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A MATTER OF SOME SENSITIVITY*

"Experience is never limited, and it is never complete; it is an immense sensibility, a kind of huge spider web of the finest silken threads suspended in the chamber of consciousness, and catching every air-borne particle in its tissue."

(Henry James 1843–1916)

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Key Word Index—Chromatographic, spectroscopic techniques; novel, unpredicted compounds; herbarium samples; plant tissue culture; antiprotozoal; cytotoxic activities; radio-ligand binding assays; traditional medicines.

Abstract—The development of sensitive chromatographic and spectroscopic techniques for the isolation and structure determination of natural products has greatly facilitated phytochemical investigations. Chemical investigations of herbarium material have resulted in the isolation of indole, quinoline and isoquinoline alkaloids from a wide number of plants. Examples of novel natural products from higher plants are given and include alkaloids, terpenoids, phenolics and quinones. Some plants investigated have not yielded the types of constituents which would have been predicted from them. Plant tissue cultures provide alternative sources of biologically active compounds and examples investigated include *Cinchona*, *Ailanthus*, *Brucea* and *Artemisia* for antiprotozoal compounds and *Datura* for tropane alkaloids. Biological tests are useful for bioassay-guided fractionation of plant extracts and examples of the isolation of a series of natural products with antiprotozoal and cytotoxic activities are given. Chemical and biological investigations into the traditional medicine Dragon's blood (*Croton lechleri*) from S. America and a Chinese prescription for the treatment of atopic eczema are described. The use of radio-ligand binding assays for the detection of a wide range of biological activities is discussed. Sensitivity of chemical and biological techniques has greatly improved prospects for finding new drug entities from plants and for investigating traditional medicines. Basic phytochemical investigations should continue to be encouraged especially in view of the rapid loss of plant species.

INTRODUCTION

Sensitivity of physical, chemical and biological methods to the analysis of natural product molecules has increased by leaps and bounds during my working life-time. In 1956, a mere 38 years ago, I commenced research into the constituents of *Equisetum* species. Identification of the alkaloid nicotine was achieved by comparison with a reference sample on paper chromatography and paper electrophoresis as well as by its UV absorption spectrum [1, 2]. At that time these techniques represented significant advances in technology over those which would have been available 30 years previously and it was exciting to record a UV spectrum even though it took 2–3 hr at a spectrophotometer.

Between 1961 and 1965 I studied the alkaloidal constituents of several species of *Mitragyna* (Rubiaceae) in a Ph.D. programme [3]. At the beginning of this work it was not possible to separate two known oxindole alkaloids, rotundifoline and rhynchophylline, by either paper chromatography or by paper electrophoresis. Separation and isolation of individual alkaloids was an extremely slow process. During 1962 the technique of thin-layer chromatography (TLC) was available for routine analytical separations mainly due to the pharmacognosist, the late Professor Egon Stahl. At once, it proved possible by this simple and sensitive analytical technique to separate a series of closely related alkaloids either by means of monitoring chromatographic columns or by the use of preparative TLC. Within the space of four years some 16 heteroyohimbine and oxindole alkaloids were isolated and five of them were novel natural products [3]. By 1968 the TLC separation of 27 heteroyohimbines [4, 5] and 12 oxindoles [4, 6, 7] had been

*This review formed the basis of a lecture which was presented at the International Congress of Natural Products Research, held in Halifax Nova Scotia, Canada, between 31 July and 4 August 1994.

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reported. Within a further two years some 30 tertiary alkaloids were separated from species of *Strychnos* mainly by the application of TLC [8]. In common with the heteroyohimbine and oxindole alkaloids it was possible to relate the adsorption process on to silica gel and alumina with the functional groups, substituents, state of oxidation and molecular stereochemistry for groups of related alkaloids.

A further sensitive analytical technique which became available during my Ph.D. research was that of proton nuclear magnetic resonance spectrometry. With amounts of 20–25 mg of alkaloid it was possible with a 60 MHz instrument to identify readily the number of aromatic protons, methoxyl groups and to differentiate between the pentacyclic heteroyohimbine/oxindole alkaloids and their *E-seco* tetracyclic analogues. Within a few years, mass spectrometry (MS) also came into routine use for natural products research and this sensitive analytical method enabled molecular weights and elemental analyses to be determined on submilligram quantities. Mass spectral fragmentation not only provided identification of specific compounds but also allowed for distinction to be made between closely related compounds including diastereoisomers. Prior to the introduction of this sensitive technique it was necessary to isolate some 15 mg of such alkaloids for elemental analysis and a further 4 mg for non-aqueous titration in order to determine, in duplicate, the molecular weight.

Comparison of UV, IR, CD, NMR and MS enabled the identification of numerous known alkaloids and the characterization of novel alkaloids so that between 1966 and 1975 some 31 novel alkaloids of the heteroyohimbine- and oxindole-types had been isolated from the closely related genera *Mitragyna* and *Uncaria* [9–19].

Gas–liquid chromatography (GLC) proved to be a further sensitive method for the separation of these alkaloids and by combining TLC and GLC with the spectroscopic techniques of UV and MS a powerful analytical method was available for the identification of some 60 closely related indole alkaloids from species of *Mitragyna* and *Uncaria* [20] and some 23 alkaloids from *Strychnos* species [21]. Further sensitivity in the analysis of natural products was achieved in the early 1980s with the advent of high-performance liquid chromatography (HPLC) and again this technique was shown to be of considerable value for the separation and identification of some 17 heteroyohimbine/oxindole alkaloids [22] and some 13 quinoline/indole alkaloids from *Cinchona* species [23]. The combination of chromatographic separations and MS as the detector system added further sensitivity and sophistication to natural product research. In the early 1990s we were able to use HPLC for the separation of *Cinchona* alkaloids and MS, interfaced by a thermospray technique, for their identification [24]. Specificity and sensitivity of detection was obtained by single ion monitoring and the alkaloids were detected in picogram amounts. Single ion monitoring does have the disadvantage that it is not necessarily adequate for the characterization of compounds within a mixture and this was overcome by the use of collision-induced dissociation (CID) in tandem MS. Protonated molecular ions formed by fast atom bombardment (FAB) were subjected to CID in the second field-free region of a double focussing mass spectrometer (CID-MIKES). In addition to the protonated M^+ of individual alkaloids other major fragment ions were readily observed allowing for a more definitive identification of each alkaloid [24]. GC-MS is undoubtedly a sensitive analytical method for the separation and identification of natural products. Quassinoids are bitter principles of the Simaroubaceae and are derived biosynthetically via triterpenoids. They usually occur in mixtures of closely related compounds which have similar chromatographic properties. Mass spectroscopy proved invaluable for the identification of quassinoids from *Brucea* species using EI, CI, FD and metastable ion techniques including mass analysed ion kinetic energy spectrometry (MIKES) [25]. Capillary GC-MS has been used by us as a sensitive method for identifying 19 tropane alkaloids in 'hairy roots' of *Datura candida* [26]. When the same technique was applied to 96 mg of total alkaloid extract from 64 g of *Erythroxylum zambesiacum* some 27 alkaloids were identified [27] and from only 50 g of *E. monogynum* root bark some 26 alkaloids were identified of which 20 had not previously been reported from this bark [28].

Sensitivity of analysis by NMR techniques also has advanced rapidly and today much higher resolution instruments than the 60 MHz spectrometer available during my Ph.D. studies are in routine use. A powerful series of NMR techniques is now available for structure determination of natural products and to supplement ^1H there is ^{13}C NMR with DEPT (distortionless enhancement by polarization transfer), COSY-45 (2D ^1H–^1H correlation spectroscopy), HETCOR (heterocorrelation between ^1H and ^{13}C spectra) with the techniques of HMBC (heteronuclear multiple bond connectivity) and HMQC (heteronuclear quantum coherence). In addition there is sophistication of NOE methods with NOESY (2D nuclear Overhauser effect spectroscopy) and ROESY (rotating frame NOE spectroscopy).

CHEMICAL SCREENING OF HERBARIUM SAMPLES

The application of sensitive chromatographic and spectroscopic techniques to the identification of natural products in plants has meant that considerable chemical information may be obtained from very small quantities of plant material. As herbaria contain collections of plant material, one logical approach to phytochemistry has been to investigate small samples of herbarium collections for the presence of chemical constituents. The application of such techniques to the occurrence of alkaloids has been reviewed previously [29]. Four hundred and forty samples of *Strychnos* representing 69 of 75 accepted African species and 36 of the 44 accepted Asian species have been examined for their tertiary alkaloids [30, 31]. Individual alkaloids obtained from herbarium specimens were identified by their chromatographic (TLC, GLC) and spectral (UV, MS) characteristics. As an

illustration of the sensitivity of the technique and the stability of compounds in herbarium specimens, it was found that one dried leaf (183 mg) of *S. nux-vomica* collected in 1675 yielded 2.2 mg of total alkaloid from which 10 tertiary alkaloids were identified [31]. Similar findings were noted for samples collected between 1695 and 1850 [31].

There are 10 species of *Mitragyna* (Rubiaceae) and over a period of years their alkaloids were subjected to intense chemical investigation [32, 33]. Comparatively little progress was made into the alkaloids of the closely related genus *Uncaria*, partly due to problems of botanical identification. Revision of the genus resulted in the reduction of some 120 specific names to 34 accepted species arranged into seven informal groups [34]. Some 400 herbarium samples representing all 34 species were investigated for their alkaloid content and some 40 alkaloids identified by their chromatographic and spectroscopic properties allowing for the chemotaxonomic significance of the alkaloids to be assessed within the botanical relationships [34]. Classically, *Mitragyna* and *Uncaria* were included within the tribe Naucleeae which included other genera which were difficult to classify. Revision of the Naucleeae resulted in the recognition of three subtribes Adininae (16 genera), Naucleinae (four genera) and Anthocephalinae (one genus) [35]. The problem genus, *Cephalanthus*, considered by some taxonomists as being so distinctly different as to belong to another subfamily, was placed in a separate tribe, the Cephalantheae [35]. *Mitragyna* and *Uncaria* were placed in the subtribe Mitragyninae which was assigned to the tribe Cinchoneae. Some 121 small samples of herbarium material representing 11 of the 16 genera of Adininae, three of the four genera of Naucleinae and the single genus *Anthocephalus* of the Anthocephalinae, comprising the then newly defined Naucleeae and also the related but separated *Uncaria* and *Mitragyna* were examined for their tertiary alkaloids [36]. The results clearly supported the reclassification which had been based on botanical considerations showing that the alkaloid chemistry differentiated between the subtribes Adininae/Naucleinae and Anthocephalinae and showed chemical affinities between *Cephalanthus*, *Mitragyna* and *Uncaria*, the latter two genera showing chemical relationships with other members of the tribe Cinchoneae.

The best known member of the Cinchoneae is undoubtedly *Cinchona* and particularly the commercial barks which are the source of the pharmaceutically important alkaloid quinine. The first planting stocks of *Cinchona* in the former Dutch East Indies were derived from plants and seeds collected by Hasskarl on his expedition to S. America in 1852/1853. Hasskarl collected harbarium material to aid the identification of the bark samples and for many years the location of this collection was not known. In 1980 the Hasskarl collection of *Cinchona* barks was relocated in the Rijksherbarium, Leiden. The identification of individual barks within the collection had been a matter of some dispute over a period of years. Small samples of some 28 of Hasskarl's 29 specimens of barks became available for chemical investigation and the results showed that although all of them contained alkaloids the yields ranged from 0.03 to 4.95% total alkaloid [37]. According to Standley in 1930, seven of these specimens were not in fact *Cinchona* species but were of other rubiaceous genera including *Pimentelia* and *Ladenbergia*, as well as *Clethra* (Clethraceae). The presence of quinine and related alkaloids outside the genus *Cinchona* is of taxonomic interest. Only two of Hasskarl's specimens of barks did not contain quinine-type alkaloids but they did contain the indole alkaloid aricine. Both of these samples were identified as *C. pubescens* by Standley [37].

Papaver is another important pharmaceutical genus particularly because of the commercial exploitation of *P. somniferum*, the opium poppy, as the source of the analgesics morphine and codeine. The capsules of two species of the section *Oxytona* obtained from herbarium specimens have been investigated chemically. Five different collections of *P. orientale* and 16 different collections of *P. pseudo-orientale* were available. The alkaloids were identified by means of TLC, mass, UV and NMR spectroscopy and the chromosome numbers of individual specimens were determined [38]. The major alkaloid of four of the samples of *P. orientale* was identified as the morphinan alkaloid oripavine whereas the fifth sample contained the tetrahydroprotoberberine alkaloid mecambridine as the major alkaloid. The promorphinan salutaridine was the major alkaloid of three samples of *P. pseudo-orientale* ($2n = 14$ or 28) whereas the aporphine isothebaine and tetrahydroprotoberberines mecambridine and orientalidine were the major alkaloids of the other 13 samples ($2n = 42$). The investigation indicated that there were at least three different chemical races of *P. pseudo-orientale* and two chemical races of *P. orientale*. Similar investigation of the section *Miltantha* has resulted in the recognition of morphinan, promorphinan/aporphine and rhoeadine chemical races for each of the three species, *P. armeniacum*, *P. fugax* and *P. tauricola* [39].

It is not easy to arrange for plant collections to be made in some countries and chemical information may be obtained from herbarium collections. Some 26 samples of Iraqi species of *Papaver* were obtained from the National Herbarium of Iraq for chemical investigations starting in 1977. Some 19 alkaloids were identified (TLC, UV, MS, NMR) from 10 species which represent five of the sections of the genus [40]. In addition, alkaloids were identified from other species of Papaveraceae including *Glaucium* and *Roemeria*. These results demonstrate that infraspecific differences exist in the alkaloids present in species of Papaveraceae. Infraspecific variation within species of *Oxytona* and *Miltantha* has been reviewed previously [41].

Such investigations utilizing sensitive analytical techniques for the identification of natural products of such well known genera as *Cinchona* and *Papaver* revealed that there is a considerable gap in our knowledge despite the many previous investigations. As techniques become even more sensitive and plant samples rarer, or even extinct, herbaria remain rich stores of plant material for chemical investigation.

SOME NOVEL NATURAL PRODUCTS FROM HIGHER PLANTS

As a result of the use of sensitive chromatographic techniques for the isolation of natural products and the application of sensitive spectroscopic techniques for determination of their chemical structures we have isolated some 110 novel compounds which are alkaloids (indoles, isoquinolines, quinolines, tropanes, quinolizidines), terpenoids (diterpenes, triterpenes, quassinoids), phenolics (flavonoids, isoflavonoids, proanthocyanidins) and quinones (naphthoquinones, anthraquinones). Some examples of these compounds are given in Fig. 1.

Between 1966 and 1975 a series of 21 novel heteroyohimbine and oxindole alkaloids was isolated from species of *Mitragyna* (Rubiaceae) [9–15, 17–19]. Systematic investigation of the alkaloids of the adjacent genus *Uncaria* led to a number of novel alkaloids including a 14-hydroxy substituted heteroyohimbine alkaloid from *U. attenuata*. Detailed investigation of the spectral properties including UV, IR, MS, ^{1}H and ^{13}C NMR led to the conclusion that the alkaloid was 14β-hydroxy-3-isorauniticine [42]. The structural arguments relied heavily on the comparison of ^{13}C NMR chemical shifts of rauniticine, 3-isorauniticine, 14β-hydroxyrauniticine and its *O*-acetyl derivative. Synthesis of 14α-hydroxy-3-isorauniticine and 14α-hydroxyrauniticine enabled direct comparison of ^{13}C NMR spectra and CD with the natural product. This resulted in revision of the structure of the novel alkaloid from *U. attenuata* to 14α-hydroxyrauniticine (Fig. 1) [43]. This is a salutary tale which serves to remind us that despite all the sensitive and sophisticated spectral techniques which are available, it is still possible to err. The related species *U. elliptica* yielded four novel alkaloids rauniticine oxindole A, rauniticine pseudoindoxyl, 3-isorauniticine pseudoindoxyl and akuammigine pseudoindoxyl (Fig. 1). The pseudoindoxyls were prepared from the corresponding heteroyohimbines by treatment with O_2 in NaOMe and characterized by their UV, mass and ^{1}H NMR spectra [44].

The majority of alkaloids isolated from higher plants are tertiary amines and during the investigations into the alkaloids of *Mitragyna* and *Uncaria* a number of polar alkaloids was isolated. Mass spectroscopy revealed that they possessed fragmentations characteristic of heteroyohimbine or oxindole alkaloids but with a $[M]^+$ of 16 amu higher than expected. The additional oxygen atom in each case was due to a N-oxide function in the molecules [45–49]. N-oxides were readily prepared from the tertiary alkaloids by treatment with either *m*-chloroperbenzoic acid or H_2O_2 thus enabling comparison of the chromatographic and spectroscopic properties of the prepared compounds with those of the isolated N-oxides. In each case it was demonstrated that N-oxidation did not result from the drying of the plant material or from the extraction process and they were deemed to be natural products and not artefacts. The question then arose as to why at that time should N-oxides be relatively rare with the exception of the pyrrolizidine alkaloids. Preparation of N-oxides of the tropane alkaloids hyoscyamine and scopolamine, of nicotine and of the morphinans, morphine, codeine and thebaine followed by chromatographic comparison of the prepared N-oxides revealed that N-oxides existed in those plants from which these well known alkaloids were obtained [50–52]. Previous lack of detection was attributed to the more polar nature of N-oxides; their presence in plants and their possible roles have been reviewed previously [53, 54].

A herbarium specimen of *Strychnos cinnamifolia* (J. F. Bourdillon, 5.1.1923) received from the Museum of Economic Botany, Royal Botanic Gardens, Kew, formed part of our screening programme for alkaloids in Asian species of *Strychnos*. TLC indicated that brucine was the major alkaloid and that strychnine was a minor component [55]. This result appeared to confirm a previous analysis which has been done in 1924 at the laboratories of the Pharmaceutical Society in which the brucine and strychnine contents were 2.23 and 0.34%, respectively. GLC analysis in our laboratories showed that the major peak of the alkaloid extract had a R_t value which was slightly longer than that of brucine. Isolation of the major alkaloid from 23.4 g of seeds enabled spectroscopic analysis to be undertaken (UV, ^{1}H NMR, MS) and the alkaloid proved to be the novel compound 4-hydroxy-3-methoxystrychnine (Fig. 1). Taxonomic revision has resulted in the inclusion of *S. cinnamonifolia* Thwaites var. *wightii* A. W. Hill into *S. wallichiana* Steud. ex DC,

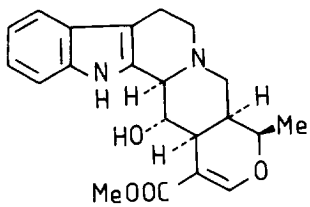
Alstonia species (Apocynaceae) are used in traditional medicine throughout south east Asia for the treatment of malaria, dysentery and other ailments. Ten alkaloids have been isolated from the root bark of *A. angustifolia* [56]. Among the minor alkaloids there was the novel compound 4′-hydroxy-3′,5′-dimethoxybenzoylvincamajine (Fig. 1). Mass spectroscopy revealed two major fragment ions at *m/z* 365 corresponding to vincamajine base and at *m/z* 181 for the aryl moiety. ^{1}H NMR spectroscopy was used to establish the structure [56]. Despite the numerous alkaloids which have been reported from *Papaver* species it is still possible to isolate novel compounds. During our investigations into the alkaloidal constituents of the *Oxytona* and *Miltantha* sections, a major alkaloid isolated from the capsules of a Turkish sample of *P. pseudo-orientale* proved to be a novel compound which was named macrantaline. A combination of UV, IR, ^{1}H NMR, mass spectroscopy and CD was used to establish its structure as 1-(2′-hydroxymethylene-3′,4′-dimethoxybenzyl)-2-methyl-6,7-methylenedioxy-8-methoxy-1,2,3,4-tetrahydroisoquinoline (Fig. 1) [57]. Chemical confirmation for the structure was obtained by converting macrantaline to the corresponding 2′-methyl analogue by Pd/C hydrogenation of the acetyl derivative which proved to be identical in its chromatographic and spectral properties to the 2′-methyl analogue prepared from (−)-α-narcotine by $LiAlH_4$ reduction to α-narcotine diol and Pd/C hydrogenation of the *O,O*-diacetyl derivative. Among the minor alkaloids was a further novel compound, named macrantoridine, which proved to be the 2′-carboxyl analogue of macrantaline (Fig. 1). $LiAlH_4$ reduction of macrantoridine yielded macrantaline. These two alkaloids are of interest from a biosynthetic viewpoint since macrantaline is a likely

precursor of the tetrahydroprotoberberine alkaloid mecambridine and macrantoridine is a likely precursor of the phthalideisoquinoline alkaloid narcotine [57].

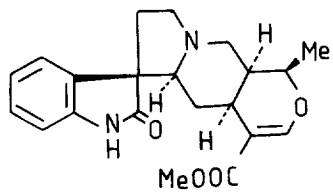
Papaver curviscapum is a member of the section *Miltantha* and is closely related to *P. fugax* and *P. tauricola*. The major tertiary alkaloid of the capsules of *P. curviscapum* from south east Turkey proved to be the novel natural product 1-methoxyallocryptopine (Fig. 1) which had been known previously as a synthetic compound. The natural alkaloid had identical chromatographic behaviour to the synthetic product [58].

By 1986, some 20 species of *Sophora* (Leguminosae) had been examined for their alkaloidal constituents, but *S. velutina* had not been investigated. The major alkaloids of the leaves of *S. velutina* Lindl. var. *zimbabwensis* Gillett and Brumitt proved to be cytisine, (+)-lamprolobine and the novel (+)-9β-hydroxylamprolobine (Fig. 1) [59]. The presence of the hydroxyl substituent was deduced from accurate mass determinations and by preparation of an acetyl derivative. Comparison of mass, ^{1}H and ^{13}C NMR spectra of (+)-lamprolobine and the novel alkaloid, together with ^{1}H–^{1}H correlation NMR spectroscopy enabled assignments to be made for each of the signals in the ^{1}H and ^{13}C NMR spectra. Previously lamprolobine-type alkaloids had not been reported as major constituents of a *Sophora* species.

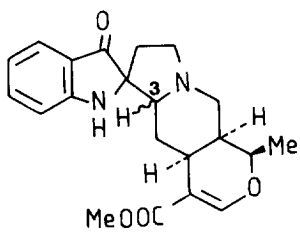
Bolusanthus speciosus (Leguminosae) is the sole member of this monotypic genus which is widespread throughout S. Africa. Ten alkaloids were isolated from the leaves and in addition to the presence of the well known quinolizidine alkaloids a novel alkaloid, 6β-hydroxylupanine (Fig. 1) was isolated [60]. The alkaloid is unstable and readily dehydrates to 5,6-dehydrolupanine. The position of the double bond at C-5/C-6 was deduced from study of the ^{1}H and ^{13}C NMR spectra. The assignment of the hydroxyl at C-6 in the novel alkaloid was established



14α-hydroxyrauniticine

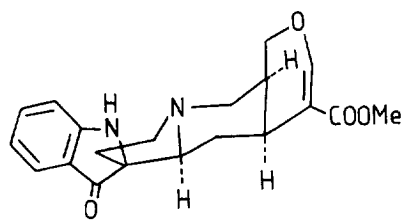


rauniticine oxindole A

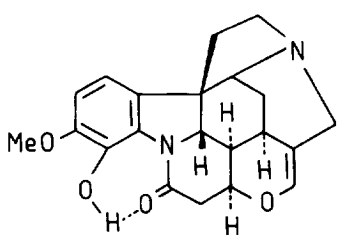


rauniticine pseudoindoxyl (C-3Hα)

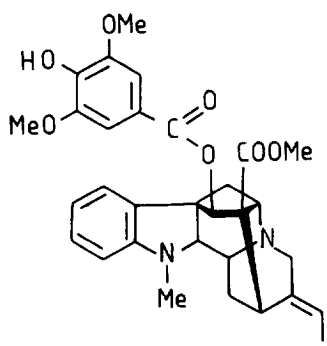
3-isorauniticine pseudoindoxyl (C-3Hα)



akuammigine pseudoindoxyl

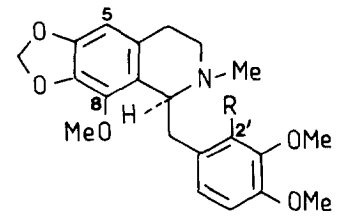


4-hydroxy-3-methoxy-strychnine



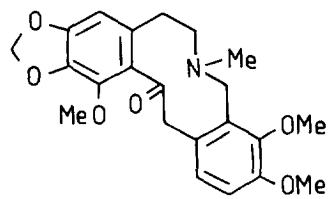
4'-hydroxy-3',5'-dimethoxy-benzoylvincamajine

Fig. 1. Examples of novel natural products isolated.

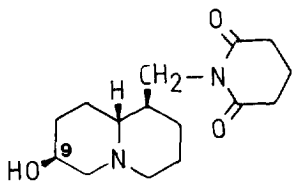


macrantaline, R = CH_2OH

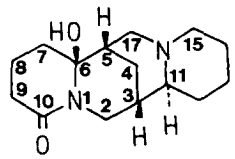
macrantoridine, R = COOH



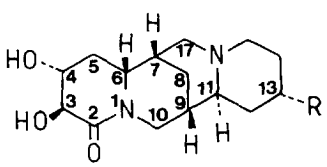
1-methoxyallocryptopine



(+)-9 β -hydroxylamprolobine



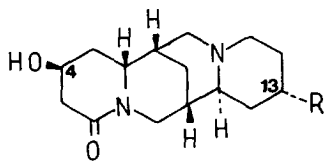
6-hydroxylupanine



3β,4α,13α-trihydroxylupanine

, R = OH

calpaurine, R = -O-CO-



4β-,13α-dihydroxy lupanine, R = OH

4β-hydroxy-13α-O-

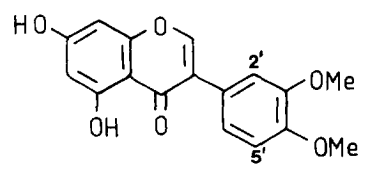
(2'-pyrrolylcarbonyl)-lupanine

R = -O-CO-

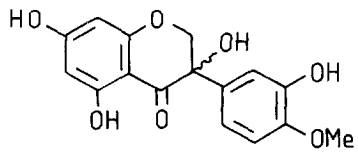
Fig. 1. *Continued*

on the basis of the lack of a signal in the δ3.3–4.0 region of the ^{1}H NMR spectrum (expected for a proton of CH–OH type), lack of signals in the δ3.0–3.40 region (position of H-6 signal in a number of 2-oxosparteines) and by $LiAlH_4$ reduction of the novel alkaloid to yield sparteine (identified by TLC, MS). Further evidence for the structure of the alkaloid was based on the presence of only three methine protons in contrast to lupanine and the ^{13}C signal for C-6 at δ60.9 in the spectrum of lupanine was replaced by a quaternary carbon signal at δ85.5 [60]. Establishment of the β-configuration of the C-6 hydroxyl was based on CD considerations. Extraction of the plant material with cold methanol yielded extracts containing the novel 6β-hydroxylupanine but yields were considerably reduced by extraction with hot methanol. 5,6-Dehydrolupanine has been identified from various species of Leguminosae mainly on the basis of GC-MS analyses. Under such conditions 6β-hydroxylupanine would readily dehydrate and, therefore, it is possible that this alkaloid may be more common than hitherto supposed.

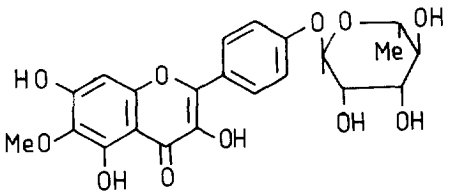
Two novel alkaloids 3β-4α,13α-trihydroxylupanine and 3β,4α-dihydroxy-13α-*O*-(2′pyrrolylcarbonyl)lupanine (calpaurine) (Fig. 1) were isolated from the leaves of Ethiopian *Calpurnia aurea* subsp. *aurea* in addition to a further 11 alkaloids [61]. ^{1}H and ^{1}H–^{1}H NMR spectrometery were used to establish the structure of these novel alkaloids in addition to IR, mass and ^{13}C spectrometry [61, 62]. The DEPT spectrum of calpaurine revealed that the ratio of methine carbons to methylene carbons was 10:7 and this contrasted with the ratio of 8:9 for the known alkaloid calpurnine. It was deduced that the two novel alkaloids differed from calpurnine by an additional two substituents in ring A. A combination of spectroscopic techniques led to the proposal that these two substituents were hydroxyl groups situated at C-3 and C-4. The configurations and conformations of these



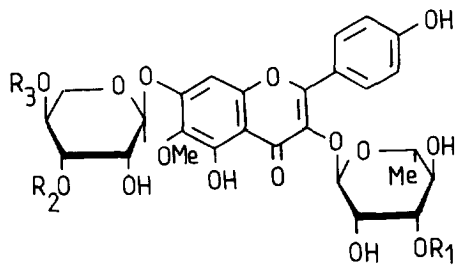
3'-O-methylpratensin



bolusanthin



eupafoline 4'-O-rhamnoside



	R_1	R_2	R_3
eupafoline 3,7-di-O-rhamnoside	H	H	H
eupafoline 3-O-rhamnosyl-7-O -(4-O-acetyl)rhamnoside	H	H	Ac
eupafoline 3-O-(3-O-acetylrhamnosyl) -7-O-(3-O-acetylrhamnoside)	Ac	Ac	H

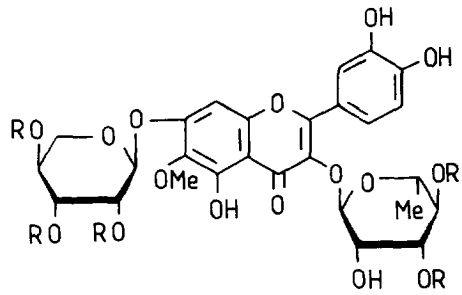
Fig. 1. *Continued*

hydroxyl substituents were established by NOE effects and consideration of coupling constants. Hydrolysis of calpaurine yielded pyrrole-2-carboxylic acid and an amino alcohol which was identical (TLC, MS) to the other novel alkaloid which had been characterized by use of the same spectroscopic techniques as 3β,4α,13α-tetrahydroxylupanine. Further investigation of the minor alkaloids of *Calpurnia aurea* subsp. *aurea* resulted in the isolation of two further novel alkaloids which were characterized by the use of the same spectroscopic techniques as 4β-hydroxy-13α-*O*-2′-pyrrolylcarbonyl)lupanine (digittine) and 4β,13α-dihydroxylupanine (Fig. 1) [63].

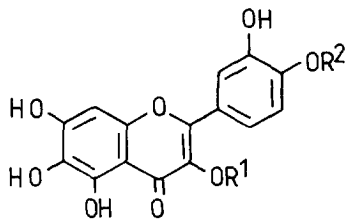
GC-MS has revealed the presence of novel tropane alkaloids 6β-benzoyloxytropan-3-one, 6-isovaleryloxytropan-3-ol and 3-(2-methylbutyryloxy)-tropan-6,7-diol from the stem bark of *Erythroxylum zambesiacum* [27] and 3α-isobutyryloxynortropane, 3α(4-methylvaleroyloxy) tropane and 3β-phenylacetoxytropane from the root bark of *E. monogynum* [28].

In addition to quinolizidine alkaloids, *Bolusanthus speciosus* Bolus (Harms) yielded isoflavonoids including the novel 3′-*O*-methylpratensin and 3,5,7,3′-tetrahydroxy-4′-methoxyisoflavanone (bolusanthin) (Fig. 1) [64]. Mass spectral fragmentation indicated the presence of the dihydroxy substituents in ring A and the dimethoxylation in ring B of 3′-*O*-methylpratensin. The positions of substitution were determined from UV shifts on addition of $AlCl_3$ and NaOAc, ^1H NMR chemical shifts and multiplicity of signals and from NOE measurements.

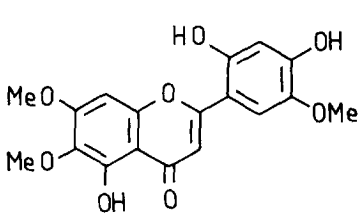
Kalanchoe gracile Hance (Crassulaceae) is used in the treatment of tissue injuries in traditional medicine in Taiwan. Nineteen flavonoids were isolated and 11 were novel flavonol glycosides of the eupafolin [65] and patuletin types [66]. A common feature of both types of flavonoid was the presence of acetylrhamnoside moieties



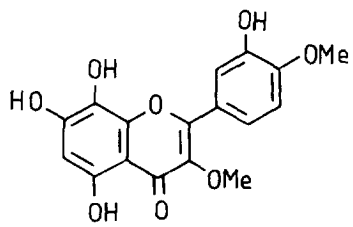
Series of patuletin dirhamnosides
R = H or Ac



	R_1	R_2
quercetagetin 3-methylether	Me	H
quercetagetin 4'-methylether	H	Me



5,2',4'-trihydroxy-6,7,5'-trimethoxyflavone



5,7,8,3'-tetrahydroxy-3,4'-dimethoxyflavone

Fig. 1. *Continued*

as indicated in Fig. 1. FAB-MS gave $[M + H]^+$ and [aglycone + H]$^+$ peaks for each compound and ^{1}H NMR spectroscopy revealed the positions of the rhamnose and acetyl moieties. We have noted that the activity of the sesquiterpene antimalarial artemisinin from *Artemisia annua* against *Plasmodium falciparum in vitro* is markedly enhanced by methoxylated flavonoids such as artemetin and casticin [67]. In contrast, the antiplasmodial activity *in vitro* of chloroquine is unaffected by these flavonoids. It was, therefore, of interest to isolate the flavonoids of *A. annua* and to assess their ability to affect the action of artemisinin against *P. falciparum*. During this investigation 14 known flavonoids and four novel ones, quercetagetin-3-methyl ether, 5,2,4′-trihydroxy-6,7,5′-trimethoxyflavone and 5,7,8,3′-tetrahydroxy-3,4′-dimethoxyflavone and quercetagetin 4′-methyl ether were isolated (Fig. 1) [69].

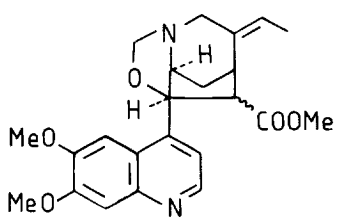
UNPREDICTED NATURAL PRODUCTS FROM HIGHER PLANTS

The vast number of natural products which have been isolated from higher plants has led to a sense of predictability about the constituents which might well be present in some species. Such assumed knowledge may well preclude chemical investigation of certain plants. Practical experience has led me to the conclusion that we cannot always be so confident in our predictions on the constituents of higher plants and some examples of unpredicted isolations are given in Fig. 2. Investigation into the alkaloids of three British species of *Equisetum* resulted in the unexpected isolation of nicotine and the failure to detect either palustrine or any of its related alkaloids which had been reported previously from the genus [2]. Similarly, the alkaloids isolated from a Polish

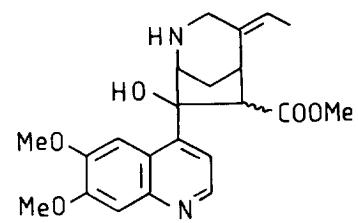
species of *Euonymus europaeus* proved to be the *p*-aryloxymacrocyclic peptide alkaloids frangulanine, fraganine and frangufoline together with the 1-benzyltetrahydroisoquinoline alkaloid armepavine and not the anticipated sesquiterpene nicotinyl ester alkaloids previously reported [70]. With hindsight the finding of *p*-aryloxymacrocyclic peptide and isoquinoline alkaloids in *Euonymus* (Celastraceae) was not too surprising from chemotaxonomic considerations even though our findings were different from those of previous workers.

No such surprises were expected from the well investigated genus *Papaver*. The major alkaloid of a Turkish sample of *P. fugax* investigated in our laboratories proved to be the morphinan thebaine with noscapine as a minor constituent [71]. This result was totally unanticipated because morphinans and phthalideisoquinolines had not previously been located in the section *Miltantha* of which *P. fugax* is a member. In an attempt to explain why our findings were different from those of previous research workers, we ascertained that our sample was obtained from an area which was at the extreme western part of the plant's distribution and was collected from the wild state, not being cultivated as previously investigated samples. This result led to the investigation of a number of species of *Papaver* and to the realization that there is considerable infraspecific variation of alkaloids within the genus [41].

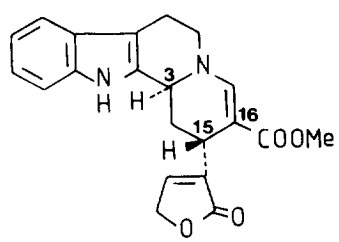
The three closely related species of section *Miltantha*, *P. armeniacum*, *P. fugax* and *P. persicum* (*P. tauricola*) exist in at least three different chemical races in which the major alkaloids are either morphinans or proaporphine-aporphines or rhoeadanes [39]. Revision of the section-*Oxytona* (*Macrantha*) resulted in the recognition of only three species which were characterized morphologically, cytologically and chemically as *P. bracteatum* Lindl. (2n = 14, thebaine), *P. orientale* L. (2n = 28, oripavine) and *P. pseudo-orientale* (Fedde) Medw. (2n = 42, isothebaine) [72]. Shortly after this revision was published, we investigated a Turkish sample of *P. pseudo-orientale* and isolated the promorphinan salutaridine and the novel alkaloid macrantaline (Fig. 1) as major alkaloids [57]. Further investigation showed that there are at least four chemical races of *P. pseudo-orientale* containing either isothebaine as major alkaloid (aporphine-type, 2n = 42), or salutaridine and thebaine (promorphinan-morphinan-type, 2n = 14) or salutaridine (2n = 28) or macrantaline (secober-



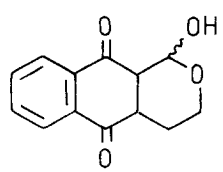
corialstonine



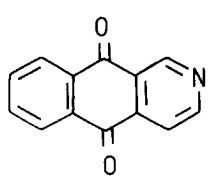
corialstonidine



vallesiachotamine lactone

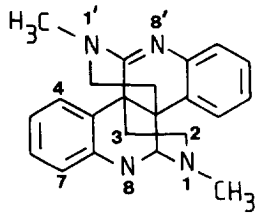


1-hydroxybenzochroman-quinone

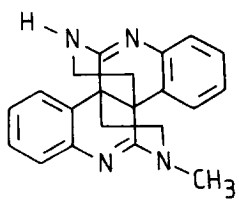


benz[g]-isoquinoline-5,10-dione

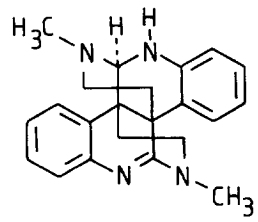
Fig. 2. Examples of unpredicted compounds from higher plants.



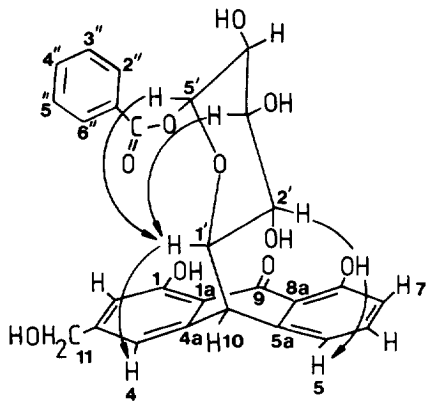
glomerulatine A



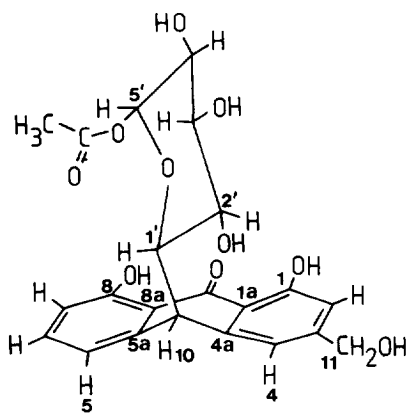
glomerulatine B



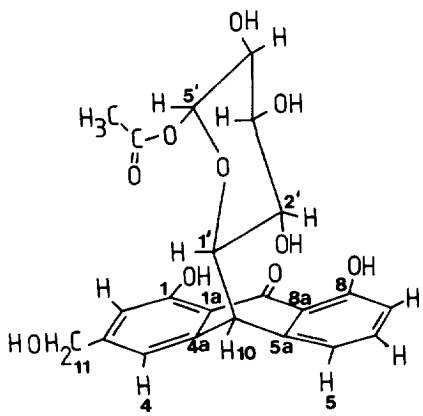
glomerulatine C



picramnioside A



picramnioside B



picramnioside C

Fig. 2. *Continued*

berine-type, 2n = 14) [38]. Similarly we were able to show that *P. bracteatum* exists in at least six different chemical races [38].

Anthocephalus chinensis (Lamk.) A. Rich ex Walp. (Rubiaceae) is located in the lowlands of the Himalayas. The plant is used medicinally for the treatment of a number of diseases including leprosy and dysentery and is known locally as 'wild cinchona'. The major alkaloid is the glycosidic indole-type cadambine which occurs together with a series of related alkaloids [73]. Investigation of the minor alkaloids led to the isolation of cinchonine and dihydrocinchonine previously known mainly from *Cinchona* species. *Anthocephalus* is within the tribe Naucleeae and the presence of *Cinchona*-type alkaloids shows affinity with the neighbouring tribe Cinchoneae.

Alstonia species (Apocynaceae) are sources of a series of indole alkaloids of which echitamine is often the major alkaloid. Some species of *Alstonia* have reputations in traditional medicine for the treatment of malaria and one New Caledonian species, *A. coriacea*, does produce the quinoline alkaloids corialstonine and corialstonidine (Fig. 2) which are chemically related to quinine [75].

The amoebicidal drug emetine is obtained from *Cephaelis ipecacuanha* (Rubiaceae) and is derived biosynthe-

tically from the monoterpene glycoside secologanin and dopamine. In searching for potential new natural products with antiprotozoal activities we decided to investigate other species of *Cephaelis* of which there are some 200 species. In Panama there are some 21 species and at the start of our investigation only *C. ipecacuanha* had been investigated chemically. We have investigated three Panamanian species, *C. dichroa*, *C. camponutans* and *C. glomerulata*. *Cephaelis dichroa* yielded the monoterpene alkaloids strictosidine, strictosamide, vallesiachotamine and angustine together with the novel alkaloid vallesiachotamine lactone (Fig. 2) [76]. The spectroscopic techniques used to determine the structure of the novel alkaloid included ^{1}H and ^{13}C NMR with COSY 45 and ^{1}H and ^{13}C heterocorrelation spectroscopy. The second species, *C. camponutans*, was submitted to bioassay guided fractionation using brine shrimps. Two major active compounds were obtained and characterized as 1-hydroxybenzoisochromanquinone and benz[*g*] isoquinoline-5,10-dione (Fig. 2) [77]. Neither of these two compounds has been isolated previously from plants although the latter compound is known as an impurity from commercial acridone. *Cephaelis glomerulata* yielded three novel quinoline alkaloids characterized as 8-8a,8′-8′a-tetradehydro-(−)-calycanthine, 8-8a,8′-8′a-tetradehydro-*N*′-demethyl(−)-calycanthine and 8-8, didehydro (−)-calycanthine which were named glomerulatine A, B and C, respectively (Fig. 2) [78]. Despite considerable use of sensitive spectroscopic techniques including COSY 45, HMQC, HMBC and ROESY NMR spectroscopy, it was not possible to establish unequivocally whether these alkaloids were of the calycanthine- or isocalycanthine-type [78]. A series of computer generated theoretical spectra was derived for each of these types of alkaloid and compared with the ^{1}H NMR spectrum of one of the alkaloids obtained experimentally. The experimentally determined spectrum matched exactly with only one of the theoretical spectra and established that the alkaloid was of the calycanthine-type [79].

The results obtained from these three Panamanian species of *Cephaelis* indicate that the dopamine-derived alkaloid emetine is rarer than hitherto supposed and that there is a diversity of structural type of natural product not previously anticipated from *Cephaelis* species. Indeed it may have seemed unnecessary to investigate *Cephaelis* species chemically because emetine-type alkaloids could have been confidently predicted. One of the reasons for the divergent chemical structures found in these three species of *Cephaelis* lies in the taxonomic position of the genus. In the north east of S. America [80] and in Venezuela [81] species of *Cephaelis* Swartz and *Psychotria* L. are polyphyletic and represent a convergent assortment of species and species groups, each being more closely related to *Psychotria* than to other species of *Cephaelis* [82]. As a result *Cephaelis* species are considered to be members of *Psychotria*. In C. America where there are fewer intermediate taxa, *Cephaelis* had been retained as a genus [83, 84]. In a monograph of Costa Rican Rubiaceae [85] *Cephaelis* species are included in *Psychotria* within the subgenus Heteropsychotria, the other subgenus being *Psychotria*. Of the three Panamanian species investigated by us *C. camponutans* Dwyer and Hayden is more correctly *P. camponutans* (Dwyer and Hayden) Hammel [85]. *Cephaelis glomerulata* J. Donnell Smith is more correctly *P. glomerulata* (J. Donnell Smith) Steyermark whilst *C. dichroa* (Standley) Standley remains as a *Cephaelis* [83].

Our interest in the chemistry of Simaroubaceae has stemmed from the presence of the bioactive quassinoids and the canthin-6-one alkaloids. *Picramnia antidesma* subsp. *fessonia* from Panama was selected because it was anticipated that it would yield a series of quassinoids which would be investigated for antiprotozoal and cytotoxic activities. Bioactivity-guided fractionation of extracts using KB cells and brine shrimps resulted in the isolation of three novel compounds named picramniosides A, B and C. Detailed spectroscopic studies including UV, IR, CD, MS, ^{1}H and ^{13}C and 2D NMR (COSY 45, HMQC, HMBC, ROESY) established that these were three closely related esterified C-glycosides of aloe-emodin. The ROESY effects for the benzoate ester picramnioside A are shown in Fig. 2. The CD spectrum showed a negative Cotton effect of 295 nm and is in agreement with that reported for (10*R*)-aloin. Picramniosides B and C are related isomeric acetates and CD established the absolute configurations as (10*S*)-picramnioside B and (10*R*)-picramnioside C (Fig. 2) [86].

Experience has shown that it is not sensible to prejudge the type of constituents present within species of higher plants and that unexpected and even novel compounds may be isolated from genera which had previously been subjected to chemical investigation. The presence of chemical races within species is a distinct possibility and this should be borne in mind when investigating plants especially for the presence of biologically active compounds.

PLANT CELL CULTURES FOR THE PRODUCTION OF BIOLOGICALLY ACTIVE COMPOUNDS

Plant cell, tissue and organ cultures possess some advantages over intact plants in that they can be grown under standard conditions for short growth cycles and are not subject to seasonal variations. Plants which are either in short supply or are difficult to obtain can be grown as cultures in order to produce biomass for chemical or biochemical investigations. The use of plants as sources of valuable products and the potential of plant tissue cultures for the production of biologically active molecules has been reviewed [87]. Our understanding of biosynthetic pathways and the enzymes involved has been facilitated by using plant cell cultures which are less complex in organization than the entire plant and hence permeability, translocation and segregation of precursors and products do not generally present problems of incorporation. Purified enzymes and active cell-free systems can be prepared more easily from plant cell cultures. Biosynthetic studies of alkaloids, phenols and terpenes utilizing plant cell cultures were reviewed by us taking literature from 1980 to 1983 [88]. At that time many of

the scientific publications dealt with *Catharanthus roseus* and the driving force was the aim to produce the expensive clinically important dimeric anticancer drugs vinblastine and vincristine. It is ironic that despite all of the efforts involved by major research groups throughout the world, this aim proved to be so elusive. Nevertheless, research was stimulated and the biosynthesis of other indole alkaloids as well as isoquinolines, quinolizidines, tropanes, flavonoids, quinolones and terpenoids were investigated. The use of plant cell culture techniques for biosynthetic studies has been greatly enhanced by the development of some of the sensitive analytical techniques referred to earlier. Further review articles prepared by us dealt with research into alkaloid production in particular [89, 90]. By 1989 it was reported by others that plant cell cultures had produced 85 novel compounds from some 30 different plant cultures. The majority of these compounds had been isolated in the previous five years and they included 23 alkaloids, 19 terpenoids, 30 quinones and 11 phenylpropanoids [91]. The possibilities of finding new products from plant cell cultures was the theme of another of our review presentations [92].

A number of plants serve as commercial sources of clinical agents and one excellent example is that of *Cinchona* species which are cultivated for the production of quinine as an antimalarial and quinidine as antiarrhythmic drug. It takes at least seven years of cultivation before the bark can be collected for the extraction of alkaloids and hence one of our initial investigations into the use of plant tissue cultures for the production of biologically active molecules featured *Cinchona*. Leaf and root organ cultures and root suspension cultures were established from two commercial varieties of *Cinchona ledgeriana* [93]. Although alkaloid yields were low, the cultures did produce quinine, quinidine, cinchonine and cinchonidine as the major alkaloids. HPLC analysis indicated that leaf organ cultures of some of the varieties produced 0.15% dry wt of cells. These results came somewhat as a surprise to us because other laboratories had published their findings and reported that their cell cultures produced only cinchonine and cinchonidine instead of the required methoxylated analogues quinine and quinidine. For practical reasons our research continued with root organ cultures of *C. ledgeriana* which remained stable over a period of years. These root organs were composed of clusters of root-like projections which grew to about 5 mm in length before they split up into smaller pieces. L-[methylene-^{14}C]Tryptophan was readily taken up by these cultures and was incorporated into quinine and quinidine [94]. Freshly subcultured root organs were fed with the precursor L-tryptophan and yields of quinine/quinidine were improved by up to five-fold. Root cultures were fed with L-[methylene-^{14}C]tryptophan, [C-5 ^{3}H]secologanin, [C-3 ^{3}H]strictosidine, [ar ^{3}H]strictosidine and [C-8 ^{3}H]quinine and -quinidine at days 1, 7, 16 and 30 of their growth cycles, corresponding to lag, exponential and stationary phases. Uptake of the labelled compounds was monitored over a 24 hr period and showed considerable differences in uptake of these compounds at different stages of the growth cycle [94]. The greatest uptake of L-[methylene-^{14}C]tryptophan was 80% which occurred at day 16 corresponding to the onset of the growth phase. The incorporation for days 1, 7 and 30 was less than 20%. Improved yields of alkaloid obtained by precursor feeding with tryptophan were shown to be particularly dependent on the stage of the growth cycle at which feeding took place [95].

In order to obtain supplies of quassinoids for our studies on the antiprotozoal activities of these compounds, cell cultures of several species of Simaroubaceae were established. Initially callus and suspension cultures of *Ailanthus altissima* were established. Quassinoids were not detected but the suspension cultures produced alkaloid yields of 1.27% dry wt of cells. The major alkaloids were identified as canthin-6-one and 1-methoxycanthin-6-one by their UV, mass and ^{1}H NMR spectra [96]. The uptake of L-[methylene-^{14}C]tryptophan into these two alkaloids and into 1-hydroxycanthin-6-one was studied and again efficient incorporation of the precursor was shown to be dependent on the phase of the growth cycle. Improved yields of the canthin-6-one alkaloids were achieved when L-tryptophan was fed during the lag phase of the growth cycle. The effect of different basal media including Murashige and Skoog, Linsmaier and Skoog, Schenk and Hildebrandt and Gamborg's B-5, on alkaloid production was also investigated with the cell suspension cultures of *A. altissima* [97]. The major alkaloid, 1-methoxycanthin-6-one, was produced in greatest yield by cells grown in LS medium (65 mg l^{-1}). Time course studies of the uptake of L-[methyl-^{14}C]methionine showed rapid uptake by these cultures fed at weekly intervals throughout the growth cycle [98].

When the cell suspensions of *A. altissima* were challenged with either yeast glucan or 8% sucrose as elicitors at different times in their growth cycle, the canthin-6-one levels increased by almost 100% during early growth stages but elicitation at the end of the growth cycle had no appreciable effect [99]. The quassinoid ailanthone was found to be present in appreciable quantities in both roots and shoots of *A. altissima* seedlings but was not detected in the cell cultures and neither could it be induced by treatment of the cultures with plant growth regulators or elicitors. Ailanthone was also not detected in transformed 'hairy' roots induced by infection of sterile seedlings with *Abrobacterium rhizogenes* or in normal root cultures. Shoot cultures grown in medium containing the plant growth regulators 6-benzylaminopurine and gibberellic acid did however accumulate ailanthone related quassinoids. Although tryptophan is incorporated into canthin-6-one alkaloids there was no incorporation of radio label when suspension cultures were fed with [side chain 2 ^{14}C]tryptamine [99].

Cell suspension cultures of *Brucea javanica* were also established in our laboratories in attempts to produce antiprotozoal and cytotoxic quassinoids. These cultures behaved in a similar manner to those of *A. altissima* in that they failed to produce quassinoids but produced high yields (2% dry wt of cells) of canthin-6-one alkaloids. The alkaloids produced by cell suspension cultures of *B.*

javanica included canthin-6-one and its 11-hydroxy-, 11-methoxy-, 5-methoxy-, 4-hydroxy-5-methoxy- and N-oxide analogues [100, 101]. The yields of these alkaloids and the major alkaloid produced in these cultures are affected by treatment with plant growth hormones [102].

Cell suspension cultures of the antimalarial plant *Artemisia annua* have also been established in our laboratories and examined for their chemical constituents and antiplasmodial activities [103]. Although artemisinin was not detected, the cell extracts did show activity against *P. falciparum in vitro*. Bioassay guided fractionation led to the isolation of a series of antiplasmodial flavonoids including artemetin, chrysoplenetin, chrysoplenol-D and circilineol [104]. The IC_{50} values of the isolated falvonoids against *P. falciparum* (K1) *in vitro* were in the order of $2.4–6.5 \times 10^{-5}$ M and they were much less active than artemisinin which has an IC_{50} value of 3×10^{-8} M. At concentrations of 5×10^{-6} M these flavonoids were not active against *P. falciparum in vitro* but they demonstrated a marked and selective potentiation effect on the antiplasmodial activity of artemisinin. These results further substantiated our earlier findings that some methoxylated flavonoids are able to potentiate the action of artemisinin against *P. falciparum in vitro* [67].

The clinically used tropane alkaloids, hyoscyamine and scopolamine produced by various species of Solanaceae are also obvious targets for production by plant cell cultures. Transformed 'hairy' root cultures of a high-yielding alkaloid hybrid of *Datura candida* were produced in our laboratories and the content of scopolamine and hyoscyamine investigated by HPLC analysis. The alkaloid yield was found to be 0.68% dry wt of hairy root culture and this is 1.6 and 2.6 times the yield found in the aerial parts and the roots, respectively, of the parent plant [105]. The composition of the minor alkaloids was investigated by GC-MS and a further 17 alkaloids, mainly tropanes were identified [26]. Two hitherto undescribed alkaloids were detected and tentatively identified as 3-hydroxy-6-propionyloxytropane and 3-hydroxy-6-butyryloxytropane.

Papaver somniferum, the opium poppy, is another obvious target for plant tissue culture techniques and a number of laboratories have attempted to produce the analgesic alkaloids morphine and codeine. Although considerable information has been obtained on the biosynthetic pathways and the enzymes involved in the production of isoquinoline alkaloids, it appears that the final goal of morphine, or more particularly codeine, production has not been achieved. In our laboratories cell suspension cultures of *P. somniferum* yielded dopamine and cryptopine but morphinan alkaloids were not detected [106].

Obviously, plant tissue cultures are effective producers of some biologically active molecules and in certain cases offer an alternative source of such compounds. It has not proved feasible to manufacture any of the major clinically useful natural products by cell culture fermentation but the technique has been invaluable for scientists who have been able to make significant contributions to our understanding of biosynthetic pathways particularly at the enzyme level.

ANTIPROTOZOAL AND CYTOTOXIC NATURAL PRODUCTS FROM HIGHER PLANTS

Sensitivity in physical techniques for structure determination of natural products has been parallelled by a development of sensitive biological techniques for assessment of biological activities. In the 1950s any phytochemist wishing to undertake pharmacological testing would be required to provide 1–2 g of pure compound for animal experimentation. Over the intervening years a series of *in vitro* assays has been developed and by using bioactivity-guided fractionation of natural product extracts many active compounds have been isolated. The prime example of this procedure has been the search for novel anti-cancer drugs carried out by the National Cancer Institute in the U.S.A. with collaborating scientists and by the pharmaceutical industry. Compounds with selective activities *in vitro* are subsequently assessed for activities *in vivo* against human tumour lines in animals.

For bioassay-guided fractionation techniques to be applied within small academic departments, inexpensive, robust cell lines are essential. We have utilized a small number of cytotoxicity tests *in vitro* and initially chose a TLX-5 mouse lymphoma cell line recommended by scientists at The Chester Beatty Cancer Research Institute, London. This cell line proved to be simple to use and sensitive, allowing for rapid screening for cytotoxicity against extracts of *Brucea javanica* fruits [107]. Cytotoxicity was assessed by the uptake of [^{3}H]thymidine into cells over a period of only 40 min and allowed for rapid identification of the quassinoid bruceine A from Fijian samples of *B. javanica*. Although this cell line proved easy to use it was by no means a common line and continuation of supplies proved problematic. Guinea-pig ear keratinocytes (GPK) proved to be more robust, readily available and a simple *in vitro* assay was developed for screening plant extracts [108]. The test procedure was effective in providing comparable cytotoxicity data on a series of alkaloids including emetine, colchicine and vinblastine. A microdilution *in vitro* assay for cytotoxicity against KB cells (human epidermal carcinoma of the human nasopharynx) has been developed in our laboratories [109]. Assessment of activity was obtained by staining with eosin and measuring absorbance at 490 nm using a microplate reader. The cytotoxicity of a series of closely related quassinoids was determined against KB cells and compared with antiplasmodial effects against *Plasmodium falciparum in vitro* in order to obtain an estimate of 'therapeutic index' (i.e. ratio of antiplasmodial activity to cytotoxicity against mammalian cells). This study showed that it was possible to select compounds with specificity of antiplasmodial activity *in vitro* prior to further investigation *in vivo*.

Brine shrimp larvae (nauplii) have been used for bioassay-guided fractionation of plant extracts containing a

variety of toxic substances. We have developed a microplate assay which gives results comparable to previously published methods but which is more sensitive [110]. The microplate method readily detected a series of 19 compounds which are toxic to KB cells and has utility as an inexpensive simple bioassay for monitoring plant extract fractionations.

Protozoal diseases are a major threat to world health and they include malaria (270 million infections per annum), leishmaniasis (12 million cases p.a.), trypanosomiasis (18 million cases p.a.), amoebiasis (42 million cases p.a.) and giardiasis (200 million cases p.a.) [111]. In addition, cryptosporidosis, pneumocystis and toxoplasmosis are becoming more prevalent in developed countries due to suppression of the immune system, particularly in AIDS patients. There is an urgent need for new antiprotozoal drugs, particularly with novel modes of action and natural products offer a potential source of such compounds. We have reviewed previously the types of natural product which have antiprotozoal activities [111–117].

During a continued investigation we have utilized tests for activity against *Plasmodium falciparum in vitro* (inhibition of incorporation of [^{3}H]hypoxanthine microplate assay) [118] and *in vivo* (four day suppressive test using *P. berghei* in mice) [119]. Assays available for antimalarial and amoebicidal testing of natural product extracts have been described in the literature [120]. In our initial tests with *Entamoeba histolytica* flat sided culture tubes were used and assessment of activity was obtained by counting the amoebae [121]; a series of cinchophylline-type alkaloids (indole analogues of emetine) were investigated for amoebicidal activity [122]. Subsequently, a more sensitive microplate assay was developed and amoebal growth was measured by staining with eosin [123]. Similarly a new microplate assay was developed for determining antigiardial activity *in vitro* utilizing *Giardia intestinalis* and measuring soluble formazan production from a tetrazolium reagent [124]. The antigiardial activities of a series of compounds including anisomycin, cycloheximide, furazolidine, metronidazole, emetine, homoharringtonine and various bruceolides have been determined.

Higher plants have yielded three clinically useful drugs, the quinoline alkaloid quinine from the bark of *Cinchona* species, the isoquinoline alkaloid emetine from the underground parts of *Cephaelis ipecacuanha* and the sesquiterpene lactone artemisinin from the aerial parts of *Artemisia annua*. The latter has been developed in more recent years and its clinical utility has done much to emphasize the potential of plants as sources of antiprotozoal drugs. Artemisinin itself is unusual chemically in that it is a sesquiterpene lactone containing an endoperoxide moiety. The cytotoxic activity of sesquiterpene lactones is well recognized and it seemed at one time that such compounds would never find clinical uses. The widespread resistance of *P. falciparum* to clinically useful antimalarial therapy including chloroquine, pyrimethamine–sulphadoxine and melfloquine has resulted in a crisis in the management of malaria. The use of artemisinin and analogues such as artemether as antimalarial drugs by the Chinese has resulted in WHO involvement and in the development of artemether by Rhone-Poulenc-Roher [125]. The role of artemisinin and its derivatives in the current treatment of malaria has been reviewed by WHO [126]. Recently published clinical trials in Vietnam and Thailand/Cambodia have shown that artemether is more effective than quinine [127].

A few years ago we asked the question whether there were more antiprotozoal drugs awaiting discovery in Nature or were there only three from the 250 000 species of higher plant. In 1947, it was reported that some 600 samples of plants representing some 126 families had been evaluated for activity *in vivo* against *P. cathemerium* and *P. lophurae* in ducklings and against *P. gallinaceum* in chicks [128]. Thirty genera yielded active species and two families in particular, Simaroubaceae and Amaryllidaceae, proved to have a number of active species. Although these avian tests were the state of the art at that time it was realized that they had doubtful predictive ability for clinical use. These findings lay dormant, partly because of the difficulties of the test procedures and partly owing to difficulties in separating and determining the chemical structures of active compounds. It was not until 1976 that *P. falciparum* was cultured successfully *in vitro* [129] and subsequently an assay procedure was developed [130].

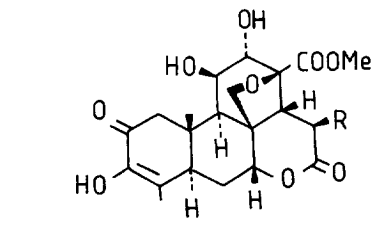
We have used a modification of the *in vitro* assay with *P. falciparum* (K1, multi-drug resistant strain) in order to follow up the antiprotozoal activities reported [108] for species of Simaroubaceae. The major active principles are a series of quassinoids (e.g. bruceine A, bruceantin, Fig. 3) which have been isolated from *Brucea javanica* (Thailand) [118, 131], *Eurycoma longifolia* (Malaysia) [132], *Ailanthus altissima* (India) [133] and *Simarouba amara* (C. America) [134]. Some 40 individual quassinoids were tested for their antiprotozoal activity against *P. falciparum in vitro* and 10 proved to have IC_{50} values of less than 0.02 $\mu g\,ml^{-1}$ (chloroquine disphosphate IC_{50} value 0.21 $\mu g\,ml^{-1}$) [135].

The effect of nine quassinoids has been determined against *P. berghei* (N strain) in mice using the four day suppressive test [111, 131]. The ED_{90} values arranged from 2.80 to 26.7 $mg\,kg^{-1}\,day^{-1}$ in comparison with an ED_{50} value of 6.02 $mg\,kg^{-1}\,day^{-1}$ for chloroquine diphosphate. Seven of the nine quassinoids caused toxic deaths in mice within dose ranges of 3–18 $mg\,kg^{-1}\,day^{-1}$. Bruceine D, 2′-acetoxyglaucarubinone and holacanthone were the least toxic of the compounds tested. Comparison of cytotoxicity to KB cells *in vitro* and activity against *P. falciparum in vitro* showed that the ratio varied from 4.2 to 77, the least toxic compound being brusatol. The mode of action of quassinoids against *P. falciparum* is by rapid onset of inhibition of protein synthesis [136]. Three quassinoids, ailanthinone, bruceantin and chaparrin have been compared for their activities against a chloroquine-sensitive strain of *P. falciparum* (T9-96) and a chloroquine-resistant strain (K1) and no differences in IC_{50} values were noted [137]. We

have also shown that quassinoids are active against *Entamoeba histolytica*, *Giardia intestinalis* and *Toxoplasma gondii in vitro* [123, 124, 138].

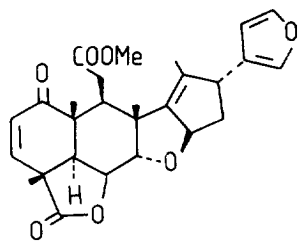
In attempts to modify the activity of quassinoids we have prepared a number of ester and ether derivatives and lipidic amino acid conjugates [139, 140]. The C-3 *t*-butoxycarbonyl-decanoate ester of brusatol and the C-3 acetyl ester have similar IC_{50} values to brusatol against *P. falciparum in vitro* but both esters are less toxic to KB cells suggesting that C-3 esterification reduces toxicity to mammalian cells but not to *P. falciparum*. Quassinoids are not readily available for further studies and they occur in mixtures of closely related compounds within the same plant. Hence synthesis is an attractive alternative and we have synthesized a number of AB bicyclic and ABC tricyclic analogues of quassinoids as well as preparing derivatives of the commercially available inactive quassin [141].

Bitter terpenoids known as limonoids which are related biosynthetically to the quassinoids are produced by species of the adjacent family Meliaceae. The neem tree *Azadirachta indica* is used widely as an antimalarial plant in Asia. We have tested 27 limonoids for antiplasmodial activity and four of them, gedunin, dihydrogedunin, nimbinin and nimbolide (Fig. 3) had IC_{50} values in the range of 0.72–1.74 $\mu g\,ml^{-1}$ against *P. falciparum* (K1) *in*

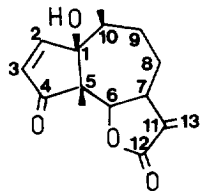


bruceine A, R = -OCO

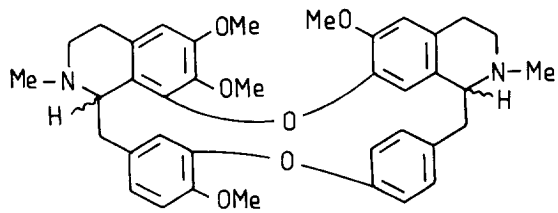
bruceantin, R = -OCO



nimbolide

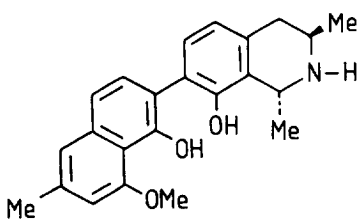


parthenin

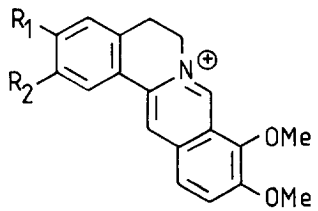


phaeanthine (R,R)

tetrandrine (S,S)

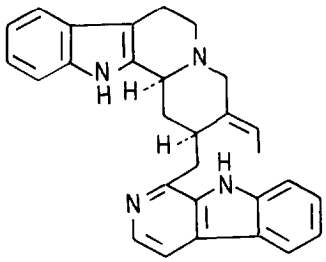


dioncophylline B

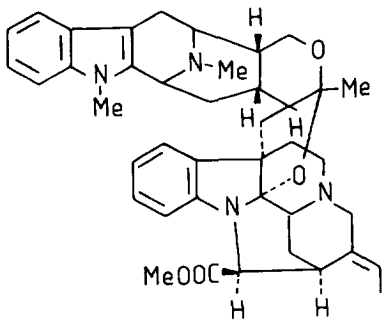


	R_1	R_2
berberine	— OCH_2 —	O
jatrorrhizine	OH	OMe

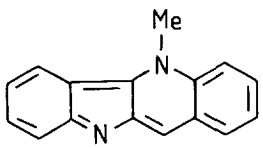
Fig. 3. Examples of natural products with antiprotozoal activites.



usambarensine



villastonine



cryptolepine

Fig. 3. *Continued*

vitro [142]. The cytotoxic activity of gedunin (IC_{50} 275 $\mu g\,ml^{-1}$) was less than that of quinine dihydrochloride (IC_{50} 174 $\mu g\,ml^{-1}$) against GPK cells *in vitro*. Hence, although the limonoids are less active against *P. falciparum in vitro* than the quassinoids they are worthy of further investigation because they are markedly less cytotoxic.

The sesquiterpene lactone parthenin (Fig. 3) has an IC_{50} value of 1.29 $\mu g\,ml^{-1}$ against *P. falciparum* (K1) *in vitro* [143]. Although parthenin is said to be too toxic to warrant further investigation, doses of up to 100 $mg\,ml^{-1}\,day^{-1}$ have been given to rats without toxic reactions. A series of 11 parthenin derivatives have been prepared and those with the exocyclic methylene lactone functionality have similar IC_{50} values to parthenin against *P. falciparum in vitro*. C-2 and C-13 adducts of parthenin with water, methanol and amines act as prodrugs as they have similar IC_{50} values to parthenin. Although parthenin is considerably less active than artemisinin, the two molecules are ampipathic and have common areas for their oxygen atoms and hydrocarbon framework [143].

Wild chimpanzees in Mahale Mountain National Park, Tanzania, have been observed to chew the young stems of *Vernonia amygdalina* (Compositae) when sick. This plant is bitter and in this respect differs from other food items used by the chimpanzees. Four sesquiterpene lactones and seven steroids have been isolated from the plant and tested for their antischistosomal, antiplasmodial, antiamoebic and antileishmanial activities. The sesquiterpene lactones vernodalin, vernolide and hydroxyvernolide had activity against *P. falciparum in vitro* and vernodalin was active against *Leishmania infantum* [144]. It is proposed that chimpanzees control their parasite-related diseases by using plants as medicines.

Extracts of 18 species of higher plants from 11 different families which are used in Sierra Leone for the treatment of malaria and/or fever have been screened for activity against *P. falciparum in vitro* [145]. *Triclisia patens* was the most active species and the active principles were identified as the bisbenzylisoquinoline alkaloids phaeanthine (Fig. 3), pycnamine and aromoline with IC_{50} values ranging from 0.15 to 1.43 $\mu g\,ml^{-1}$. We have screened some 24 bisbenzylisoquinoline alkaloids for antiplasmodial, antiamoebic and cytotoxic activities by the use of *in vitro* microtests [146]. Eight of these alkaloids had antiplasmodial activity with IC_{50} values of less than 1 μM against *P. falciparum in vitro* in comparison to chloroquine with an IC_{50} value of 0.2 μM. Three of these alkaloids had IC_{50} values of 5–11 μM against *E. histolytica in vitro* (metronidazole had an IC_{50} value of 1.87 μM). None of the 24 alkaloids possessed any significant cytotoxicity against KB cells *in vitro*. Tetrandrine (Fig. 3) has been reported to be more active against chloroquine-resistant strains of *P. falciparum* than against chloroquine-sensitive strains [147] and in our hands phaeanthine, the enantiomer of tetrandrine, was twice as potent against chloroquine-resistant strain K1 than against the chloroquine-sensitive strain T9-96 with IC_{50} values of 0.37 and 0.71 $\mu g\,ml^{-1}$, respectively [148]. Tetrandrine and related alkaloids have been reported to be effective in reversing the resistance of *P. falciparum* to chloroquine [149]. Bisbenzylisoquinoline alkaloids merit further investigation as potential novel antimalarial agents.

Ancistrocladus abbreviatus and *A. barteri* (Ancistrocladaceae) and *Triphyophyllum peltatum* (Dioncophyllaceae) are used in traditional medicine for the treatment of malaria and other diseases. The active principles of these three species have been identified as naphthylisoquinoline alkaloids [150]. Dionocophylline B (Fig. 3), for example, is active against chloroquine-resistant strain (K1) and chloroquine-sensitive strain (NF/56/64) with IC_{50} values of 0.06 and 0.22 $\mu g\,ml^{-1}$, respectively. These alkaloids represent a new class of natural product with antiprotozoal activity and further investigation is warranted.

Berberine (Fig. 3) is used in some countries to treat malaria, amoebiasis and leishmaniasis. IC_{50} values

against three chloroquine-resistant strains of *P. falciparum in vitro* are in the range of 0.14–0.36 $\mu g\,ml^{-1}$ whilst jatrorrhizine (Fig. 3) has IC_{50} values of the order of 0.42–1.6 $\mu g\,ml^{-1}$ [151, 152].

Alkaloids with similar structures to the antiamoebic cinchophylline-type alkaloids of some *Cinchona* species are found in some species of *Strychnos*. The IC_{50} values of usambarensine (Fig. 3), a constituent of the arrow poison *Strychnos usambarensis*, against *P. falciparum*, *E. histolytica* and *G. intestinalis in vitro* are 0.88, 1.13 and 1.40 μM, respectively [153]. Six related alkaloids were also investigated and shown to possess significant activities against these three species of protozoa, *in vitro*. These alkaloids have structural similarities to emetine but are in the order of some 100 times less cytotoxic to KB cells. To date, some 20 *Strychnos* alkaloids have been tested against these three species of protozoa and the most active compounds were assessed for cytotoxicity against KB cells. 3',4'-Dihydrousambarensine was the most active against *P. falciparum* and was relatively non-toxic to KB cells (cytotoxic/antiplasmodial ratio of 1474) [154]. This alkaloid was also the most selective of the compounds tested against *G. intestinalis* whilst usambarensine proved to be the most selective compound against *E. histolytica*. The different antiprotozoal profiles in activity seen with these *Strychnos* alkaloids suggest that there may be subtle differences in site of action of these compounds between the three species of protozoa.

Alstonia angustifolia is used in Malaysia as an antimalarial and we have identified dimeric indole alkaloids as the active principles [155]. Nine alkaloids were tested for antiprotozoal activities with macrocarpamine, macralstonine acetate and villastonine (Fig. 3) showed significant activities against *P. falciparum* and *E. histolytica in vitro*. These alkaloids are four to eight times less potent than emetine against *E. histolytica* and 15 to 50 times less potent than chloroquine against *P. falciparum*. The monomeric alkaloids alstonerine, 11-methoxyakuammicine, norfluorocurine, pleiocarpamine and vincamajine are all considerably less active than the dimers.

Many other species of *Alstonia* have reputations as antimalarials in traditional medicines. The monomeric alkaloid echitamine is the major alkaloid of many species of *Alstonia* and it has been assumed to be the active antiprotozoal principle. In our hands, echitamine is only weakly active against *P. falciparum* with an IC_{50} value of 43 μM *in vitro* and we have made a reappraisal of the antiprotozoal activity of *Alstonia* species [156].

The roots of *Cryptolepis sanguinolenta* are used in Ghana for the treatment of malaria. The major alkaloid, cryptolepine (Fig. 3) is highly active against *P. falciparum* (K1) *in vitro* with an IC_{50} value of 0.134 μM [157]. When administered to mice infected with *P. berghei* no reduction in parasitaemia was observed [158]. Further investigation of this apparant anomaly is merited particularly in view of the continued clinical use of the root extract in Ghana.

The interdisciplinary approach to the study of natural products with antiprotozoal activity has resulted in a number of interesting lead compounds and in some instances has provided scientific evidence to explain the use of traditional medicines. A number of questions have arisen as a result of these studies and none of them have been fully answered [111]. Bioassay-guided fractionation has led to the identification of lipid soluble active principles but in traditional medicines plants are used mainly as aqueous extracts. Our approach has been to search for compounds with direct chemotherapeutic action but plant extracts contain complex mixtures of compounds which may possess other activities including anti-inflammatory, immunostimulant and antipyretic which may contribute to the overall activity of an extract. In order to avoid unnecessary duplication of effort in this area of research, collaborative programmes of research between different countries and between scientists and clinicians need to be established. It is hoped that this area of research will not only lead to new drug entities but will also help in the selection and standardization of plants used in traditional medicine.

INVESTIGATIONS INTO TRADITIONAL MEDICINES

It is estimated that the majority of the World's population cannot afford Western pharmaceutical drugs and have to rely on their own indigenous systems of medicine which are mainly plant based. In the section of antiprotozoal natural products, reference was made to our investigations into a number of species of higher plant which are used in traditional medicine for the treatment of malaria, amoebic dysentery and other diseases caused by protozoal infections. We have also researched some other areas of traditional medicine and two further examples will be discussed.

Dragon's blood

The blood red sap from the bark of several tree species of *Croton* is used in traditional medicine in S. America. One of these species, *C. lechleri* yields a sap known as Dragon's Blood which is used to treat a number of diseases including cancer and rheumatism as well as being used to aid wound healing. So popular is this traditional medicine that the trees from which it is obtained are in danger of extinction owing to the excessive demand in countries such as Peru and Ecuador. We have investigated the chemical constituents and biological activities of Dragon's Blood obtained from *C. lechleri* collected in Ecuador. More than 90% dry wt of the sap consists of mixtures of proanthocyanidins varying from monomers to heptamers although we do have evidence to show that oligomers of up to 20 flavan-3-ol units are present [159]. In addition to (+)-catechin, (−)-epicatechin, (+)-gallocatechin, (−)-epigallocatechin and dimeric procyanidins B-1 and B-4, five novel dimers and trimers were isolated (general structure, Fig. 4). The novel compounds were characterized as catechin-(4$\alpha \rightarrow$ 8)-epigallocatechin, gallocatechin-(4$\alpha \rightarrow$ 8)-epicatechin, gallocatechin-(4$\alpha \rightarrow$ 6)-epigallocatechin, catechin-(4$\alpha \rightarrow$ 8)gallocatechin-(4$\alpha \rightarrow$ 8)-gallocatechin and

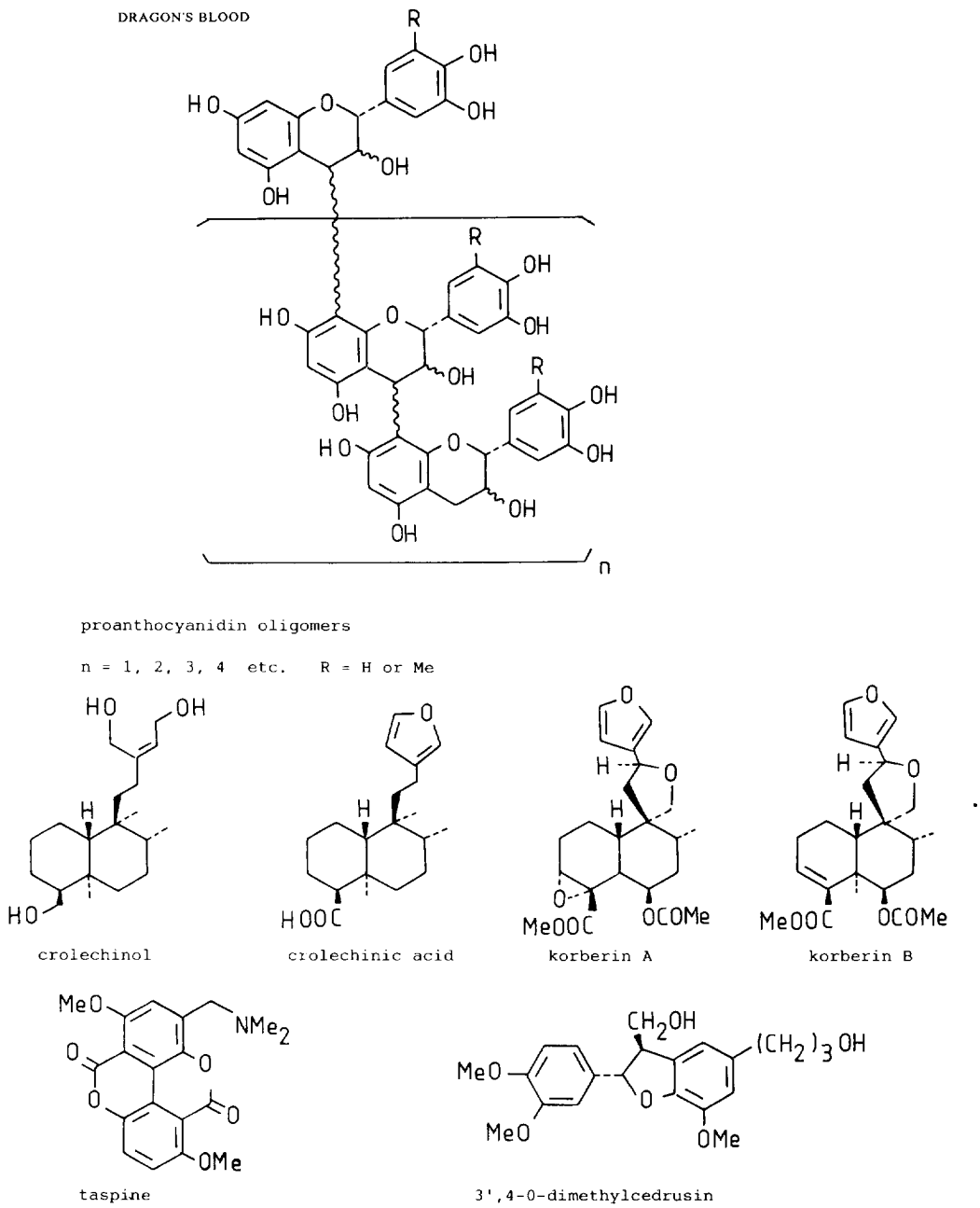
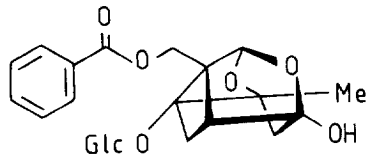


Fig. 4. Examples of constituents isolated from the traditional medicines Dragon's Blood (S. America) and Red Paeony root (China).

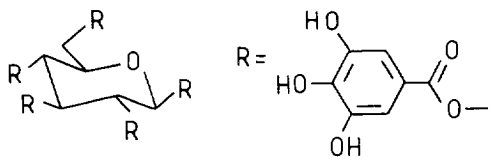
gallocatechin-(4α → 8)-gallocatechin-(4α → 8)-epi-gallocatechin. Structure determination was established mainly by the use of mass, ^{1}H NMR spectroscopy and thiolytic degradation with toluene α-thiol which yields benzylthioethers of extension units and the monomeric underivatized flavan-3-ol terminal unit. Each compound was separated chromatographically and characterized individually.

A new procedure for rapid analysis of these oligomers was developed by combining thiolytic degradation and direct ^{1}H NMR spectroscopy without separation of the components within the mixture. This procedure has proved to be of considerable value when dealing with the more polar fractions of the sap which contain higher oligomers. The H-2 signals of the individual flavan-3-ols and their thioether derivatives are sufficiently separated to allow for identification of each compound. The method requires quantification of the integrals for the H-2 signals which gives the relative amounts of each monomeric unit to be determined. The ratio of extension units to terminal units enabled average M_r estimations. The mean degree of polymerization of two fractions was 4,5-6 and 6–7 with M_r of up to 2130. Higher oligomers have an M_r of *ca* 6000. The homogeneity of the oligomers was clearly indicated

RED PAEONY ROOT



paeoniflorin



1,2,3,4,6-penta

-O-galloyl

gluco-D-pyranose

Fig. 4. *Continued*

by the presence of high proportions of gallocatechin and epigallocatechin as the monomeric units.

The chloroform extract of the sap yielded a mixture of small molecular weight aromatic compounds including 1,3,5-trimethoxybenzene, 2,4,6-trimethoxyphenol, 3,4-dimethoxyphenol, 3,4-dimethoxybenzylalcohol, 4-hydroxyphenethylalcohol and its acetate together with the common steroids sitosterol, sitosterol D-glucopyranoside, β-sitostenine and a series of diterpenoids [160]. Two of the diterpenoids were identified by mass, ^{1}H and ^{13}C NMR spectroscopy as the known clerodanes hardwickiic acid and bincatriol. Extensive use of ^{1}H and ^{13}C NMR including ^{1}H–^{1}H, ^{1}H–^{13}C COSY, ^{13}C DEPT pulse sequences and NOE experiments led to the structrual elucidation of four novel clerodane diterpenes named crolechinol, crolechinic acid [160], korberin A and korberin B [161] (Fig. 4).

The alkaloid taspine (Fig. 4) has been isolated by other workers from *C. lechleri*, *C. palanostigma* and *C. draconoides* obtained from Peru. The sap obtained from Ecuador yielded taspine only as a trace component and comparison of the ^{1}H NMR spectra of chloroform extracts of saps from Ecuador and Peru showed that the Peruvian sap contains taspine as a major component, whereas it was not detected in the sample from Ecuador.

Croton lechleri sap from Ecuador, various extracts and individual compounds have been evaluated for their cytotoxicity against KB cells. The results show that neither the sap nor the crude extracts were cytotoxic. Furthermore, the flavan-3-ols and proanthocyanidins which are the major components of the sap are not cytotoxic [162]. The IC_{50} values of the minor components of the chloroform fraction of the sap had IC_{50} values generally greater than 20 $\mu g\,ml^{-1}$ with the exception of 1,3,5-trimethoxybenzene which was the most cytotoxic compound having an IC_{50} value of 7.13 $\mu g\,ml^{-1}$. Studies by other workers have demonstrated that taspine is highly cytotoxic and it has been identified as the anticancer principle of *C. draconoides* of Peru being present at 1–2% dry wt of sap. Our findings show that the Ecuadorian sap does not contain any appreciable quantity of the anticancer principle taspine and if it is indeed effective in cancer chemotherapy, it may derive its activity from a mechanism other than by direct effects on cancer cells [162].

Antibacterial testing showed that the minor components 1,3,5-trimethoxybenzene and 2,4,6-trimethoxyphenol were some 30 times more active than penicillin or chloramphenicol with minimum inhibition amounts (IA_{min}) of 0.0003 μg. They are also highly inhibitory against *E. coli*. The diterpenoids korberin A and B were active against *B. subtilis* (IA_{min} 0.04, 0.05 μg, respectively).

The effect of different components against proliferation of endothelial cells was determined by assessing incorporation of [^{3}H]thymidine into their DNA as part of an investigation into the wound healing properties of the sap. Gallocatechin, epigallocatechin and procyanidin B-4 slightly stimulated cell proliferation whereas some of the other constituents, including 1,3,5-trimethoxybenzene were inhibitory. The dried sap as a whole was inhibitory to the proliferation of endothelial cells. Other workers have reported that 4,3′-*O*-dimethylcedrusin (Fig. 4) is the wound healing principle of Peruvian sap but that it does not stimulate proliferation of endothelial cells although it is said to protect cells against degradation in starvation medium [163]. The alkaloid taspine has also been reported to be the active principle responsible for wound healing [164]. In the samples of sap from Ecuador which we examined 4,3′-*O*-methylcedrusin was not detected and taspine was only a very minor component. Since it is accepted by us that the sap from Ecuador does promote wound healing the explanation must be different from those reported. We conclude that the wound healing properties of the sap may result from a number of factors including the strong antimicrobial activity of polyphenols, the occlusive layer which the sap forms over a wound, the anti-inflammatory and the radical scavenging activities of the proanthocyanidins. There is considerable variation in the chemical composition between samples of Dragon's Blood from two different countries and for medicinal use quality assurance procedures need to be established.

Traditional chinese medicine (TCM) in the treatment of eczema

In the late 1980s clinicians from Great Ormond Street Hospital for Sick Children in London noted marked improvements in some of their young patients who were suffering from severe atopic eczema. The beneficial effects were not due to their therapy but to that of a practitioner

of TCM in the centre of London. Agreement was reached between the clinicians and the TCM practitioner in setting up hospital based clinical trails of a formulation of a single prescription of 10 herbs. This prescription was reported to be clinically effective when given orally as an aqueous extract to 37 young patients suffering from non-exudative atopic eczema [165]. The herbs used in the prescription include *Ledebouriella seseloides, Potentilla chinensis, Akebia clematidis, Rehmannia glutinosa, Paeonia lactiflora, Lophatherum gracile, Dictamnus dasycarpus, Tribulus terrestris, Glycyrrhiza uralensis* and *Schizonepeta tenuifolia.*

When we originally discussed this prescription it was thought that one of the herbs must be the active ingredient and that its effect might well be due to a single active compound. Since no single biological test is available in laboratory animals for assessing atopic eczema it was decided to test each of the herbs separately for anti-inflammatory effects using the writhing test in mice. Aqueous extracts of four of the 10 herbs proved to be anti-inflammatory as assessed by this test but treatment of a small number of patients suffering from atopic eczema with these four herbs had no clinical effect. Increasing the number of herbs to seven on the basis of an observed mild sedative effect in mice and literature reports of use in the treatment of itch, again resulted in no great significant improvement of the eczema. It was, therefore, apparent that there was no single active herb and that it required the combination of herbs to produce full clinical effectiveness. The chemical constituents, pharmacological and clinical effects of these 10 herbs have been reviewed in the context of eczema treatment [166].

Each of the 10 herbs is the subject of a monograph in the current edition of the Pharmacopoeia of the People's Republic of China [167]. Although standards of quality are laid down in monographs only two of them, Schizonepeta herb (volatile oil) and Red Paeony root ($\geqslant 2\%$ paeoniflorin) include quantitative assays. There are no mandatory requirements for TCM herbs used in the U.K. and we have assessed the quality of some of them. The roots of several *Paeonia* species are common ingredients in TCM prescriptions and it is known that samples may vary in quality owing to species differences, harvesting and processing. We developed quantitative HPLC and ^{1}H NMR assays for paeoniflorin and 1,2,3,4,6-penta-*O*-galloyl-*O*-glucopyranose (PGG) (Fig. 4) [168]. Twelve samples of red paeony roots were purchased from Chinese herb shops in London and two authentic samples were obtained from the Institute of Materia Medica, Huaxi Medical University, China. An authentic sample of *P. veitchii* had the highest levels of paeoniflorin and of PGG (5.10 and 0.96%, respectively). The paeoniflorin content from the 12 commercial samples ranged from 0.01–4.57% and for the PGG from < 0.01 to 0.81%. There is no requirement for PGG content in the Chinese Pharmacopoeia but the 2% requirement for paeoniflorin was met by only five of the 12 samples tested [168]. It is obvious that there is considerable variation in the quality between different commercially available batches of one of the ingredients present in the TCM eczema prescription and it is our belief that quality assurance standards should be set and adhered to for all herbal material which is intended for medicinal use. Quantitative ^{1}H NMR was carried out on crude extracts of the red paeony root and it proved to be a rapid method giving comparable values to the lengthier HPLC assay.

RADIO-LIGAND RECEPTOR BINDING ASSAYS FOR EVALUATION OF BIOLOGICAL ACTIVITIES OF PLANT EXTRACTS

Pharmacological evaluation of plant extracts and of isolated compounds has been limited for many years owing to the problems perceived by pharmacologists in dealing with 'sticky green extracts' and also to the general small weights of isolated compounds available for testing. There has been a revolution in the sensitive techniques which are available for the assessment of biological activities and particularly in the proliferation of *in vitro* assays for specific enzymes and receptors. The resurgence of interest of the pharmaceutical industry and of biotechnological companies in plant products may owe something to the public perception that 'natural' equates with 'goodness' but it is firmly based on the ability to use fast throughput automatic screens for a range of specific biological targets.

In order to identify potential CNS active principles from plants we selected 10 Chinese medicinal herbs which were chosen from literature reports. Extracts were prepared with 70% ethanol and screened for activity using a series of radio-ligand receptor binding assays which included adrenoceptors (α_1, α_2, β), 5-HT (1, 1A, 1C, 2), opiate, benzodiazepine, ion channels (Ca^{2+}, K^+), dopamine (1, 2), adenosine (1), muscarinic, histamine, Na^+/K^+ ATPase, GABA (A&B). All of the extracts (50 μl) were able to inhibit binding of ligands to at least one receptor at concentrations of 1 mg ml^{-1} [169]. Applications of bioactivity-guided fractionation of extracts resulted in the isolation of a series of biologically active compounds with selective binding to specific receptors.

In a parallel investigation, receptor binding assays were used to ascertain whether or not ethnomedical data conferred any advantages in selection of plants for novel leads to analgesic agents [170]. More than 20 endogenous neuropeptides including bradykinin, choleocystokinin, opiates, substance P and calcitonin gene-related peptide are implicated in the mediation of pain. Human cloned bradykin (BKII) receptor binding site expressed in Chinese Hamster ovary cells (CHO) was selected for the investigation of 300 species of plant which were chosen at random and a further 300 species which were selected from the scientific and medicinal literature of China, S. America, S. Africa and W. Indies on the basis that the plants are used traditionally for the relief of pain. Methanol extracts were prepared and the relative affinity of each extract for BKII binding sites was determined by assessing the abilities of extracts to inhibit binding of [^{3}H]BK to BKII sites in CHO cells. Of the 300 plants chosen at random only two proved to be positive in the BKII assay contrasting with 20 from the selected group.

These results provide some scientific support for a number of plant species which are used in traditional medicine for the relief of pain. This data indicated that ethnomedical selection significantly improved the possibility of obtaining a novel ligand. Although receptor ligand binding assays are sensitive and specific, they are not necessarily predictive of activity *in vivo* and any positive findings would require further verification by the use of functional assays in whole animals or with isolated organs. Nevertheless these sensitive receptor binding assays form the basis of screening methodologies which are in current use.

CONCLUSIONS

Plants continue to be sources of clinically used drugs in Western Medicine and this is exemplified by the fact that they are represented in 14 of the 15 therapeutic categories of pharmaceuticals which are recommended by medical practitioners in U.K. [171]. Throughout the developing world there are more than 20 000 species of higher plant which are used in traditional medicines and they, together with the other 230 000 species of higher plant, form a vast reservoir of potential new drugs. During my working lifetime the sensitivity of methods to investigate plant constituents has improved enormously. New, sensitive and sophisticated chromatographic techniques (e.g. TLC, GLC, HPLC) are used for separation and isolation procedures whilst X-ray crystallography and spectroscopic techniques (e.g. UV, IR, CD, ^{1}H and ^{13}C NMR) are used for structure elucidation. Not that long ago pharmacologists would require gram quantities of a pure compound to undertake whole animal or isolated organ experiments in order to establish pharmacological activity. The development of sensitive *in vitro* assays based on enzymes or receptors (isolated or cloned) using radioactive ligand binding, now means that minute amounts of compounds can be rapidly screened for a whole series of biological activities. Industry has established automated high-throughput *in vitro* screens for biological activities which are capable of examining thousands of compounds or extracts in very short periods of time. Hence the race is now on to locate new drug entities from natural sources.

As a result of these new technologies it has become current fashion to use bioactive-guided fractionation of plant extracts in attempts to isolate biologically active compounds. In fact some scientists and grant awarding authorities believe that it is the only way to proceed and it forms the basis of our perceived current wisdom. Industry is undoubtedly concerned with the development of new drug entities and is particularly interested in the detection of compounds with specific activity against a single enzyme or receptor but with no activity against closely related targets. The high throughput screens provide masses of data and only those active compounds which show specificity will be developed further. Thus the possibility exists that information on other active natural products will remain buried in company files. Academic institutions may wish to collaborate with industry and/or pursue their own interests. If they are undertaking their own bioactivity-guided fractionation then their number of screens will mainly be far less than those used for industry. It is, therefore, likely that academics stand more chance of missing active compounds in comparison with industry. For small laboratories in academic institutes bioassay-guided fractionation coupled with phytochemistry slows down the rate of chemical progress. In view of the rapid loss of forests and of species it is critical that the rate of research into plant constituents be increased. In the light of diminishing resources, it would seem evident that more emphasis be placed on phytochemistry itself. Modern sensitive techniques allow for rapid isolation and structure determination and novel natural products will inevitably be obtained. Novel chemical structures are themselves of interest to industry and they may be screened for a wide range of biological activities by negotiation. Wherever possible, more use should be made by phytochemists of herbarium collections which represent wide collections of plants.

In pursuing the theme of sensitivity, sight should not be lost of the needs of developing countries. The majority of their populations rely on plant traditional medicines and most of these plants require to be verified for efficacy by *in vitro*, *in vivo* and clinical assessments, for their active principles, for their quality and for their lack of toxicity. Today's technology could be used and should be used to answer these needs.

Those methods which seem to be so sensitive to us as judged by today's criteria will inevitably become outmoded and one can only wonder about the advances which will be made in the coming decades and put to use in order to further our understanding of Phytochemistry.

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