column, collected and identified by spectral methods (IR PMR and MS).

Although most of the essential oils yielded mixtures of terpenoids commonly encountered in the genus, several oils contained very high proportions of one or two components and these may in time attract

commercial interest. For example, the oil from *A. cerefolium* consists chiefly of anethole (61,7%) and estragole (24,6%), while the oils from *A. capensis*, *A. ciliaris* and *A. imbricata* contain large amounts of linaloöl (31,8%; 51,5%; 52,9%).

In spite of Pillans[14] reducing *A. barosmaefolia* to synonymy under *A. bisulca*, there are marked differences in both the yield and composition of their essential oils. *A. barosmaefolia*, yielding 1,3% of oil, contains α-pinene, β-ocimene, γ-terpinene, terpinen-4-ol and α-terpineol —— terpenoids not reported from *A. bisulca* oil, while the essential oil (yield 0,2%) of *A. bisulca* contains 1,8- cineol, linaloöl, anethole and safrole which are not present in the oil of *A. barosmaefolia*. Although methyleugenol and trimethoxyallylbenzene are the main components of the oils of both species, *A. barosmaefolia* contains more of these components than *A. bisulca*. These differences may yet prove to have some chemotaxonomic significance provided that ecological parameters are also taken into consideration.

1.2.1.3 Sulphur compounds

Sulphur compounds in the form of *S*-thioesters have only recently been recognized as a potentially important class of natural sulphur compounds. These have been shown to occur in the plant genera *Ferula* L. and *Agathosma* Willd. as well as in the marine algal genus *Dictyopteris* Lamx.[24].

Early investigations of the essential oils of *A. apiculata* G.F.W.Mey and *A. puberula* (Steud.) Fourc. showed these to contain 8-11% and 2,5% of sulphur respectively. The sulphur content of the former species was considered on very tenuous evidence to be a mixture of butyl 1-pentenyl disulphide and an unsaturated ester, $C_{10}H_{16}O_2$, each constituting 30% of

the oil, whereas the sulphur component of the latter species was described as bis(1-pentenyl-2-)tetrasulphide[25,26].

Recently Rivett[27] corrected these previous reports and established the main sulphur component of these two species to be an *S*-thioester the structure of which was established, by spectral evidence and synthesis, to be *S*-prenyl thioisobutyrate. This compound also occurred in the oil of *A. clavisepala* R.A.Dyer, but was absent in that of *A. ovata* (Thunb.) Pillans. As energy-rich prenyl compounds enjoy prominence in the biosynthesis of isoprenoids, the isolation of this *S*-thioester suggests that high-energy *S*-prenyl thioesters may occur more abundantly in Nature[22].

During the investigation by Kaiser *et al.*[21], a very interesting odour complex was isolated and shown to be a mixture of two diastereoisomeric ketothiols *viz.* 8-mercapto-*p*-methan-3-ones. These were the first terpenoid ketothiols to be found in Nature and were shown to be derived from (-)-pulegone[28,29]. Two diastereoisomeric pairs, closely related to 8-mercapto-*p*-menthan-3-ones, *viz.* 8-acetylthio-*p*-menthan-3-one and 8-methylthio-*p*-menthan-3-one were found in Nature for the first time. In addition to the aforementioned thio-compounds, four other sulphur-containing terpenoids were detected of which one was positively identified as 8-acetylthiopiperitone. The remaining three occurred in small quantities and are probably diosphenol derivatives. The biogenetic pathway by which the diastereoisomeric *p*-menthan-8-thiol-3-ones accompanying (-)-pulegone and other monoterpenoid ketones in Buchu leaf oil are produced, would certainly evoke much interest[24].

1.2.1.4 Coumarins

The coumarins are typical compounds of the Rutaceae and occur in

many genera and species belonging to the sub-families Rutoideae, Toddalioideae, Aurantioideae and Flindersioideae. Almost 200 of the 600 plus coumarins reported from natural sources occur in members of the Rutaceae and all, with one possible exception, appear to be biogenetically derived from cinnamic acid[30]. There seems to be a tendency for rutaceous coumarins to be substituted with isoprenoid moieties which are sometimes converted to furano- and pyranocoumarins.

In a comprehensive review of the coumarins in the Rutaceae, Gray and Waterman[30] list the known sources within the family. The coumarins thus listed in some 45 rutaceous genera, were extended by a recent report[23] from two further genera *viz.* *Diosma* L. and *Phyllosma* H.Bolus *ex* Schltr. (sub-family: Rutoideae).

Four coumarins were isolated from *D. acmaeophylla* Eckl. & Zeyh. and were identified as 6,7-dimethoxycoumarin, 7,8-methylenedioxycoumarin, 7-*O*-(3,3-dimethylallyl)coumarin and 6-methoxy-7-*O*-(3,3-dimethylallyl)-coumarin. *D. pilosa* I.Williams was reported to contain two coumarins, one of which was identified as 5,6,7-trimethoxycoumarin. Work on the structure of the second coumarin is still in progress.

In the present work an *O*-prenyl coumarin, puberulin, was isolated from *A. puberula* (Steud.) Fourc. and *A. clavisepala* R.A.Dyer. The structure was established by spectral evidence and synthesis to be 2*H*-1-benzopyran-6,8-dimethoxy-7-(3'-methyl-2'-butenyloxy)-2-one or 6,8-dimethoxy-7-prenyloxycoumarin. This is the first report of a coumarin from the endemic genus *Agathosma*[31].

A novel coumarin, capensin, has subsequently been isolated from *Phyllosma capensis* H.Bol. and its identity established spectrally and chemically as 8-hydroxy-6-methoxy-7-prenyloxycoumarin. Methylation of capensin with diazomethane yielded puberulin[23]

1.2.1.5 Alkaloids

The only occurrence of an alkaloid from this genus was a report[32] of the furoquinoline alkaloid, skimmianine, in *Agathosma virgata* (Lam.) Bartl. & Wendl. *f*. Just recently, however, the same alkaloid, so ubiquitous in the rutaceous genera, was also found in *A. capensis* (L.) Duemmer and *A. bisulca* (Thunb.) Bartl. & Wendl. *f*.[23]

1.2.2 Chemotaxonomic aspects

Two commercially important buchu species, *A. betulina* (Berg.) Pillans and *A. crenulata* (L.) Pillans, commonly referred to as round-leaf and oval-leaf buchu respectively, were previously distinguished on the basis of leaf form only[14]. Since the establishment of extensive buchu plantings in the western Cape, considerable hybridization has occurred resulting in plants with intermediate leaf forms. A taxonomic problem of distinguishing between these two species by visual identification is therefore evident. Important economic implications are also involved, because the round-leaf species, commanding higher prices, is preferred by overseas buyers[33].

In an effort to positively identify the two buchu species, oil distillates from the leaves of 24 individual plants were analysed by gas-liquid chromatography and u.v. spectrophotometry[33]. Only oil originating from the round-leaf buchu and containing ψ-diosphenol and diosphenol in reasonable quantities, shows a u.v. absorbance peak at 272 nm. By contrast, oil from the oval-leaf buchu which contains onl trace amounts of diosphenols, shows a characteristic peak at 252 nm. This absorption is identical with that of pulegone, which in turn is the major constituent in both species. Whereas the presence of diosphenols in round-leaf buchu seemed to be a reliable criterion for

distinguishing botanically between the two species of commerce, this does not hold true for hybrid buchu. Chemical analyses of the oil indicated that hybrid buchu contained all the major components including both the diosphenols occurring in round-leaf oil, albeit in lower concentration. Although the main components of the oil of both species are qualitatively similar, there are striking quantitative differences, including a large variation in the percentage of oil obtained from individual plants (4,0%-1,4%).

These chemical studies clearly indicate that it is important in future to identify and propagate only those plants with both a high oil and diosphenol content; hence a more standardized product of high quality could consequently be available to buyers and consumers. As buchu plants cannot be propagated vegetatively, only seed from chemically selected plants, grown in isolated localities, should be made available to growers[33].

Following work by Spreeth, the commercially important species of *Agathosma* were sub-divided into three species. Although marked differences in leaf morphology are used in the key to distinguish between these species, a biochemical investigation of the leaves for amino acids, sugars and organic acids was conducted for chemotaxonomic verification. While no differences were noted with respect to succinic acid and citric acid, a semi-quantitative determination of the malic acid content showed wide differences. It was conceded that there is a greater difference between the two taxa combined by Pillans[14] than between *A. betulina* (Berg.) Pillans and *A. serratifolia* (Curt.) Speeth. The malic acid content of the leaves of *A. crenulata* (L.) Pillans (excluding *A. serratifolia*) renders this species clearly distinguishable from the other two species[16].

By reference to the chemical composition of the oil, *A. betulina* is immediately distinguishable from the other two species in terms of the content of pulegone and diosphenols. There are, however, no noteworthy distinguishing differences in the oils of the other two species[16].

In this work, four eastern Cape species of *Agathosma* were investigated for coumarins. The coumarin, puberulin, only occurred in two species *viz. A. puberula* (Steud.) Fourc. and *A. clavisepala* R.A. Dyer[31]. As further species of this genus are surveyed, the presence of coumarins could likewise provide additional chemical criteria for consideration in taxonomic problems.

1.3 The genus *Zanthoxylum* L.

According to Engler's system[8], the closely related genera *Fagara* L. and *Zanthoxylum* L. belong to the sub-tribe Euodiinae, tribe Xanthoxyleae (Zanthoxyleae) and sub-family Rutoideae.

Nomenclature: The confusion in nomenclature between *Zanthoxylum* L. and the closely allied genus *Fagara* L., seems to have been settled after a chemotaxonomic assessment of the secondary metabolites of taxa from both genera[4]. On the chemical evidence available, *Fagara* L. has now been put into synonymy under *Zanthoxylum* L.[34] In order to further rationalize the genus, Waterman corrected several nomenclatural errors and proposed new combinations for most of the well-known taxa[34,35].

In South African botanical circles, the confusion in nomenclature has also largely been resolved; however, an authoritative publication on the genera of Southern African plants has retained *Fagara* L.[10] A later work[36] also conserves this genus as *Fagara* L., though an editor's note draws the reader's attention to Waterman's publication

where *Fagara* L. was put into synonymy under *Zanthoxylum* L.[34] It can therefore be assumed that had the book been compiled subsequent to the revision, the formerly conserved *Fagara* L. would have been replaced by *Zanthoxylum* L.

South African species: *Zanthoxylum* L. in which is included *Fagara* L., is a large genus consisting of about 255 species[4] and occurs in Africa to the East Indies and the Americas[10]. This pan-tropical genus is represented in Africa by some 40 species[6] while in South Africa six species are recognized *viz.*:

1. *Zanthoxylum capense* (Thunb.) Harv.[37] (= *Fagara capensis* Thunb.)
   (= *Fagara armata* Thunb.)
   (= *Fagara multifoliolata* Engl.)
   (= *Fagara magalismontana* Engl.)
   (= *Xanthoxylon** *capense* Harv.)
   (= *Xanthoxylon** *thunbergii* DC. var. *obtusifolia* Harv.)
2. *Zanthoxylum davyi* (Verdoorn) Waterm.[34] (= *Fagara davyi* Verdoorn)
   (= *Xanthoxylon** *thunbergii* DC. var. *grandiflora* Harv.)
3. *Zanthoxylum humile* (E.A.Bruce) Waterm.[34] (= *Fagara humilis* E.A.Bruce)
4. *Zanthoxylum leprieurii* Guill. & Perr.[34,36] (= *Fagara leprieurii* (Guill. & Perr.) Engl.)
5. *Zanthoxylum thorncroftii* (Verdoorn) Waterm.[34] (= *Fagara thorncroftii* Verdoorn)

+6. *Zanthoxylum ovatifoliolatum* (Engl.) Finkelstein *comb. nov.*[38] (= *Fagara ovatifoliolata* Engl.)
   (= *Zanthoxylum citriodorum* Welw. *ex* Hiern)

* Variant spelling of *Zanthoxylum*

\+ Occurs in the mandated territory of South West Africa - to be known as Namibia after independence.

Morphology: The genus *Zanthoxylum* L. is distinguished from other rutaceous genera by the large and small prickles on the stem, leaf stalks and sometimes flower stalks, the pinnate leaves, unisexual flowers, 4 stamens and globose, capsular fruit[10].

The species in this genus occur as deciduous or evergreen trees or shrubs, the stems of which usually possess conspicuous woody or corky bosses (knobs) and are aculeate. The lemon-scented leaves are alternate, compound, imparipinnate, the leaflets being 3-9-jugate with scattered aromatic, translucent glands. The inflorescences of unisexual flowers are arranged in terminal or axillary panicles on the plants which are monoecious or dioecious. The ovary, vestigial in the male flowers, is raised on a short basal gynophore and is obliquely obovate, with 2 sutures distinct, unilocular, with 2 apical ovules and immersed glands in the case of the female flowers. The simple style, shorter than the ovary, is obliquely inserted with the stigma capitate[10,36].

Botanical keys: There are several keys[9,36,39] to the species based on the number of petals in the flowers, the shape, apex and number of lateral veins in the leaflets or the position of the pellucid glands. Von Breitenbach[9] uses the pentamerous flowers of *Z. humile* as a means of distinguishing this species from the tetramerous flowers purported to occur in the flowers of *Z. davyi*, *Z. thorncroftii* and *Z. capense*. Most keys distinguish *Z. capense*, *Z. davyi* and *Z. leprieurii* morphologically on the basis of their leaflets[9,36,39]. The first species has ovate, elliptic or obovate leaflets with an obtuse or rounded apex and 4-8 pairs of lateral veins, whereas the second species has lanceolate, oblong or narrowly elliptic leaflets with a tapering apex and 16-20 pairs of lateral veins. In contrast to the other species

of *Zanthoxylum*, the leaflets of the third species (*Z. leprieurii*) are opposite or subopposite, ovate to elliptic in shape and have an apex attenuate to form a slender tip. Unlike *Z. capense* and *Z. davyi*, which have their pellucid glands confined along the leaf margins only, the whole surface of the leaflets of *Z. leprieurii* is dotted with these translucent glands.

Verdoorn[40] separated *Z. thorncroftii* from *Z. capense* and *Z. davyi* by the inflorescences of the former bearing both male and female flowers while the inflorescences of the other two species are reputed to have flowers of one sex only. Von Breitenbach[9] acknowledges the existence of *Z. thorncroftii* and distinguishes this species from *Z. capense* by the shape of the apices of their leaflets. The former species is reported to have a subacuminate and retuse apex whereas that of the latter is obtuse or rounded. Palmer and Pitman[39], however, suggest that *Z. thorncroftii* might prove to be inseparable from *Z. capense* and Palgrave's recent work has reduced these two species to synonymy[36]. The Botanical Research Institute has not unreservedly accepted Palgrave's work and since this uncertainty has arisen, Dr. I.C.Verdoorn and the staff of B.R.I. are re-examining the taxonomy of *Z. thorncroftii* with a view to seeking more reliable morphological features to justify its continued status or reduce it to synonymy as suggested by other workers.

Plant habit and Southern African distribution: *Z. capense* (Small Knobwood; Woodland Knobwood) occurs as a shrub or much-branched tree up to 10 m in height and is found in a variety of habitats ranging from dry woodland or bush, rocky hill-slopes at higher altitudes among rocky outcrops and even into the mist-belt. It is distributed from the southern Cape coast (Knysna) eastwards through Transkei, Natal and Swaziland, the central, northern and eastern Transvaal and southern

Mozambique to Rhodesia[9,36,39].

Z. davyi (Forest Knobwood) is a tree 8-30 m in height and is a fairly common species in montane forest. It occurs in the eastern forest belt, extending northwards from the Cape Midlands through Transkei, Natal, Swaziland, eastern and northern Transvaal to Rhodesia[9,36].

Z. humile is a shrub or small tree 2-3 m in height. This species was originally collected in the Kruger National Park but also occurs in open woodland in the north-eastern Transvaal as well as in Mozambique and Rhodesia[9]. It is closely allied to *Z. capense* with respect to the small leaflets, nevertheless it is easily distinguished from this species by its dwarfer habit, pubescent branches and leaf rhachides, dull-surfaced leaves and fasciculate inflorescences[41].

Z. thorncroftii is a small tree about 7 m high and occurs in the mist-belt forest region of the eastern Transvaal at an altitude of 1300 m[9].

Z. leprieurii (Sand Knobwood) is a small to large tree depending upon its habitat, being 5-10 m in height, but reaching 15-20 m in forests. It occurs in isolated populations as part of the under-storey in evergreen forest, at forest margins, at low altitudes in hot, dry alluvial soils, in river valleys, near pans often on termite mounds or in sand forest near the coast. This species is distributed in northern Zululand, north-eastern Transvaal and Rhodesia. In South Africa it is a protected plant[36].

Z. ovatifoliolatum is a shrub or tree about 3 m in height having curved thorns on the twigs and leaf rhachides. It occurs in northern South West Africa (Namibia), the type species having been collected in Angola[42].

Economic botany: The bark (both stem and root), leaves, and fruit of the plants represented by species of *Zanthoxylum* have been widely used in folklore medicine. The diversity of these ethnobotanical uses has been summarized in an earlier work[43]

The wood of *Z. capense* is light yellow, very close-grained, compact and heavy (density 0,94). Though the timber is not very durable, it makes good yokes as well as axe- and pick-handles[9]. By contrast, the light-yellow wood from *Z. davyi* has a fairly pronounced grain and a fine texture. It is hard, strong and heavy (density 0,80) and is one of the best of the indigenous woods with respect to elasticity and bending properties. The wood polishes well and is used as handles for various tools and implements, walking sticks and is reputed to make good fishing rods[9,39].

Z. leprieurii from Nigeria has been investigated phytochemically[3,6] but material from the South African counterpart is not readily available since this species is a protected plant[36]. The collection of plant material from *Z. ovatifoliolatum* is presently rather hazardous since it occurs in the military operational area of South West Africa. *Z. capense* had been studied previously[44,45] and these phytochemical studies were further extended[3,46].

In view of the foregoing, it was therefore decided to chemically investigate the three remaining South African species *viz. Z. humile*, *Z. thorncroftii* and *Z. davyi* in order to supplement the existing data on the secondary metabolites of *Zanthoxylum* species as well as possibly utilize this chemical information chemotaxomically to clarify the position of *Z. thorncroftii*.

1.3.1 Phytochemical Review

Phytochemical studies on the genus *Zanthoxylum* L. have shown that it abounds with fascinating chemical compounds covering a wide range of skeletal patterns *e.g.*, alkaloids, coumarins, lignans, triterpenes and other secondary metabolites. Although experimental biosynthetic work on *Zanthoxylum* has been minimal[47], most of the probable biosynthetic pathways are known as a result of investigations of other rutaceous plants. The secondary metabolites of the genus are basically derived via three pathways involving anthranilic acid, tyrosine and cinnamic acid and have a common precursor in chorismic acid[4].

In 1970 a limited review of the alkaloids isolated from *Zanthoxylum* L. appeared[48]. Hegnauer[13] later dealt with the chemotaxonomy of the Rutaceae which was followed by several comprehensive reviews on the alkaloids[5,12,49] and coumarins[30] present in this family. Joshi[50] also reviewed the alkaloids, terpenoids, flavonoids and coumarins of some Indian plants belonging to the Rutaceae.

These reviews have recorded the phenomenal progress that has occurred in the phytochemistry of the secondary rutaceous metabolites by listing not only the new compounds isolated, but also covering their distribution in the family. To avoid unnecessary repetition, it was deemed prudent in this work to present only information pertaining to the genus *Zanthoxylum* which was not covered in the reviews. Such information should therefore be regarded as being complementary to the existing reviews and will be discussed under appropriate headings.

1.3.1.1 Alkaloids

Several quinoline alkaloids were isolated from the leaves (0,1%) of *Z. mayu* Bertol. *ex* Hook. and Arn., a tree found in the rain forest

of Robinson Crusoe Island (Juan Fernández Group), and were identified as the 2-quinolone, (-)-edulinine, the furoquinoline, skimmianine, and the linear pyranoquinolone alkaloid, ribalinine. *Z. mayu* seems to be the only member of the genus which to date is able to synthesize a linear pyranoquinolone alkaloid; a characteristic not surprising since this species is morphologically somewhat removed from the rest of the genus having been placed by Engler in the monotypic section, *Mayu*. Although the alkaloids were extracted under weakly basic conditions, the possibility does exist that (-)-edulinine could be an artefact formed by alkali attack on the pyranoquinolone alkaloid[51]. In a recent personal communication[52], it is reported that in addition to certain alkaloids known to be present in *Z. mayu*, the benzophenanthridine bases, bocconoline and 11-(2'-ketobutane)-dihydrochelerythrine, have also been isolated.

Since *Zanthoxylum* species of the West Indies and Central America have not been intensively studied, Stermitz and Sharifi[53] investigated two Puerto Rican species *viz.* *Z. monophyllum* P. Wilson belonging to the American subsection *Neogaeae* Engl. section *Macqueria* and *Z. punctatum* Vahl placed in the section *Tobinia* Desv. Whilst a few species of the former subsection have been chemically studied, *Z. punctatum* is the first species in the latter section to be investigated.

The stems and branches of *Z. monophyllum* contained berberine as the major alkaloid along with a novel pyrano-2-quinolone alkaloid, zanthophylline, and its demethyl analogue. In contrast to the linear pyrano-2-quinolone isolated from *Z. mayu*, zanthophylline is an angular pyrano-2-quinolone and contains a rare acetoxymethyl grouping which has only been reported once before from another rutaceous plant, *Boronia ternata* Endl. Although the authors asserted that this was

the first report of an angular pyran-2-quinolone from *Zanthoxylum*, Hostettmann *et al.*[54] in fact had previously isolated *N*-methylflindersine, also an angular pyrano-2-quinolone, by preparative liquid chromatography from *Z. chalybeum* Engl. The *N*-demethyl analogue is interesting biogenetically. *N*-demethylation of quinolones apparently proceeds through conversion of N-Me to N-CH_2OH especially if acid is used during the isolation. In this analogue, it is apparent that acetylation had intercepted the intermediate leading to *N*-demethylation.

The ground stems and branches of *Z. punctatum* yielded two relatively common quaternary aporphine alkaloids, (+)-*N*-methylcorydine and magnoflorine. A benzophenanthridine base, assigned the trivial name punctatine, was also isolated and from spectral data it was suggested that it possesses one hydroxyl and three methoxyl groups in a chelerythrine-sanguinarine type arrangement[53].

Although the stem bark of *Z. elephantiasis* Macfad., a tree indigenous to the Caribbean and Central America, had been previously subjected to chemical investigation, Fish *et al.*[55] decided to study the root bark since experience of this genus has shown that the root bark alkaloidal yields are greater than those of the stem bark. The root bark extracts yielded canthin-6-one, candicine, laurifoline, (all previously reported from the stem bark) chelerythrine chloride and dihydrochelerythrine. The presence of two further alkaloids, tembetarine and magnoflorine, was demonstrated by thin-layer chromatography.

In an effort to identify the minor components in the root bark of *Z. rubescens* Planch. *ex* Oliv., Fish *et al.*[56] isolated and identified the benzophenanthridine base, dihydrochelerythrine. No alkaline conditions prevailed during the isolation of this base and

hence there is no possibility of it being an artefact of chelerythrine. Horstettmann *et al.*[54] also avoided the use of alkali during their isolation of the same alkaloid from another African species, Z. *chalybeum* Engl.

Subsequent work on the root bark of Z. *rubescens* by Dadson and Minta[57] resulted in the isolation, from the light petroleum extract, of *N*-(3,4-methylenedioxyphenethyl)-cyclopropanecarboxamide which was assigned the trivial name of rubesamide. The identity of this amide was determined spectrally and was confirmed by unambiguous synthesis.

A comparative study of the alkaloids in three African *Zanthoxylum* species[6] showed that dihydrochelerythrine was also present in Z. *leprieurii* Guill. and Perr. Since none of the samples of Z. *rubescens* could be shown to contain the previously reported acridone or furoquinoline alkaloids, the Nigerian material previously investigated as Z. *rubescens* was probably Z. *leprieurii*.

Further studies on the constituents of Z. *tsihanimposa* H. Perr., a plant growing in Madagascar (Malagasy Republic), showed that the trunk bark contains (+)-tembetarine as the major alkaloid. The furoquinolines, skimmianine and γ-fagarine, as well as the benzophenanthridines, chelerythrine, *O*-demethylchelerythrine and *O*-demethylnitidine, were also isolated. The latter alkaloids were isolated with difficulty due to their transformation at C-6 during extraction and purification[58].

Chemical work done on the bark of Z. *tingoassuiba* L. showed the presence of 1-hydroxy-2,9,10-trimethoxyaporphinium chloride and a weakly potassium iodoplatinate - positive compound, which was shown to be tembamide[59]. This amide has only been isolated previously in Z. *conspersipunctatum* Merr. and Perry[60], Z. *hyemale* St.-Hil.[61a] and Z. *ocumarense* (Pittier) Steyerm.[62]

Tiwari and Masood[63] chemically examined the roots of the Himalayan plant *Z. oxyphyllum* Edgew. the stem bark of which had been examined by other workers and found to contain rhetsinine. They isolated two aporphine alkaloids, corydine and zanthoxyphylline, and assigned the tentative structure of 4,5,6-trimethoxy-*N*:*N*-dimethylaporphinium hydroxide to the latter base.

Further studies on the chemical constituents of the stem and root barks of *Z. arnottianum* Maxim.[64] yielded six known alkaloids *viz.* chelerythrine chloride, *N*-demethylchelerythrine, oxychelerythrine, decarine, arnottianamide and skimmianine. A new phenolic amide alkaloid (m.p. 271°-3°) named iwamide was also isolated and its structure determined spectrally as well as by synthesis from decarine[65].

The chemical studies on two Asian *Zanthoxylum* species seem to have been inadvertently omitted from the alkaloidal reviews[12,49], consequently these are now included for the sake of completeness. The stem bark and root of *Z. piperitum* DC. yielded skimmianine, γ-fagarine, menisperine, magnoflorine and laurifoline[66,67], while the wood of the Formosan rutaceous plant, *Z. cuspidatum* Benth.[68], contained dictamnine, skimmianine, robustine, haplopine, γ-fagarine and nitidine chloride. The bark of the latter species also yielded nitidine chloride as well as oxynitidine, de-*N*-methylavicine, arnottianamide, isoarnottianamide and an unknown base (as chloride) with a m.p. 268°-272°.

1.3.1.2 Lignans

These compounds, thought to be biogenetically derived from the oxidative coupling of two *p*-coumaric acid units or more highly substituted cinnamic acid units, have been reported from several

Zanthoxylum taxa. Lignans of the asarinin type appear to be quite common, particularly in African taxa, but they also occur in Asian, Australian and American taxa. In a review on the chemosystematics of the *Zanthoxylum/Fagara* complex, the distribution of lignans known at that time was reported in tabular form[4]. Since then, several new reports have appeared and these are summarized in Table I.

TABLE I: Lignans occurring in *Zanthoxylum* species

Species	*Plant Part(s)	Lignan(s)	Ref.
Z. *acanthopodium* DC.	B	(+)-sesamin	69
		(±)-fargesin	69
		(±)-eudesmin	69
		(-)-acanthotoxin	70
Z. *acanthopodium* DC.	B,S	(+)-sesamin	71
		(±)-eudesmin	71
		(+)-epieudesmin	71
		syringaresinol	71
	B	(+)-episesamin	72
		(±)-methyl piperitol	72
Z. *ailanthoides* Sieb. & Zucc. (including Z. *inerme* Koidz.)	W	(±)-syringaresinol	73
Z. *alatum* Roxb.	B,S	(+)-sesamin	71
		(+)-fargesin	71
		eudesmin	71
		epieudesmin	71
Z. *arnottianum* Maxim.	R	(-)-asarinin	74
		syringaresinol	74
	R,B	(-)-sesamin	64
Z. *conspersipunctatum* Merr. & Perr.	B	(+)-sesamin	60
Z. *decaryi* H. Perr.	B	(-)-asarinin	75
Z. *dinklagei* Waterm.	R,B	(+)-sesamin	76
Z. *leprieurii* Guill. & Perr.	R	sesamin	6
Z. *oxyphyllum* Edgew.	B,S	sesamin	71
		eudesmin	71
		epieudesmin	71
		syringaresinol	71
Z. *piperitum* DC.	B	(-)-xanthoxylol	67
		(-)-xanthoxylol γ,γ-dimethylallyl ether	67
		(-)-piperitol	67
		(-)-piperitol γ,γ-dimethylallyl ether	67
		(-)-sanshodiol	67
		(-)-asarinin	67
	B,R	(-)-sesamin	66,67
Z. *rubescens* Planch. *ex* Oliv.	R	(-)-sesamin	56

*B = stem bark; S = branches and stems; R = root bark or root; W = wood.

1.3.1.3 Miscellaneous Compounds

Coumarins: Although simple cinnamic acid derivatives *e.g.* the prenylpropanoid, cuspidiol, and parvifloral have been reported from *Zanthoxylum*, the coumarins preponderate among cinnamic acid metabolites encountered in this genus[4,30]. Since Waterman & Gray's extensive review[30] of the coumarins in the Rutaceae, no additional reports of these compounds from *Zanthoxylum* have appeared.

Flavonoids: Morita reviewed the distribution of flavonoids from the results of studies covering 1100 plant species occurring in *Zanthoxylum* and three other genera[77]. It seems that hesperidin and diosmin are the most characteristic flavonoids in this genus[4].

Resulting from work by Chatterjee *et al.*[78], the doubt concerning the structures of tambuletin and tambulin has been satisfactorily resolved. Tambulin was isolated from Z. *alatum* Roxb. seeds and Z. *acanthopodium* DC. defatted fruits and shown to be a herbacetin derivative. This new evidence is contrary to that of Harborne *et al.*[79] who postulated that tambulin belonged to the gossypetin series. Tambulol, previously reported by Bose and Bose[80], was also shown by Chatterjee *et al.* to be identical to tambuletin and its structure proposed as the 8-glucoside of gossypetin 7,4'-dimethyl ether[78].

Terpenoids: The major monoterpene constituents reported from the volatile oils of the *Zanthoxylum/Fagara* complex are summarized in tabular form in Waterman's review[4]. A rare, new monoterpene triol has since been reported to occur in the volatile oil of Z. *budrunga* Roxb. fruits. From spectral data and synthetic experiments, this triol was assigned the structure as 1S, 2S, 4S-trihydroxy-*p*-menthane[81].

A preparative liquid chromatographic investigation of the bark of Z. *chalybeum* Engl. showed the presence of a sesquiterpene,

germacrone[54]. The blossoms, leaves, pericarp and seeds of another African tree, *Z. decaryi* H. Perr., were analyzed for the chemical composition of its essential oils. α-Pinene(I) linaloöl(II) and eucalyptol(III) were identified though the major component in the blossoms was citronellol acetate(IV). Compound II predominated in the leaves (56%) followed by caryophyllene epoxide (6,5%), III (6%), 4-hydroxyterpinene (5%), I (4%), IV (4%), β-pinene (3%) and α-terpineol (1,5%) while compound IV was the main component of the seeds[82].

The distribution of the pentacyclic triterpene, lupeol, and the tetracyclic phytosterol, β-sitosterol, probably does not warrant reviewing because of their ubiquity in Nature. However, a novel pentacyclic triterpenoid ketone, xanthoxylone, has been isolated from *Z. rhetsa* DC. and its structure and stereochemistry determined[83], while β-amyrenone and β-amyrin were reported from the stem bark of *Z. acanthopodium* DC.[84]

Anthraquinones: Among the unusual compounds recently detected in *Zanthoxylum* species, were compounds showing characteristics for condensed aromatics and which did not appear to belong to any of the structural classes already known in the Rutaceae. The anthraquinones, 1,8-dihydroxy-6-methoxy-3-methylanthraquinone and barbaloin, were isolated from *Z. acanthopodium* DC. Whilst anthraquinones are fairly widely distributed in both higher and lower plants, no anthraquinone had previously been isolated or characterized from the Rutaceae or for that matter from *Zanthoxylum*[85].

1.3.2 Previous phytochemical studies on South African *Zanthoxylum* species

During a phytochemical investigation of *Z. capense*, the following

compounds were isolated and characterized: the benzophenanthridine alkaloids, chelerythrine and nitidine, the furoquinoline alkaloid, skimmianine[44], the tetahydroprotoberberine alkaloid, *N*-methyltetrahydropalmatine[45], and the lignan, (+)-sesamin[46]. A further chromatographic and electrophoretic examination of the same species demonstrated the presence of quaternary alkaloids which included candicine, tembetarine, magnoflorine, *N*-methylcorydine and/or *N*-methylisocorydine[3].

The remaining South African *Zanthoxylum* species have, however, been subjected to rather superficial investigation. A survey of several indigenous plant families for alkaloids incorrectly indicated the absence of alkaloids in Z. *capense* while Z. *thorncroftii* and Z. *davyi* were stated to give positive alkaloidal tests[32]. It was further reported that the bark of the last-mentioned species contains a resin[86].

In a preliminary investigation[87], the hexane and ether extracts of the trunk bark, sapwood and heartwood of Z. *davyi* were shown to give a positive Liebermann-Burchard test for tetra- and pentacyclic triterpenes. The acetone and methanol extracts both exhibited strongly positive alkaloidal reactions and were also positive to Shinoda's test for flavones, flavonols, xanthones *etc.*

In the present work, bark from Z. *davyi*, Z. *thorncroftii* and Z. *humile* was examined phytochemically and many compounds, *inter alia* tertiary and quaternary alkaloids, a triterpene, a phytosterol, and a lignan were isolated and their structures confirmed spectrally and/or chemically.

1.3.3 Medicinal properties

As a result of secondary metabolism within the plant, Nature

successfully provides an impressive variety of complex compounds spanning a wide range of pharmacological activity.

The experimental pharmacology of *Zanthoxylum* extracts as well as the alkaloids isolated from the genus has already been discussed in an earlier work[88]. Subsequently the essential oil from Z. *budrunga* Roxb. has shown anti-inflammatory activity[89] as well as local anaesthetic activity[90] in test animals. No mortality or behavioural changes occurred when mice were given 1000 mg oil/kg.[90] The present survey will, however, be confined to a précis of the medicinal potential resulting from the more recent work on *Zanthoxylum* isolates.

A number of aromatic benzophenanthridine alkaloids possess interesting biological activity and have shown enough promise to warrant clinical evaluation[91]. Whilst sanguinarine and chelerythrine are devoid of useful human antitumour activity[2], nitidine and fagaronine (obtained from the roots of Z. *xanthoxyloides* Waterm.[12]) in particular have shown such promise as potent antitumour agents[2,92] that there has been significant development and interest in their synthesis[93-95] as well as in the production of isomers and analogues[96]. Nevertheless, sanguinarine, chelerythrine and chelirubine (bocconine) are nematocides[97].

In spite of the structural similarity of fagaronine and nitidine, they differ in their spectrum of anti-cancer activities[91]. Both compounds show high activity in the P-388 and L-1210 test systems, though only nitidine shows cytotoxicity. Fagaronine is a potent inhibitor of RNA-directed DNA-polymerase activity from avian myeloblastosis virus, Rauscher leukaemia virus and simian sarcoma virus, apparently by preventing the elongation reaction[2]. A simple addition reaction product of nitidine, methoxydihydronitidine, obtained as an

artefact from plant material, showed activity in Lewis lung tumour[98].

An aqueous extract from the roots of Z. *xanthoxyloides* Waterm. (= *Fagara zanthoxyloides* Lam.) was reported as having an anti-sickling effect, a phenomenon later attributed to a compound known as 2-hydroxymethylbenzoic acid[99]. This claim has since been repudiated[100]. The reported action of a chemically modified derivative of xanthoxylol, 3,4-dihydro-2,2-dimethyl-2*H*-1-benzopyran-6-butyric acid (DBA), which was isolated from the same plant and reputed to prevent as well as reverse sickle cell anaemia, has recently been discounted[101].

In a screening programme designed to discover new antibiotics, extracts of Z. *elephantiasis* Macfad. (= *Fagara elephantiasis* Kr. and Urb.) showed consistent *in vitro* activity against a range of test micro-organisms. This antimicrobial activity was ascribed to an alkaloid, canthin-6-one, which in turn showed remarkable specificity, since other canthinones were found to be devoid of meaningful antibiotic activity[102].

1.3.4 Chemotaxonomy

The use of secondary metabolites as chemotaxonomic markers in the Rutaceae[5,30] and more specifically in *Zanthoxylum*, has been the subject of several comprehensive reviews[3,4,6].

Numerous African species of the genus have been investigated and these appear to constitute a relatively homogeneous group in the subsection *Gerontogaeae* Engl.[4] The anthranilic acid pathway yields not only the ubiquitous furoquinoline bases, but also occasionally acridones (Z. *leprieurii* Guill. & Perr.) canthinones (Z. *viride* Waterm.) or β-indoloquinazolines (Z. *dinklagei* Waterm.)[6]

Several pathways occur in phenylalanine metabolism resulting in

the formation of aporphine, benzophenanthridine, protoberberine and tetrahydroprotoberberine ring systems, usually via a 1-benzyltetrahydroisoquinoline precursor[3].

Perusal of available data reveals that the African species have the ability to synthesize both 1,2,10,11- and 1,2,9,10-substituted aporphines via oxidative coupling *ortho* and *para* to the hydroxyl function in the 1-benzyl substituent of the 1-benzyltetrahydroisoquinoline presursor. *Ortho* or *para* coupling of another spatial arrangement of the precursor produces protoberberine/tetrahydroprotoberberine intermediates which, in turn, yield benzophenanthridine alkaloids by fission and re-arrangement[4]. Of the benzophenanthridine alkaloids, chelerythrine (*ortho* coupled) generally predominates in African species though nitidine (*para* coupled) also occurs, albeit in less abundance[3].

In the present work three South African *Zanthoxylum* species were investigated for secondary metabolites, the data for which provide additional evidence of homogeneity in African *Gerontogaeae*. The *N*-methylated tetrahydroprotoberberine alkaloid previously reported from only one African taxon[3,45] was conclusively detected in the three other taxa. The South African species of the genus are therefore uniform in their ability to synthesize quaternary alkaloids having a *N*-methyltetrahydroprotoberberine nucleus, a feature so far undetected in taxa occurring elsewhere on the continent.

2A. EXPERIMENTAL (*AGATHOSMA* WILLD. INVESTIGATION)

The ultraviolet spectra were recorded in ethanol on a Unicam SP800 spectrophotometer and the infrared spectra in chloroform on a Beckman IR 8 infrared spectrophotometer. Proton magnetic resonance (60 MHz) spectra were recorded in $CDCl_3$ on a Perkin-Elmer R-12 instrument with tetramethylsilane as internal standard (δ = 0 p.p.m.). Mass spectra were determined by the N.C.R.L. of the CSIR on an AEI-MS 9 double-focusing mass spectrometer at 70 eV. Melting points (uncorrected) were determined on a Kofler block and elemental analyses performed by the microanalytical laboratory of Dr. Franz Pascher. Thin-layer chromatograms were done on silica gel (Kieselgel G - Merck) and developed with *n*-hexane/ethyl acetate (1:1) at ambient temperature.

2A.1 Preliminary extractions

2A.1.1 Plant materials

2A.1.1.1 *Agathosma puberula* (Steud.) Fourc. The aerial parts of the plants (i.e. stems and leaves severed approximately 10 cm from soil level) were collected on the farm 'Upper Gletwyn' 13 km east of Grahamstown on 21-12-1974.

Voucher specimen: A1745*

2A.1.1.2 *Agathosma ovata* (Thunb.) Pillans. The aerial parts of the plants were collected 0,5 km beyond the Leather Industries Research Institute, Grahamstown on 18-12-1974.

Voucher specimen: A1739*

2A.1.1.3 *Agathosma apiculata* G.F.W. Mey. The aerial parts of the plants were collected at Port Alfred in the Rainbow Park caravan site 50-100 m along the foot-path obliquely behind the cafe on 14-9-1975.

Voucher specimen: A1981*

2A.1.1.4 *Agathosma clavisepala* R.A. Dyer. The aerial parts of the plant were collected on the road to Alexandria 2,1 km beyond the farm 'Longford Grange' on a high ridge on the right-hand side of the road on 16-9-1975.

Voucher specimen: A1982*

*The plants were authenticated by Mrs. E. Brink of the Albany Museum, Grahamstown, and voucher specimens were lodged in the Albany Herbarium under the numbers indicated.

2A.1.2 Drying and comminution

With the exception of *A. clavisepala* which was dried in a hot-air oven (Botany Department) at 55°, all plant material was air-dried under cover for approximately six weeks. The dried aerial parts of all four species as well as the leaves, separated carefully by hand-picking from the stems and twigs in the case of *A. puberula*, were each separately powdered in a Retsch cross-beater mill (type SK 1) using a 2 mm sieve.

2A.1.3 Extraction

The powdered aerial parts (1,56 kg) stems and twigs only (2 kg) and leaves (1,70 kg) of *A. puberula*, the aerial parts (1,60 kg) of *A. ovata*, the aerial parts (1,60 kg) of *A. apiculata* and the aerial parts (2 kg) of *A. clavisepala* were each in turn macerated in 2 ℓ of *n*-hexane for 4 hours and then continuously extracted in a Soxhlet extractor (Quickfit large-scale extractor IIEX) with a total of 15 ℓ of solvent for 48 hours.

The extracts were each concentrated under reduced pressure to approximately 2 ℓ. All the extracts from *A. puberula* deposited crystals, whereas the extract from *A. clavisepala* only deposited

crystals after some six weeks in a cold room. No crystallization occurred in the concentrated extracts of the other two species and these extracts were thus not investigated further. The yields obtained are summarized in Table 2.

TABLE 2: YIELDS OF PUBERULIN IN *AGATHOSMA* SPECIES

Species	Plant Parts	Quantity Extracted (kg)	Crude Crystalline Residue (g)	Pure Puberulin (g)	Yield of Puberulin (%)
A. puberula (Steud.) Fourc.	Aerial parts	1,56	7,8	2,808	0,180
	Stems and twigs	2,00	1,7	0,410	0,021
	Leaves	1,70	21,4	10,200	0,600
A. clavisepala R.A. Dyer	Aerial parts	2,00	7,1	0,682	0,034

2A.2 Isolation and purification of crystalline material

2A.2.1 Small scale

A portion of crystalline solid (4 g) from the aerial parts of *A. puberula* in boiling ethanol (30 mℓ) was decolourized with charcoal and the solution evaporated under reduced pressure. The residual dark brown oil was crystallized from benzene/light petroleum (b.p. 40°-60°) m.p. 78°-83° (1,44 g).

2A.2.2 Large scale

The concentrated extract (2 ℓ) of the stems and twigs of *A. puberula* was poured onto an alumina column (Merck - Activity II-III) 400 g, 19,5 cm x 5,5 cm and eluted with *n*-hexane, *n*-hexane/benzene (1:1) and benzene. The fractions as indicated in the table overleaf were collected.

Fraction	Eluting Solvent	Volume (mℓ)	Mass (g)
1 - 5	*n*-hexane	2000	11,6
6	*n*-hexane	400	1,1
7	*n*-hexane	400	0,7
8	*n*-hexane/benzene (1:1)	400	1,2
9	benzene	400	2,6
10	benzene	400	1,05

2A.2.2.1 Fraction 8

The residue was dissolved in benzene (5 mℓ) and light petroleum (b.p. 40°-60°; 70 mℓ) added. A greenish solution resulted which when concentrated under reduced pressure to 60 mℓ yielded a crystalline residue, m.p. 84°-85° (113 mg). This showed no blue fluorescence (UV 366 nm) on thin-layer chromatoplates.

2A.2.2.2 Fractions 9 and 10

The combined residue (3,65 g) was dissolved in benzene (16 mℓ) and light petroleum (b.p. 40°-60°) added until cloudiness persisted to afford crystals, m.p. 75°-78° (1,25 g). Thin-layer chromatography revealed a single blue fluorescent (under UV light, 366 nm) spot (R_f 0,67) which appeared as an orange colour in daylight when sprayed with Dragendorff's spray reagent[103].

2A.2.3 "Alkali-conversion" method

A portion of the crystalline solid (500 mg) from Fractions 9 and 10 (2A.2.2.2 refers) in methanol (20 mℓ) and 10% aqueous sodium hydroxide (10 mℓ) was heated on a water-bath for one minute and allowed to stand at ambient temperature for 24 hours. The insoluble precipitate (26 mg) was removed by filtration and rejected. The filtrate was extracted

with ether (20 mℓ) to remove neutral impurities (15 mg), then concentrated under reduced pressure to remove residual methanol and cooled to 0°. The solution was acidified by the slow addition of conc. HCℓ/H_2O (1:1) and the resultant fine white precipitate extracted with ether (3 x 20 mℓ) to afford a yellow oil (470 mg) which after chromatography on neutral alumina (10 g), elution with *n*-hexane (20 mℓ) and crystallization from benzene-light petroleum (b.p. 40°-60°) yielded colourless platelets of puberulin, m.p. 90°-92°, (120 mg).

UV spectrum: λ_{max} (ε) 342 (7200), 298 (10400), 228 sh. (18000) and 210 nm (35600);

IR spectrum: ν_{max} 1720, 1610, 1565, 1485, 1460, 1410, 1350, 1290, 1150, 1125, 1085, 1035, 970 cm^{-1};

PMR spectrum: δ 1,73 (3H, s); 1,79 (3H, s); 3,91 (3H, s); 4,05 (3H, s); 4,66 (2H, d, J = 6Hz); 5,60 (1H, t, J = 6Hz); 6,35 (1H, d, J = 9,5Hz); 6,71 (1H, s); 7,66 (1H, d, J = 9,5Hz);

MS $^{m}/_{e}$ (relative intensity): M^+ 290 (3); 223 (61), 222 (100), 207 (54), 194 (27), 179 (18), 176 (22), 79 (18), 78 (63), 69 (96), 68 (8).

Analysis: A sample was dried (P_2O_5) at 40°/0,2 mmHg for 1 hour.

Found: C = 65,93 ; H = 5,93%

$C_{16}H_{18}O_5$ required: C = 66,19 ; H = 6,25%

2A.3 Hydrogenation of puberulin

A solution of puberulin (500 mg) in ethanol (150 mℓ) was hydrogenated over Paal catalyst[104] for 15 minutes during which time 38,5 mℓ of hydrogen (equivalent to 1,20 moles per mole of puberulin) were absorbed. The solution, after filtration through a Celite 545

pad, was evaporated to dryness under reduced pressure. The residue was dissolved in hot benzene, passed through a short column of neutral alumina (10 g) and eluted with benzene. Concentration of the solution gave slightly yellowish needles, m.p. 146°-148° (75 mg). Recrystallization from aqueous ethanol (charcoal) yielded slender colourless needles, m.p. 147°-148°, which produced a single yellowish-green fluorescent (UV light 366 nm) spot (R_f 0,32) on t.l.c. The compound also produced a green precipitate with ferric chloride solution.

IR spectrum: ν_{max} 3530, 1720, 1610, 1565, 1500, 1460, 1410, 1370, 1310, 1250, 1150, 1115, 1080, 1030, 970 cm^{-1};

PMR spectrum: δ 3,96 (3H, s); 4,10 (3H, s); 6,33 (1H, d, J = 9,5Hz), 6,33 (1H, s; disappears with D_2O); 6,74 (1H, s); 7,68 (1H, d, J = 9,5Hz).

MS spectrum: M^+ 222; Calc. for $C_{11}H_{10}O_5$: M^+ 222

Analysis: A sample was dried (P_2O_5) at 80°/0,2 mmHg for 12 hours.

Found: C = 59,73 ; H = 4,35%

Calculated for $C_{11}H_{10}O_5$: C = 59,46 ; H = 4,54%

2A.4 Acetylation of "hydrogenated" puberulin

The "hydrogenated" product of puberulin (30 mg) was allowed to stand overnight in a mixture of acetic anhydride (1 mℓ) and dry pyridine (0,75 mℓ). The solvents were removed under reduced pressure and the residue dissolved in dry ethanol (2 mℓ). Off-white prisms formed (24 mg) which on recrystallization from ethanol yielded colourless prisms, m.p. 142°-143° (18,5 mg). On t.l.c. this derivative gave a blue fluorescent (UV light 366 nm) spot (R_f 0,48).

Analysis: A sample was dried (P_2O_5) at 80°/0,2 mmHg for 7 hours.

Found: C = 59,38 ; H = 4,54%

Calculated for
$C_{13}H_{12}O_6$: C = 59,09 ; H = 4,58%

2A.5 Methylation of "hydrogenated" puberulin

A solution of "hydrogenated" puberulin (67 mg) in ether (50 mℓ) was treated with diazomethane (prepared from 2 g of nitrosomethylurea) and left in ice overnight. The mixture was concentrated to dryness under reduced pressure. The dried residue in benzene (10 mℓ) was passed through a short column of neutral alumina (700 mg) and crystallized from ethanol (3 mℓ) to afford slender yellow needles, m.p. 104°-105° (40 mg) the melting point of which was not raised on recrystallization.

Analysis: A sample was dried (P_2O_5) at 50°/0,2 mmHg for 14 hours.

Found: C = 61,48 ; H = 5,08%

Calculated for
$C_{12}H_{12}O_5$: C = 61,02 ; H = 5,12%

2A.6 Ethylation of "hydrogenated" puberulin

A mixture of puberulin (70 mg), finely powdered, anhydrous potassium carbonate (200 mg), ethyl iodide (0,4 mℓ) and dry, redistilled acetone (10 mℓ) was refluxed on a glycerin bath for 10 hours. The solvents were removed under reduced pressure, the residue dissolved in hot benzene (20 mℓ) and the mixture passed through a short column of neutral alumina (700 mg) to afford a yellowish-green oil (64 mg) which on crystallization from ethanol (2 mℓ) gave an off-white crystalline solid, m.p. 82°-84° (42 mg). Recrystallization from aqueous ethanol (charcoal) gave colourless plates, m.p. 82°-83°. Since t.l.c. still

revealed some impurities, a portion (25 mg) in benzene (2 mℓ) was rechromatographed on alumina and crystallized from aqueous ethanol to afford colourless platelets, m.p. 82,5°-83,5° (14 mg) which produced a single blue fluorescent (UV light, 366 nm) spot (R_f 0,56) on t.l.c. When the plate was sprayed with Dragendorff's spray reagent[103], the spot showed a mauve colour in daylight.

Analysis: A sample was dried (P_2O_5) at 37°/0,3 mmHg for 16 hours.

Found: C = 62,42 ; H = 5,74%

Calculated for $C_{13}H_{14}O_5$: C = 62,39 ; H = 5,64%

2A.7 Prenylation of "hydrogenated" puberulin

A mixture of "hydrogenated" puberulin (30 mg), finely powdered, anhydrous potassium carbonate (50 mg), prenyl bromide[105] (30 mg) and dry acetone (4 mℓ) was refluxed for 6 hours. Solvent was removed under reduced pressure, the residue extracted with hot benzene (5 mℓ) and filtered. Crystallization from light petroleum (b.p. 40°-60°; 3 mℓ) afforded fine colourless needles, m.p. 89°-90° (11 mg) which had the same R_f and exhibited the identical blue fluorescence under UV light (366 nm) as puberulin. The melting point of this compound was unchanged on admixture with puberulin and their infrared spectra were superimposable.

2A.8 Acid fission of ether linkage of puberulin

A mixture of puberulin (302 mg), ethanol (10 mℓ) and concentrated hydrochloric acid (10 mℓ) was refluxed for 30 minutes and concentrated under reduced pressure to approximately 10 mℓ. Water (5 mℓ) was added and the mixture extracted with chloroform (1 x 20 mℓ and 1 x 10 mℓ). The combined chloroform extract was dried (Na_2SO_4), concentrated and

passed in benzene through a short column of neutral alumina. Recrystallization from aqueous ethanol afforded slender, faintly yellow needles, m.p. 147°-148° (47 mg), identical (mixed m.p., t.l.c. and IR) with "hydrogenated " puberulin.

2B. EXPERIMENTAL (*ZANTHOXYLUM* L. INVESTIGATION)

The ultraviolet spectra were recorded in ethanol or methanol on a Unicam SP800 spectrophotometer and the infrared spectra (KBr disc or in carbon tetrachloride) on a Perkin-Elmer Model 180 spectrophotometer. Proton magnetic resonance spectra (80MHz) were recorded on a Bruker Spectrospin by the Flavonoid Chemistry Research Unit of the University of the Orange Free State in the solvent indicated. Tetramethylsilane was used as internal standard (δ = 0 p.p.m.) except in spectra using D_2O as solvent when the internal standard was 2,2,3,3-tetradeutero-3-(trimethylsilyl)propionic acid sodium salt (Merck Uvasol 8652). Mass spectra were determined by the National Chemical Research Laboratory of the CSIR as well as by the Chemistry Department of Stellenbosch University on an AEI-MS9 double-focusing mass spectrometer at 70eV. Melting points (uncorrected) were determined on a Kofler block and elemental analyses were performed by the microanalytical laboratories of Dr. Franz Pascher and the N.C.R.L. The optical rotations were measured in a 1 dm tube on a Perkin-Elmer Model 141 polarimeter using the solvent indicated. Thin-layer chromatograms, prepared as described previously[106], were carried out on the following absorbents and developed at ambient temperature for a distance of 15 cm using the stated developing solvents.

System	Adsorbent	Developing Solvent
S 1	Silica Gel (Kieselgel G Merck)	*n*-hexane/ethyl acetate (4:1)
S 1B	Silica Gel (Kieselgel G Merck)	benzene/ethyl acetate (4:1)
S 2	Silica Gel (Kieselgel G Merck)	chloroform/ethanol (99:1)
S 3	Alumina (DS-5 Camag)	chloroform/cyclohexane/ethanol/ diethylamine (70:30:0,5:0,5)
S 4	Alumina (DS-5 Camag)	chloroform/ethanol (99:1)
S 5	Cellulose (CC41 Whatman)	0,1M aq. hydrochloric acid
S 6	Cellulose (CC41 Whatman)	*n*-butanol/pyridine/water (6:4:3)

The dried chromatograms were visualized under screened UV light (366 nm) before and after exposure to ammonia vapour. Alkaloids were detected by the use of spray reagents *e.g.* modified Dragendorff's reagent[103], iodoplatinic acid reagent[107] and diazotised sulphanilic acid[108].

2B.1 Preliminary extractions

2B.1.1 Plant materials

2B.1.1.1 *Zanthoxylum davyi* (Verdoorn) Waterm.

The stem and root bark were collected by Mr. Gordon Young, Government Forester, in the Hogsback Forest Reserve (Auckland Indigenous Forest) on a south-west aspect along the Tyumie river near Madonna and Child waterfall on the 11-8-1971.

Voucher specimens:++ RUH 21837 and 21838

2B.1.1.2 *Zanthoxylum thorncroftii* (Verdoorn) Waterm.

The root bark was collected by Messrs. B. J. Durand and J. N. Pienaar of the Botanical Research Institute in the Louis Trichardt district (start of Wyliespoort) in stony ground having a north-eastern slope on the 20-12-1974.

Voucher specimens:* B20 and 494

2B.1.1.3 *Zanthoxylum humile* (E.A. Bruce) Waterm.

The root bark was collected by Mr. B. J. Durand of the Botanical Research Institute at Malonga in the Kruger National Park on an eastern slope of a limestone hill.

Voucher specimen:* B7

++ The plant material was authenticated by Dr. A. Jacot Guillarmod of the Botany Department, Rhodes University and voucher specimens were lodged in the Rhodes Herbarium under the numbers indicated.

* The plant material was authenticated by the Botanical Research Institute, Pretoria and voucher specimens were lodged in the National Herbarium under the numbers indicated.

2B.1.2 Drying and comminution

With the exception of *Z. davyi* which was dried in an oven at 60° for 3 days, all the other plant material was air-dried under cover for approximately 2 months.

The dried stem and root bark of *Z. davyi* and the root barks of *Z. thorncroftii* and *Z. humile*, freed from adhering epiphytes, were each separately ground to a medium powder in a Retsch cross-beater mill (type SK 1) using a 2 mm sieve.

2B.1.3 Extraction

The powdered materials from each species were each successively extracted to exhaustion (negative to modified Dragendorff's reagent[103]) in a Soxhlet apparatus (Quickfit large-scale extractor IIEX) with *n*-hexane or light petroleum (b.p. 40°-60°), chloroform and methanol (20 litres of each solvent).

The extracts were each concentrated under reduced pressure at 50° to give brown, viscous residues. The details are summarized in Table 3.

TABLE 3: Extracts of three South African *Zanthoxylum* species

Species, Plant portion, and Mass extracted	Mass and Fraction Designation of Concentrated Extracts *					
	Light Petroleum (b.p. 40°-60°) or *n*-Hexane		Chloroform		Methanol	
	Fraction	Mass(g)	Fraction	Mass(g)	Fraction	Mass(g)
Z. davyi (Verdoorn) Waterm.						
Stem bark (2,0 kg)	ZD/SB1	127,55	ZD/SB2	68,34	ZD/SB3	604,3
Root bark (1,8 kg)	ZD/RB1	136,97	ZD/RB2	92,09	ZD/RB3	341,1
Z. thorncroftii (Verdoorn) Waterm.						
Root bark (1,26 kg)	ZT/RB1	62,89	ZT/RB2	36,50	ZT/RB3	339,3
Z. humile (E.A. Bruce) Waterm.						
Root bark (800 g)	ZH/RB1	41,33	ZH/RB2	28,30	ZH/RB3	156,0

* All extracts gave a positive reaction with modified Dragendorff's reagent[103]

2B.2 Isolation and characterization of compounds

2B.2.1 *Z. davyi* concentrated extracts

2B.2.1.1 *n*-Hexane extract of stem bark (ZD/SB1)

After storage for approximately six months, the concentrated brown, viscous extract deposited a crystalline solid which was washed with *n*- hexane (25 mℓ) to give a yellowish-white residue (4,2 g).

Recrystallization from *n*-hexane afforded colourless needles, m.p. 195°-196° (3,6 g). Repeated recrystallization of a portion (500 mg) from methanol yielded colourless plates of lupeol, m.p. 212°-214° (100 mg). The combined mother liquors yielded further crystals m.p. 213°-214° (270 mg) $[\alpha]_D^{25}$ + 26,9° (*c*, 1,07 in chloroform) [lit.[109] m.p. 214°; $[\alpha]_D$ + 26° (*c*, 0,7 $CHCl_3$)].

IR spectrum: $\nu_{max}^{CCl_4}$ 3650 [-OH] 2960, 1640, 1450, 1380, 1040, 885 [$-C(CH_3)=CH_2$] cm^{-1}.

Analysis: A sample (6 mg) was dried (P_2O_5) at 100°/0,5 mmHg for 4 hours.

Found: C = 84,16 ; H = 11,86%

Calculated for $C_{30}H_{50}O$: C = 84,44 ; H = 11,81%

2B.2.1.1.1 Acetylation of lupeol

A portion (100 mg) of the above material was allowed to stand overnight in a mixture of acetic anhydride (3 mℓ) and dry pyridine (0,5 mℓ). As no solution had occurred, the mixture was refluxed at 100° (glycerin bath) for 15 minutes. On the addition of water (5 mℓ), a crystalline solid formed. This solid was washed with water, dried (silica gel) and recrystallized twice from ethanol to afford colourless needles, m.p. 217° (42 mg) [lit.[60] m.p. 217°]. The combined mother liquors yielded a further crop of needles, m.p. 214°-215° (31 mg). A mixture of the acetate and the starting material (lupeol) had a m.m.p. 181°-183°.

IR Spectrum: $\nu_{max}^{CCl_4}$ 2960, 1730 [>C=O], 1640, 1385 & 1370 [$>C(CH_3)_2$], 1250 (acetate), 1030, 980, 885 [$-C(CH_3)=CH_2$] cm^{-1}.

2B.2.1.1.2 Benzoylation of lupeol

Lupeol (60 mg) was allowed to stand for two days at ambient

temperature in a mixture of benzoyl chloride (0,75 mℓ) and pyridine (3 mℓ). Water (1 mℓ) was added and the mixture poured into saturated sodium carbonate solution (10 mℓ). The mixture was warmed gently for 2 minutes, cooled, filtered and the residue (65 mg) washed with water (5 x 5 mℓ). Crystallization from ethanol, and then from *n*-propanol afforded slender, colourless needles, m.p. 257°-258° (18 mg). [lit.[110] m.p. 257°-259°].

2B.2.1.1.3 Saponification of lupeol acetate

A mixture of lupeol acetate (31 mg), potassium hydroxide (50 mg), water (0,5 mℓ) and ethanol (3 mℓ) was refluxed for 1 hour with occasional agitation. Water was added and the precipitate washed and dried m.p. 213°-214° (22 mg).

The m.p. was undepressed when mixed with the lupeol originally isolated. (*vide* 2B.2.1.1)

2B.2.1.2 Chloroform extract of stem bark (ZD/SB2)

A portion of the concentrated extract (30 g) was dissolved in chloroform (150 mℓ) and extracted with M hydrochloric acid (2 x 100 mℓ and 3 x 50 mℓ). The yellowish precipitate (YPI) collecting at the interface was removed by filtration and the acid filtrates bulked, washed with chloroform (1 x 100 mℓ and 1 x 50 mℓ) and reserved for further study (AFI). The remaining chloroform fraction and washings (300 mℓ) were also reserved (CFMI).

2B.2.1.2.1 Yellowish precipitate (YPI)

The yellowish precipitate was refluxed with methanol (15 mℓ) and conc. hydrochloric acid (0,3 mℓ) for 30 minutes. The resultant crystalline solid was removed, washed first with chloroform (10 mℓ), then with methanol (4 mℓ) and dried (silica gel) (102 mg). Several

recrystallizations from dry methanol afforded greenish-yellow needles (NF/1) of nitidine chloride, m.p. 277°-278° (dec.) (36 mg) [lit.[111] 275°-277° (dec.)], which produced a single, greenish-yellow, fluorescent spot (R_f 0,27-S3; R_f 0,00-S5) on t.l.c. When the plates were separately sprayed with Dragendorff's reagent[103] and iodoplatinic acid reagent[107], the yellow spot (visible light) gave orange and light-brown colours respectively. The ultraviolet and infrared spectra, m.m.p. and chromatographic characteristics of NF/1 were identical with those of an authentic sample of nitidine chloride.

2B.2.1.2.2 Chloroform fraction and washings (CFM 1)

This fraction was concentrated *in vacuo* to about one-third of its volume and extracted with M hydrochloric acid (1 x 50 mℓ). The bright orange precipitate collecting at the interface was removed, washed with 0,1 M hydrochloric acid (3 x 30 mℓ), dissolved in ethanol (15 mℓ) and ether added dropwise to yield slender, orange needles (NF/2) of chelerythrine chloride, m.p. 197°-198° (dec.) (131 mg) [lit.[112] 198°-199°], which produced a single orange fluorescent spot (R_f 0,78-S4; R_f 0,05-S5) on t.l.c. When the plates were separately sprayed with Dragendorff's reagent and iodoplatinic acid reagent, the yellow spot (visible light) gave orange and brownish colours respectively.

The ultraviolet and infrared spectra, m.m.p. and chromatographic characteristics of NF/2 were identical with those of an authentic sample of chelerythrine chloride.

2B.2.1.2.3 Liquid-liquid extraction of combined acid filtrate (AF 1)

This fraction was extracted with chloroform (400 mℓ) in a liquid-liquid extractor for 12 hours. The chloroform extract was dried (Na_2SO_4) overnight, evaporated to dryness under reduced pressure and the residue (5 g) dissolved in dry methanol (10 mℓ). Dry ethyl acetate

was added to the solution until the resulting cloudiness just persisted. The crystalline solid (2,7 g) which formed after refrigeration was recrystallized several times from the same solvents to yield colourless plates, (NF/3), of (-)-α-canadine methochloride which softened at 165°, resolidified at about 210°, started decomposing at 230°-233° and finally melted at 244°-246° (dec.), $[\alpha]_D^{25}$ -158,7° (*c*, 1,005 in methanol). [lit.[113] m.p. 249°-250°; $[\alpha]_D^{25}$-160° (*c*, 0,408 in methanol)] MS *m/e* (relative intensity): (M^+ - CH_3Cl) 339 (64), 176 (10), 174 (25), 164 (100), 149 (61). The crystals produced a single, non-fluorescent (before and after exposure to ammonia vapour) spot (R_f 0,85-S5; R_f 0,00-S3) on t.l.c. When the plates were separately sprayed with Dragendorff's reagent and iodoplatinic acid reagent, the spot gave orange and purple colours respectively.

2B.2.1.2.4 Conversion of crystals NF/3 to iodide salt

Crystals NF/3 (50 mg) were dissolved in water (1,5 mℓ), and an aqueous saturated solution of potassium iodide (0,6 mℓ) added dropwise to produce a curdy white precipitate which, after washing with water (3 x 3 mℓ), was dried overnight (silica gel). The dried solid (45 mg) was crystallized from dry methanol (ice-cold) to yield slender, colourless needles (33 mg) of (-)-α-canadine methiodide which melted at 160°-162°, resolidified at about 175°-180° and finally melted at 248°-249° (dec.) [lit.[114] 249°-251° (dec.)].

UV spectrum: λ_{max}^{EtOH} (log ε) 207 (4,87), 233 (sh.) (4,22), 287 nm (3,77);
λ_{min}^{EtOH} (log ε) 258 nm (3,03);

[lit.[115] λ_{max}^{EtOH} (log ε) 207 (4,86), 233 (sh.) (4,20), 287 nm (3,77);
λ_{min}^{EtOH} (log ε) 258 nm (3,00)].

The chromatographic characteristics of this iodide were identical to those described under crystals NF/3 (*vide* 2B.2.1.2.3)

2B.2.1.3 Methanol extract of stem bark (ZD/SB3)

A portion of the concentrated extract (528 g) was mixed with Celite 545 (500 g), agitated with M hydrochloric acid (3 x 1 ℓ) in a reciprocal shaker for 30 minutes and filtered. The bulked acidic filtrate was adjusted to pH 6 using 5 M sodium hydroxide and the resultant brownish, resinous residue removed by filtration. A portion (1 ℓ) of this filtrate was now adjusted to pH 4 with hydrochloric acid and an aqueous solution of 2% m/v ammonium reineckate added with constant stirring until no further precipitation occurred. The precipitated reineckate was removed by centrifugation and washed with water (4 x 500 mℓ), the washings being discarded. The dried reineckate was dissolved in a mixture of methanol/acetone (5:1) and chromatographed on a column (30 mm x 500 mm) of Amberlite resin IRA-400 (Cl) (Analytical grade-BDH). The column was eluted with a mixture of methanol/acetone (1:1) and the eluate (420 mℓ) concentrated to dryness *in vacuo* to yield an alkaloidal residue (R_1) (3,55 g).

2B.2.1.3.1 Horizontal cellulose chromatography of alkaloidal residue (R_1)

A horizontal column (25 mm x 680 mm), identical to that described by Nunn *et al.*[116] was packed with prewashed cellulose powder (Whatman Cellulose Chromedia CF 11). A portion of residue R_1 (1 g) was admixed with cellulose (3 g) and applied to the column's supply tube. The column was eluted with 0,1 M hydrochloric acid at 27°. After 4½ hours, the nylon tube was slit down its entire length and the damp, exposed column cut into 51 roughly equal portions. Each portion was monitored on t.l.c. using system S5. Homogeneous fractions were bulked and then worked up as follows:-

2B.2.1.3.2 Fractions 1 - 8

As no alkaloids were detected in this fraction, it was not examined further.

2B.2.1.3.3 Fractions 9 - 17

This fraction was extracted with methanol, filtered (Celite 545), and the filtrate taken to dryness (56 mg). The residue was dissolved in methanol, decolourized and concentrated to a volume of 1 mℓ. Ether was added dropwise until cloudy and the solution then refrigerated. The crystalline solid which had formed overnight was recrystallized from absolute ethanol to yield prisms (NF/4) of (+)-laurifoline chloride, m.p. 251°-253° (dec.), (12 mg) [lit.[117] 253° (dec.)], which produced a single, non-fluorescent spot (R_f 0,07-S5; R_f 0,49-S6) on t.l.c. When the plate run on system S5 was exposed to ammonia vapour, the spot exhibited a pale blue fluorescence. In the case of the plate run on system S6, the spot already exhibited a blue fluorescence. On spraying the plates with Dragendorff's reagent and iodoplatinic acid reagent, the spot gave orange and bluish-violet colours respectively.

The ultraviolet and infrared spectra, m.m.p. and chromatographic characteristics of NF/4 were identical with those of an authentic sample of (+)-laurifoline chloride.

2B.2.1.3.4 Fractions 18 - 32

Since this fraction was a mixture of two compounds, one of which had already been isolated in fractions 9 - 17, it was not examined further.

2B.2.1.3.5 Fractions 33 and 34

Thin-layer chromatograms of this fraction showed that it consisted of one predominant spot. This fraction was extracted with

methanol, filtered (Celite 545) and the filtrate taken to dryness (61 mg). The yellowish residue was dissolved in methanol (1,5 mℓ) and an aqueous saturated solution of potassium iodide (0,1 mℓ) added. Concentration of this solution to 1 mℓ yielded a crystalline deposit which, on recrystallization from dry ethanol, afforded yellowish prisms (NF/5) of (+)-magnoflorine iodide, m.p. 249°-252° (dec.), (24 mg) [lit.[118] 248° (dec.)], which produced a single, non-fluorescent spot (R_f 0,27 - S5) on t.l.c. When the plate was exposed to ammonia vapour, the spot exhibited a dark blue fluorescence. With system S6, the spot (R_f 0,34) exhibited a blue fluorescence. Both plates gave an orange and a bluish-violet colour when sprayed with Dragendorff's and iodoplatinic acid reagents respectively.

The ultraviolet and infrared spectra, m.m.p. and chromatographic characteristics of NF/5 were identical with those of an authentic sample of (+)-magnoflorine iodide.

2B.2.1.3.6 Fractions 35 - 51

Thin-layer chromatograms of fraction 35 - 46 showed three alkaloid-positive spots ($R_{f's}$ 0,27; 0,77; 0,87-S5) one of which was already isolated and characterized as magnoflorine (*vide* 2B.2.1.3.5). Fraction 47 - 51 was a mixture of two alkaloids ($R_{f's}$ 0,85 and 0,90-S5 and $R_{f's}$ 0,35 and 0,63-S6). The one spot (R_f 0,85-S5 and R_f 0,63-S6) gave a mauve colour with iodoplatinic acid reagent and exhibited identical R_f values to that of α-canadine methochloride isolated from fraction ZD/SB2 (*vide* 2B.2.1.2.3). The other spot (R_f 0,90-S5 and R_f 0,35-S6) gave a distinctive greenish colour with iodoplatinic acid reagent indicative of tembetarine.

2B.2.1.4 *n*-Hexane extract of root bark (ZD/RB1)

A portion (50 g) of the extract was chromatographed on an alumina

(Merck; activity II-III) column (67 mm x 330 mm; 1000 g) and eluted with solvents of increasing polarity as depicted below:

Fraction No.	Solvent	Vol. of Eluate (mℓ)	Mass (g)
1-6	*n*-hexane	1430	4,06
7	benzene	260	0,20
8	benzene	260	0,30
9	benzene	260	3,90
10	benzene	260	6,20
11	benzene	260	1,30
12	benzene	260	0,45
13	benzene	260	0,15
14-22	chloroform	1820	3,25
23-28	chloroform/methanol (99:1)	910	0,80
29-31	chloroform/methanol (98:2)	390	0,50
32-36	chloroform/methanol (95:5)	650	0,80

2B.2.1.4.1 Fraction 10

The residue was recrystallized several times from methanol to yield plates of lupeol, m.p. 213°-214° (1,52 g) which produced a single, non-fluorescent spot (R_f 0,58 - S1B) on t.l.c. When the plate was sprayed with antimony trichloride 300% m/v in conc. hydrochloric acid and heated at 110° for ± 3 minutes, an orange spot was produced which changed to purple on standing.

2B.2.1.4.2 Fractions 9 and 11 (bulked)

Since thin-layer chromatograms revealed that lupeol was the predominant constituent of this fraction, further investigation was abandoned.

2B.2.1.4.3 Fractions 12 and 13 (bulked)

This fraction was dissolved in ethanol (10 mℓ), decolourized with charcoal (200 mg) and filtered. When the filtrate was concentrated to about 5 mℓ, it yielded fine needles, m.p. 130°-133° (54 mg). Several recrystallizations from methanol afforded colourless needles (NF/6) of β-sitosterol, m.p. 135°-136°, (25 mg) [lit.[75] 136°], which produced a single, non-fluorescent spot (R_f 0,39-S1) on t.l.c. When the plate was sprayed with $SbCl_3$/HCl and heated as described previously a bright crimson spot was produced which faded on standing to a light mauve colour.

Analysis: A sample (3 mg) was dried (P_2O_5) at 68°/0,4 mmHg for 3 hours.

Found: C = 81,71 ; H = 12,25%

Calculated for $C_{29}H_{50}O.\frac{1}{2}H_2O$ C = 82,20 ; H = 12,13%

The mass and infrared spectra, m.m.p. and chromatographic characteristics of NF/6 were identical with those of an authentic sample of β-sitosterol.

2B.2.1.4.4 Acetylation of β-sitosterol (NF/6)

β-sitosterol (34 mg) was dissolved (water-bath) in a mixture of acetic anhydride (1 mℓ) and pyridine (0,4 mℓ) and allowed to stand overnight. The resulting crystalline deposit was recrystallized from methanol to yield fine needles (NF/7), m.p. 122°-123°, (20 mg) [lit.[119] 118°-120°], which produced a single, non-fluorescent spot (R_f 0,81-S1) on t.l.c. When the plate was sprayed with $SbCl_3$/HCl and heated, a faint pink spot was produced which changed to mauve on standing.

Analysis: A sample (2,6 mg) was dried (P_2O_5) at 65°/0,2 mmHg for 3 hours.

Found: C = 81,07 ; H = 11,62%

Calculated for $C_{31}H_{52}O_2$: C = 81,52 ; H = 11,48%

2B.2.1.4.5 Fractions 14 - 16 (bulked)

This fraction yielded a further quantity of β-sitosterol (34 mg).

2B.2.1.4.6 Fractions 17 - 36

As no crystalline material could be obtained from any of these fractions, further investigation was therefore abandoned.

2B.2.1.5 Chloroform extract of root bark (ZD/RB2)

This extract was extracted with M hydrochloric acid (2 x 100 mℓ and 2 x 25 mℓ) and the acidic fractions bulked. The yellow deposit (YD2) which formed in the acidic fraction after standing for ± 3 weeks, was removed. The acidic filtrate was then subjected to a liquid-liquid extraction with chloroform (600 mℓ) for 4 hours. This chloroform extract was concentrated to dryness under reduced pressure to afford an alkaloidal residue (R2) (2,25 g).

2B.2.1.5.1 Yellow deposit YD2

Thin-layer chromatography of this deposit showed that it consisted of a mixture of two yellow alkaloids which fluoresced greenish-yellow and orange respectively (R_f = 0,00 and 0,11 - S5; R_f 0,18 and 0,79 - S3). These chromatographic characteristics were identical with authentic samples of nitidine and chelerythrine; however, as these compounds had previously been isolated and characterized from the stem bark (*vide* 2B.2.1.2.1 and 2B.2.1.2.2), the investigation was not pursued further.

2B.2.1.5.2 Residue R2

A portion (1 g) of R2 was dissolved in dry methanol (8 mℓ) and dry ethyl acetate (70 mℓ) added. The mixture was concentrated to

approximately two-thirds of its original volume and refrigerated overnight whereupon yellowish prisms crystallized out (700 mg). Several recrystallizations from MeOH/EtOAc yielded colourless prisms, (NF/8), m.p. 245°-246° (dec.), which produced a single, non-fluorescent spot (R_f 0,86 - S5) on t.l.c. and a purple colour with iodoplatinic acid reagent.

MS: Found: 339,1462; Calculated for $C_{20}H_{21}O_4N$: 339,1470 (M^+ -15).

The spectra were identical with those of (-)-α-canadine methochloride which had previously been isolated from the stem bark (*vide* 2B.2.1.2.3).

2B.2.1.5.3 Conversion of R2 to iodide salt

A portion (100 mg) of R2 was dissolved in water (5 mℓ) and an aqueous solution of potassium iodide 20% $^m/_v$ (0,5 mℓ) added. The resultant precipitate was dried, and recrystallized several times from methanol to yield prisms, (NF/9), m.p. 251°-252° (dec.). The spectra were identical with those of (-)-α-canadine methiodide which has already been described under the stem bark. (*vide* 2B.2.1.2.4).

2B.2.1.6 Methanol extract of root bark (ZD/RB3)

Since t.l.c. showed that this extract contained the same alkaloids as those in the corresponding extract obtained from the stem bark, it was decided not to pursue this investigation further.

2B.2.2 *Z. thomsoftii* concentrated extracts

2B.2.2.1 Light petroleum (b.p. 40°-60°) extract of root bark (ZT/RB1)

A portion (38 g) of the amber-coloured, viscous extract was dissolved in ether (200 mℓ) and extracted with 0,3 M hydrochloric acid (2 x 100 mℓ) and 0,1 M HCl (50 mℓ). The orange precipitate (OP_1) collecting at the interface was removed by filtration and the acid filtrates (±250 mℓ) bulked, washed with ether (50 mℓ) and reserved for

further study (AF2). The bulked ether fraction (±250 mℓ) was then extracted with 5% $^{m}/_{v}$ aqueous sodium bicarbonate solution (2 x 100 mℓ) to give fraction (P2) followed by 5% $^{m}/_{v}$ aqueous sodium carbonate solution (200 mℓ) to give fraction (P3).

2B.2.2.1.1 Orange precipitate (OP_1)

A sample of this precipitate (188 mg) was monitored on t.l.c. (system S2) and was shown to contain a predominant, brown-fluorescing spot (R_f 0,31). When the plate was sprayed with Dragendorff's reagent[103], the spot gave an orange-brown colour which changed to deep blue when the sprayed plate was subsequently exposed to ammonia vapour.

As identical characteristics were observed in the chloroform extract (ZT/RB2), identification of this compound was deferred and done in conjunction with the corresponding alkaloid isolated from ZT/RB2 (*vide* 2B.2.2.2.4 *et seq.*)

2B.2.2.1.2 Combined acid filtrate (AF2)

This fraction was adjusted with strong ammonia solution (12 mℓ) to pH 8 and extracted with ether (1 x 100 mℓ and 2 x 50 mℓ) to give fraction E1, followed by chloroform (1 x 100 mℓ and 2 x 75 mℓ) to give fraction CFM2.

The dried (Na_2SO_4) combined chloroform extract (CFM2) was concentrated under reduced pressure to yield a yellowish crystalline residue (200 mg) which, after two recrystallizations from ethanol, afforded colourless prisms, (NF/10), m.p. 178°-179° (105 mg). The mother liquor yielded a further crop of colourless prisms, (NF/10a), m.p. 175°-177° (23 mg).

The dried (Na_2SO_4) combined ether extract (E1) was concentrated under reduced pressure to yield a yellow crystalline residue (73 mg).

This residue was dissolved in benzene (10 mℓ), chromatographed on a short column of neutral alumina (500 mg) and eluted with the same solvent until the eluate no longer exhibited a blue fluorescence in UV light (366 nm). All fractions exhibiting a blue fluorescence were bulked, concentrated *in vacuo* to approximately 5 mℓ and light petroleum (b.p. 40°-60°) added until the resulting cloudiness just persisted. Colourless prisms, (NF/10b), m.p. 175°-177° (30 mg) formed after refrigeration overnight. NF/10, NF/10a and NF/10b each gave a single, turquoise-blue spot ($R_{f's}$ 0,32-S2; 0,79-S4) on t.l.c. When the plates were sprayed with Dragendorff's reagent[103], the spots gave an orange colour which in turn became a deep rose colour when the sprayed plates were subsequently exposed to ammonia vapour. The infrared and ultraviolet spectra, melting point, m.m.p. and chromatographic characteristics of NF/10 - NF/10b were identical with those of an authentic sample of skimmianine, m.p. 175°-176°[112].

2B.2.2.1.3 Fractions P2 and P3

As no crystalline material could be obtained after acidification of each of these fractions, further study was abandoned.

2B.2.2.2 Chloroform extract of root bark (ZT/RB2)

A portion (31,3 g) of the extract was dissolved in chloroform (100 mℓ). Approximately half of this solution was then extracted with M hydrochloric acid (3 x 100 mℓ and 2 x 50 mℓ). The yellow precipitate - YP2 (720 mg) collecting at the interface was removed by filtration and the orange-coloured acid filtrate (AF3) reserved (±400 mℓ).

2B.2.2.2.1 Yellow precipitate (YP2)

Since t.l.c. of this precipitate showed that it consisted largely of chelerythrine and nitidine ($R_{f's}$ 0,89 and 0,20-S4; 0,06 and 0,00-S5),

it was decided not to continue this investigation further.

2B.2.2.2.2 Combined acid filtrate (AF3)

A portion (250 mℓ) of this fraction was extracted with ether (2 x 50 mℓ) and the ether layer discarded. The acid fraction was then adjusted to pH 8 with strong ammonia solution (20 mℓ) and the resultant brown precipitate extracted with ether (1 x 100 mℓ and 2 x 50 mℓ). The ether phase was discarded and the aqueous phase was subjected to a liquid-liquid extraction with chloroform (400 mℓ) for 10,5 hours. The chloroform extract was dried (Na_2SO_4) and concentrated to dryness *in vacuo* to yield a brown crystalline residue (244 mg). This residue was dissolved in methanol (3 mℓ) and an aqueous saturated solution of potassium iodide (0,15 mℓ) added. After decolourization (charcoal), the dried iodide was recrystallized from methanol to yield colourless plates of (-)-α-canadine methiodide (44 mg) which melted at 161°-165°, resolidified at about 230° and finally melted at 251°-252° (dec.) [lit.[114] m.p. 249°-251° (dec.)]; $[\alpha]_D^{21}$ - 114° (*c*, 0,22 in methanol) [lit.[115] $[\alpha]_D^{18}$ - 114° ±3° (*c*, 0,27 in methanol)]. The ultraviolet and infrared spectra, m.m.p. and chromatographic characteristics were identical with those of an authentic sample of (-)-α-canadine methiodide.

2B.2.2.2.3 Column chromatography of remaining ZT/RB2

The remaining half of the chloroform solution (*vide* 2B.2.2.2) was diluted with an equal volume of chloroform and chromatographed on a silica gel (Kieselgel 60 extra pure; Merck) column (45 mm x 450 mm; 400 g) which was packed dry. The column was eluted with chloroform and 28 x 200 mℓ fractions were collected. The yellow, insoluble residue (YP3) which remained at the top of the column was carefully

removed and reserved for further study. The column was then eluted with a mixture (500 mℓ) of chloroform/methanol (9:1) to give Fraction 29, followed by elution with a mixture of chloroform/methanol (1:1) to give Fractions 30 (300 mℓ), 31 (350 mℓ), 32 (150 mℓ), 33 (280 mℓ) and 34 (400 mℓ).

2B.2.2.2.4 Fractions 23 - 26

Methanol (40 mℓ) was added to this fraction (1,67 g) and the resulting solution filtered to yield an amber-coloured filtrate and a brownish residue - R3 (403 mg). R3 was relatively insoluble in methanol (5 mg was soluble in ±2 mℓ of hot methanol), produced a brown precipitate with ferric chloride solution, gave a bright orange precipitate with hydrochloric acid and exhibited an emerald-green colour when heated with gallic acid/conc. sulphuric acid (Labat test[120]). On t.l.c. the residue (R3) showed a predominant spot (R_f 0,33-S2) which gave a brownish fluorescence under UV light. When the plate was sprayed with Dragendorff's reagent, the spot gave an orange-brown colour which was transformed to deep blue when the sprayed plate was subsequently exposed to ammonia vapour.

A solution of R3 (50 mg) in methanol (30 mℓ) was filtered, concentrated to about one-third of its volume and water (20 mℓ) added while the solution was hot. The resulting precipitate (27 mg) was re-precipitated from chloroform/light petroleum (b.p. 40°-60°) and the buff residue (15 mg) dried (silica gel). Sublimation of the buff solid at 165°/0,3 mmHg produced fine, buff needles (NF/11) of decarine, m.p. 244°-245°, [lit.[121] 243°].

UV spectrum: λ_{max}^{EtOH} (log ε) 383 (3,50), 326 (4,20), 277 (4,71), 257 sh. (4,60), 247 nm. (4,59);

(Addition of NaOH produced a bathochromic shift)

[lit.[121] λ_{max}^{EtOH} (log ε) 384 (3,46), 335 sh. (4,11), 326 (4,20), 285 sh. (4,52), 277 (4,67), 257 (4,55), 249 nm. (4,54)].

IR spectrum: ν_{max}^{KBr} 3440 (-OH), 2910, 1620, 1590, 1530, 1500, 1470, 1400, 1370, 1350, 1320, 1280, 1250, 1220, 1190, 1130, 1110, 1040, 1010, 1000, 945, 870, 860, 840, 825, 795 cm^{-1}.

PMR spectrum: $[(CD_3)_2CO]$ δ4,09 (3H,s); 6,16 (2H,s); 7,34 (1H,s); 7,56 (1H,d, J = 9Hz); 7,91 (1H,d, J = 9Hz); 8,44 (1H,d, J = 9Hz); 8,47 (1H,d, J = 9Hz); 8,62 (2H,s); 9,59 (1H,s).

Mass spectrum: Found: M^+ 319,0837; $C_{19}H_{13}O_4N$ requires: 319,0844

The infrared and p.m.r. spectra are virtually identical with those obtained by Dr. J. Vaquette for decarine.

Analysis: A sample (3,4 mg) was dried (P_2O_5) at 84°/0,2 mmHg for 4 hours.

Found:	C = 71,81 ;	H = 4,09 ;	N = 4,42%
Calculated for $C_{19}H_{13}NO_4$:	C = 71,47 ;	H = 4,10 ;	N = 4,39%

2B.2.2.2.5 Conversion of R3 to the hydrochloride

A portion (84 mg) of R3 was dissolved in a mixture of methanol/chloroform 1:1 (30 mℓ) and filtered. Concentrated hydrochloric acid (0,3 mℓ) was added to the filtrate. The resultant orange precipitate was washed with water (2 x 3 mℓ) and the washings discarded. The dried precipitate (64 mg) was dissolved in a mixture (40 mℓ) of M hydrochloric acid in methanol/chloroform (1:1) and the solution refrigerated to afford slender orange needles (NF/12) of decarine hydrochloride, m.p. 243°-245° (32 mg), [lit.[122] 244°-246°].

IR spectrum: ν_{max}^{KBr} 3100, 3040, 2705, 1620, 1590, 1560, 1530, 1510 1480, 1415, 1400, 1360, 1320, 1290, 1265, 1230, 1180, 1150, 1120, 1040, 1020, 1005, 940, 915, 880, 860, 825, 800, 770, 745 cm^{-1}.

PMR spectrum: $[(CD_3)_2SO]$ δ4,03, (3H,s); 6,19 (2H,s); 7,50 (1H,s); 7,62 (1H,d, J = 9Hz); 7,97 (1H,d, J = 9Hz); 8,44 (1H,d, J = 9Hz); 8,50 (1H,d, J = 9Hz); 8,56 (2H,s); 9,53 (1H,s).

The ultraviolet spectrum as well as the chromatographic characteristics were virtually identical with those obtained for the buff needles, NF/11.

Analysis: A sample (2,4 mg) was dried (P_2O_5) at 84°/0,2 mmHg for 4 hours.

Found:	C = 64,18 ;	H = 3,96 ;	N = 3,97%
Calculated for $C_{19}H_{14}NO_4Cl$:	C = 64,14 ;	H = 3,97 ;	N = 3,94%

2B.2.2.2.6 Acetylation of R3

A portion (20 mg) of R3 was dissolved (water-bath) in a mixture of acetic anhydride (2 mℓ) and pyridine (1 mℓ) and the solution allowed to stand for 4 hours. The solvents were removed under reduced pressure and the residue (23 mg) was dissolved in chloroform (3 mℓ). Methanol was added dropwise until cloudy. The resultant precipitate (12 mg) was dried (silica gel), dissolved in a mixture (4 mℓ) of chloroform/ethanol (1:1) and the solution concentrated to approximately half of its original volume. Refrigeration of this solution overnight afforded buff needles, (NF/13), m.p. 238°-240° (9 mg), which gave a single, yellow fluorescent spot (R_f 0,59-S2) on t.l.c. When the plate was sprayed with Dragendorff's reagent, the spot gave an orange colour which in turn changed to greenish-

yellow when the sprayed plate was subsequently exposed to ammonia vapour.

IR spectrum: ν_{max}^{KBr} 2940, 1750 (>C = O), 1585, 1525, 1505, 1460, 1450, 1420, 1365, 1250 (acetate), 1220, 1110, 1030, 1020, 1000, 985, 940, 880, 850, 785 cm^{-1}.

Analysis: A sample (3,4 mg) was dried (P_2O_5) at 110°/0,3 mmHg for 16 hours

Found: C = 69,50 ; H = 4,00 ; N = 3,90%

$C_{21}H_{15}NO_5$ requires: C = 69,80 ; H = 4,18 ; N = 3,88%

2B.2.2.2.7 Methylation of R3

A portion (50 mg) of R3 was dissolved in a mixture (20 mℓ) of chloroform and methanol (1:1), the solution cooled in ice, treated with diazomethane (prepared from 1 g of nitrosomethylurea) and allowed to stand for 2,5 hours when faintly yellow prisms crystallized out. Concentration of the supernatant solution to 10 mℓ afforded fine, faintly yellow needles, m.p. 214°-216° (21 mg). The prismatic crystals were recrystallized from *n*-pentanol to afford faintly yellow needles of norchelerythrine, m.p. 215°-216° (17 mg) [lit.[123] m.p. 215°-216°], which gave a single, yellow fluorescing spot (R_f 0,73-S2) on t.l.c. When the plate was sprayed with Dragendorff's reagent, the spot gave an orange colour which in turn changed to a pinkish colour when the sprayed plate was exposed to ammonia vapour.

IR spectrum: ν_{max}^{KBr} 3000, 2940, 2900, 2840, 1590, 1575, 1530, 1495, 1460, 1420, 1385, 1355, 1315, 1280, 1270, 1245, 1190, 1160, 1130, 1110, 1075, 1035, 1000, 990, 860, 850, 825, 800 cm^{-1}.

Analysis: A sample (3 mg) was dried (P_2O_5) at 140°/0,3 mmHg for 2 hours.

Found: C = 71,76 ; H = 4,56 ; N = 4,30%

Calculated for $C_{20}H_{15}NO_4$: C = 72,06 ; H = 4,54 ; N = 4,20%

2B.2.2.2.8 Fraction 31

This fraction (344 mg) was dissolved in ethanol (5 mℓ), M hydrochloric acid (20 mℓ) added, followed by chloroform (20 mℓ). The mixture was shaken, the aqueous layer separated, filtered and the orange precipitate dried (60 mg). Recrystallization from ethanol/2 M hydrochloric acid afforded orange needles of chelerythrine chloride, m.p. 199°-202° (dec.) (36 mg), which gave a single, orange fluorescent spot (R_f 0,06-S2) on t.l.c. The infrared spectrum was superimposable with that of an authentic sample of chelerythrine chloride.

2B.2.2.2.9 Fraction 33

This fraction (1,076 g) was dissolved in methanol (15 mℓ), filtered and an aqueous saturated solution of potassium iodide (0,3 mℓ) added to the filtrate. The resultant crystalline solid (415 mg) in methanol (25 mℓ) was decolourized (charcoal) and the solution cooled in ice to yield colourless platelets of (-)-α-canadine methiodide, m.p. 249°-251° (dec.) (314 mg), which gave a single, non-fluorescent, Dragendorff-positive spot (R_f 0,02-S2) on t.l.c. The infrared spectrum was superimposable with that of an authentic sample of (-)-α-canadine methiodide.

2B.2.2.2.10 Yellow insoluble solid (YP3)

This solid (2B.2.2.2.3 refers) was dissolved in methanol (100 mℓ) and the solution filtered. The filtrate was concentrated

under reduced pressure to 40 mℓ, conc. hydrochloric acid (2 mℓ) was added and the mixture refluxed for 40 minutes with constant stirring. Refrigeration of the solution overnight afforded yellowish needles, (NF/14), m.p. 275°-278° (91 mg). The ultraviolet and infrared spectra, melting point, m.m.p. and chromatographic characteristics of NF/14 were identical with those of an authentic sample of nitidine chloride.

2B.2.2.3 Methanol extract of root bark (ZT/RB3)

A portion (10,7 g) of this extract was agitated with 0,1 M hydrochloric acid and the resultant brown precipitate extracted with ether (50 mℓ). The ether fraction was discarded and the acidic fraction was adjusted to pH 6 with 10% $^{m}/v$ aqueous sodium hydroxide solution (±5 mℓ). After filtration and rejection of the unwanted brown precipitate, the filtrate was gently stirred with 'Amberlite' resin IRC-50; analytical grade BDH-20 g (50% H^+ and 50% Na^+ form by volume) for 24 hours. The "suspension" was then transferred to a glass column where the filtrate was slowly passed through the resin several times. The resin column was washed with water, 50% $^{v}/v$ aqueous methanol, and methanol (100 mℓ of each solvent). The alkaloids were then eluted with 5% $^{v}/v$ conc. hydrochloric acid in methanol (200 mℓ) and the eluate concentrated *in vacuo* to dryness. Absolute ethanol (30 mℓ) was added to the residue and the mixture refrigerated overnight. The insoluble sodium chloride was removed by filtration and the filtrate concentrated to dryness. This residue (914 mg) was dissolved in dry methanol and the solution refrigerated for about a month. The resultant crystalline solid (100 mg) was recrystallized from dry methanol to yield prisms (NF/15) of (+)-laurifoline chloride, m.p. 252°-253° (dec.) (43 mg),

[lit.[117] 253° (dec.)]; $[\alpha]_D^{24}$ + 17,3° (*c*, 1,037 in water) [lit.[124] $[\alpha]_D$ + 17°].

UV spectrum: λ_{max}^{MeOH} (log ε) 228 (4,56), 283 (4,09), 308 nm (4,19);

[lit.[117] λ_{max}^{MeOH} (log ε) 228 (4,61), 282 (4,17), 308 nm (4,26)].

MS spectrum: Found: (M^+) 342, 1683

$C_{20}H_{24}O_4N^+$ requires: (M^+) 342, 1705

Characteristic fragments at $^m/_e$ 341 (11%) [M^+ - 1] and $^m/_e$ 58 (100%)

The infrared spectrum and chromatographic characteristics were identical with those of an authentic sample of (+)-laurifoline chloride.

2B.2.2.3.1 Low pressure liquid chromatography of ZT/RB3

A portion (10,3 g) of the methanol extract of the root bark was extracted with 0,2 M hydrochloric acid. The unwanted precipitate was removed by extraction with ether (50 mℓ). The aqueous acidic fraction was neutralized by passage through an ion-exchange column ('Amberlite' resin IR-4B (OH) - BDH) and the eluate plus washings freeze-dried. The dry residue, dissolved in the minimum of solvent "A" [methanol/water/strong ammonia solution (15:9:1)] was applied to a preparative low pressure liquid chromatography system consisting of a "scrubber" column (15 mm x 250 mm; 18,3 g Kieselgel Woelm 0,032 - 0,063 mm ICN Pharmaceuticals) and a main column (25 mm x 1000 mm; 296 g Kieselgel Woelm 0,032 - 0,063 mm ICN Pharmaceuticals).

Using 500 kPa pressure, the columns were then eluted with solvent "A" at a rate of approximately 4 mℓ/minute and the eluate collected in 10 mℓ fractions on a fraction collector. All fractions were monitored for alkaloids on t.l.c. using system S5 and like

fractions were bulked.

2B.2.2.3.2 Fractions 57 - 66

This fraction (±200 mℓ) was freeze-dried to yield an alkaloid-positive residue (1,16 g). This residue was dissolved in methanol (25 mℓ) and the insoluble material removed and discarded. The filtrate was concentrated *in vacuo* to yield a brownish residue (757 mg) which, in turn, was dissolved in methanol (5 mℓ), an aqueous saturated solution of potassium iodide (0,2 mℓ) added and the mixture refrigerated. The resultant precipitate (196 mg) dissolved in methanol (20 mℓ), was decolourized (charcoal) and the pale yellow filtrate was concentrated to dryness to yield a yellow residue (129 mg). The residue crystallized from dry methanol to afford colourless prisms (NF/16) of (+)-magnoflorine iodide, m.p. 248°-250° (dec.) (8 mg), [lit.[118] 248° (dec.)]. The yellow mother liquor, after concentration to 1 mℓ and cooling in ice, yielded a further crop of prisms (36 mg) which gave a single, non-fluorescent spot (R_f 0,32-S5) on t.l.c. The spot exhibited a blue fluorescence after exposure of the plate to ammonia vapour.

The ultraviolet and infrared spectra, m.m.p. and chromatographic characteristics of NF/16 were identical with those of an authentic sample of (+)-magnoflorine iodide.

2B.2.2.3.3 Fractions 84 - 86

The residue from this fraction was refluxed with methanol (30 mℓ) and the insoluble material removed and rejected. The filtrate was concentrated *in vacuo* to yield a brown oil. This oil was dissolved in dry methanol (2 mℓ) and dry ethyl acetate added to yield a deposit (80 mg) which gave two non-fluorescent, alkaloid-positive spots ($R_{f's}$ 0,86 and 0,75-S5) on t.l.c. The predominant spot (R_f 0,86) gave a

distinctive green colour with iodoplatinic acid reagent[107] indicative of tembetarine. All attempts to crystallize this material were unsuccessful.

2B.2.3 *Z. humile* concentrated extracts

2B.2.3.1 Light petroleum (b.p. 40°-60°) extract of root bark (ZH/RB1)

A portion (36,33 g) of the dark brown, viscous extract was dissolved in ether (150 mℓ) and extracted with M hydrochloric acid (50 mℓ), 0,2 M hydrochloric acid (2 x 50 mℓ) and 0,1 M hydrochloric acid (3 x 50 mℓ). The orange precipitate (OP2) collecting at the interface after each addition of acid, was removed by filtration and the acid filtrates (AF4) bulked (±300 mℓ). The ether fraction (E2) was dried ($CaCl_2$) and concentrated to dryness to yield a dark brown oil (25,24 g).

2B.2.3.1.1 Orange precipitate (OP2)

A portion of this solid (920 mg) was chromatographed on a silica gel (Kieselgel 60 - Merck) column (19,5 mm x 340 mm; 50 g) using chloroform as the packing solvent. The column was eluted at a rate of 4 mℓ/minute using the stipulated solvents, and the fractions indicated in the table below were collected.

Fraction	Eluting Solvent	Volume (mℓ)	Mass (mg)
1 - 3	Chloroform	275	590
4 - 6*	Chloroform	375	30
7 - 9*	Chloroform	450	30
10 -11*	Chloroform	300	50
12 - 15	Chloroform/ethanol (9:1)	600	27
16 - 17	Chloroform/ethanol (8:2)	300	54
18 - 19*	Chloroform/ethanol (1:1)	450	100

* Yielded no crystalline residue, hence further investigation was abandoned.

2B.2.3.1.2 Fractions 1 - 3

This fraction was recrystallized from chloroform/ethanol to afford colourless platelets (NF/17) of *N*-norchelerythrine, m.p. 212°-214° (66 mg) [lit.[125] 211°-213°], which gave a single, yellow fluorescent spot (R_f 0,84-S2) on t.l.c. When the plate was sprayed with Dragendorff's reagent[103], the spot gave a brownish-orange colour which in turn changed to rose-pink when the sprayed plate was subsequently exposed to ammonia vapour.

The m.m.p., chromatographic characteristics and infrared spectrum of NF/17 were identical with those of *O*-methyldecarine, (*N*-norchelerythrine) a compound synthesized by methylation of decarine, an alkaloid previously isolated from *Z. thorncroftii* (*vide* 2B.2.2.2.7).

2B.2.3.1.3 Fractions 12 - 15

This fraction was dissolved in a mixture (15 mℓ) of chloroform/methanol (1:1) and dilute hydrochloric acid (0,1 mℓ) added. The resultant orange precipitate (8 mg) was dried and sublimed at 175°/0,3 mmHg to yield a crystalline solid, (NF/18), m.p. 230°-235°. When this solid was spotted on filter paper and then sprayed with Dragendorff's reagent, a brownish-orange colour was produced which changed to deep blue when the sprayed paper was subsequently exposed to ammonia vapour.

An infrared spectrum of the sublimate (NF/18) was virtually superimposable with that of decarine hydrochloride. Further purification could not be effected due to an insufficiency of material.

2B.2.3.1.4 Fractions 16 - 17

A solution of this fraction in alcohol (2 mℓ), was treated with

6 M nitric acid (0,15 mℓ) and cooled in ice. The resultant yellow precipitate (36 mg) was dissolved in absolute ethanol (3 mℓ) and chromatographed on a short column (8 mm x 140 mm; 5 g) of silica gel (Kieselgel 60 - Merck). The column was eluted with chloroform/ethanol (99:1) and the eluate (65 mℓ) discarded. The yellow material adsorbed on the column was then eluted with methanol (25 mℓ) and the eluate concentrated to dryness *in vacuo*. The residue was recrystallized from absolute ethanol to yield fine needles (NF/19) of chelerythrine nitrate, m.p. 239°-240°, (7 mg) [lit.[126] 240°], which gave a single, orange fluorescent spot (R_f 0,05-S5) on t.l.c. On spraying the plates with Dragendorff's reagent[103] and iodoplatinic acid reagent[107], the spot gave orange and brownish colours respectively.

The ultraviolet and infrared spectra, m.m.p. and chromatographic characteristics of NF/19 were identical with those of an authentic sample of chelerythrine nitrate.

2B.2.3.1.5 Isolation of chelerythrine chloride from OP2

Another portion of OP2 (944 mg) was boiled in methanol (10 mℓ). The insoluble, neutral white solid, (NS/1), m.p. 144°-145° (143 mg), was removed and reserved for further study while the filtrate was chromatographed on a silica gel (Kieselgel 60 - Merck) column (19,5 mm x 340 mm; 50 g) which had been packed dry. The column was eluted successively with chloroform, chloroform/ethanol (99:1), chloroform/ethanol (8:2) using 100 mℓ of each solvent. The column was finally eluted with methanol (100 mℓ), the solid (300 mg) dissolved in hot 5% $^{v}/_{v}$ conc. hydrochloric acid in methanol (20 mℓ) and the solution refrigerated to afford fine, orange needles (NF/20) of chelerythrine chloride, m.p. 202°-203° (dec.), (135 mg) [lit.[127]

203°-205° (dec.)], which gave a single, orange fluorescent spot (R_f 0,02-S2; R_f 0,06-S5) on t.l.c. On spraying the plates with Dragendorff's and iodoplatinic acid reagents, identical colours as described under 2B.2.3.1.4 were obtained. The mother liquor yielded a further crop of orange needles, m.p. 204°-205° (dec.) (42 mg).

The infrared spectrum, m.m.p. and chromatographic characteristics of NF/20 were identical with those of an authentic sample of chelerythrine chloride.

The neutral solid (NS/1) gave a bluish fluorescent spot (R_f 0,26-S1) on t.l.c. When plates were separately exposed to iodine vapour or sprayed with $SbCl_3$/HCl and then heated, the spot gave a yellow colour with both reagents. This substance was not examined further.

2B.2.3.1.6 Combined acid filtrate (AF4)

This fraction was adjusted to pH 8 with strong ammonia solution. The resultant precipitate was extracted with chloroform (100 mℓ; 2 x 50 mℓ). The bulked chloroform fraction (±200 mℓ) was dried ($CaCl_2$) and concentrated to dryness under reduced pressure. The residue (400 mg) was chromatographed on a silica gel (Kieselgel 60 - Merck) column (10 mm x 400 mm; 20 g) using chloroform as the packing solvent. The column was eluted with the same solvent and the first 70 mℓ of eluate was reduced to dryness *in vacuo* (186 mg). The residue was recrystallized from benzene to afford colourless prisms (NF/21) of skimmianine, m.p. 177°-178° (41 mg) [lit.[112] 175°-176°], which gave a single, blue fluorescent spot (R_f 0,38-S2) on t.l.c. When the plate was sprayed with Dragendorff's reagent,

the spot gave an orange colour which changed to rose-pink when the sprayed plate was subsequently exposed to ammonia vapour.

The ultraviolet and infrared spectra, m.m.p. and chromatographic characteristics of NF/21 were identical with those of an authentic sample of skimmianine.

2B.2.3.1.7 Low pressure liquid chromatography of E2

A portion (3 g) of E2 (2B.2.3.1 refers) was dissolved in benzene (3 mℓ) and applied to a low pressure preparative liquid chromatography system consisting of a "scrubber" column (15 mm x 250 mm; 18,3 g Kieselgel Woelm 0,032 - 0,063 mm; ICN Pharmaceuticals) and a main column (15 mm x 950 mm; 71 g identical adsorbent). The columns were eluted at 500 kPa with *n*-hexane/ethyl acetate (4:1) and the eluate collected in 10 mℓ fractions on a fraction collector. Homogeneous fractions were bulked after they had been monitored by t.l.c. System 1.

2B.2.3.1.8 Fractions 17 - 23

This fraction (367 mg) was decolourized (charcoal) and recrystallized from acetone to afford colourless needles (NF/22) of lupeol, m.p. 195°-198° (59 mg) [lit.[109] 196°], which gave a single, non-fluorescent spot (R_f 0,49-S1) on t.l.c. When the plate was sprayed with 300% $^m/_v$ antimony trichloride in conc. hydrochloric acid and the plate heated at 110° for 3 minutes, the spot gave an orange colour which changed through crimson to purple after several hours.
MS : Found : M^+ 426,3892 ; $C_{30}H_{50}O$ requires: 426,3862

The ultraviolet and infrared spectra as well as the chromatographic characteristics of NF/22 were identical with those of an authentic sample of lupeol.

2B.2.3.1.9 Fractions 30 - 38

This fraction (82 mg) was recrystallized from *n*-hexane (5 mℓ) to

afford faintly yellow needles of sesamin, m.p. 125° (30 mg). The mother-liquor after concentration to ±1 mℓ afforded colourless plates of β-sitosterol (NF/23), m.p. 135°-137° (6 mg) [lit.[75] 136°], which gave a single, non-fluorescent spot (R_f 0,31-S1) on t.l.c. When the plate was treated with $SbCl_3$/HCl as described previously, the spot gave a pink colour which changed to mauve after several hours.

MS: Found: M^+ 414,3903 ; $C_{29}H_{50}O$ requires: 414,3861

The ultraviolet and infrared spectra, m.m.p. and chromatographic characteristics of NF/23 were identical with those of an authentic sample of β-sitosterol.

2B.2.3.1.10 Fractions 50 - 59

This fraction (278 mg) crystallized readily from methanol to afford slender, colourless needles of (-)-sesamin (NF/24), m.p. 124°-125°, [lit.[67] 124°-126°]; $[\alpha]_D^{24}$ -35,3° (*c*, 1,11 in chloroform) [lit.[67] $[\alpha]_D^{24}$ -68,6° (*c* 0,53 in chloroform)], which gave a single, non-fluorescent spot (R_f 0,32-S1) on t.l.c. When the plate was treated with $SbCl_3$/HCl, the spot gave a greyish-blue colour.

MS: Found: M^+ 354,1152 ; $C_{20}H_{18}O_6$ requires: 354,1103

The ultraviolet and infrared spectra as well as the chromatographic characteristics of NF/24 were identical with those of an authentic sample of (+)-sesamin.

2B.2.3.2 Chloroform extract of root bark (ZH/RB2)

A portion (22,9 g) of the extract was dissolved in chloroform (200 mℓ) and extracted with M hydrochloric acid (3 x 150 mℓ). After filtration, the bulked acid fraction (±450 mℓ) was adjusted to pH 8 by the addition of strong ammonia solution (30 mℓ) and subjected to a liquid-liquid extraction with chloroform (1 ℓ) for 48 hours. The chloroform extract was dried ($CaCl_2$) and reduced to dryness *in vacuo*

(1,45 g).

A portion (1,049 g) of the dry residue in chloroform (2 mℓ) was chromatographed on a silica gel (Kieselgel 60; Merck) column (30 mm x 260 mm); (100 g) which had been packed dry. The column was initially eluted with chloroform, followed by chloroform containing increasing percentages of methanol and finally with methanol containing 1% $^{v}/_{v}$ conc. hydrochloric acid. Fractions as depicted below were collected.

Fraction	Eluting Solvent	Volume of Eluate (mℓ)	Mass (mg)
1-6	Chloroform	1000	50
7-12	Chloroform/methanol (95:5)	600	166
13-16	Chloroform/methanol (90:10)	400	40
17-18	Chloroform/methanol (80:20)	200	60
19-21	Chloroform/methanol (80:20)	300	350
22-23	Chloroform/methanol (50:50)	200	84
24-26	Chloroform/methanol (50:50)	325	40
27-28	Methanol/hydrochloric acid (99:1)	150	170

2B.2.3.2.1 Fractions 19 - 23 (bulked)

The residue dissolved in methanol (15 mℓ) was decolourized (charcoal) and the filtrate concentrated to dryness under reduced pressure. The residue (408 mg) was dissolved in methanol (1,5 mℓ) and an aqueous saturated solution of potassium iodide (0,2 mℓ) added. The resultant crystalline solid was washed with ice-cold water (2 x 5 mℓ), dried (silica gel) and the off-white residue (106 mg) recrystallized from methanol to afford colourless platelets of (-)-α-canadine methiodide (NF/25), which melted at 164°-165°, solidified at 180°-220° and finally remelted at 251°-252° (dec.) [lit.[114] 249°-251° (dec.)]. This alkaloid produced a green colour

with gallic acid and sulphuric acid (Labat's test[120]) and gave a single, non-fluorescent spot (R_f 0,78-S5) on t.l.c. Chromatography on silica gel thin-layer chromatoplates using methanol/water/conc. ammonia (15:9:1) as developing solvent gave a single, non-fluorescent (Dragendorff-positive) spot (R_f 0,30), which became purple with iodoplatinic acid reagent[107].

The ultraviolet and infrared spectra, m.m.p. and chromatographic characteristics of NF/25 were identical with those of an authentic sample of (-)-α-canadine methiodide.

No further crystalline material could be obtained from any of the remaining fractions.

2B.2.3.3 Methanol extract of root bark (ZH/RB3)

A portion (100 g) of the extract was mixed with Celite '545' (50 g) and the suspension continuously stirred with 0,1 M hydrochloric acid for 12 hours. After four days, the suspension was filtered. The residue was similarly extracted with additional 0,2 M hydrochloric acid (200 mℓ).

The bulked acid filtrate (±450 mℓ) was adjusted to pH 6 with 2 M sodium hydroxide solution and stirred with 'Amberlite' resin IRC-50 analytical grade - BDH; 260 g (75% Na^+ and 25% H^+ form by volume) for 24 hours. The "suspension" was transferred to a glass column (40 mm x 600 mm) where the filtrate was passed slowly through the resin several times. The resin column was washed with water, 50% $^v/_v$ aqueous methanol and methanol (1 ℓ of each solvent). The alkaloids were then eluted with 5% $^v/_v$ conc. hydrochloric acid in methanol until the eluate was negative to Dragendorff's reagent. The eluate (2 ℓ) was concentrated under reduced pressure to 200 mℓ and the mixture refrigerated overnight. The insoluble sodium

chloride was removed by filtration, the filtrate further reduced to 50 mℓ and refrigerated again. After further removal of sodium chloride, the filtrate was reduced to dryness *in vacuo* (12,5 g).

The residue, dissolved in dry ethanol (30 mℓ), was refrigerated for 10 days to yield a crystalline solid (S1) (605 mg) which, on recrystallization from absolute ethanol, afforded fine needles (NF/26) of candicine chloride, m.p. 273°-274° (dec.) (45 mg) [lit.[113] 276°-278° (dec.)]. NF/26 gave a single, non-fluorescent (before and after exposure to ammonia vapour) spot (R_f 0,91-S5) on t.l.c. When the chromatoplates were separately sprayed with Dragendorff's reagent [103], iodoplatinic acid reagent[107] and diazotised sulphanilic acid[108], the spot gave orange, greyish and red colours respectively. The ethanolic filtrate from S1 was concentrated to dryness under reduced pressure and reserved for further study (R4; 6,38 g).

The residue (317 mg) from the mother-liquors of NF/26 was dissolved in dry methanol (3 mℓ) and ether added until the resultant cloudiness just persisted. A further crop of slender, colourless needles (NF/26a) of candicine chloride, m.p. 276°-277° (dec.) (77 mg) [lit.[128] 278°-279° (dec.)], resulted.

MS: $^{m}/_{e}$ ($M^{+}-59$) 121, 120, 107, 91 and 59.

PMR spectrum: (D_2O) δ3,16 (11H, s); 3,16-3,72 (2H, m); 6,83 (2H, d, J = 9Hz); 7,19 (2H, d, J = 9Hz).

Analysis: A sample (3 mg) was dried (P_2O_5) at 82°/0,3 mmHg for 6 hours.

Found: N = 6,43%

Calculated for $C_{11}H_{18}N^{+}OCl^{-}$: N = 6,49%

The ultraviolet and infrared spectra, m.m.p. and chromatographic characteristics of NF/26 and NF/26a were identical with those of an authentic sample of candicine chloride.

2B.2.3.3.1 Conversion of candicine chloride to iodide salt

Candicine chloride (30 mg) was dissolved in water (0,1 mℓ) and an alcoholic saturated solution of potassium iodide (0,25 mℓ) added. The resultant solid was recrystallized from dry ethanol to afford colourless needles of candicine iodide, m.p. 229°-230° (15 mg) [lit.[129] 229°-230°].

2B.2.3.3.2 Column chromatography of R4

A portion (1 g) of R4 (*vide* 2B.2.3.3), dissolved in methanol (3 mℓ), was chromatographed on a column (30 mm x 210 mm) of silicic acid - BDH (100 g) mixed with Celite '545' (25 g) prepared as described[113], and packed into the column as a slurry in chloroform.

The column was eluted with chloroform/methanol mixtures and finally with methanol. The results are tabulated below.

Fraction	Eluting Solvent	Volume of Eluate (mℓ)	Alkaloid	mass (mg)
1	Chloroform/methanol (3:1)	200	-	-
2	Chloroform/methanol (3:1)	200	Mixture	110
3	Chloroform/methanol (1:1)	150	-	-
4	Chloroform/methanol (1:1)	150	tembetarine chloride	377
5	Chloroform/methanol (1:1)	150	-	-
6	Methanol	150	-	-
7	Methanol	150	candicine chloride	128

2B.2.3.3.3 Fraction 4

Concentration of this fraction to dryness under reduced pressure yielded a cream-coloured, very hygroscopic solid (NF/27) which gave a single, non-fluorescent (before and after exposure to ammonia vapour) spot (R_f 0,86-S5) on t.l.c. When the chromatoplates were separately sprayed with iodoplatinic acid reagent[107] and diazotised sulphanilic acid[108], the spot gave green and orange colours respectively. Co-spotting of NF/27 with an authentic sample of (+)-tembetarine chloride produced a single Dragendorff-positive spot on two different t.l.c. systems. Additionally, the infrared spectrum of NF/27 was superimposable with that of an authentic sample of (+)-tembetarine chloride. However, all attempts to crystallize NF/27 were unsuccessful.

3A. DISCUSSION (*AGATHOSMA* WILLD. INVESTIGATION)

3A.1 Coumarins

The interest in coumarins probably stems from their real or potential medicinal virtues in such areas as anticoagulants, dermal photosensitizers, vasodilators and antibacterial agents, (*e.g.* novobiocin) as well as other uses, *e.g.* flavouring agents (constituent of vanilla extract)[130,131]. It is therefore not surprising that phytochemical investigations for naturally-occurring coumarins have accelerated over the last decade. Additionally, there has been a parallel and prodigious output of synthetic coumarin derivatives aimed at providing compounds specially tailored for more selective or even antagonistic activity[130]. Some of these, *e.g.* 3-pyridyl- and 3-aminocoumarins, have been reported to act as central nervous system depressants and to possess antibacterial properties respectively. A hitherto unknown class of 3-phenoxycoumarin derivatives has recently been synthesized and the majority of these compounds show anti-tubercular activity[132].

As the thioester *S*-prenylthioisobutyrate was reported to occur in *Agathosma puberula* (Steud.) Fourc., *A. clavisepala* R.A.Dyer and *A. apiculata* G.F.W.Mey[22], it was likely that prenylated coumarins may also occur in these species. The present work describes the isolation and characterization of a novel prenylated coumarin, puberulin, obtained in reasonable yield from *A. puberula* and in a much lower yield from *A. clavisepala*. This compound was neither detected in *A. apiculata* nor in *A. ovata* (Thunb.) Pillans though it is noteworthy that the latter species contains no thioester[22].

3A.2 Extraction and purification of puberulin

Puberulin, like most free (non-glycosidic) coumarins, was easily extracted from the dried, powdered plant material with *n*-hexane, but the crude crystalline material from the *n*-hexane extracts was purified with some difficulty.

Whilst column chromatography initially seemed a reasonable method, it was subsequently found to be unsatisfactory when employed as the sole technique because it did not effect complete separation of puberulin from other secondary plant metabolites, *e.g.* waxes. This problem was further compounded by the fact that these waxes exhibited a similar melting point to that of puberulin. Although puberulin gave a blue fluorescence (UV light; 366 nm) on t.l.c. plates while the waxes did not, it was, nevertheless, not possible to rely on t.l.c. as an indication of sample purity. After several rather variable elemental microanalyses, it was concluded that waxes were in fact co-crystallizing with the puberulin thereby causing a marked depression in melting point.

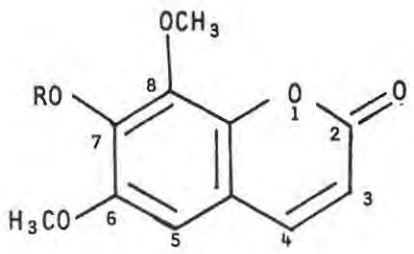
In order to remove the neutral compounds co-crystallizing with puberulin, the "alkali-conversion" method was utilized. The lactone ring was opened by dilute sodium hydroxide solution to give a water-soluble coumarinate which on acidification at 0°, cyclized to regenerate the original coumarin. This method, in combination with column chromatography on neutral alumina, afforded analytically acceptable puberulin.

3A.3 Locality of puberulin

In order to establish the locality of maximum puberulin occurrence, the aerial parts of *Agathosma puberula* (Steud.) Fourc.

were separated into leaves and stems/twigs. Reference to Table 2 (page 32) confirms that the leaves are the site of maximum puberulin deposition. This is in accordance with the work of Gorz and Haskins[131] who found that the primary site of coumarin synthesis is in the young, actively growing leaves and that the stems and roots are relatively unimportant sites. There is no doubt, however, that both roots and aerial organs of the plants can form coumarins, but there is strong evidence that under normal growth conditions, synthesis predominates in the leaves[131].

3A.4 Structure elucidation of puberulin



[1] R = $(CH_3)_2C{=}CHCH_2$

[2] R = H

[3] R = CH_3

[4] R = CH_3CH_2

[5] R = CH_3CO

Puberulin [1] crystallized as colourless platelets, m.p. 90°-92°, and was shown to have the formula $C_{16}H_{18}O_5$ (M^+ 290). Like most other coumarins, it exhibited a blue fluorescence under UV light (366 nm). It also gave a positive orange spot on t.l.c. with Dragendorff's reagent[103]. This false-positive reaction with an essentially alkaloidal reagent, has been reported for non-nitrogenous compounds possessing ketone, aldehyde or lactone functions[133].

The ultraviolet absorption bands at 210, 228, 298 and 342 nm are suggestive of a coumarin substituted in the benzene ring only[130]. The addition of alkali produced no bathochromic shift in the spectrum and is indicative of the lack of -OH substituents.

As the position of the carbonyl band in the infrared spectra of coumarins is generally more constant when recorded in chloroform[130], the spectrum of puberulin was accordingly recorded in this solvent.

The presence of a strong absorption band at 1720 cm^{-1} in the spectrum of puberulin is characteristic of a coumarinyl lactone while strong, sharp absorption at 1610 cm^{-1} is due to the C=C stretching frequency of a pyrone ring[130]. The lack of absorption in the 3500 cm^{-1} region confirms the absence of hydroxyl groups.

In an extensive review, Steck and Mazurek[134] showed that p.m.r. spectroscopy is a most useful technique in the characterization of coumarins. Based on chemical shifts and coupling constants of over a hundred natural coumarins and their derivatives, spectra-structure correlations were proposed. Although other n.m.r. techniques, *e.g.* internal nuclear Overhauser effect and ^{13}C n.m.r. spectroscopy, have also been successfully applied to structural problems, a simple, rapid, non-degradative method utilizing the n.m.r. shift reagent $Eu(fod)_3$ to aid elucidation of the substitution pattern of coumarins, was recently described[135].

The p.m.r. spectrum of puberulin [1] defined all eighteen protons. The two doublets (δ6,35 and 7,66; J = 9,5 Hz) are typical of a coumarin nucleus unsubstituted in the pyrone ring and are attributed to the *cis* protons H-3 and H-4 of this ring. In coumarins lacking an oxygen function at C-5, the H-4 doublet occurs at δ7,5-7,9

(in $CDCl_3$), whereas the H-4 resonance is shifted downfield to δ7,9-8,2 ($CDCl_3$) should C-5 bear an oxygen function[134]. On this basis, it appears that puberulin having an H-4 doublet at δ7,66 obviously lacks a C-5 oxygen function and this is further supported by the presence of a one proton singlet at δ6,71.

The two signals consisting of three protons each (δ4,05 and δ3,91) are assigned to two aromatic methoxyl groups. The methylene doublet (δ4,66; J = 6 Hz), coupled olefinic triplet (δ5,60) and two non-equivalent methyl resonances at δ1,73 and δ1,79 strongly suggest the presence of a 3-methyl-2-butenyl(prenyl) group or its oxygenated congener. The three alkyl substituents must occupy the three vacant positions on the benzene ring (C-6, C-7 and C-8), the exact positions of which were firmly established by chemical methods.

The mass spectrum of puberulin supported the structural evidence already available. The low relative abundance (3%) of the molecular ion (M^+) at $^m/_e$ 290 and the intense fragments at $^m/_e$ 222 (100%) and 69 (96%), suggest a facile fragmentation of the prenyl substituent[136,137].

Naturally-occurring coumarins containing isoprenoid residues attached to the ring through oxygen are often characterized by the extreme ease of decomposition under electron bombardment[137,138] thus confirming that puberulin is definitely an *O*-prenyl coumarin.

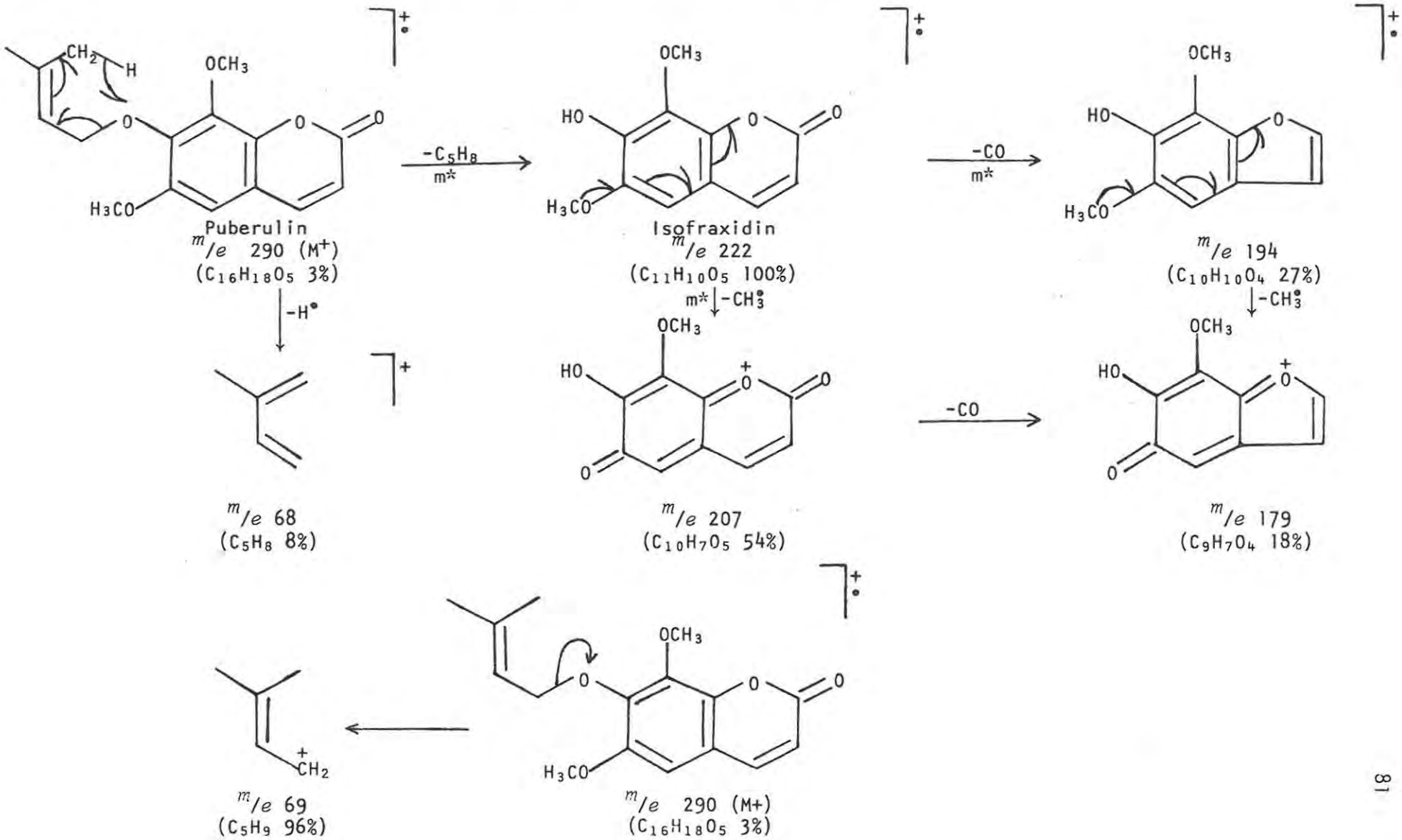
The remaining fragmentation pattern of puberulin is characteristic of the coumarins as a whole where several eliminations of 28 atomic mass units (CO) occur. The expected loss of 15 a.m.u. occurs as a result of ejection of a methyl ($-CH_3$) group from the aromatic methoxyl substituents.

The assignment of structures corresponding to $[M^+ - 28]$ ions arising from loss of the lactone carbonyl function as CO seems to be quite controversial. Comparison of the fragmentation pattern of benzofuran derivatives caused several authors to suggest a benzofuran-type structure for the $[M^+ - 28]$ ion in the case of coumarins. A study of the electron impact fragmentations of four monodeuterio-2-pyrones has shown that the distribution of deuterium among the various cations allows confident exclusion of a furan-like structure for the 2-pyrone $[M^+ - CO]$ ion; consequently no single structure can be assigned to the $[M^+ - CO]$ ion obtained from coumarins[130]. For the sake of simplicity and in accordance with most representations, a benzofuran structure for the $[M^+ - 28]$ ion is used in Scheme 1 (overleaf).

Reference to Scheme 1 shows that puberulin readily loses the side-chain as a result of rupture of the carbon-oxygen bond with concomitant migration of a hydrogen atom to form a base peak at $^m/_e$ 222 (isofraxidin). The intense peak at $^m/_e$ 69 is obviously due to the 3,3-dimethylallyl ion from the prenyl side-chain. Although the elimination of CO is still the initial stage of decomposition of isofraxidin to give an ion at $^m/_e$ 194 (27%), the further elimination of a methyl group affords a stable quinonoid structure $^m/_e$ 179 (18%) which predominates in the second stage. The metastable peak at $^m/_e$ 170 is probably associated with the loss of the prenyl side-chain, while the metastable peaks at $^m/_e$ 169 and $^m/_e$ 193 are attributed to the loss of CO and $-CH_3$ respectively, resulting in the formation of the corresponding benzofuran and benzopyrone derivatives.

SCHEME 1

MASS SPECTRAL FRAGMENTATION OF PUBERULIN



Although spectral evidence showed quite conclusively that puberulin was a simple coumarin possessing three (two methoxyl and a prenyl) substituents on the benzene ring, the exact position of these was finally confirmed by the application of chemical methods.

Since the p.m.r. spectrum of puberulin showed two doublets at δ6,35 and δ7,66 ($J_{3,4}$ = 9,5 Hz) attributed to vicinally coupled protons, hydrogenation of the double bond over Paal catalyst[104] was attempted. The p.m.r. spectrum of the "hydrogenated" product [2] resembled that of puberulin in that it possessed similar methoxyl singlets (δ3,96 and δ4,10), a one proton singlet (δ6,74) assigned to H-5 and the two doublets (δ6,33 and δ7,68; J = 9,5 Hz) attributed to H-3 and H-4 respectively of the pyrone ring. The methylene doublet (δ4,66) coupled olefinic triplet (δ5,60) and two methyl singlets (δ1,73 and δ1,79) present in puberulin had disappeared and were replaced by a one proton singlet (δ6,33) which, in turn, readily exchanged with D_2O. The facile hydrogenolysis of the prenyl to a hydroxyl group strongly suggests that puberulin is present as the *O*-prenyl rather than a *C*-prenyl group.

The above evidence is further corroborated by the mass spectrum of puberulin which shows a low relative abundance (3%) of the parent ion and intense fragments at $^{m}/_{e}$ 69 (96%; prenyl) and $^{m}/_{e}$ 222 (100%) - evidence consistent with that of an *O*-prenyl coumarin[136]. Since allylic ethers seem to readily undergo hydrogenolysis[137], it was not surprising that after several attempts with both high and low pressure hydrogenation, no dihydro-derivative could be isolated. This is similar to the experience of Noguti and Kawanami[139] who were unable to form

the dihydro-derivative of phellopterin by catalytic reduction. It was also possible to effect fission of the ether linkage by acid treatment. In addition to the p.m.r. evidence for a phenolic group, the "hydrogenated" product [2] also gave a green precipitate with ferric chloride solution and showed typical -OH absorption at 3530 cm^{-1} in the infrared spectrum.

The isolated phenol, $C_{11}H_{10}O_5$ [2], had a melting point of 147°-148°. The melting points of all three 6,7,8-dimethoxyhydroxycoumarins are given in Table 4.

TABLE 4: Melting point data of 6,7,8-dimethoxyhydroxycoumarins

Coumarin	Reported m.p. (°C)	Reference
6,8-dimethoxy-7-hydroxycoumarin	148° - 149°	140
7,8-dimethoxy-6-hydroxycoumarin	184°	141
6,7-dimethoxy-8-hydroxycoumarin	195°	142

It is evident from Table 4 that the phenol [2] produced by the hydrogenolysis of puberulin [1] is probably 6,8-dimethoxy-7-hydroxycoumarin (isofraxidin). This was confirmed by preparation of the methyl [3] and ethyl [4] ethers of the phenol and comparison of their melting points with literature values. (*vide* Table 5).

TABLE 5: Melting point data of ethers of "hydrogenated" puberulin compared with known compounds

Isolated compound	m.p. (°C)	Compound (literature)	m.p. (°C)	Ref.
methyl ether of "hydrogenated" puberulin [3]	104°-105°	methyl ether of isofraxidin	104°-105°	140
ethyl ether of "hydrogenated" puberulin [4]	82,5°-83,5°	ethyl ether of isofraxidin	82°	141

The phenol [2] was also acetylated to afford the unrecorded acetyl derivative, $C_{13}H_{12}O_6$, [5], m.p. 142°-143°.

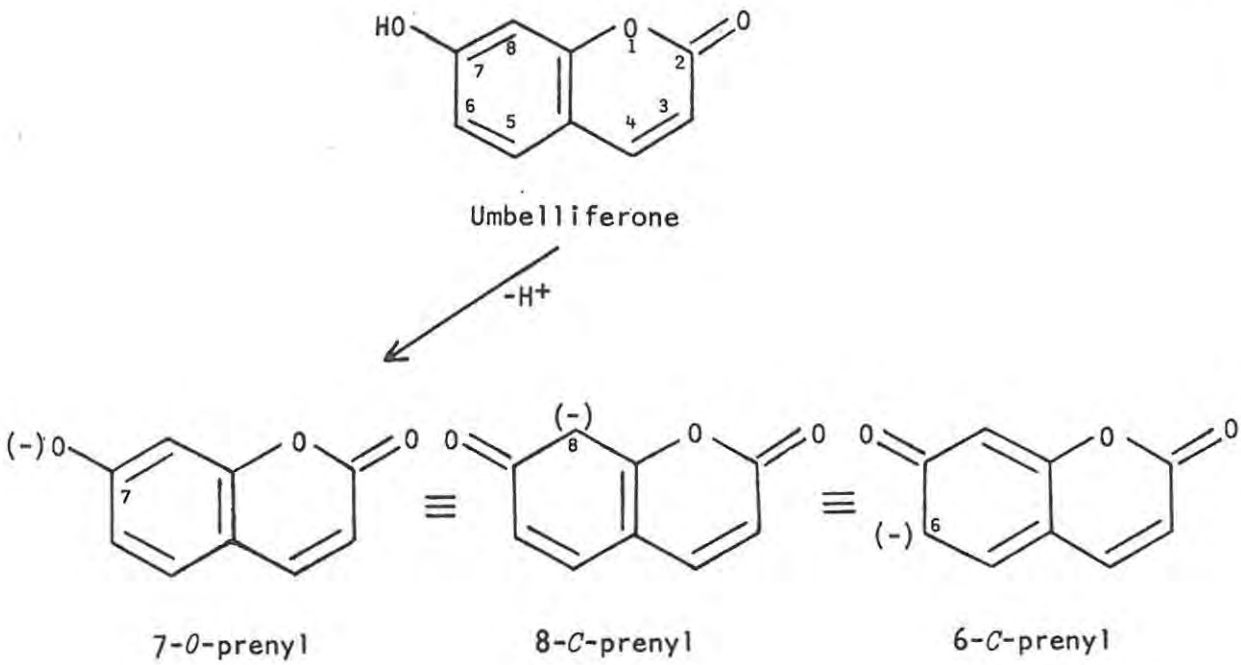
Puberulin was synthesized by the treatment of isofraxidin [2] with prenyl bromide[105]. The product was indistinguishable by infrared spectroscopy, thin-layer chromatography and mixed melting point from puberulin [1].

3A.5 Coumarins with isoprenoid substituents

Naturally-occurring coumarins have been isolated from many plant families, but those having isoprenoid substituents (prenyl, geranyl, farnesyl) occur chiefly among members of the Rutaceae, Umbelliferae[134] and Compositae[136,143,144,145]. The isoprenoid chains are of biosynthetic significance and these may be present as either *O*- or *C*- substituents in either the benzene or pyrone ring.

Prenylation has been shown to occur at the umbelliferone stage where the addition of the prenyl unit at C-6 is controlled by a specific enzyme. *O*- and C-8 prenylation is probably mediated by other similar enzyme systems that have yet to be resolved. Prenylation of umbelliferone is represented as involving the formation of a stable anion which will permit the electrophilic attack of a prenyl carbonium ion at either C-6 or C-8 to yield *C*-prenyl coumarins or on the phenoxide to give *O*-prenyl coumarins (*vide* Scheme 2)[30].

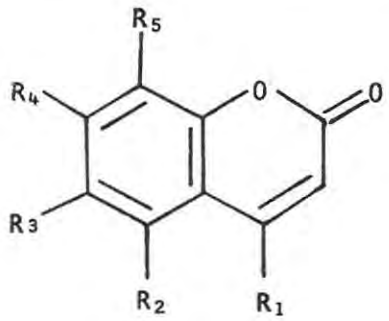
SCHEME 2: ORIGINS OF PRENYLATED COUMARINS



The simple coumarin, puberulin, isolated from *Agathosma puberula* (Steud.) Fourc. and in much lower yield from *A. clavisepala* R.A. Dyer, is an *O*-prenyl coumarin and is closely related to the sesquiterpene ethers of isofraxidin obtained from certain *Artemisia* L.[145] and *Anthemis* L.[136] species (Compositae). Subsequent to our work[31], Bohlmann and his co-workers reported the isolation of an impure oil from *Pteronia ciliata* Thunb. (Compositae) for which, based entirely on spectroscopic evidence, they suggested the same structure as puberulin[143].

Table 6 lists the known simple coumarins having unmodified *O*-prenyl side-chains, their melting points and distribution.

TABLE 6 : *O*-PRENYL SUBSTITUTED SIMPLE COUMARINS AND THEIR DISTRIBUTION



Coumarin	Structure	m.p.(°C)	Plant Source (Family)	Ref.
7-isopentenyloxy-8-isopentenyl-coumarin	R_1=R_2=R_3= H R_4= O-CH_2-CH=C(CH_3)$_2$ R_5= CH_2-CH=C(CH_3)$_2$	66,5-66,8	*Choisya arizonica* Standl. *Choisya mollis* Standl. (Rutaceae) *Choisya ternata* H.B.K.	146
Umbelliferone 3,3-dimethylallyl ether (Auraptene?)	R_1=R_2=R_3=R_5= H R_4= O-CH_2-CH=C(CH_3)$_2$	75 - 76° 77 - 78*	*Seseli libanotis* (L.)Koch } (Umbelliferae) subsp. *intermedium* (Rupr.) } P.W.Ball° } **Euodia vitiflora* F.Muell. } (Rutaceae) *Diosma acmaeophylla* Eckl. & Zeyh. }	147 148 149 23
Scopoletin 7-γ,γ-dimethylallyl ether	R_1=R_2=R_5= H R_3= OCH_3 R_4= O-CH_2-CH=C(CH_3)$_2$	81 - 83	*Artemisia dracunculoides* Pursh. (Compositae) *Diosma acmaeophylla* Eckl. & Zeyh. (Rutaceae)	150 23
Puberulin	R_1=R_2= H R_3=R_5= OCH_3 R_4= O-CH_2-CH=C(CH_3)$_2$	90 - 92	*Agathosma puberula* (Steud.)Fourc. } (Rutaceae) *Agathosma clavisepala* R.A.Dyer } *Pteronia ciliata* Thunb. (Compositae)	31 31 143

TABLE 6 (cont.)

Coumarin	Structure	m.p. (°C)	Plant Source (Family)	Ref.
5-Isopentenyloxy-7-methoxycoumarin	$R_1=R_3=R_5=$ H $R_4=$ OCH_3 $R_2=$ $O\text{-}CH_2\text{-}CH=C(CH_3)_2$	90 - 92	*Citrus limon* Burm. *f.* (Rutaceae)	151
Brayleyanin	$R_1=R_2=$ H $R_3=$ OCH_3 $R_4=$ $O\text{-}CH_2\text{-}CH=C(CH_3)_2$ $R_5=$ $CH_2\text{-}CH=C(CH_3)_2$	95	*Flindersia brayleyana* F.Muell. (Rutaceae)	152
Gerberacoumarin	$R_1=$ $O\text{-}CH_2\text{-}CH=C(CH_3)_2$ $R_2=$ CH_3 $R_3=R_4=R_5=$ H	99	*Gerbera crocea* (L.) Kuntze (Compositae)	144
7-Desmethyl-7-isopentenyl-isoaculeatin	$R_1=R_3=$ H $R_2=$ OCH_3 $R_4=$ $O\text{-}CH_2\text{-}CH=C(CH_3)_2$ $R_5=$ $CH_2\text{-}CH(O)\text{-}C(CH_3)_2$	105,5	*Libanotis buchtormensis* (Fisch.)DC. (Umbelliferae)	153
Sesibiricin	$R_1=R_3=$ H $R_2=$ $O\text{-}CH_2\text{-}CH=C(CH_3)_2$ $R_4=$ OCH_3 $R_5=$ $CH_2\text{-}CH=C(CH_3)_2$	120-122	*Seseli sibiricum* (L.) Garcke (Umbelliferae)	154
Capensin	$R_1=R_2=$ H $R_3=$ OCH_3 $R_4=$ $O\text{-}CH_2\text{-}CH=C(CH_3)_2$ $R_5=$ OH	135-136	*Phyllosma capensis* H.Bol. (Rutaceae)	23
Prenyletin [7-*O*-(3,3-dimethylallyl) aesculetin]	$R_1=R_2=R_5=$ H $R_3=$ OH $R_4=$ $O\text{-}CH_2\text{-}CH=C(CH_3)_2$	145-146	*Ptaeroxylon obliquum* (Thunb.) Radlk. (Ptaeroxylaceae)	155

3B. DISCUSSION (*ZANTHOXYLUM* L. INVESTIGATION)

3B.1 Preliminary extractions and separations

3B.1.1 Materials

The starting materials for this work were authenticated samples of the dried stem and root bark of *Z. davyi* (Verdoorn) Waterm. as well as the root bark of *Z. thorncroftii* (Verdoorn) Waterm. and *Z. humile* (E.A. Bruce) Waterm. (2B.1.1 refers). The investigation selectively centred on the root bark of the species mentioned, because previous phytochemical work[55,156,157,158] had demonstrated that the root bark is the richest site of alkaloid deposition in most *Zanthoxylum* species. Adhering epiphytes or soil particles were carefully removed from the samples lest extraneous matter vitiate the results.

3B.1.2 Extraction

The removal of alkaloids by Soxhlet extraction utilizing organic solvents of increasing polarity was adopted, as this procedure had previously been found to be satisfactory[156,159]. Successive extraction of the powdered bark samples with *n*-hexane or light petroleum (b.p. 40°-60°), followed by chloroform and finally methanol, not only removed both tertiary and quaternary alkaloids, but also extracted large quantities of other secondary plant constituents.

n-Hexane or light petroleum extracted some tertiary alkaloids and some quaternary benzophenanthridine alkaloids, particularly chelerythrine. Non-alkaloidal compounds, *e.g.* phytosterols, lignans and pentacyclic triterpenes, were also removed at this stage.

The remaining tertiary alkaloids were removed by chloroform. With the exception of nitidine (quaternary benzophenanthridine alkaloid), the occurrence of other quaternary alkaloids, *e.g.* tetrahydroprotoberberines, in the chloroform extracts, can probably be ascribed to the presence of 1-2% $^{v}/_{v}$ of ethanol in the commercial solvent.

The majority of quaternary alkaloids and other polar plant constituents, *e.g.* tannins, flavonoids, *etc.*, were extracted with methanol.

All extracts were concentrated under reduced pressure in order to prevent possible decomposition of any thermolabile compounds.

3B.2 Screening of concentrated extracts

Thin-layer chromatography of the crude extracts was extensively used as a preliminary screening technique to establish the number of tertiary and quaternary alkaloids in each fraction. Other secondary plant metabolites were likewise screened by this technique. Light petroleum/*n*-hexane and chloroform extracts were monitored on alumina and silica gel G chromatoplates, whilst cellulose seemed to be the most suitable adsorbent for screening the methanol extracts.

All chromatograms were developed at ambient temperature using the appropriate solvent system detailed on page 39. Variable ambient temperatures and tank saturation times obviously reduce the reproducibility of R_f values[159,160], but this was deemed to be of minor consequence as t.l.c. was merely used as a qualitative screening technique. In characterization studies, however,

isolates were co-spotted with samples of the corresponding authentic compounds for valid comparisons in preference to placing reliance on published R_f values.

3B.2.1 Alumina chromatograms

Thin-layer chromatography on alumina is a useful technique for distinguishing between tertiary and quaternary alkaloids, since all quaternary alkaloids, other than the quaternary benzophenanthridines, remain at the point of application. The choice of the solvent system, chloroform/cyclohexane/ethanol/diethylamine (70:30:0,5:0,5), for the development of these chromatograms has been previously discussed[159].

3B.2.2 Silica gel G chromatograms

T.l.c. on silica gel G plates is routinely used to characterize both alkaloids and non-alkaloids; however, in phytochemical studies on *Zanthoxylum* species, this adsorbent has been specifically used to differentiate between similar acridone alkaloids[161]. In the present work, this technique was extended to differentiate between tertiary (decarine, *N*-norchelerythrine) and quaternary (chelerythrine, nitidine) benzophenanthridine alkaloids. Chromatoplates developed with *n*-hexane/ethyl acetate (4:1) effected satisfactory separation of lupeol, β-sitosterol and (-)-sesamin, while plates developed with chloroform/ethanol (99:1) clearly distinguished between *N*-norchelerythrine, decarine and chelerythrine.

3B.2.3 Cellulose chromatograms

Owing to the short time required for the development of cellulose chromatoplates with 0,1 M hydrochloric acid, this technique was routinely adopted in all studies involving quaternary alkaloids.

However, separation of the quaternary benzophenanthridine alkaloids, nitidine and chelerythrine, by this system was relatively inefficient. Although the developing solvent *n*-butanol/pyridine/water (6:4:3) was occasionally used, it presented practical problems due to the interaction of pyridine with routine alkaloid-detecting reagents which resulted in diminished colour contrast between the spots and the background.

3B.2.4 Detection of compounds

Visualization of unsprayed chromatograms by screened ultraviolet light (366 nm) is an invaluable technique for the initial localization of fluorescent components. Several alkaloids isolated from *Zanthoxylum* species exhibit characteristic colours on t.l.c., *e.g.* skimmianine, chelerythrine and nitidine emit a blue, an orange and a lime-green fluorescence respectively. Some non-fluorescent, quaternary alkaloids, however, only exhibit fluorescence when the unsprayed chromatograms are exposed to ammonia vapour, *e.g.* the aporphines, (+)-magnoflorine and (+)-laurifoline, both fluoresce blue provided that the chromatoplate is not developed with a basic solvent. Thus, when pyridine-containing developing solvents are used, (+)-magnoflorine gives an immediate blue fluorescence.

Alkaloids were generally detected on chromatograms using either modified Dragendorff's reagent[103] or iodoplatinic acid reagent[107]. The former produced orange to brown spots against a pale yellow background, whereas the latter gave a variety of colours (purple, green, brown and grey) against a pink background. Dragendorff's reagent is still regarded as the most sensitive

reagent for the detection of alkaloids[162] though it lacks specificity. It produces false-positive reactions with certain non-alkaloids possessing lactone or conjugated carbonyl functions[133]. Some alkaloids, *e.g.* skimmianine and decarine, gave characteristic rose-pink (alumina) and blue (silica gel G) colours respectively when plates were sprayed with Dragendorff's reagent and then exposed to ammonia vapour. Iodoplatinic acid reagent also lacks specificity; nevertheless it is a useful diagnostic reagent in the detection and identification of certain quaternary *Zanthoxylum* alkaloids. These show a wide range of characteristic colours with the reagent[61].

Diazotised sulphanilic acid reagent[108] was occasionally used to detect compounds possessing phenolic groups since several quaternary alkaloids occurring in *Zanthoxylum* species, have been reported to give characteristic colours with this reagent[61].

Detection of the lignan, (-)-sesamin, the pentacyclic triterpene, lupeol, and the phytosterol, β-sitosterol, was achieved by spraying the chromatoplates with 300% ^{m}/v antimony trichloride in conc. hydrochloric acid[163]. These compounds exhibited a variety of colours after the sprayed plates were heated; however, after several hours, these colours either faded or were transformed to other colours.

3B.3 Compounds isolated from *Z. davyi* extracts

3B.3.1 Stem bark extracts

3B.3.1.1 Concentrated *n*-hexane extract (ZD/SB1)

During storage for approximately six months, this viscous extract deposited a crystalline, neutral solid, which on repeated

crystallization from methanol, afforded colourless plates of lupeol, m.p. 212°-214°.

Lupeol [32](Fig.6 p.125)

The infrared spectrum showed typical -OH absorption at 3650 cm^{-1}, while the peak at 885 cm^{-1} indicated a vinyl group (>C=CH_2)[164]. The compound analyzed satisfactorily for $C_{30}H_{50}O$ which is suggestive of a pentacyclic triterpene. Optical rotation and melting point values are in close agreement with reported literature data[109] for lupeol.

As no authentic lupeol was available for comparison, the acetate and benzoate were prepared. The infrared spectrum of the former derivative showed typical carbonyl absorption at 1730 cm^{-1}, while the strong peak at 1250 cm^{-1} was ascribed to acetate absorption[164]. The doublet of equal intensity at 1370 and 1385 cm^{-1} due to the *gem*-dimethyl groups at C-4[164] was not observed in the infrared spectrum of lupeol, but was clearly defined in that of the acetate. The acetate's melting point (217°) was identical to the reported value[60] and showed, as expected, marked depression when admixed with lupeol. Saponification of lupeol acetate afforded a product identical with lupeol. The benzoate's melting point (257°-258°) is also in close agreement with the reported literature value[110].

3B.3.1.2 Concentrated chloroform extract (ZD/SB2)

This extract was agitated with dilute hydrochloric acid to convert the alkaloids to their corresponding hydrochloride/chloride salts. The yellow, insoluble precipitate (YP1) collecting at the interface suggested the presence of insoluble salts of benzo-

phenanthridine alkaloids, since this phenomenon was encountered by several researchers examining other species of *Zanthoxylum*[158, 163,165,166,167]. Because benzophenanthridine alkaloids were suspected, the use of alkali was avoided due to the possible production of artefacts[58,168]. YPI was therefore refluxed with methanolic hydrochloric acid and was converted to greenish-yellow needles (NF/1), m.p. 277°-8° (dec.). Further extraction of the chloroform fraction with acid gave an orange precipitate at the interface which on crystallization afforded slender, orange needles (NF/2), 197°-198° (dec.).

Liquid-liquid extraction of the combined acid filtrate (AFI) with chloroform yielded a solid which formed colourless plates (NF/3), m.p.244°-246° (dec.).

Nitidine chloride [28] (Fig.5 p.124)

NF/1 was identified as nitidine chloride by comparison (m.p., m.m.p., UV, IR, TLC) with an authentic sample.

Chelerythrine chloride [23] (Fig.5 p.124)

NF/2 was identified as chelerythrine chloride by comparison (m.p., m.m.p., UV, IR, TLC) with an authentic sample.

(-)-α-*N*-methylcanadine [22] (Fig.4 p.123)

Crystals NF/3 exhibited a "double" melting point, 165° and 244°-246 (dec.), which has also been observed by other workers for (-)-α-*N*-methylcanadine salts[114,169]. The melting point and optical rotation are in close agreement with those reported in the

literature for (-)-α-canadine methochloride[113]. Owing to the loss of methyl chloride, the mass spectrum showed no parent peak, but rather an (M^+-15) ion at $^m/_e$ 339 in marked similarity with that reported for another tetrahydroprotoberberine alkaloid, (-)-*N*-methyltetrahydropalmatine[159]. Retro-Diels-Alder fission gave the expected non-nitrogenous base peak ion at $^m/_e$ 164 (*c.f.* Ref. 159) and the isoquinolinium ion at $^m/_e$ 174. The fact that this fragment is 16 a.m.u. lower than the fragment observed for (-)-*N*-methyltetrahydropalmatine, indicates that in (-)-α-canadine methochloride a methylenedioxy-group replaces the two methoxyls of (-)-*N*-methyltetrahydropalmatine. The high relative abundance (61%) of the $^m/_e$ 149 ion confirms the similarity of (-)-α-*N*-methylcanadine and (-)-*N*-methyltetrahydropalmatine with reference to substitution in the non-nitrogenous fragment[170].

As the chloride salt of (-)-α-*N*-methylcanadine was very hygroscopic, the less soluble iodide was prepared. This salt also showed the characteristic "double" melting point, 160°-162° and 248°-249° (dec.). The melting point and ultraviolet spectrum of (-)-α-canadine methiodide were in close agreement with the literature values[114,115]. Thin-layer chromatography of either the chloride or the iodide salts of (-)-α-*N*-methylcanadine on alumina chromatoplates gave a single, non-fluorescent, Dragendorff-positive spot which did not move from the point of application. As mentioned previously, this feature is characteristic of most quaternary alkaloids. On cellulose chromatoplates, however, the compound showed good mobility which is to be expected of a water-soluble alkaloid.

3B.3.1.3 Concentrated methanol extract (ZD/SB3)

The isolation of quaternary alkaloids has always been fraught with problems due to their hygroscopic nature, solubility in polar solvents and relative insolubility in most non-polar solvents. Several traditional methods have been employed to effect purification of quaternary alkaloids *inter alia* precipitation as insoluble picrates[171] or reineckates[172] and liquid-liquid partition into a relatively polar, yet water-immiscible solvent, *e.g.* *n*-butanol[172]. Ion-exchange chromatography has been used successfully by several workers[61,158], though not without risk, *e.g.* a quaternary compound can undergo a Hofmann degradation[173].

Mindful of the aforementioned, this extract was therefore worked up as follows: After agitation with M hydrochloric acid, the acid filtrate was adjusted to pH 6 to facilitate precipitation of resinous, non-alkaloidal material. The water-soluble alkaloids in the filtrate were then precipitated as insoluble reineckates and subsequently converted to chlorides by ion-exchange. Separation of the mixture of alkaloid chlorides was then attempted on a horizontal cellulose column. This technique had previously been successfully employed to separate sugar mixtures, as it has the advantage of avoiding the use of large volumes of eluting solvent[116]. The horizontal column was developed with 0,1 M hydrochloric acid since similar separation was obtained when the alkaloid mixture was chromatographed on cellulose thin-layer chromatoplates developed with the same solvent. Where the R_f values of the individual alkaloids were distinctly separate on t.l.c., good separation was obtained on the horizontal column. Two homogeneous fractions

(9-17 and 33-34), each consisting of a single alkaloid,were obtained and one was converted to a less soluble crystalline alkaloidal salt *viz.* NF/4 (as chloride), m.p. 251°-253° (dec.), and NF/5 (as iodide), m.p. 249°-252° (dec.). The remaining fractions consisted chiefly of an unresolved mixture of two alkaloids having similar R_f values. One of these exhibited chromatographic characteristics identical to that of (-)-α-*N*-methylcanadine (already isolated from ZD/SB2), while the other gave a distinctive green colour with iodoplatinic acid reagent which was indicative of tembetarine.

(+)-Laurifoline chloride [19] (Fig.4 p.123)

NF/4 was identified as (+)-laurifoline chloride by comparison (m.p., m.m.p., UV, IR, TLC) with an authentic sample.

(+)-Magnoflorine iodide [16] (Fig.4 p.123)

NF/5 was identified as (+)-magnoflorine iodide by comparison (m.p., m.m.p., UV, IR, TLC) with an authentic sample.

3B.3.2 Root bark extracts

3B.3.2.1 Concentrated *n*-hexane extract (ZD/RB1)

A portion of this weakly Dragendorff-positive extract was chromatographed on an alumina column and eluted with solvents of increasing polarity. Two homogeneous fractions (10 and 12-13), each consisting of a single compound, were collected. The former fraction yielded colourless plates of lupeol, m.p. 213°-214°, while the latter afforded colourless needles (NF/6), m.p. 135°-136°.

Lupeol [32] (Fig.6 p.125)

The crystalline solid obtained from Fraction 10 was identified as lupeol by comparison (m.p., m.m.p., TLC) with an authentic sample.

β-Sitosterol [33] (Fig.6 p.125)

NF/6 gave a satisfactory elemental analysis for $C_{29}H_{50}O.\frac{1}{2}H_2O$ and was identified as β-sitosterol by comparison (m.p., m.m.p., IR, MS, TLC) with an authentic sample. In order to confirm this identification, the acetate, m.p. 122°-123°, was prepared. The recorded m.p. for this derivative is variable[119,174,175], but it nevertheless gave a satisfactory elemental analysis.

3B.3.2.2 Concentrated chloroform extract (ZD/RB2)

This extract contained the same alkaloids as the stem bark *viz.* chelerythrine, nitidine and (-)-α-*N*-methylcanadine. The last was identified as the chloride (NF/8) and the iodide (NF/9) by the same comparative criteria described under 3B.3.1.2.

3B.3.2.3 Concentrated methanol extract (ZD/RB3)

When the extract was monitored on t.l.c., no alkaloids additional to those already observed in the corresponding stem bark extract were detected.

3B.4 Compounds isolated from *Z. thorncroftii* extracts

3B.4.1 Root bark extracts

3B.4.1.1 Concentrated light petroleum extract (ZT/RB1)

Agitation of an ethereal solution of this extract with dilute

hydrochloric acid produced an orange precipitate (OP_1) which collected at the interface. As t.l.c. of OP_1 showed identical alkaloids to those observed in the chloroform extract (ZT/RB2), characterization of this precipitate was deferred until ZT/RB2 was worked up. (*vide* 3B.4.1.2)

The acid filtrate, adjusted to pH 8, was extracted with ether and then with chloroform. Concentration of each extract to dryness yielded a solid which after crystallization afforded colourless prisms (NF/10), m.p. 178°-179°, (NF/10a), m.p. 175°-177°, and (NF/10b), m.p. 175°-177°.

The original ether fraction was then successively extracted with sodium bicarbonate and sodium carbonate solutions to yield two fractions (P2 and P3 respectively) which,after acidification, afforded no crystalline material,thus confirming the absence of acidic components.

Skimmianine [6] (Fig.2 p.121)

NF/10 - NF/10b was identified as skimmianine by comparison (m.p., m.m.p., UV, IR, TLC) with an authentic sample. The orange,Dragendorff-positive spot which became deep rose to mauve in colour when the sprayed plates were exposed to ammonia vapour, is indicative of furoquinoline alkaloids[159].

3B.4.1.2 Concentrated chloroform extract (ZT/RB2)

A solution of the dry extract in chloroform was divided into two equal portions. The first was agitated with dilute hydrochloric acid whereupon a yellow precipitate (YP2) collected at the interface. Thin-layer chromatograms revealed that YP2

consisted largely of chelerythrine and nitidine.

The acid filtrate was adjusted to pH 8 and the resultant brown resinous precipitate was removed. Liquid-liquid extraction of the acidic fraction with chloroform yielded a crystalline residue which was converted to the iodide. Comparison of this salt with authentic (-)-α-canadine methiodide showed them to be identical (*c.f.* 3B.3.1.2).

The second portion of the chloroform solution was chromatographed on silica gel as thin-layer chromatography of YP2 had indicated the presence of benzophenanthridine alkaloids. Alumina was avoided as an adsorbent because the benzophenanthridines can form many artefacts under alkaline conditions[58,168]. The column was initially eluted with chloroform and the bright yellow, insoluble residue (YP3) remaining at the top of the adsorbent was removed and reserved for further study. Elution with chloroform/methanol mixtures yielded three relatively homogeneous fractions *viz.* 23-26, 31 and 33.

Addition of methanol to fraction 23-26 gave an insoluble brownish residue (R3) which was virtually insoluble in most organic solvents. R3 produced a brown precipitate with ferric chloride solution (phenolic group(s)), while the positive Labat test indicated the presence of a methylenedioxy-function. Although attempts to crystallize R3 were unsuccessful, marked purification was achieved by precipitation of a methanolic solution with water followed by reprecipitation from chloroform with light petroleum (b.p.40°-60°). The amorphous buff precipitate produced a single brownish fluorescent spot on silica gel G thin-layer plates. When the chromatograms were

sprayed with Dragendorff's reagent, the spot gave an unusual brownish-orange colour which was transformed to deep blue when the plate was subsequently exposed to ammonia vapour. Crystalline material, in the form of fine, buff-coloured needles (NF/11), m.p. 244°-245°, could only be obtained when the amorphous precipitate was sublimed.

Decarine [29] (Fig.5 p.124)

The ultraviolet spectrum of NF/11 (λ_{max}^{EtOH} 247, 257sh., 277, 326, 383nm) showed absorption typical of a benzophenanthridine nucleus[176] and on the addition of sodium hydroxide, showed a marked bathochromic shift suggestive of a phenolic hydroxyl function. Absorption at 3440 cm^{-1} in the infrared spectrum coupled with the positive ferric chloride reaction confirmed the presence of phenolic group(s). The p.m.r. spectrum of NF/11 integrated for thirteen protons and gave signals for aromatic methoxy-(δ4,09) and methylenedioxy-(δ6,16) substituents. The four coupled doublets, (δ7,56; 7,91; 8,44; 8,47; J = 9 Hz) accounting for a single proton each, were assigned to vicinally-coupled protons (two AB quartets), *i.e.* H-9, H-10, H-11 and H-12. The remaining three aromatic proton signals (δ7,34 and δ8,62) were assigned to H-1, H-4 and H-6, while the downfield singlet (δ9,59) was attributed to the hydroxyl function.

The melting point and ultraviolet spectrum of NF/11 agreed closely with that reported for decarine[121] while the infrared and p.m.r. spectra were virtually superimposable with those recorded for decarine. NF/11 analyzed satisfactorily for $C_{19}H_{13}NO_4$ and accurate mass measurement supported this formula. However, this

compound occurred as fine, buff-coloured needles in contrast to the cotton-like orange needles reported by other authors[121,122].

Treatment of NF/11 with hydrochloric acid gave an orange precipitate which on crystallization afforded bright orange needles (NF/12) of decarine hydrochloride, m.p. 243°-245°, which analyzed satisfactorily for $C_{19}H_{14}NO_4Cl$. While the melting point of this salt was being determined, the orange colour of the crystals disappeared prior to melting. This suggested a facile loss of HCl resulting in a melting point virtually indistinguishable from decarine *per se*. The p.m.r. spectra of decarine and decarine hydrochloride showed minor differences in chemical shifts; however, their infrared spectra differed appreciably.

The difference in physical appearance (colour) observed in the present work for decarine (buff) and its hydrochloride (orange) suggests that the cotton-like orange needles reported by Vaquette *et al.*[121] for decarine contained a small amount of the hydrochloride. This suggestion is supported by the relatively poor elemental analysis (H and N) obtained by them for decarine. The decarine hydrochloride isolated as orange needles from *Zanthoxylum viride* Waterm.[122] was erroneously reported as decarine because it seems unlikely that when decarine was crystallized from "MeOH/N HCl", the alkaloid would not form the hydrochloride. An accurate mass determination of the molecular ion is also misleading as HCl would be lost in the same way as during melting.

Two further derivatives of decarine were prepared to confirm its identity. One was obtained by methylation of R3 with diazomethane to form an *O*-methyl derivative which analyzed

satisfactorily for $C_{20}H_{15}NO_4$ and had a melting point (215°-216°) identical to that reported for *N*-norchelerythrine. The other derivative was prepared by acetylation of R3 to afford buff needles (NF/13) of decarine acetate, m.p. 238°-240°, which analyzed satisfactorily for $C_{21}H_{15}NO_5$. The infrared spectrum of this derivative showed typical carbonyl absorption at 1750 cm^{-1}, while the strong peak at 1250 cm^{-1} was ascribed to acetate[164]. Although Vaquette *et al.* also prepared *O*-acetyldecarine, no melting point was recorded[121].

It is clear that in decarine, substitution of the benzophenanthridine nucleus occurs at C-2, C-3, C-7 and C-8 and that the methylenedioxy-substituent definitely occupies C-2 and C-3 due both to the similar substitution pattern in decarine and its *O*-methyl derivative, *N*-norchelerythrine, and the fact that this group occurs at C-2 and C-3 in all other benzophenanthridines isolated from the Rutaceae. By observation of the nuclear Overhauser effect (NOE) between the methoxy-group and the C-6 imine proton, Vaquette *et al.* assigned the methoxy-group to C-7. The hydroxyl group therefore occupies the remaining position at C-8; however, there was no chemical proof to support this spectral proposal.

Waterman[122] quaternized decarine with methyl iodide and found that the product's melting point and spectral data were at variance with those published for fagaridine [24]. As fagaridine had been shown[177] to posses methoxyl and hydroxyl substituents at C-8 and C-7 respectively, it is reasonable to deduce that the converse substitution pattern, with respect to those positions, must apply to decarine. The structure of decarine has recently been unequivocally established

by Ishii *et al.* during their synthesis of ethyl isodecarine which differed markedly from ethyl decarine[178].

Chelerythrine [23] (Fig.5 p.124)

This alkaloid was isolated from Fraction 31 as orange needles of chelerythrine chloride, m.p. 199°-202° (dec.). The characterization of this compound has been previously discussed. (3B.3.1.2 refers)

(-)-α-Canadine methiodide [22] (Fig.4 p.123)

The residue obtained from Fraction 33 was converted to the iodide and on crystallization afforded colourless platelets of (-)-α-canadine methiodide, m.p. 249°-251° (dec.). Characterization of this compound has been previously discussed. (3B.3.1.2 refers)

Nitidine [28] (Fig.5 p.124)

The yellow insoluble solid (YP3) was treated as described previously to afford yellow needles (NF/14) of nitidine chloride, m.p. 275°-278° (see 3B.3.1.2). Characterization of this compound was discussed under the same reference.

3B.4.1.3 Concentrated methanol extract (ZT/RB3)

A portion of this extract was agitated with dilute hydrochloric acid to form the chlorides of the water-soluble quaternary alkaloids. Adjustment of the acidic solution to pH 6 precipitated unwanted, non-alkaloidal, resinous material. The quaternary alkaloids in the acid filtrate were then preferentially

adsorbed on a cationic exchange resin from which they were finally eluted with methanolic hydrochloric acid. Concentration of the eluate to dryness and extraction of the residue with boiling absolute ethanol removed appreciable quantities of insoluble sodium chloride. Final crystallization from dry methanol afforded prisms (NF/15), m.p. 252°-253° (dec.).

Another portion of the extract was agitated with dilute hydrochloric acid, neutralized and freeze-dried. Since a preliminary investigation on t.l.c. plates using methanol/water/strong ammonia (15:9:1) as developing solvent showed reasonably efficient separation of the quaternary alkaloid mixture, low pressure liquid chromatography of this mixture,utilizing the same adsorbent/solvent system,was attempted. All homogeneous fractions as established by t.l.c. were bulked. The alkaloid-positive residue obtained from Fractions 57-66 was converted to the insoluble iodide and, on crystallization,afforded colourless prisms (NF/16), m.p. 248°-250° (dec.). Whilst no further crystalline material could be obtained, Fractions 84-86 were shown by t.l.c. to consist of a mixture of two alkaloids, one of which gave a distinctive green colour with iodoplatinic acid reagent indicative of tembetarine.

(+)-Laurifoline chloride [19] (Fig.4 p.123)

Identification of NF/15 as (+)-laurifoline chloride was achieved by comparison with an authentic sample (m.p., m.m.p., UV, IR, TLC and optical rotation). Accurate mass measurement of the molecular ion matched the theoretical value for $C_{20}H_{24}O_4N^+$.

The characteristic fragment at $^{m}/e$ 341 [M^{+}-1] was probably due to loss of the angular hydrogen atom, while the base peak at $^{m}/e$ 58 is attributed to the $(CH_3)_2\overset{+}{N}=CH_2$ ion.

(+)-Magnoflorine iodide [16] (Fig.4 p.123)

NF/16 was identified as (+)-magnoflorine iodide by the same comparative criteria described under 3B.3.1.3.

3B.5 Compounds isolated from *Z. humile* extracts

3B.5.1 Root bark extracts

3B.5.1.1 Concentrated light petroleum extract (ZH/RB1)

A solution of this extract in ether was agitated with hydrochloric acid whereupon an orange precipitate (OP2) collected at the interface. Because benzophenanthridine alkaloids were suspected, the orange precipitate was chromatographed on a silica gel column for the same reasons as outlined previously (*vide* 3B.4.1.2). Elution with chloroform yielded a fraction (1-3) which on crystallization afforded colourless plates (NF/17), m.p. 212°-214°. Further elution afforded an amorphous alkaloid which was converted to the orange hydrochloride. Sublimation of this orange salt gave crystalline material (NF/18), m.p. 230°-235°. The alkaloid in Fractions 16-17 was converted to the nitrate and purified by chromatography on silica gel. Elution with chloroform/ethanol (99:1) removed all the unwanted impurities while the yellow alkaloid remained strongly adsorbed to the column. The alkaloid was removed by methanol and crystallized to afford fine,

yellow needles (NF/19), m.p. 239°-240°. Treatment of another portion of OP2 in boiling methanol resulted in the separation of an insoluble,neutral white solid (NS/1), m.p. 144°-145°, the identity of which has not yet been established. Purification of the filtrate was achieved by chromatography on silica gel as described for Fractions 16-17 (see previous page). Final crystallization afforded slender, orange needles (NF/20), m.p. 202°-203° (dec.).

The combined acid filtrate (AF4) was adjusted to pH 8 and the resulting precipitate was extracted with chloroform. Final crystallization afforded colourless prisms (NF/21), m.p. 177°-178°.

The neutral compounds present in a portion of the remaining ether extract (E2) were successfully separated by low pressure liquid chromatography on silica gel. All fractions were monitored on t.l.c. and homogeneous fractions were bulked to afford colourless needles (NF/22), m.p. 195°-198° (Fractions 17-23), colourless plates (NF/23), m.p. 135°-137° (Fractions 30-38), and slender, colourless needles (NF/24), m.p. 124°-125° (Fractions 50-59).

N-norchelerythrine [30] (Fig.5 p.124)

Identification of NF/17 as *N*-norchelerythrine was achieved by comparison (m.p., m.m.p., IR, TLC) with a sample of *O*-methyldecarine (see discussion on decarine under 3B.4.1.2).

Decarine [29] (Fig.5 p.124)

NF/18 was obtained as slightly impure decarine hydrochloride but due to insufficient material, further purification could not be effected. However, the virtually superimposable infrared

spectrum and characteristic chromatographic behaviour confirm the identity of this compound.

Chelerythrine [23] (Fig.5 p.124)

NF/19 was identified as chelerythrine nitrate by comparison (m.p., m.m.p., UV, IR, TLC) with an authentic sample, while NF/20 was identified as chelerythrine chloride by the same comparative criteria outlined in 3B.3.1.2.

Skimmianine [6] (Fig.2 p.121)

Characterization of NF/21 as skimmianine has been previously discussed. (3B.4.1.1 refers)

Lupeol [32] (Fig.6 p.125)

NF/22 was identified as lupeol by comparison (UV, IR, MS, TLC) with an authentic sample. The m.p. of NF/22 agreed with that reported for lupeol[109], but was nevertheless some 20° lower than that recorded for the lupeol isolated from the extract ZD/SB1 (3B.3.1.1 refers).

The discrepancy in melting point is due to the fact that lupeol can exist in two crystalline forms[109], the one melting at about 196° being obtained from acetone,and the other melting at about 215° from methanol.

β-Sitosterol [33] (Fig.6 p.125)

Fractions 30-38 consisted of two compounds virtually indistinguishable on t.l.c. but crystallization from *n*-hexane

resulted in preferential deposition of sesamin, whereas the mother liquor afforded colourless plates (NF/23) the melting point of which agreed closely with that published for β-sitosterol. Accurate mass measurement matched the theoretical value for $C_{29}H_{50}O$. The other criteria used for the characterization of β-sitosterol have been previously discussed. (*vide* 3B.3.2.1)

(-)-Sesamin [34] (Fig.6 p.125)

The melting point of NF/24 was in close agreement with that recorded for (-)-sesamin, while accurate mass measurement matched the theoretical value for $C_{20}H_{18}O_6$. Although the ultraviolet and infrared spectra were superimposable with those of an authentic sample of (+)-sesamin, the optical rotation obtained for NF/24 was laevorotatory (-35,3°) and some 33° lower than the literature value[67]. However, the rotation of the (-)-sesamin occurring in another African species[56] was only half of the value (-16°) now recorded in *Z. humile*. In accordance with the findings of Carnmalm *et al.*[179], it is therefore clear that some of the sesamin must be dextrorotatory.

3B.5.1.2 Concentrated chloroform extract (ZH/RB2)

A solution of this extract in chloroform was agitated with dilute hydrochloric acid, the aqueous phase adjusted to pH 8, and extracted with chloroform. The residue was chromatographed and converted to the iodide (NF/25), m.p. 251°-252° (dec.).

(-)-α-Canadine methiodide [22] (Fig.4 p.123)

Characterization of NF/25 as (-)-α-canadine methiodide has

been previously discussed (3B.3.1.2 refers).

3B.5.1.3 Concentrated methanol extract (ZH/RB3)

This extract was worked up by the ion-exchange method described for ZT/RB3 (3B.4.1.3 refers).

After removal of several crops of insoluble sodium chloride, the filtrate was reduced to dryness. A solution of the dried residue in dry ethanol was refrigerated whereupon a crystalline solid (S1) deposited. Recrystallization of the solid afforded slender needles (NF/26), m.p. 273°-274° (dec.). The residue from the mother liquors afforded another crop of slender, colourless crystals (NF/26a), m.p. 276°-277° (dec.).

After the removal of S1, the filtrate was concentrated to dryness (R4). Thin-layer chromatograms of R4 showed that it consisted of two forward-running alkaloids the R_f's of which virtually merged. The slower-running spot gave a green colour with iodoplatinic acid characteristic of tembetarine, while the other spot gave an identical colour to that shown by NF/26 and subsequently identified as candicine chloride.

Although Kuck *et al.*[61] claimed to separate candicine and tembetarine on a cellulose column, this method was found ineffective in the present work. After many unsuccessful attempts with several other adsorbents and solvent systems, these alkaloids were finally separated on a silicic acid column. Chloroform/methanol (1:1) preferentially eluted the tembetarine, (NF/27), while methanol eluted the candicine.

Candicine [14] (Fig.3 p.122)

The identity of NF/26 and NF/26a as candicine chloride was

achieved by comparison (m.p., m.m.p., UV, IR, TLC) with an authentic sample. The p.m.r. spectrum in D_2O showed a singlet (δ3,16) accounting for three *N*-methyl groups (9H). The complex multiplet between δ3,16-3,72 integrated for a further four protons and was assigned to the two methylene groups in the *N*-trimethylethylamine side-chain. The two equivalent doublets (δ6,83 and 7,19; J = 9 Hz) showed that the aromatic ring was *para* substituted. The mass spectrum showed no parent peak at $^{m}/e$ 180, but prominent lines at $^{m}/e$ 121, 120, 107, 91 and 59. The $^{m}/e$ 59 fragment (due to the trimethylamine ion) indicated facile cleavage of the N-C bond, while the fragment at $^{m}/e$ 121 accounted for the *p*-hydroxyphenethyl moiety. The fragments at $^{m}/e$ 120 and 91 were also described by Waterman[161] who proposed a tentative pathway for their origin. NF/26 gave an acceptable analysis for nitrogen and the colours obtained with iodoplatinic acid reagent (greyish) and diazotised sulphanilic acid (red) were in accordance with those previously reported[61]. The iodide salt was prepared from NF/26 and the melting point was identical to the literature value[129] for candicine iodide.

Tembetarine [15] (Fig.3 p.122)

NF/27 was a very hygroscopic solid which could not be crystallized and was identified as tembetarine chloride by comparison (TLC and IR) with an authentic specimen.

3B.6 Distribution of alkaloids in African *Zanthoxylum* species

Zanthoxylum L. is represented in Africa by some forty species[3] many of which have been subjected to phytochemical studies. The secondary metabolites characterized in these investigations appear to originate via three main biogenetic pathways (anthranilic acid, phenylalanine/1-benzyltetrahydroisoquinoline or cinnamic acid) the details of which have been comprehensively reviewed[4,5].

Table 7 summarizes the distribution of alkaloids in African *Zanthoxylum* species and is based on previous reports. These include *Z. capense* (Thunb.) Harv.[3,44,45], *Z. chalybeum* Engl.[3,54,156,165], *Z. decaryi* H.Perr.[121], *Z. dinklagei* Waterm.[76], *Z. gilletii* Waterm.[3,112,168,185], *Z. lemairei* Waterm.[6,184], *Z. leprieurii* Guill. and Perr.[3,6,158,180], *Z. rubescens* Planch. *ex* Oliv.[6,56,57], *Z. tsihanimposa* H. Perr.[58,182,183], *Z. xanthoxyloides* Waterm.[3,92,177] and *Z. viride* Waterm.[3,122,181]. The three South African species, *i.e. Z. davyi* (Verdoorn) Waterm., *Z. thorncroftii* (Verdoorn) Waterm. and *Z. humile* (E. A. Bruce) Waterm., are now included in this table in order to assess the relationship of these South African species to the other African species.

3B.7 Chemotaxonomic significance of South African *Zanthoxylum* taxa

Reference to Table 7 shows that the South African *Zanthoxylum* taxa generally resemble the other African taxa to form a relatively homogeneous group in the African subsection *Gerontogaeae* Engl. With respect to anthranilic acid-derived alkaloids, the furoquinoline skimmianine occurs in all the South African taxa with the exception of *Z. davyi*. Although skimmianine commonly occurs in the majority of

TABLE 7: DISTRIBUTION OF ALKALOIDS IN AFRICAN *ZANTHOXYLUM* SPECIES

ALKALOID TYPE	ALKALOID	SPECIES														
		Z. capense	*Z. chalybeum*	*Z. davyi*	*Z. decaryi*	*Z. dinklagei*	*Z. gilletii*	*Z. humile*	*Z. lemairei*	*Z. leprieurii*	*Z. rubescens*	*Z. thomaroftii*	*Z. tsihanimposa*	*Z. xanthoxyloides*	*Z. viride*
[I] ANTHRANILATE SKELETON:	Figures in brackets refer to structural formulae (pp.121-124)														
(a) 2-Quinolones	Atanine [1]													+	
	4-Methoxy -1-methyl-2-quinolone [2]				+										
(b) Angular Pyranoquinolones	*N*-methylflindersine [3]		+												
(c) Furoquinolines	Dictamnine [4]				+										
	γ-Fagarine [5]												+		
	Skimmianine [6]	+	+		+	+	+	+		+		+	+	+	
(d) Canthinones	Canthin-6-one [7]														+
(e) Acridones	Arborinine [8]						+			+					
	1-Hydroxy-3-methoxy-*N*-methylacridan-9-one [9]									+					
	3-Dimethylallyl-4-methoxyacridan-9-one(?) [10]												?		
(f) Indoloquinazolines	Mixt. β-indoloquinazolines [11]					+									

TABLE 7 (continued)

ALKALOID TYPE	ALKALOID	SPECIES													
		Z. capense	*Z. chalybeum*	*Z. davyi*	*Z. decaryi*	*Z. dinklagei*	*Z. gilletii*	*Z. humile*	*Z. lemairei*	*Z. leprieurii*	*Z. rubescens*	*Z. thomarofii*	*Z. tsihanimposa*	*Z. xanthoxyloides*	*Z. viride*
[II] CINNAMIC ACID SKELETON:	Figures in brackets refer to structural formulae (pp.121-124)														
Amides	Fagaramide [12]		+				+							+	
	Rubesamide [13]										+				
[III] PHENETHYLAMINES	Candicine [14]	+	+					+	+	+	+				
[IV] 1-BENZYLTETRAHYDROISOQUINOLINE SKELETON	(+)-Tembetarine [15]	+	+	?			+	+	+	+	+	+	+	+	+
(a) Aporphines	(+)-Magnoflorine [16]	+	+	+			+		+	+	+	+		+	+
	(+)-*N*-methylcorydine [17]/*N*-methylisocorydine [18]	+	+				+		+	+	+			+	+
	(+)-Laurifoline [19]			+								+			
(b) Protoberberines	Berberine [20]	+	+				+							+	
(c) Tetrahydroprotoberberines	(-)-*N*-methyltetrahydropalmatine [21]	+													
	(-)-α-*N*-methylcanadine [22]			+				+				+			

TABLE 7 (continued)

ALKALOID TYPE	ALKALOID	SPECIES													
	Figures in brackets refer to structural formulae (pp.121-124)	*Z. capense*	*Z. chalybeum*	*Z. davyi*	*Z. decaryi*	*Z. dinklagei*	*Z. gilletii*	*Z. humile*	*Z. lemairei*	*Z. leprieurii*	*Z. rubescens*	*Z. thomsonii*	*Z. tsihanimposa*	*Z. xanthoxyloides*	*Z. viride*
(d) Benzophenanthridines	Chelerythrine [23]	+	+	+			+	+	+	+	+	+	+	+	+
	Fagaridine [24]												?	+	
	Fagaronine [25]													+	
	O-desmethylchelerythrine [26]												?		
	O-desmethylnitidine [27]												+		
	Nitidine [28]	+	+	+		+	+		+	+	+	+			+
	Decarine [29]				+			+				+			+
	N-norchelerythrine [30]							+							
	Dihydrochelerythrine [31]		+				+		+	+	+		+	+	+

African taxa, there are taxa where skimmianine is absent (Z. *lemairei*[6] and Z. *rubescens*[6]) or replaced by a canthinone (Z. *viride*[181]). Such absence should be treated with some circumspection since the alkaloid could have been present in such a low concentration as to have escaped detection.

Phenylalanine metabolism via the 1-benzyltetrahydroisoquinoline precursor gives rise to three alkaloidal nuclei, *viz.* aporphines, benzophenanthridines and protoberberines/tetrahydroprotoberberines. In spite of a report[3] asserting that only 1,2,10,11-substituted aporphines (due to oxidative coupling *ortho* to the hydroxy-group of the 1-benzyl substituent of the precursor) occur in African species, the detection of (+)-laurifoline in two South African taxa (Z. *davyi* and Z. *thorncroftii*) confirms that *para* coupling, which gives rise to 1,2,9,10-substituted aporphines, is also possible. It is significant that Z. *humile* contains considerable quantities of an early product of phenylalanine metabolism (candicine) as well as the 1-benzyltetrahydroisoquinoline alkaloid, tembetarine, at the expense of aporphine, and to a certain extent, benzophenanthridine production (absence of nitidine). Waterman made a similar observation for Z. *chalybeum*[3].

With the exception of Z. *humile*, the South African taxa show both *ortho* and *para* coupling of the tetrahydroprotoberberine intermediate leading to the formation of the benzophenanthridine alkaloids, chelerythrine and nitidine. Both compounds occur in virtually all African taxa; however, in addition to such quaternary alkaloids, an intermediate form (usually tertiary *N*-group) may occur, *e.g.* decarine in Z. *humile*, Z. *viride* and Z.

thorncroftii. The occurrence of decarine, *N*-chelerythrine (first report from African taxa) and chelerythrine in Z. *humile* and decarine and chelerythrine in Z. *thorncroftii* is noteworthy because it highlights the efficient methylating system prevailing in the genus. It could therefore be postulated that decarine is first *O*-methylated to yield the tertiary alkaloid *N*-norchelerythrine, which in turn quaternizes to form the ubiquitous chelerythrine. This mechanism is obviously different in Z. *xanthoxyloides* where fagaridine occurs together with chelerythrine. The former alkaloid already exists as a quaternary *N*-methylated benzophenanthridine having a phenolic group which, after *O*-methylation, is converted to chelerythrine.

The South African taxa appear to be unique among the African taxa in their ability to produce compounds which originate from an alternative pathway (*N*-quaternization) of the tetrahydroprotoberberine intermediate. (-)-*N*-methylcanadine was isolated from three taxa (Z. *humile*, Z. *davyi* and Z. *thorncroftii*), while Z. *capense* is the only taxon in which (-)-*N*-methyltetrahydropalmatine occurs. Although protoberberine alkaloids have been reported from African taxa[3], it is significant that *N*-methyltetrahydroprotoberberines seem to prevail in taxa occurring in the southern hemisphere *e.g.* Australian taxa[186,187,188] and South American taxa[62,169].

Coumarins, lignans, flavonoids and amides are among the metabolites derived from cinnamic acid that have been reported from African *Zanthoxylum* taxa[4,6,56,57,75,76]. Whilst the lignan (+)-sesamin has been previously found in several African *Zanthoxylum* taxa[6,46,76], (-)-sesamin has now been isolated from Z. *humile*. As the recorded

optical rotation was much lower than that reported in the literature, it is obvious that (-)-sesamin was in fact a mixture of the dextro- and laevorotatory forms. This observation is similar to the findings of Fish *et al.* for the same lignan occurring in Z. *rubescens*[56].

Two non-shikimate derivatives, *viz.* lupeol and β-sitosterol, were isolated from Z. *davyi* and Z. *humile*. Owing to their ubiquity in other plant families, their presence is not regarded as having any chemotaxonomic significance.

The value of chemical data from an interspecific point of view is normally viewed with reserve, but when such data are used to complement other characters (*e.g.* morphological and/or anatomical), it may furnish the necessary confirmatory evidence in a taxonomic controversy. In the light of present chemical knowledge, a tentative chemical key, based on alkaloidal patterns, is proposed for the South African *Zanthoxylum* species. (Fig.1 refers) As and when South African material of Z. *leprieurii* becomes available, it would be interesting to ascertain whether this species also contains acridone alkaloids like its Ghanian and Nigerian counterparts. Should this feature be confirmed, it would render Z. *leprieurii* quite distinct from the other South African taxa.

3B.7.1 Concluding remarks

Whilst the results of this phytochemical investigation show distinctive chemical differences favouring retention of the *status quo* of South African *Zanthoxylum* species, further comparative studies are indicated to determine whether these differences are in fact genetic or environmental, *i.e.* whether these are merely ecotypes

of a single species. A further collaborative, comparative study will be undertaken as soon as samples of Z. *capense* and Z. *thorncroftii*, occurring in the same locale, are collected. It is then hoped that this study will establish once and for all whether to reduce Z. *thorncroftii* to synonymy with Z. *capense* or to retain them as two distinct species.

FIGURE 1: TENTATIVE CHEMICAL KEY TO SOUTH AFRICAN *ZANTHOXYLUM* SPECIES

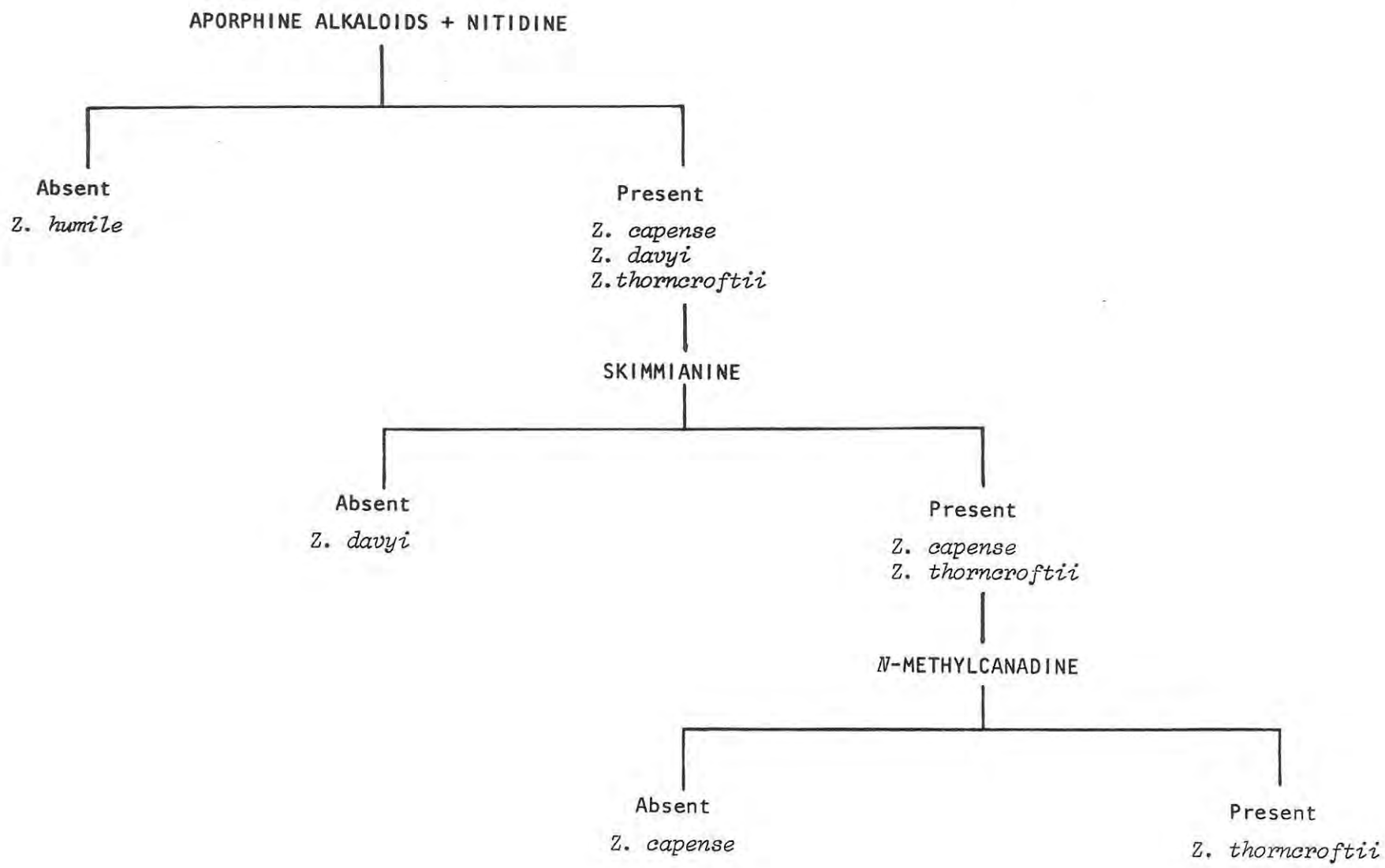
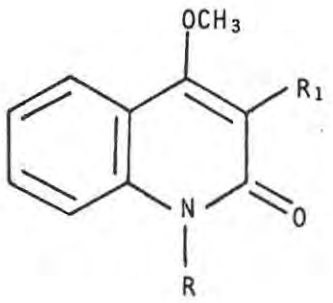


FIGURE 2: STRUCTURAL FORMULAE OF ALKALOIDS OCCURRING IN AFRICAN *ZANTHOXYLUM* SPECIES

[I] ANTHRANILATE SKELETON

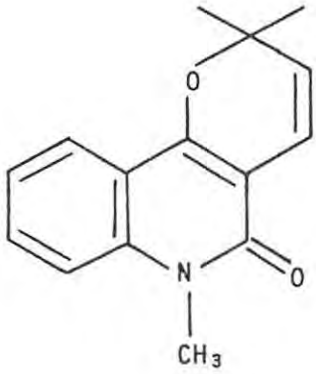
(a)



[1] R = H; R_1 = CH_2-CH=$C(CH_3)_2$

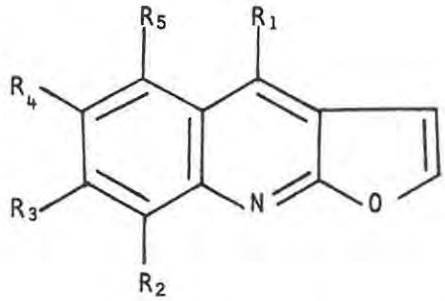
[2] R = CH_3; R_1 = H

(b)



[3]

(c)

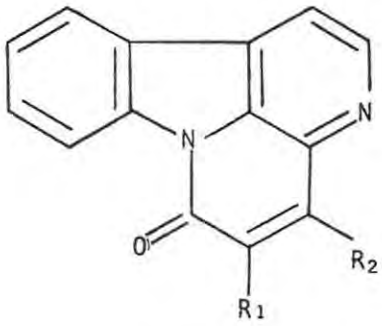


[4] R_1 = OCH_3; R_2 = R_3 = R_4 = R_5 = H

[5] R_1 = R_2 = OCH_3; R_3 = R_4 = R_5 = H

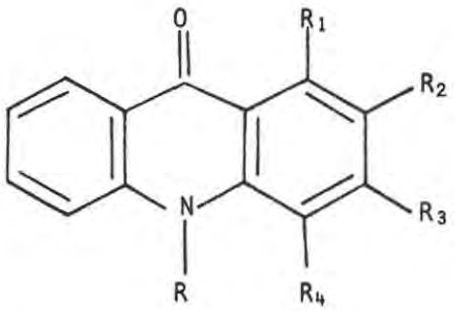
[6] R_1 = R_2 = R_3 = OCH_3; R_4 = R_5 = H

(d)



[7] R_1 = R_2 = H

(e)

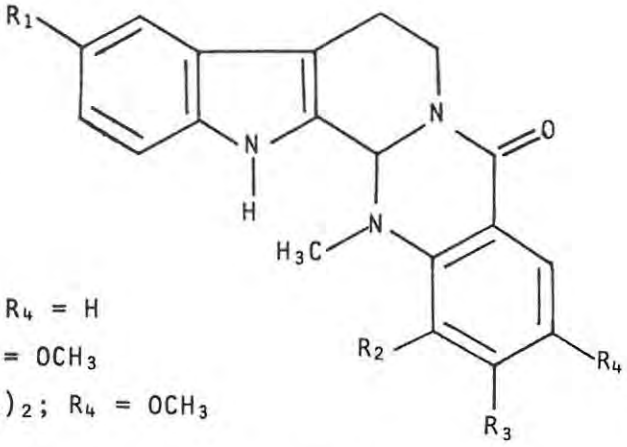


[8] R = CH_3; R_1 = OH; R_2 = R_3 = OCH_3; R_4 = H

[9] R = CH_3; R_1 = OH; R_2 = R_4 = H; R_3 = OCH_3

[10] R = R_1 = R_2 = H; R_3 = CH_2-CH=$C(CH_3)_2$; R_4 = OCH_3

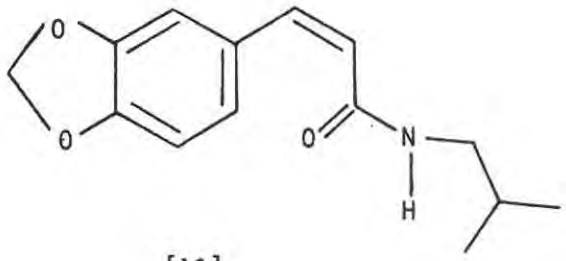
(f)



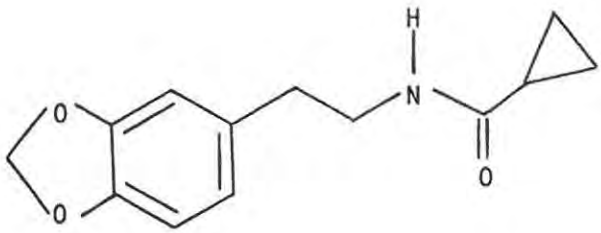
[11]

FIGURE 3: STRUCTURAL FORMULAE OF ALKALOIDS OCCURRING IN AFRICAN *ZANTHOXYLUM* SPECIES

[II] CINNAMIC ACID SKELETON

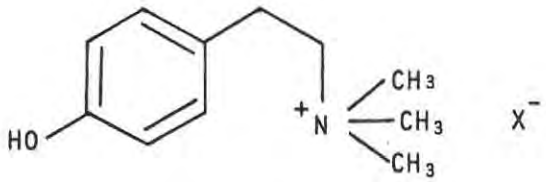


[12]



[13]

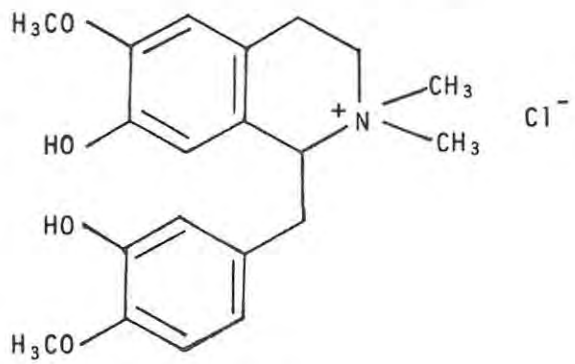
[III] PHENYLETHYLAMINES



[14]

(X^- = Cl^- or I^-)

[IV] 1-BENZYLTETRAHYDROISOQUINOLINES

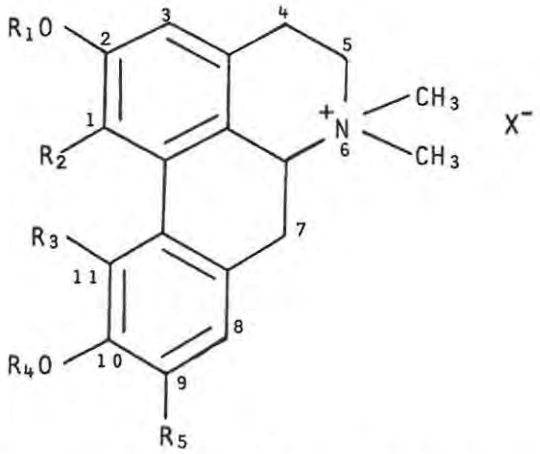


[15]

FIGURE 4: STRUCTURAL FORMULAE OF ALKALOIDS OCCURRING IN AFRICAN *ZANTHOXYLUM* SPECIES

[IV] 1-BENZYLTETRAHYDROISOQUINOLINE SKELETON

(a) Aporphines



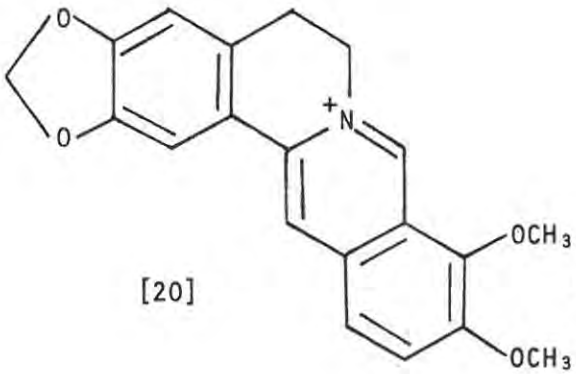
[16] $R_1 = R_4 = CH_3$; $R_2 = R_3 = OH$; $R_5 = H$; $X^- = I^-$

[17] $R_1 = R_4 = CH_3$; $R_2 = OH$; $R_3 = OCH_3$; $R_5 = H$

[18] $R_1 = R_4 = CH_3$; $R_2 = OCH_3$; $R_3 = OH$; $R_5 = H$

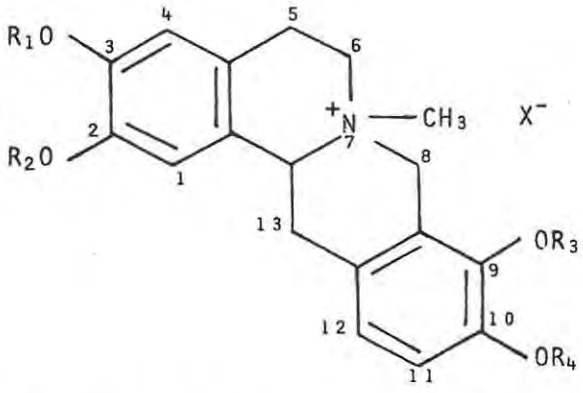
[19] $R_1 = R_4 = CH_3$; $R_2 = R_5 = OH$; $R_3 = H$; $X^- = Cl^-$

(b) Protoberberines



[20]

(c) Tetrahydroprotoberberines



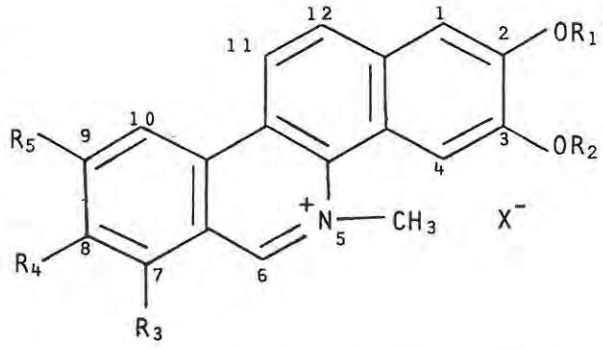
[21] $R_1 = R_2 = R_3 = R_4 = CH_3$; $X^- = NO_3^-$ or I^-

[22] $R_1 + R_2 = CH_2$; $R_3 = R_4 = CH_3$; $X^- = Cl^-$ or I^-

FIGURE 5: STRUCTURAL FORMULAE OF ALKALOIDS OCCURRING IN AFRICAN *ZANTHOXYLUM* SPECIES

[IV] 1-BENZYLTETRAHYDROISOQUINOLINE SKELETON

(d) Benzophenanthridines



[23] $R_1 + R_2 = CH_2$; $R_3 = R_4 = OCH_3$; $R_5 = H$; $X^- = Cl^-$ or NO_3^-

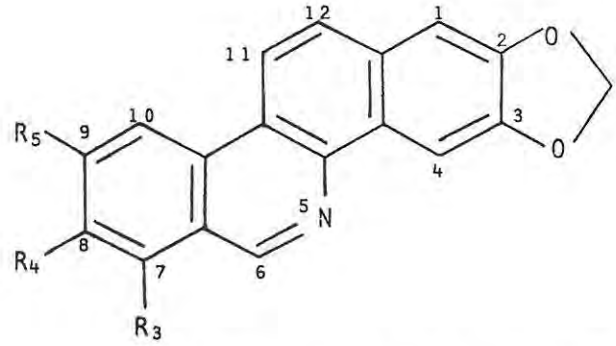
[24] $R_1 + R_2 = CH_2$; $R_3 = OH$; $R_4 = OCH_3$; $R_5 = H$; $X^- = OH^-$

[25] $R_1 = R_3 = H$; $R_2 = CH_3$; $R_4 = R_5 = OCH_3$; $X^- = Cl^-$

[26] $R_1 + R_2 = CH_2$; $R_3 = OCH_3$; $R_4 = OH$; $R_5 = H$; $X^- = OH^-$

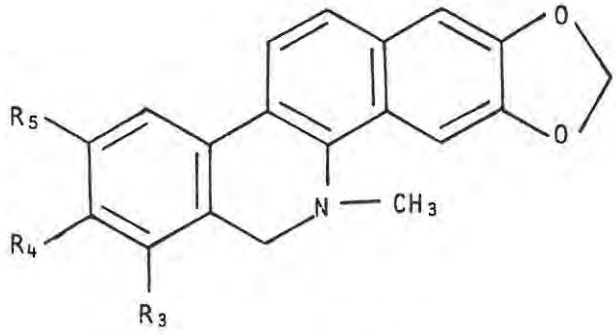
[27] $R_1 + R_2 = CH_2$; $R_3 = H$; $R_4 = OCH_3$ or OH; $R_5 = OH$ or OCH_3

[28] $R_1 + R_2 = CH_2$; $R_3 = H$; $R_4 = R_5 = OCH_3$; $X^- = Cl^-$



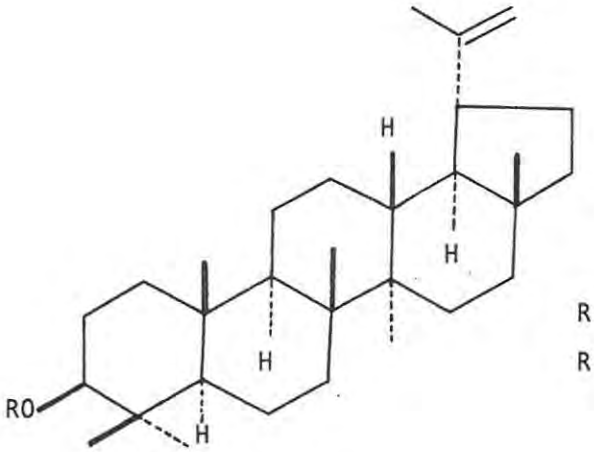
[29] $R_3 = OCH_3$; $R_4 = OH$; $R_5 = H$

[30] $R_3 = R_4 = OCH_3$; $R_5 = H$



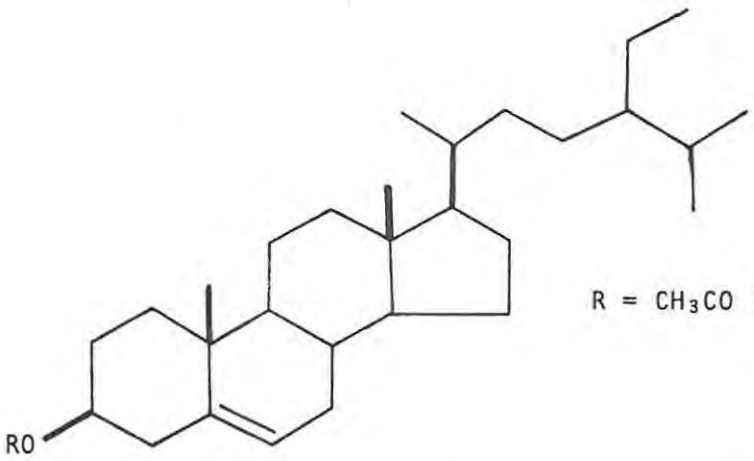
[31] $R_3 = R_4 = OCH_3$; $R_5 = H$

FIGURE 6: STRUCTURAL FORMULAE OF NON-ALKALOIDS OCCURRING IN SOUTH AFRICAN *ZANTHOXYLUM* SPECIES



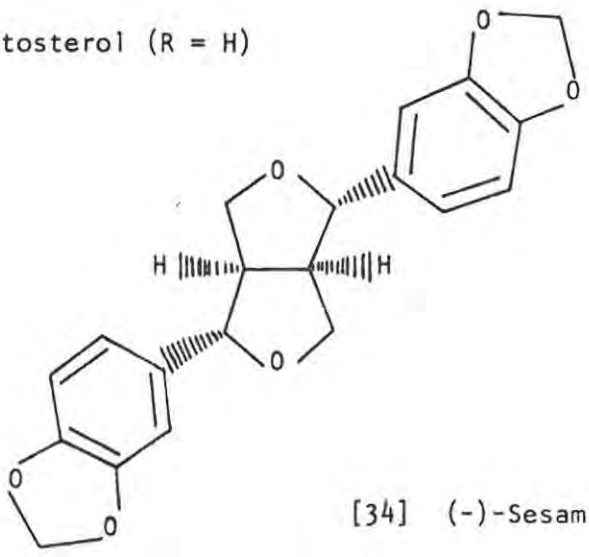
R = CH_3CO (Lupeol acetate)
R = C_6H_5CO (Lupeol benzoate)

[32] Lupeol (R = H)



R = CH_3CO (β-Sitosterol acetate)

[33] β-Sitosterol (R = H)



[34] (-)-Sesamin

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