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The constituents of Schefferomitra and Boletus species from New Guinea

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THE CONSTITUENTS OF SCHEFFEROMITRA AND BOLETUS SPECIES FROM NEW GUINEA

A THESIS

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This Thesis is dedicated to my wife

Anna

for her continual cooperation and
encouragement through all the years
of study.

S U M M A R Y

The constituents of the bark and leaves of Schefferomitra subaequalis belonging to the family Annonaceae and those of an allegedly hallucinogenic Boletus species, section Ixocomus, group Nudi, both originating from New Guinea, were investigated.

Eight alkaloids were isolated from the bark of Sch. subaequalis. Two of these: liriodenine and anonaine, were already known to be present in Sch. subaequalis. In this investigation three other aporphine alkaloids, asimilobine, isoboldine and anolobine were isolated and identified. In addition two new tetrahydroprotoberberine type alkaloids, aequaline and schefferine and a third new alkaloid, described as alkaloid "Y" have been isolated. The structures of aequaline and schefferine were elucidated as L-3, 9-dihydroxy-2, 10-dimethoxy tetrahydroprotoberberine and L-9-hydroxy-2,3,10-trimethoxy tetrahydroprotoberberine respectively, and their absolute configurations established as S. The structure of the alkaloid "Y" is not yet known.

From the leaves of Sch. subaequalis six of the above eight alkaloids viz. anonaine, asimilobine, isoboldine, anolobine, aequaline and schefferine were isolated. Only the two coloured alkaloids: liriodenine and alkaloid "Y" were absent.

From the Boletus species there were isolated mannitol and ergosterol and the known amino acids: leucine, isoleucine, valine, methionine, tyrosine, alanine, threonine, glycine, arginine, lysine and histidine.

One of the major constituents of the free amino acid pool in this Boletus was found to be a new unsaturated α-amino acid, the structure of which has been elucidated as 2(S)-amino-4-methylhex-5-enoic acid.

In addition two apparently new steroids have been isolated and characterised but due to shortage of available material no structural assignments have been made for these compounds.

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EXPERIMENTAL

I N T R O D U C T I O N

A. THE AUSTRALIAN PHYTOCHEMICAL SURVEY

A systematic survey of the chemical and pharmacological potentialities of Australian native plants began in 1944.[1] The pharmacological survey at that time centred mainly on alkaloids, because they were believed to offer the most promising approach to pharmacologically useful materials.

The project, however, was soon extended to other natural products such as steroids, triterpenes, antibiotics, cardiac-glycosides, saponins, coumarins,plant pigments, etc. This expanded project, under the direction of Dr. C. Barnard of the Council for Scientific and Industrial Research (later the Commonwealth Scientific and Industrial Research Organisation: - C.S.I.R.O.), became known as the Australian Phytochemical Survey, and was conducted in cooperation with the interested University Departments. Most of the early plant screening was carried out by C. Barnard and L.J. Webb of the Division of Plant Industry, C.S.I.R.O. while the chemical studies were under the supervision of J.R. Price of the then Division of Industrial Chemistry, C.S.I.R.O.

The initial investigations were carried out almost entirely with the flora of Queensland which, because of its varied nature - from that of the tropical North to that of the dry West - seemed to offer the richest field for investigation. The results of chemical screening tests for

alkaloids of numerous plant species, principally from tropical rain-forests, were reported by Webb.[1,2] These reports were followed by a bulletin setting out the results of a preliminary survey for saponins, mainly among plants of rain-forest areas in Eastern Australia.[3]

Little attention was paid to the phytochemical resources of the Territory of Papua - New Guinea until the mid 1950's, although many of the alkaloid-bearing families, such as Rutaceae, Apocyanaceae, Lauraceae, Loganiaceae, Monimiaceae, Menispermaceae are well represented in New Guinea.[2] In 1951 a brief reconnaissance of native plants for alkaloids and various other classes of chemical compounds was made by Barnard and Webb in various parts of Territory of Papua - New Guinea. In 1955 Webb published both the collected information, and his original findings in "A Preliminary Phytochemical Survey of Papua - New Guinea."[4] Since then the work there has been continued by the C.S.I.R.O. and by botanists of the Department of Forests.

The achievements of the Australian Phytochemical Survey up to 1960 have been briefly summarized by Price in "Australian Natural Product Research"[5] showing that entirely new classes of compounds have been discovered and many known ones identified. Several new alkaloids have been shown to have therapeutic promise in the treatment of human diseases, including leukaemia and hypertension.

Over 4,000 species of higher plants in Australia and New Guinea were screened for the presence of alkaloids and for other substances with physiological activity in man and animals. The approximate numbers of new and already known selected compounds, identified before 1969 in Australian and New Guinean plants, are given as 421 and 622-672 respectively by Webb in "Australian Plants and Chemical Research".[6]

The present study involved a detailed examination of the alkaloidal constituents of Schefferomitra subaequalis, a New Guinea plant species belonging to the family Annonaceae and the investigation of the constituents of a Boletus species which is alleged to cause hallucinations in New Guinean natives.

B. THE ANNONACEAE FAMILY

The presence of alkaloids in plants of the Annonaceae family was reported as early as 1855.[7] Subsequent chemical investigations of a number of plant sources have shown that alkaloid-containing members of this family produce alkaloids belonging to the most widespread group of alkaloids in the plant kingdom classified as derivatives of benzylisoquinoline, e.g. aporphines, protoberberines and, to a lesser extent, oxoaporphines (table I).

Many of the Annonaceae species of alkaloids are physiologically active but, unlike the isoquinoline type papaverine from Papaveraceae, are not used in medicine.

Various plants of the Annonaceae family, and extracts prepared from them are used by the natives of Southern and Eastern Africa for a variety of purposes ranging from the treatment of coughs and fevers to homicidal poisoning. Thus, the leaf of *Annona chrysophylla* is used in Tanganyika as a snake-bite remedy, while the root serves as a homicidal poison. The whole plant of *Annona muricata* is used in Angola for the treatment of coughs, spasms and fevers, the bark and root are employed as remedies for dysentery and intestinal worms.[8]

The fruit of the small tree *Asimina triloba* (papaw) is cultivated, and eaten in the Eastern and Southern United

GENUS AND SPECIES	ALKALOIDS ISOLATED	REF.
Alphonsea ventricosa	alphonsine	9
Annona chrysophylla	anonaine	10
" muricata	anonaine, anoniine	9
	muricine, muricinine	11
" reticulata	anonaine	10
" squamosa	anonaine	10,12
	anolobine, liriodenine	12
	michelalbine, L-(+)-reticuline	
Artabotrys suaveolens	artabotrinine, artabotrine	13,14
	suaveoline	
Asimina triloba	anolobine, asimilobine	15,16
	coreximine, isocorydine	
	liriodenine, michelalbine	
Enantia chloranta	palmatine	17
" polycarpa	palmatine	18
	hydroquinidine, quinidine	19
Guatteria psilopus	atherospermidine, guatterine	20
Melodorum punctulatum	asimilobine, liriodenine	21
	michelalbine	
Mitrella kentii	anonaine, asimilobine	22
	liriodenine	
	alkaloid A (identical with aequaline)	

TABLE I

GENUS AND SPECIES	ALKALOIDS ISOLATED	REF.
Phaeanthus ebracteolatus	phaeantharine	23
	phaeanthine	24
" macropodus	limacine, phaeantine	25
Polyalthia nitidissima	liriodenine	26
Popowia cyanocarpa	asimilobine, 1-hydroxy-2,10,11-trimethoxynoraporphine, O-methyldauricine, (+)-wilsonirine	27
Pseudovaria dolichonema	2-hydroxy-1,9,10-trimethoxynoraporphine, glaucine, norglaucine	26
" grandifolia	anonaine, 1,2-dimethoxynoraporphine, liriodenine	26
" T.G.H. 10,530	anonaine, liriodenine	26
Xylopia discreta	discretamine, discretine discretinine, xylopine xylopinine	28
" macrocarpa	berberine	29
" papuana	anonaine, coclaurine laurolitsine, reticuline roemerine, xylopine	30
" polycarpa	berberine	7

TABLE I (Continued)

States and Southeastern Canada. Nevertheless, a small fraction of the population is sensitive to this plant, and reacts with contact dermatitis. Certain individuals may even exhibit severe gastrointestinal symptoms after ingestion of the fruit though they may be insensitive to contact of the plant with their skin.[31]

The green fruit of Annona reticulata, extensively cultivated in the Philippines, is used to check diarrhoea and dysentery, but the juice of its trunk is an irritant. The leaves and fruit are listed in the first and fourth editions of the Mexican Pharmacopoeia.[10]

The stem bark and fruit of the tropical tree Annona squamosa are used in Taiwan in folk medicine as amoebicide, tonic and astringent.[32] In addition, the dried fruit and leaves of this plant have been reported[33,34] to possess activity against Ehrlich ascites cancer cells in mice.

To our knowledge, there are no records indicating the use of plants belonging to the Annonaceae family in folk medicine practised by Australian aborigines or New Guinean natives.

C. HALLUCINOGENIC MUSHROOMS

In the mythology of extinct tribes, as well as in early historical chronicles, including the Bible, there are reports on divine visions guiding the progress of their respective personages towards their goal. It is well known that certain mushrooms cause hallucinations, i.e., distortion in perception accompanied by vivid dreams and imaginations.

Recently, on basis of philology, even the development of the Eastern (Jewish and Christian) religions was attributed to the effect of mushroom lore.[35] Still, the most fascinating story of this kind is the fully authenticated effect of the "Sacred Mushrooms" of Mexico.[36,37] The Aztecs named these "teo-nanácatl" and used the name as a collective noun for all mushroom species employed by them and other tribes in Mexico for the purpose of producing ecstasy during their ritual gatherings.

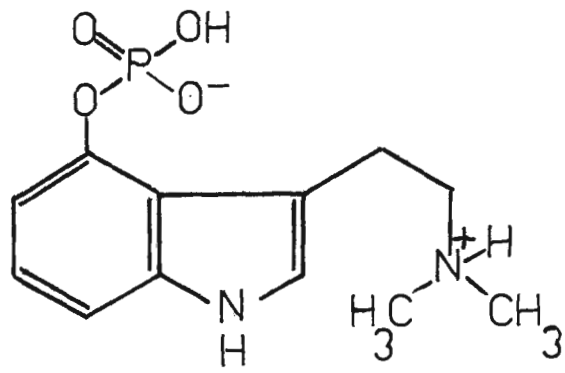
The chronicles of the 16th century mention that, long before the conquest of Mexico by the Spaniards (1521 A.D.), the Aztecs and neighbouring tribes used "teo-nanácatl" in their religious ceremonies and royal feasts.[38,39]

Dúran[39] (p. 225) records that "teo-nanácatl" was distributed at the coronation ceremony of Montezuma II in 1502 A.D. to make the ceremony seem more spectacular, and describes the events as follows: "When the sacrifice was

finished and the steps and courtyard were bathed with human blood, everyone went to eat raw mushrooms and were in a worse state than if they had drunk a great quantity of wine. They became so inebriated and witless that many of them took their lives in their hands. With the strength of these mushrooms they saw visions and had revelations about the future, since the devil spoke to them in their madness".

The fact that the "sacred" mushroom cult in Central America must have extended much further back in history than the advent of the conquistadors is evidenced by the discovery, in Guatemala, of the so called "mushroom stones". These are mushroom shaped stone carvings with the figure of a god worked into the stalk. The oldest of these stone carvings dates back more than 3,000 years.[37]

Surprisingly, however, the nature and the active constituent of the "sacred" mushrooms remained unknown, and their use was not mentioned in Western literature until recently, when it was discovered that such mushrooms are still used by Indians in the remote mountainous areas of Southern Mexico for the purpose of divination and in religious rites superimposed on Christian ceremonies.[40] A detailed description of the ritual consumption of the "sacred" mushrooms by Mexican Indians, and of the psychotomimetic effects these mushrooms produce on the participants is given by Wasson.[37] Seven different species of mushrooms were



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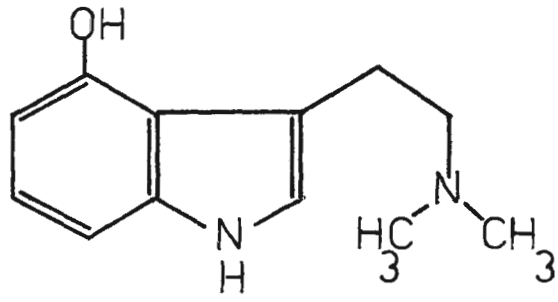

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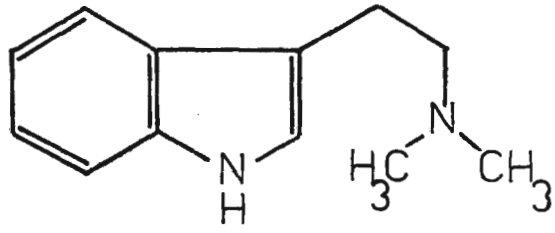

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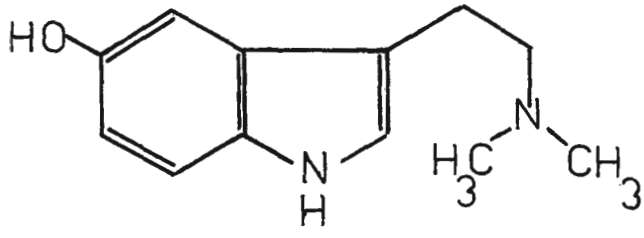

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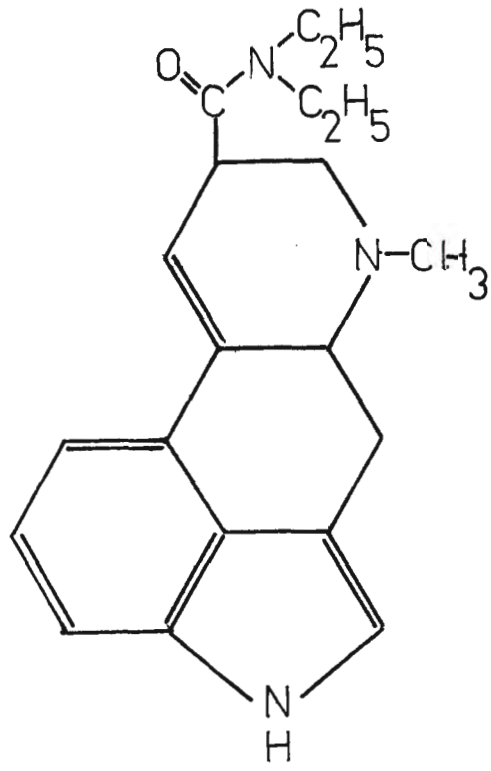
found by Wasson and Heim to have pronounced hallucinogenic effects. These mushrooms were later identified and botanically classified by Heim[37] as Psilocybe mexicana Heim, Ps. zapotecorum Heim, Ps. caerulescens Murill var. nigripes Heim, Ps. caerulescens Murill var. mazatecorum, Conocybe siligineoides Heim, and Stropharia cubensis Earle. Lifesize water colour paintings of these seven mushroom species were provided by Heim for illustrations of Wasson's articles.[36,41]

The history, anthropology, taxonomy and cultivation of the Mexican hallucinogenic mushrooms are described in the extensive monograph by Heim and Wasson[37], and in the publications by Singer.[42,43]

The substances responsible for the psychotomimetic activity of the Mexican hallucinogenic mushrooms were first isolated from the laboratory cultures of Ps. mexicana Heim by Hofmann and his co-workers.[44] The compounds have been named psilocybin (1) and psilocin (2) respectively.

Psilocybin was shown to be the phosphoric acid ester of 4-hydroxy-N,N-dimethyltryptamine and this structural assignment was confirmed by total synthesis via a route starting from 4-benzyloxyindole.[45] Psilocin, which in small amounts accompanies psilocybin in the mushrooms, was identified as dephosphorylated psilocybin, i.e., 4-hydroxy-N,N-dimethyltryptamine.[46] Its identity was confirmed by phosphorylation to psilocybin.[47]



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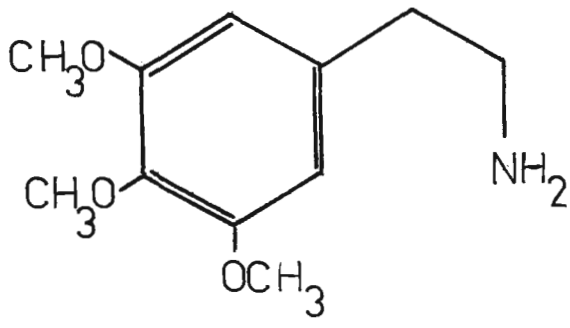

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These two psychotropic substances, psilocybin and psilocin, both being tryptamine derivatives, are structurally closely related to N,N-dimethyltryptamine (3) and bufotenine (4), both of which have been found to occur in hallucinogenic plants. Thus, N,N-dimethyltryptamine occurs in the pods of the leguminous shrubs Piptadenia peregrina Benth, and P. macrocarpa Benth,[48] in the roots of Mimosa hostilis[49] and in the leaves of Lespedeza bicolor var. japonica.[50] N,N-dimethyltryptamine produces in normal persons psychotic effects similar to those observed with lysergic acid diethylamide (LSD) (5) or mescaline (6),[51] and it has been shown to be the active principle present in the leaves of Prestonia amazonicum, used by some South American Indians for the preparation of a hallucinogenic drink.[52]

Bufotenine, a 5-hydroxytryptamine derivative, first isolated from certain toad species[53], was found later also in the seeds of Piptadenia colubrine[49], P. peregrina and P. macrocarpa.[48] A snuff prepared from the ground seeds of P. peregrina and P. macrocarpa is used by some Indian tribes of South America and the Caribbean in rituals to produce hallucinations. Taken in small quantities, the snuff is reported[48] to produce a kind of intoxication during which visions are reputed to occur, but it produces a violent temporary derangement when taken in excessive doses.

It is of special interest that another group of

substituted tryptamine derivatives, i.e., the 4-alkyl substituted one viz. lysergic acid group, shows practically the same type of hallucinogenic activity as the 4-hydroxy-tryptamine derivatives, psilocybin and psilocin. While the physiological activity of simple 4-alkyltryptamines are of no major significance, its more complicated derivative, lysergic acid diethylamide, is the most active hallucinogenic agent known. Both psilocybin and lysergic acid derivatives are of fungal origin and their hallucinogenic activity is approximately one hundred times, and thousand times, respectively, greater than the long ago recognized activity of the cactus alkaloid mescaline. Although the effective dose rates for the adult human being differ considerably (mescaline 400 mg, psilocybin 4-8mg, LSD 0.1-0.5mg), the type of hallucinations produced seem practically indistinguishable.[54]

The pharmacological action of psilocin was shown to be identical with that of psilocybin.[47] Both these psychotropic substances produce psychotomimetic symptoms (oral dose 4-8mg/man) similar to those of mescaline and LSD and lasting several hours.[55] These effects are identical with those elicited by the fresh or dried mushrooms and have been vividly described, from their own experience, by Hofmann[56] and Graves[57]. The outstanding symptoms following psilocybin ingestion are (i) distortion of sense percep-

tions which involves all senses, (ii) coloured visions, pseudo-hallucinations and hallucinations, and (iii) thought-flow dreams. Many other symptoms, such as depression alternating with euphoria, anxiety, and fear have been reported, but these vary with the individual.[58]

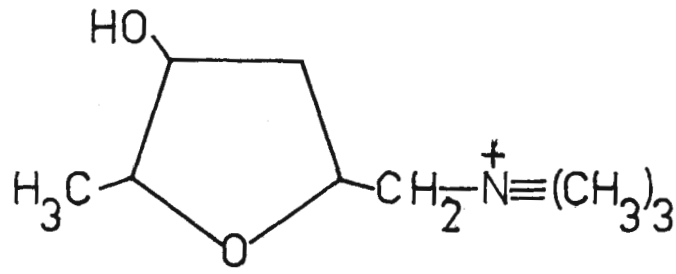
Various modifications in the molecular structure of psilocybin and psilocin have been made to obtain insight into the relationship between structure and psychotropic action of these substances.[59] It is not yet known with certainty whether any of these compounds show psychotomimetic activity, but pharmacological data for many of them have been reported.[60] It is, however, noteworthy that to our knowledge none of these derivatives have been introduced into current medical practice.

The occurrence of hallucinogenic mushrooms of the Psilocybe and Stropharia species is not entirely limited to Mexico and Central America. Stropharia cubensis Earle has been found in Pnom-Penh (Cambodia) and Bangkok (Thailand) and the species have been shown to contain psilocybin and psilocin[61], but there does not appear to be any record of these mushrooms being used by the inhabitants of these countries for the purpose of intoxication.[56] Three psilocybin containing Psilocybe species are stated to have been discovered in Western Oregon and also in Seattle.[62] Some unidentified species of Stropharia found in Kenya

(Africa) have been reported to cause temporary mental disorder, the symptoms being delusion and euphoria, and laughter alternating with depression.[8]

Hallucinogenic mushrooms or infusions prepared from them have for a long time been used in Northern Europe and Eastern Siberia as intoxicants. It has been suggested that the fly agaric, Amanita muscaria (L. ex Fr.) Hooker which has effects similar to those of Cannabis sativa[8] might possibly have played in these countries a role similar to that played by the Psilocybe species in Pre-Columbian cultures of Central America.[36] The use of A. muscaria as an intoxicant by many primitive tribes of Northern and Northeastern Siberia was first reported by Filip J. von Strahlenberg in 1730 and later confirmed by other sources.[36,63] Some Scandinavian circles believe even now that the berserk raging of the vikings was due to consumption of A. muscaria. Wasson, however, points out that this is only an erroneous myth as the symptoms of fly agaric inebriation are just the opposite of berserk raging.[63]

The effect of A. muscaria does not extend to coloured visions, but when consumed fresh and in small quantities it can produce agreeable sensations limited to contemplative well being.[64] It can also call forth hilarious manifestations, similar to the convulsive laughter experienced by Huxley[65] under the influence of mescaline.



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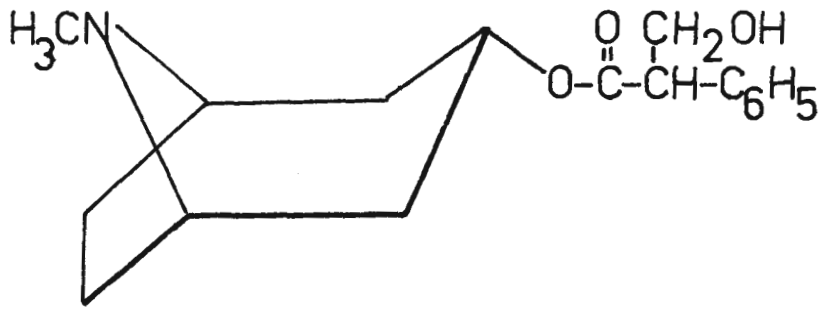

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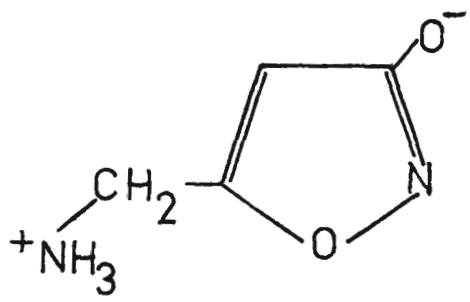

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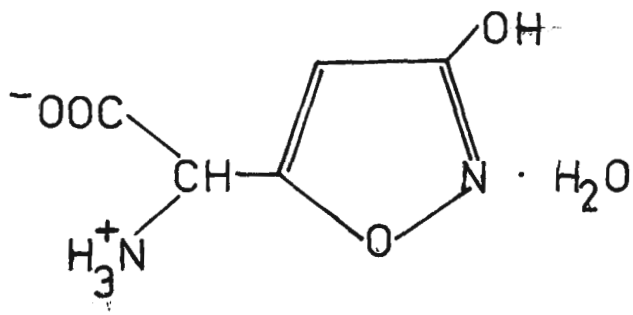

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In spite of extensive chemical investigations of A. muscaria, the active principle which imparts to the mushroom its psychopharmacological properties has not yet been ascertained. The chemistry and pharmacological aspects of muscarine (7), one of the physiologically active constituents of A. muscaria, have been reviewed by Wilkinson.[66]

Muscarine could be responsible for some of the psychotic symptoms associated with the ingestion of the mushroom, as its central effects result in acute excitement,[8] but does not explain the reported hallucinogenic properties of the mushroom.

Bufotenine and (-)-hyoscyamine (8) have also been reported[67] to occur in A. muscaria, but the small amount in which these psychotropic substances are present in the mushroom is considered insufficient for its psychotomimetic effect, even if allowance is made for the added activity of muscarine.[68]

Muscimol (9) another physiologically active substance isolated from A. muscaria,[69] was later shown to be an artifact formed from the labile compound premuscimol, which has the same pharmacological activity as muscimol.[70]

Ibotenic acid (10), recognised as identical with premuscimol, was isolated from A. muscaria, A. pantherina and A. strobiliformis.[71] It is markedly insecticidal and

was proved to be the fly killing principle of the Amanita species[72], but there are no records of it having any psychotropic activity.

In Australia, the literature on psychotic effects following mushroom consumption is sparse. The mushroom Panaeolus ovatus (now renamed Anellaria sepulchralis) has been suspected in several poisonings of humans, the symptoms being similar to those of acute alcohol intoxication-drowsiness, hilarity and in some cases vomiting.[73,74] The occurrence of the hallucinogenic mushroom Psilocybe cubensis Singer in certain parts of Australia was first described by Aberdeen.[75] This species is found in the same areas of Queensland and in the coastal ranges of New South Wales where cases of mushroom poisoning, allegedly associated with P. ovatus, have occurred. Aberdeen and Jones observed that Ps. cubensis contaminated ordinary collections of P. ovatus and suggested that a) Ps. cubensis is more likely to be the toxic agent and b) that the symptoms ascribed to P. ovatus are really due to the contamination by Ps. cubensis.[75] As a matter of fact, the species Ps. cubensis was found responsible for a case of mushroom poisoning in Lismore area (Australia) in 1961, the symptoms of which closely resembled those described by Wasson and Heim for the Mexican mushrooms.[76] This suggests that the previous evidence[73,74] implicating P. ovatus might have

been mistaken, and that Ps. cubensis, and not P. ovatus, was responsible for the poisonings.

To our knowledge there are no references to mushrooms in accounts of Australian aboriginal folklore, except the use of puff-balls found in subtropical N.S.W. in the treatment of wounds.[77]

The use of hallucinogenic mushrooms as intoxicants by the natives in certain areas in New Guinea is mentioned in three anthropological publications.[78,79,80] According to a passage by Vicedom and Tischner[78] which refers to the natives of Mt. Hagen tribes, "..when people eat of certain mushrooms then they become mad and run around without heart and understanding. In this case the mushroom has caused the madness." A brief reference by Wasson[41] to the use of hallucinogenic mushrooms by Mt. Hagen natives can probably be attributed to Gitlow's "Economics of the Mt. Hagen Tribes."[79] The mushroom referred to is known as "nondo" and its ingestion has been reported to lead to frenzy and death. Other extremely rare, as yet unidentified, mushrooms which grow in the Wahgi Valley area are known to cause temporary insanity which manifests itself in frenzied rage and hysteria of the affected person.[81] A species of Boletus mushrooms, which is found only in the Mt. Hagen area, is reputed to possess hallucinogenic properties. It is reported that this mushroom, eaten either raw or cooked in

quantity, affects people in different ways, some more than others. One physiological effect of the mushrooms upon the person who consumes it is described as multiple vision or, in some cases, inverted vision. The hallucinations begin an hour or so after eating and last for one or more hours depending on the amount eaten. The sufferer however does not, apparently, regain full mental control for from one to five days.[82]

As these effects on the human brain differ markedly from those produced by the Mexican "sacred" mushrooms, we were interested in the investigation of the constituents of this Boletus from the Mt. Hagen area of New Guinea.

D I S C U S S I O N

A. THE CONSTITUENTS OF SCHEFFEROMITRA SUBAEQUALIS

Schefferomitra subaequalis (Scheff.), Diels (family Annonaceae) is a climbing liana found on rain-forest trees in New Guinea. The species was originally collected in New Guinea by T. G. Hartley, and designated by the herbarium voucher number TGH 11636. Field tests indicated the presence of alkaloids in the bark while those with the leaves gave doubtful results.[83] The bark alkaloids have been examined by S. R. Johns and co-workers[26] who reported the presence of seven or eight constituents in the crude alkaloid mixture. They isolated two of these and identified them as the known aporphine alkaloids liriodenine (11) and anonaine (12) respectively. The other constituents were not investigated.

The plant material used in this investigation was recollected by J. Womersley, and is the same species as TGH 11636.[83]

The procedure used for the isolation of the crude alkaloids was essentially the same for both bark and leaves, and consisted of extraction of the dried, milled plant material by continuous percolation with methanol at room temperature. The methanolic extracts were concentrated to smaller volumes in a climbing film evaporator, and then diluted by the addition of dilute sulphuric acid. The

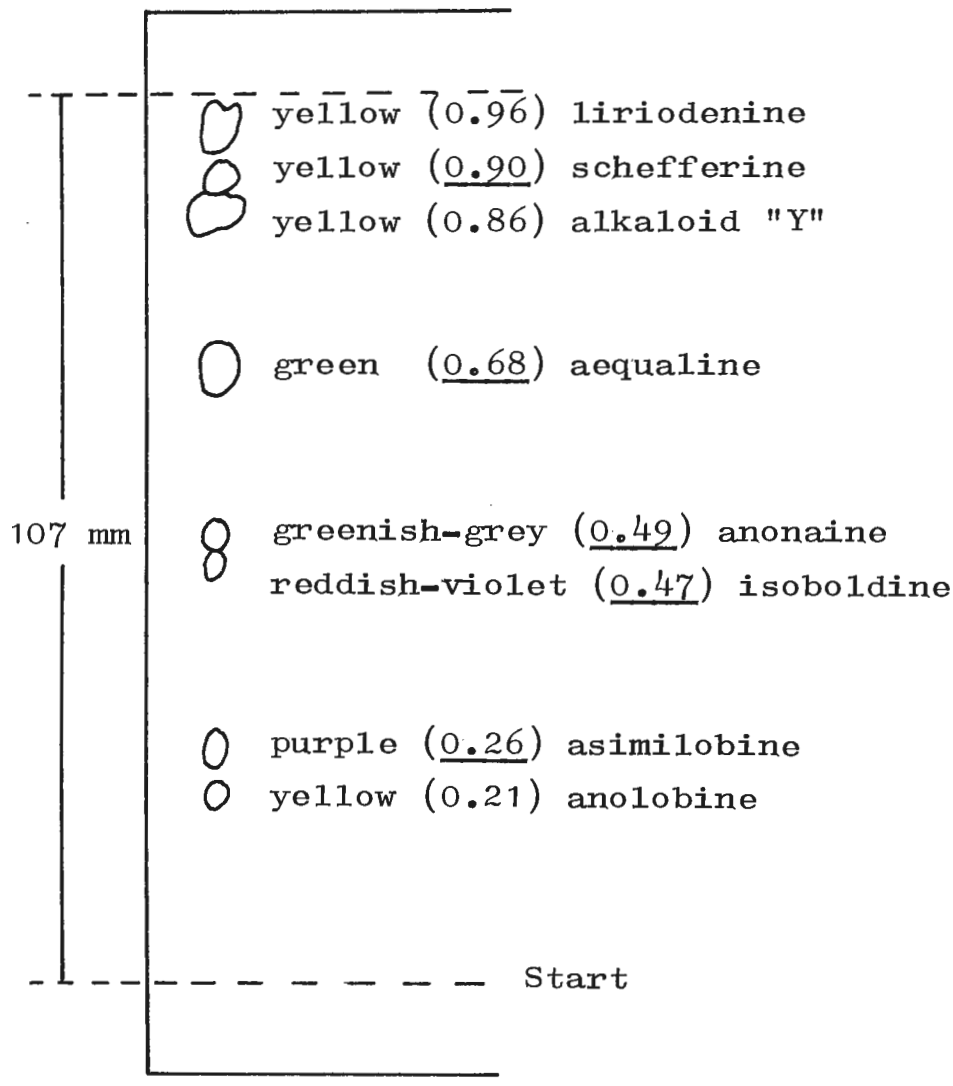
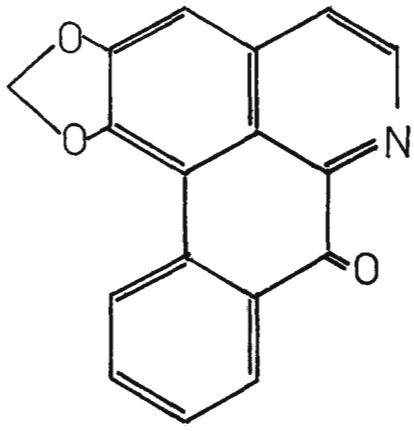


FIGURE 1. TLC of Crude Alkaloid Mixture.

Schematic representation but travelling distances are shown in actual lengths.

The R_f values of the most prominent spots are underlined.



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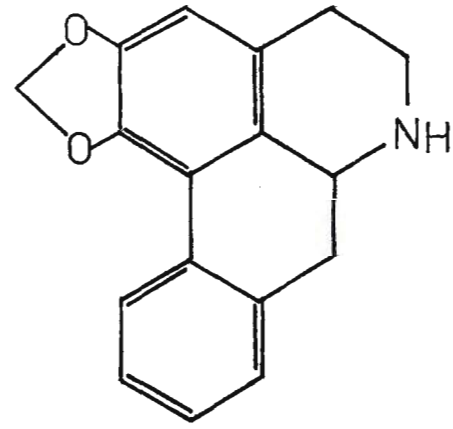

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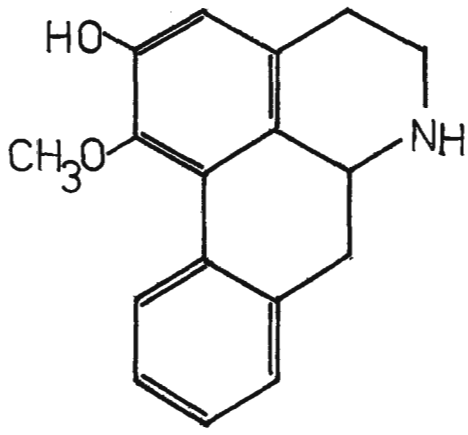

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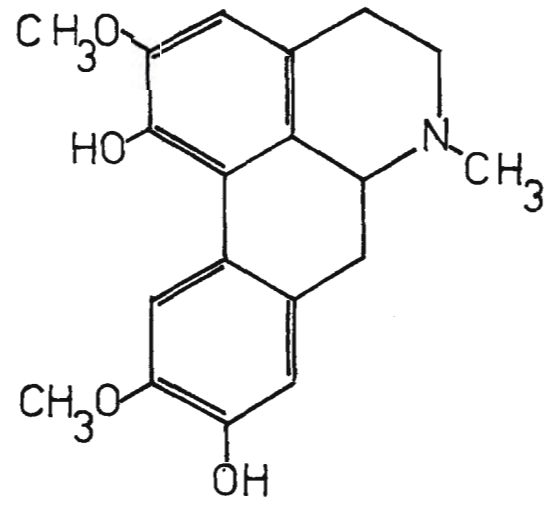

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aqueous acidic solution was clarified by filtration, basified with ammonia, and the crude bases were extracted into chloroform. Evaporation under reduced pressure of the washed and dried chloroform extract gave the crude alkaloids in the form of a very dark gum which still contained appreciable amounts of other plant constituents. Some purification of the crude alkaloid mixture could be achieved by extracting the crude alkaloids from chloroform solution into dilute acid and then re-extracting them into chloroform.

Alkaloids of the Bark

The crude alkaloids were obtained in 0.032% yield. Analytical thin-layer chromatography on silica with chloroform-methanol (9:1) gave apparently good resolution of the crude alkaloid mixture into eight components. Fig. 1 shows the thin-layer chromatogram developed by spraying with a solution of iodine in chloroform.

From observations of the t.l.c. behaviour of the alkaloid mixture on silica and alumina plates with chloroform - methanol mixtures, a separation procedure was developed involving column chromatography on neutral alumina grade II/III. Alumina of activity grade II/III was preferred to grade I as the latter adsorbed the lower

R_f value alkaloids too strongly to be eluted with reasonable quantities of solvent. The fractions obtained from chromatography on alumina were then re-chromatographed on columns of silica followed, where necessary, by further separation or purification on preparative thick-layer plates. Finally, all the alkaloidal constituents were obtained as pure crystalline compounds according to m.p. and t.l.c. behaviour in several solvent systems.

Identification of the Known Alkaloids

Liriodenine and Anonaine

The alkaloids liriodenine (R_f 0.96) and anonaine (R_f 0.49) previously isolated from Schefferomitra subaequalis, were identified by direct comparison with authentic samples.

Asimilobine (13)

Asimilobine crystallised from acetone or benzene as colourless, transparent plates m.p. 175-7° (dec.), R_f 0.26. The alkaloid was shown to have the molecular formula $C_{17}H_{17}O_2N$ by elementary analysis and by the presence of a molecular ion peak at m/e 267 in its mass spectrum. The ultra violet absorption spectrum was consistent with the alkaloid being a 1,2-disubstituted aporphine[84] while a bathochromic shift in the presence of alkali indicated that it contains a phenolic hydroxyl group.

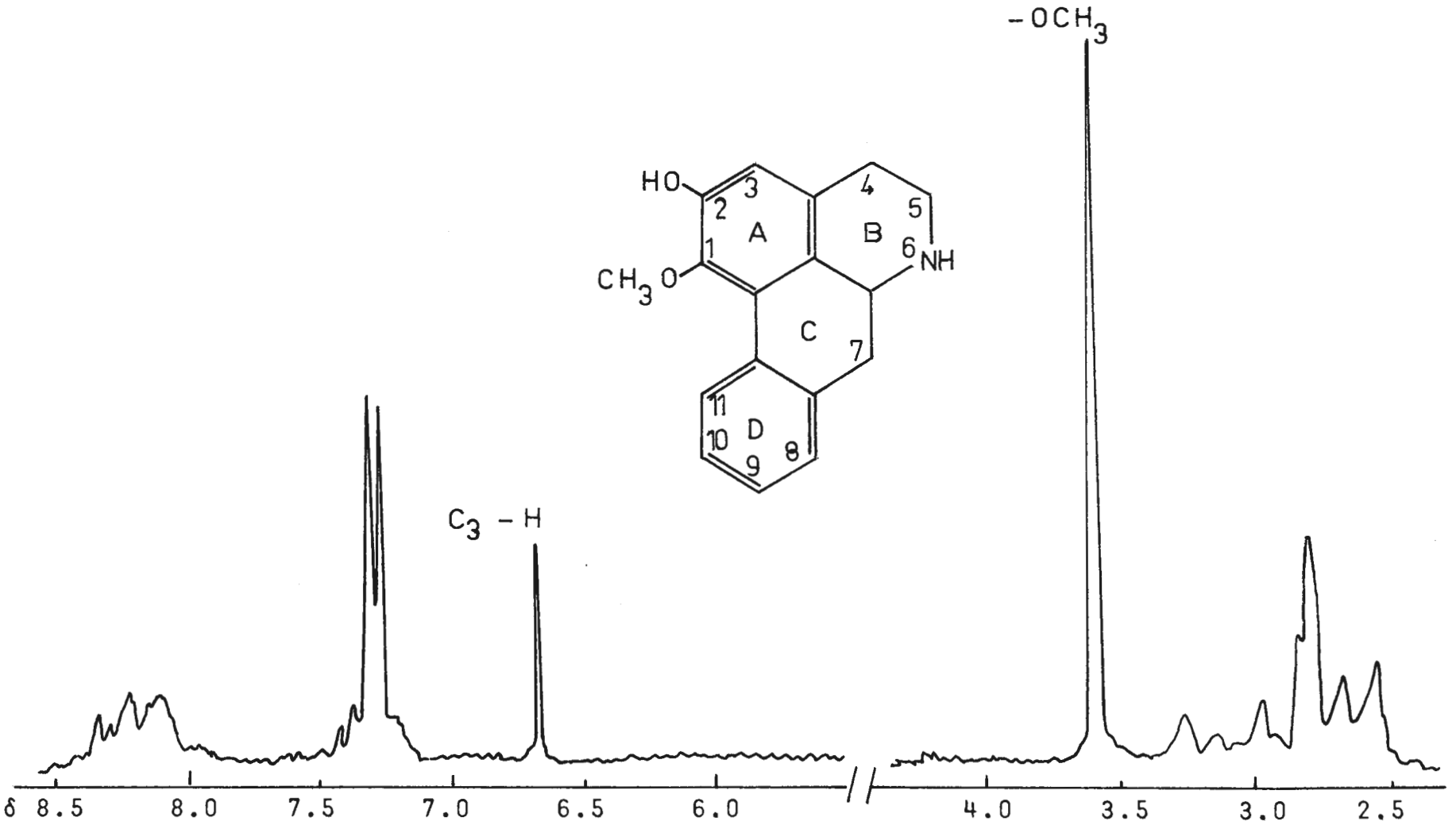


FIGURE 2. N.M.R. Spectrum of Asimilobine

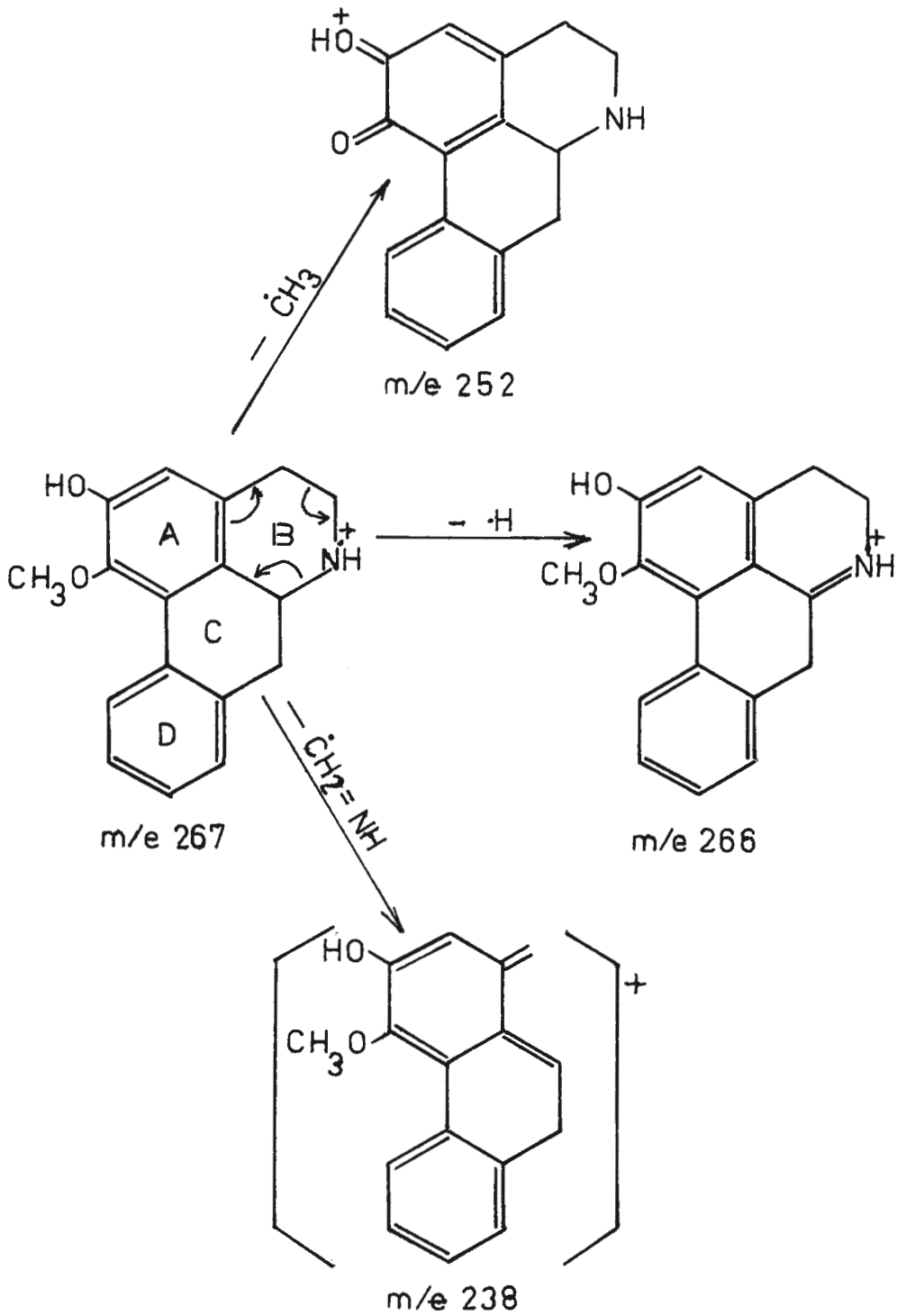


FIGURE 3. Main Fragment Ions of Asimilobine

The n.m.r. spectrum (Fig. 2) showed a three-proton singlet at δ 3.62 which can be interpreted as due to a methoxyl group. The chemical shift of the methoxyl protons indicated that the methoxyl group is at C1, where it is shielded by the aromatic ring D. A one-proton singlet at δ 6.74 could be assigned to C3-H, and a pattern for the other aromatic ring protons was typical of aporphines (e.g. nuciferine) which have no substituents on ring D.[85]

The mass spectrum of the alkaloid showed peaks at m/e 267 (parent and base peak), 266 (M-1), 252 ($M-CH_3$), 250, 238 ($M-H_2C=NH$), 236, 223, 208 and 178. The peak at m/e 238 (M-29) arises through the opening of ring B as shown in Figure 3, and is typical of noraporphines.[86]

On the spectroscopic evidence the structure of the alkaloid was assigned as 2-hydroxy-1-methoxy-noraporphine, identical with asimilobine, an alkaloid originally obtained from the wood and the root of the tree Asimina triloba Dunal (Annonaceae).[16] The physical constants of the alkaloid were in agreement with those recorded for asimilobine[16], and direct comparison with an authentic specimen of asimilobine confirmed their identity.

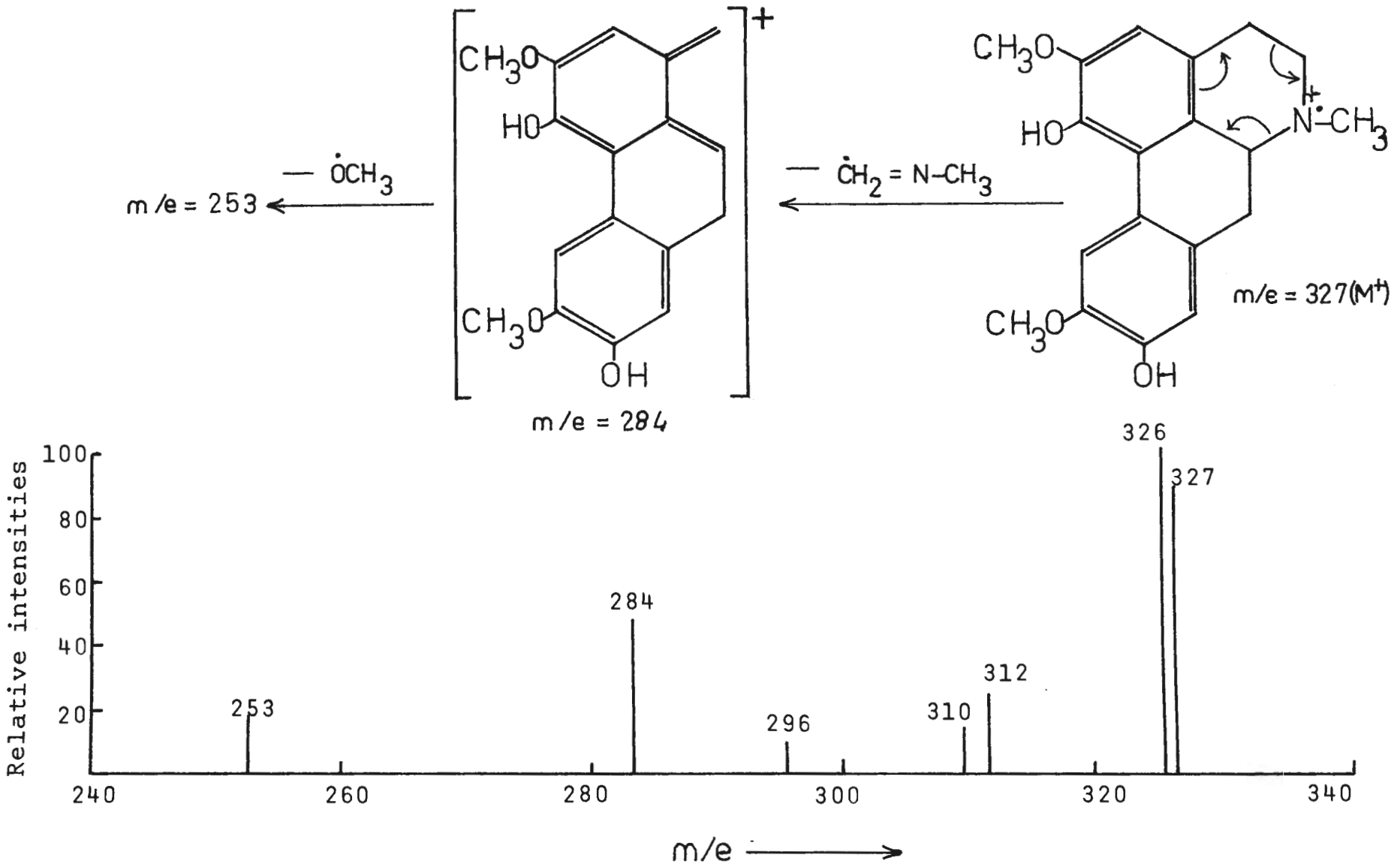
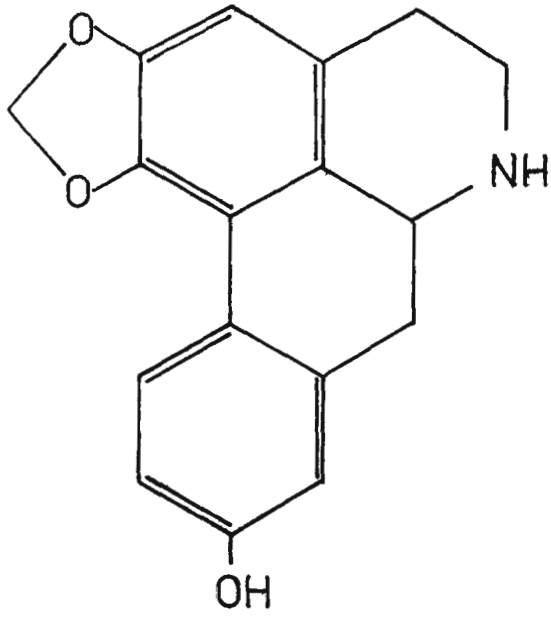


FIGURE 4. Mass Spectrum of Isoboldine (Major Peaks)



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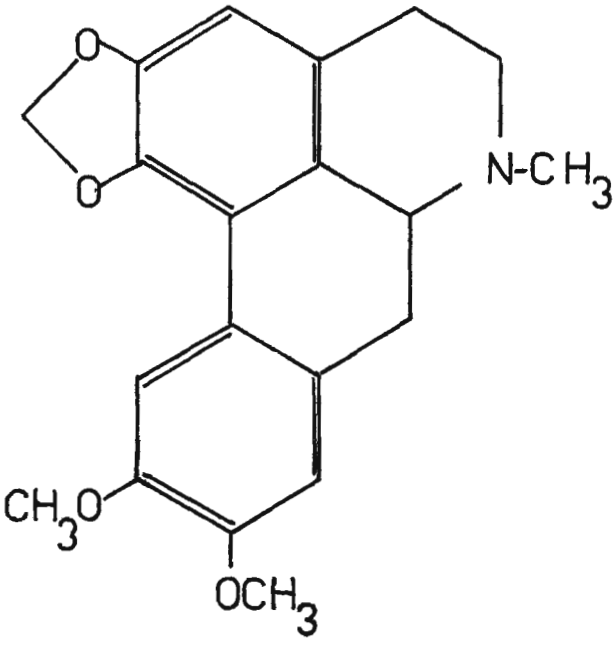

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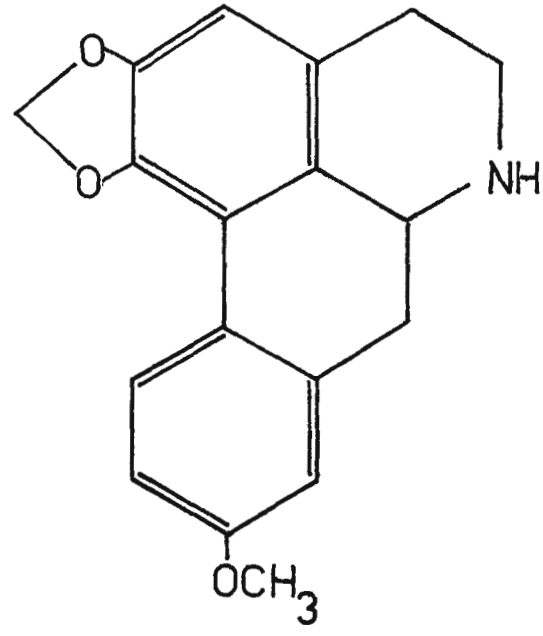

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Isoboldine (14)

Isoboldine was obtained as colourless plates which turned pink on standing, m.p. 122-4°, R_f 0.47. Micro-analyses and the presence of a molecular ion peak at m/e 327 in the mass spectrum established the molecular formula $C_{19}H_{21}O_4N$. The alkaloid was characterised spectroscopically as a phenolic tetra-substituted aporphine.[84] Its mass spectrum (Fig. 4) was identical with that reported[87] for isoboldine (1,9-dihydroxy-2,10-dimethoxy aporphine).

The identification of the alkaloid as isoboldine was confirmed by comparison with an authentic specimen. Ultra violet and infrared spectra and the chromatographic behaviour of the two compounds were identical and there was no depression of m.p. in a mixed m.p. determination.

Anolobine (15)

The alkaloid, $C_{17}H_{15}O_3N$, crystallised from methanol as colourless needles, m.p. 310°, R_f 0.21. It was characterised from its u.v. spectrum as an aporphine with position 11 free of substituents (boldine type)[88], and was shown to be phenolic by a bathochromic shift of the ultra violet absorption maxima on the addition of alkali. The infrared spectrum showed a band at 3330 cm^{-1} indicative of hydrogen bonded hydroxyl absorption[89], and bands at

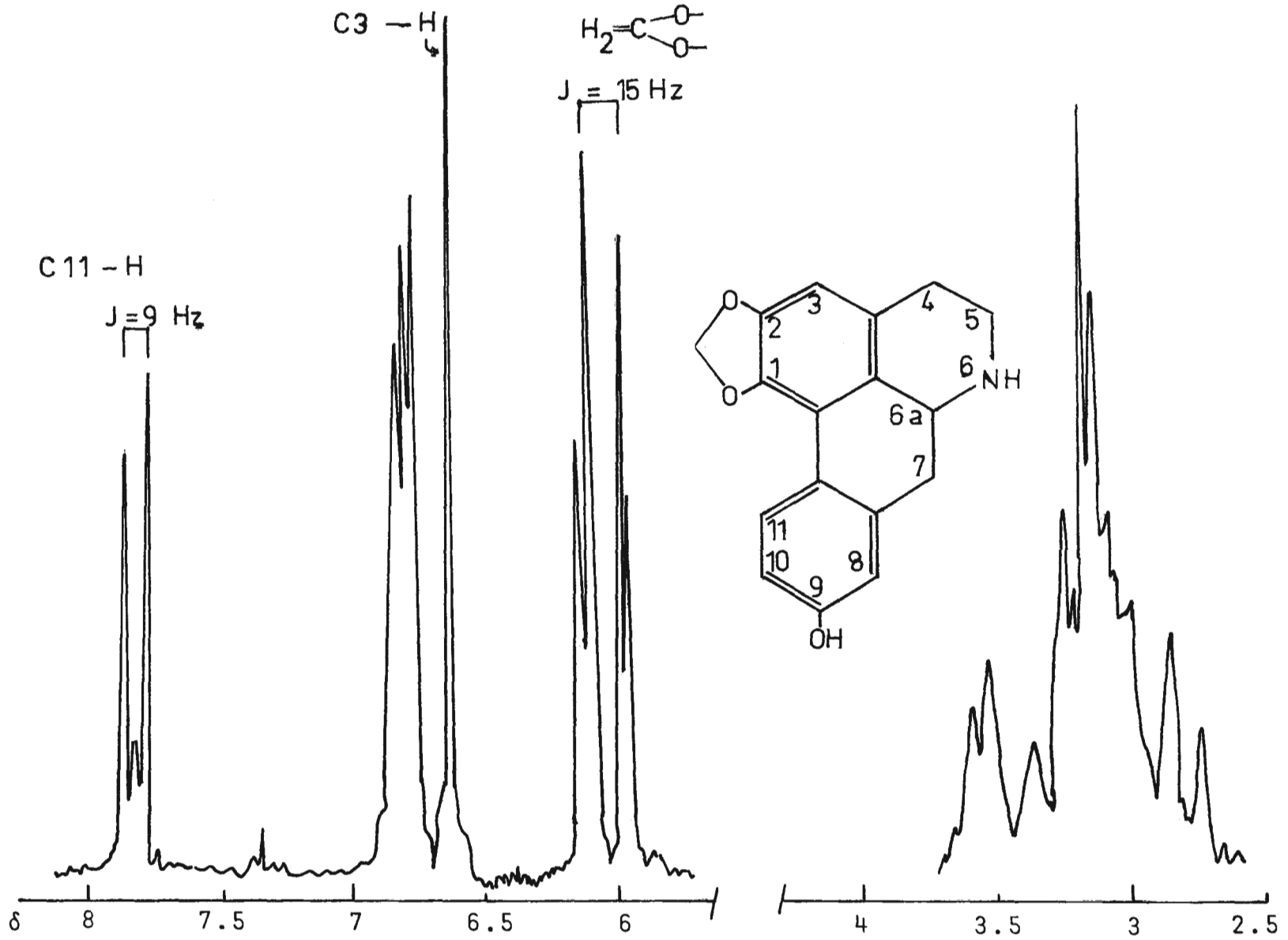
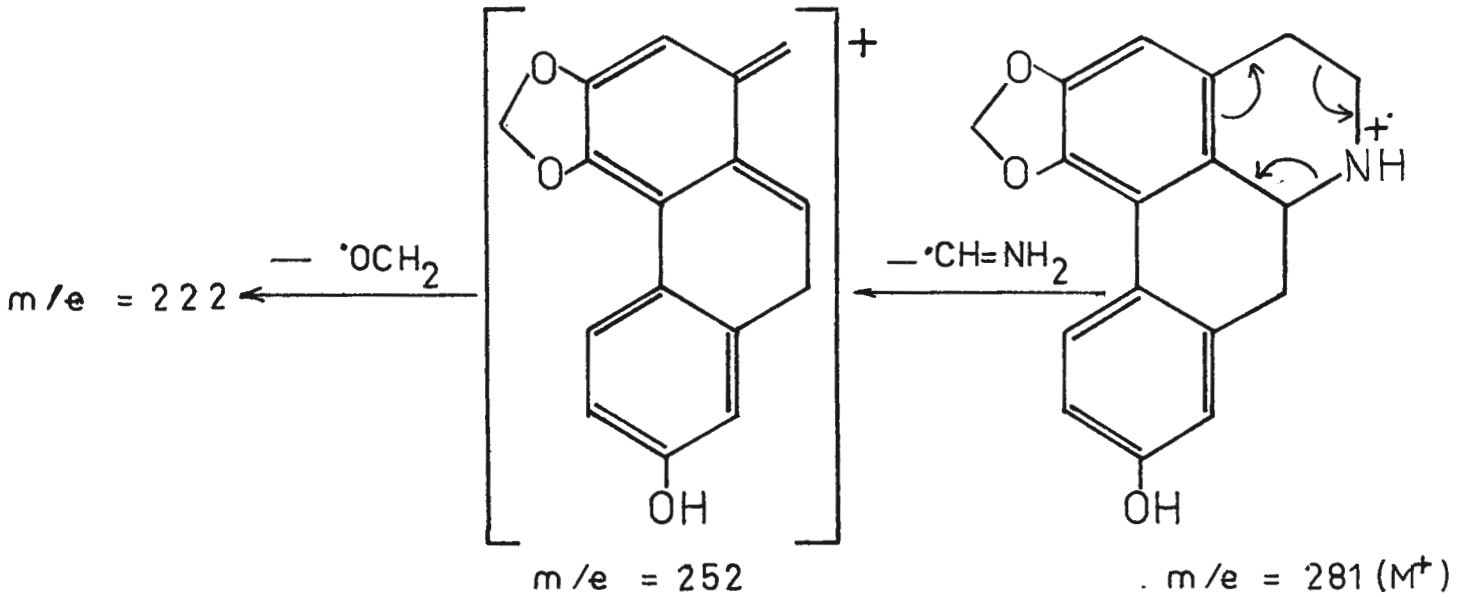


FIGURE 5. N.M.R. Spectrum of Anolobine



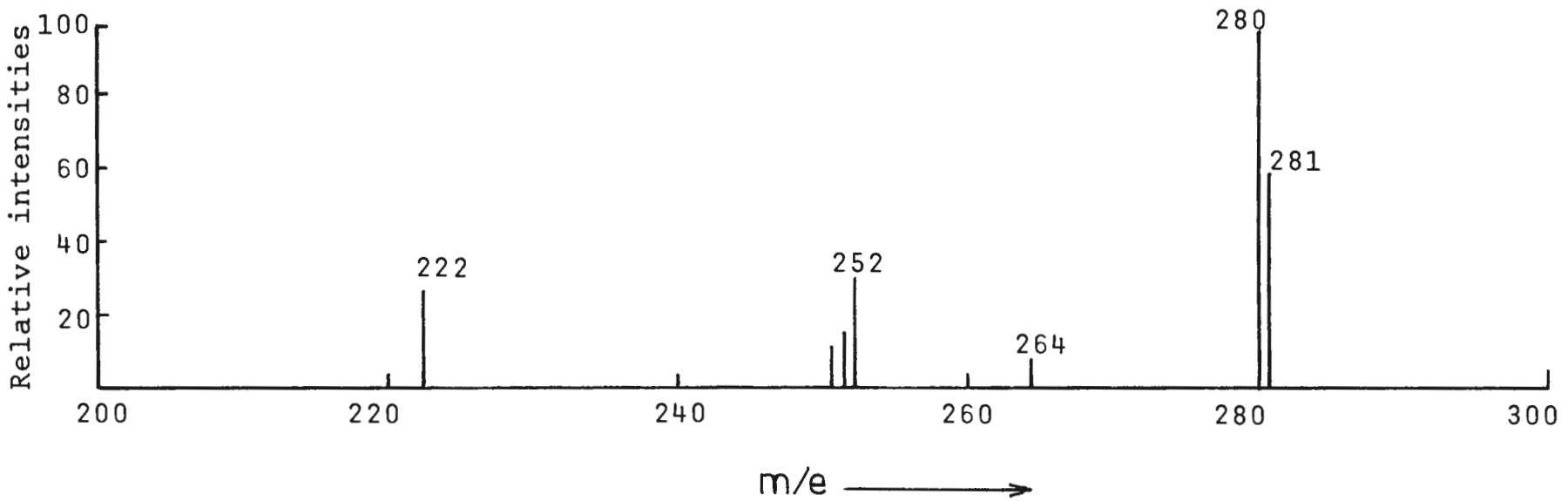


FIGURE 6. Mass Spectrum of Anolobine (Major Peaks)

1500, 1418, 1345, 1110, 940 cm^{-1} which are compatible with the presence of a methylenedioxy group.[90,91]

The two one-proton doublets at δ 6.0 and δ 6.15 (J = 15 Hz) in the n.m.r. spectrum of the alkaloid (Figure 5) are similar to those found in the n.m.r. spectra of dicentrine (16) and related alkaloids,[85] and can be assigned to methylenedioxy group attached to the non-planar aporphine ring in the 1,2-position.[92] The highest field aromatic proton signal (δ 6.66) was assigned to the proton in position 3, as a single one-proton peak at δ 6.54 - 6.75 has been observed in the spectra of all those aporphine alkaloids in which no spin-spin coupling of the protons in position 3 could occur.[92] The low-field aromatic proton signal at δ 7.86 (1H, d, J = 9 Hz) was assigned to the sterically hindered C11 proton because in the n.m.r. spectra of aporphines with substituents at both positions 1 and 2, the one-proton peak of C11 is observed to occur at characteristically low field (δ 8.74 - δ 7.68).[93] The remaining two aromatic ring proton signals appear as a triplet centred at δ 6.81. The absence of an N-methyl signal characterises the alkaloid as a noraporphine.

The mass spectrum of the alkaloid (Figure 6) is characterised by an abundant molecular ion peak at m/e 281, as well as significant peaks at m/e 280 (base peak), 264,

252 and 222. The base peak at m/e 280 (M-1) is characteristic of aporphines which have no substituents in position 11.[87] The peak at m/e 252 (M-29) is found in all aporphines which have the NH grouping.[86] The peak at m/e 264 (M-17) arises from fragmentation by loss of OH. This fragmentation is much less pronounced than the principal cleavage.[86]

On the basis of the spectroscopic evidence the alkaloid was identified as a noraporphine with one methylenedioxy and one hydroxyl group as the only substituents. This is in accordance with its molecular formula.

As all the known aporphine alkaloids are invariably substituted at positions 1 and 2[88], and since the n.m.r. spectrum of the alkaloid indicated that position 3 is unsubstituted the methylenedioxy group was assigned to positions 1 and 2. The phenolic hydroxyl group can be located at either positions 8 or 9 or 10 as both the u.v. and the mass spectra of the alkaloid are characteristic of aporphines with a free 11 position. Accordingly, the alkaloid can be only one of the following compounds:

1,2-methylenedioxy-10-hydroxynoraporphine

or

1,2-methylenedioxy-9-hydroxynoraporphine (anolobine)

or

1, 2-methylenedioxy-8-hydroxynoraporphine.

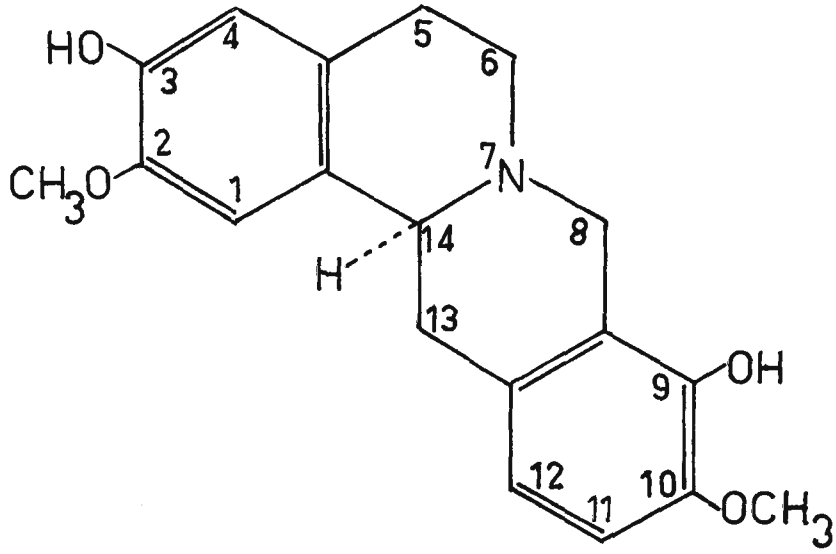
O-methylation of the alkaloid with excess diazomethane gave the known alkaloid xylopine (17), identified by direct comparison with authentic sample. The identification was confirmed by N-methylating both the O-methyl derivative of the alkaloid, and an authentic sample of xylopine. Direct comparison of the N-methylation products showed that they are identical. This chemical correlation settled the position of the hydroxyl group in the alkaloid at C9. Consequently the alkaloid has the structure 1,2-methylene-dioxy-9-hydroxynoraporphine, which has previously been assigned to anolobine, an alkaloid originally isolated from the bark of the North American papaw Asimina triloba Dunal.[15] The discrepancy in the melting points observed here and reported in the literature can be explained by different crystalline structures.

Elucidation of the Structure of the New Alkaloids Isolated

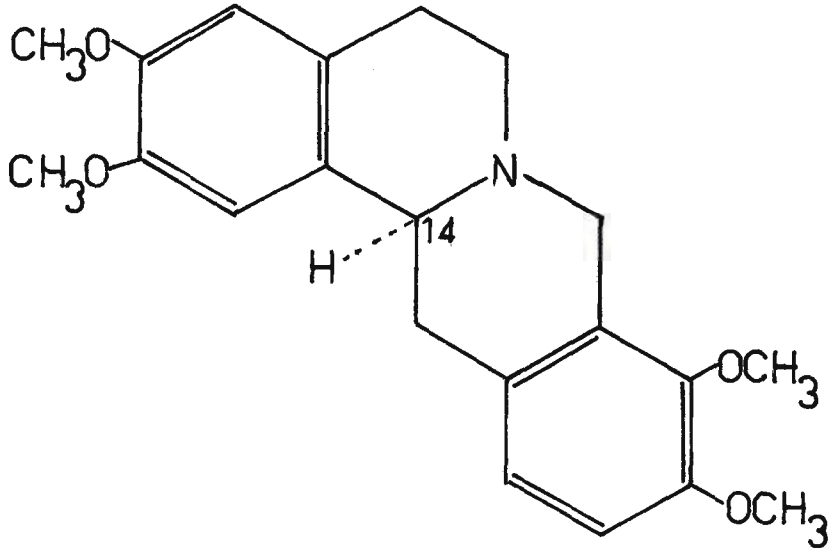
Aequaline (18). 3,9-Dihydroxy-2,10-dimethoxy tetra-hydroprotoberberine

This new alkaloid was obtained as colourless needles from methanol, m.p. 232°(dec.), R_f 0.68, $(\alpha)_D$ -283. Its molecular formula $C_{19}H_{21}O_4N$ was established by microanalyses and by the presence of a molecular ion signal at m/e 327 in its mass spectrum.

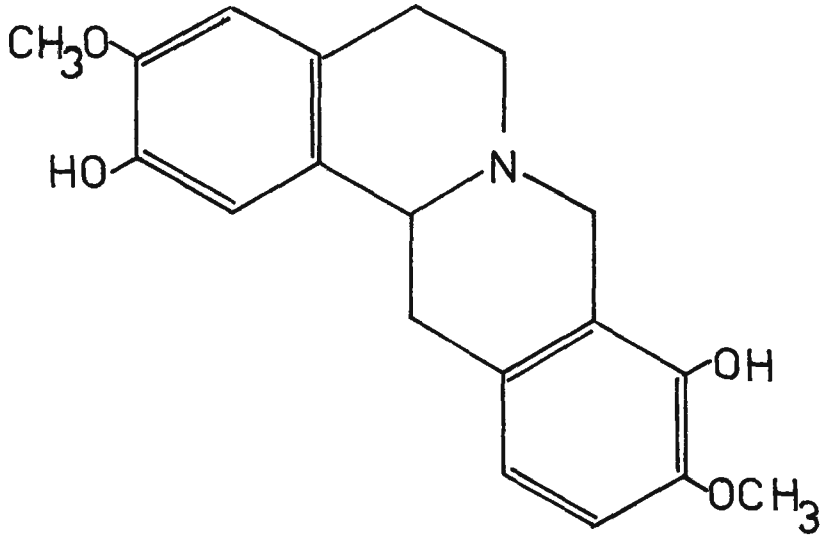
The ultra violet absorption spectrum showed maxima at


HO
3
4
5
6
2
7
N
CH_3O
1
14
8
H
13
9
OH
12
11
10
OCH_3

18


CH_3O
CH_3O
N
14
H
OCH_3
OCH_3

19


CH_3O
HO
N
OH
OCH_3

20

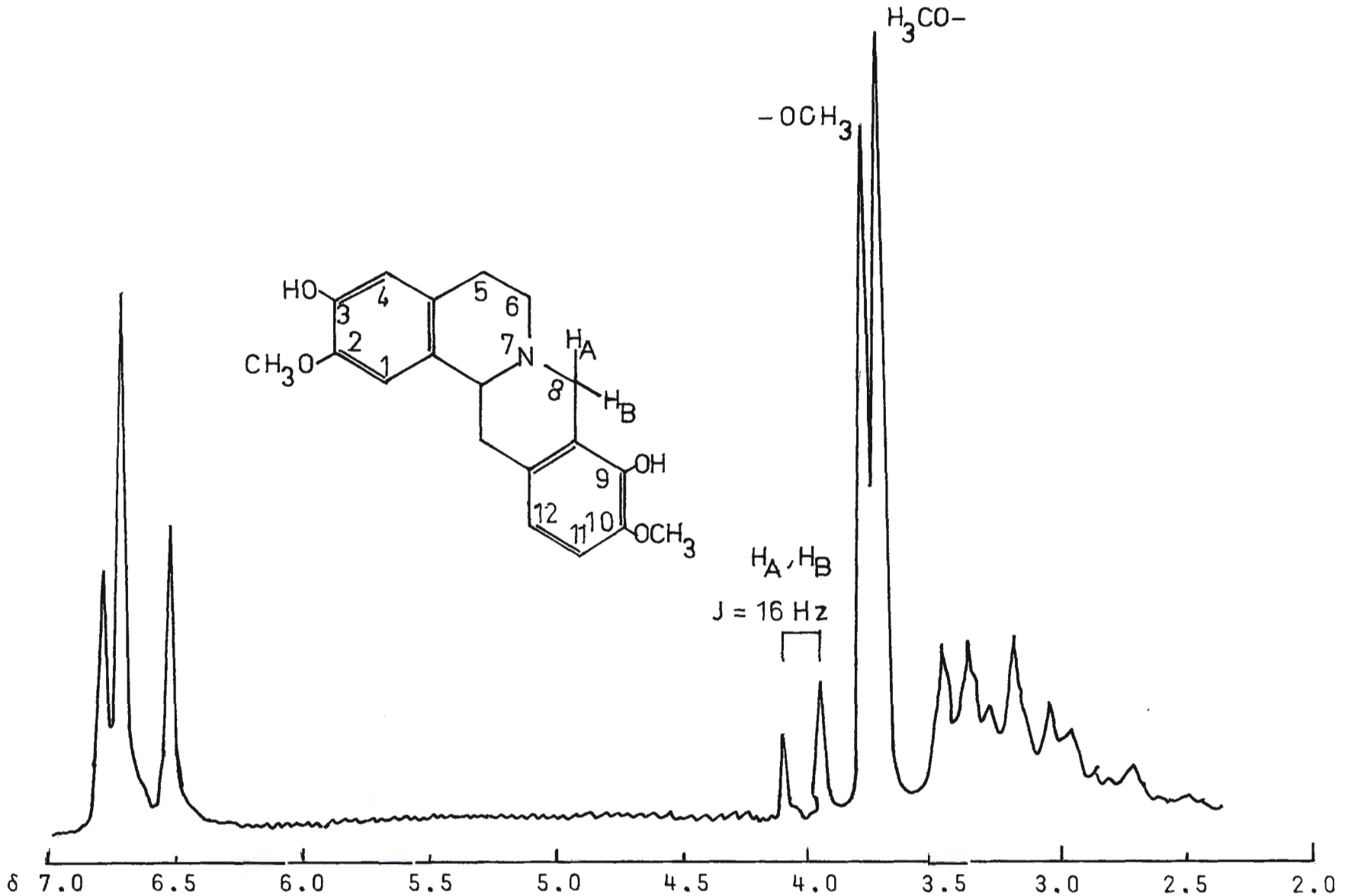


FIGURE 7. N.M.R. Spectrum of Aequaline (after deuterium exchange)

212 and 284 nm (log ε 4.5 and 3.79) and a shoulder at 225 nm (log ε 4.1), typical of a tetrahydroprotoberberine system.[94] Addition of alkali caused a bathochromic shift indicating the presence of at least one phenolic hydroxyl group. While the u.v. spectra can provide information regarding the oxygenation pattern of members belonging to the aporphine group, this is not the case with protoberberine type alkaloids.

The n.m.r. spectrum of the alkaloid (Figure 7) shows two three-proton signals at δ 3.74 and δ 3.77, a broad two-proton peak at δ 8.80 which disappears on exchange with deuterium, and signals for four aromatic protons which appear as two one-proton singlets at δ 6.48 and δ 6.80 and a two-proton singlet at δ 6.68. There is also half of an AB pair of doublets at δ 4.02 (J = 16 Hz), the high field half being obscured by the methoxyl signals. These signals can be assigned to the C8 protons.[95] The other aliphatic protons appear as a complex pattern centred around δ 3.20.

The oxygenation pattern of ring D can be deduced from an examination of the protons at C8. These protons are separated from the rest of the alicyclic protons by the nitrogen atom and give a simple pattern. It has been shown[95] that in tetrahydroprotoberberine alkaloids with 9,10- substitution the C8 protons appear as an AB quartet (J = 16 Hz), or half the AB quartet when the high field half is obscured by

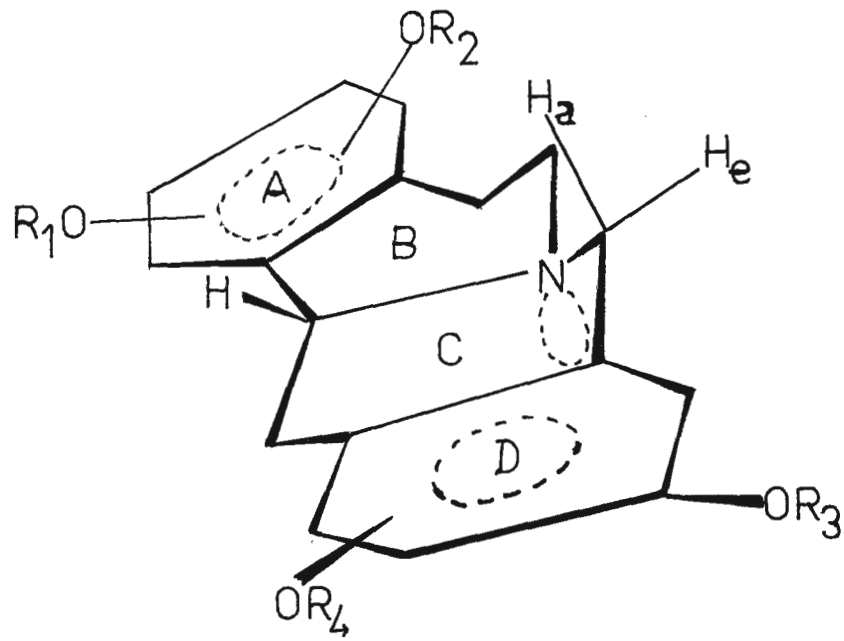
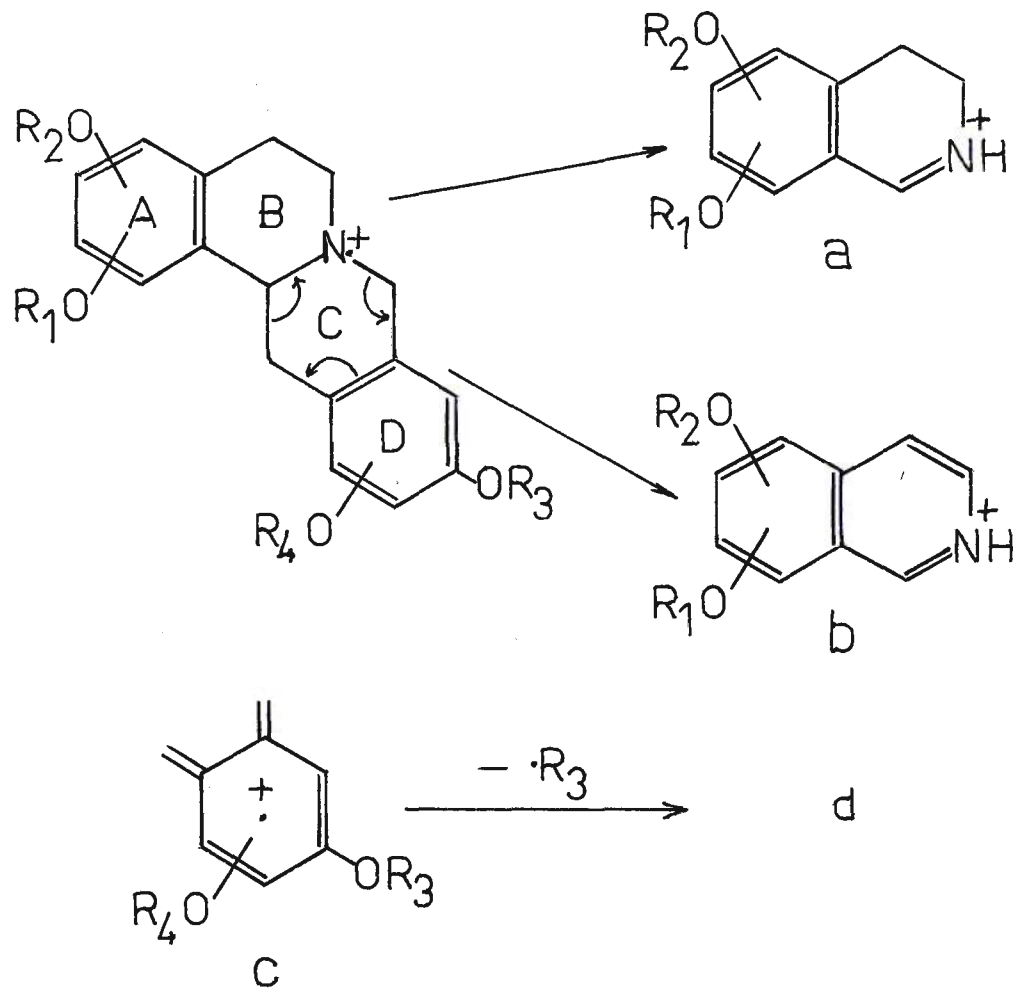


FIGURE 8. Conformation of Tetrahydroproto-berberines.



If $R_1, R_3 = CH_3$ and $R_2, R_4 = H$

then parent m/e = 327

a. m/e = 178

c. m/e = 150

d. m/e = 135

FIGURE 9. Main Fragment Ions of Tetrahydroprotoberberines.

methoxyl signals. On the other hand, in tetrahydroprotoberberine alkaloids with 10,11- substitution the C8 protons appear as a broad singlet underlying the O-methyl signals.

Figure 8 shows the proposed conformation for tetrahydroprotoberberine alkaloids[95], regardless of the substitution patterns of rings A and D. Since a broad singlet is observed for the protons at C8 in the n.m.r. spectrum of those alkaloids which are unsubstituted at C9, the axial and equatorial protons at C8 coincidentally have the same chemical shift. The introduction of an oxygen substituent at C9, however, apparently causes a downfield shift of the equatorial proton and an upfield shift of the axial proton at C8. As a result the n.m.r. signals of the C8 protons appear as an AB quartet.

Thus the n.m.r. data establishes the presence of two hydroxyl groups, two methoxyl groups, and provides evidence for 9,10- substitution in ring D. Additional information regarding the structure of the alkaloid was obtained from its mass spectrum.

The mass spectra of a number of tetrahydrorprtoberberine alkaloids have been studied and their significant fragmentation features correlated.[95] Figure 9 shows the main fragment ions of tetrahydroprotoberberine type alkaloids. The predominant decomposition is the retro Diels-Alder opening

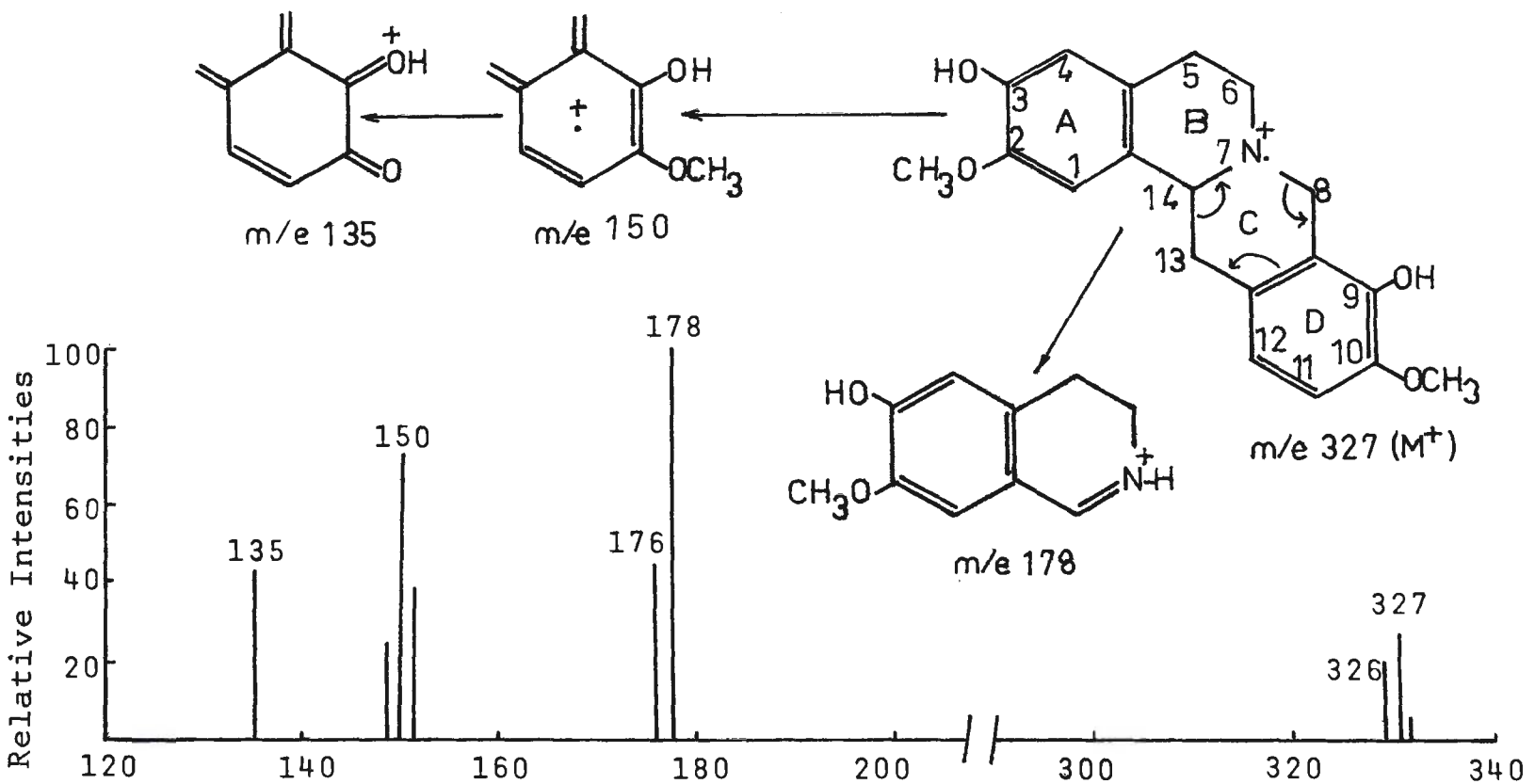


FIGURE 10. Mass Spectrum of Aequaline (Major Peaks)

of ring C with the production of an ion of type (c). This ion can then lose one of its R groups to give the fragment ion (d). The fragmentation pathways of the nitrogen containing fragment formed in the retro Diels-Alder ring opening have been shown to depend on the substitution pattern of ring D:

(i) With no hydroxyl substituents in ring D the fragment loses a single hydrogen to give an ion of the type (b).

(ii) If ring D carries hydroxyl group(s), a hydrogen transfer from ring C or D takes place, to give a fragment ion of type (a).

Hence, a base peak corresponding to ion (a) in the mass spectrum of a tetrahydroprotoberberine alkaloid is indicative of the presence of one or more hydroxyl groups in ring D.

Moreover, it is possible to differentiate between 9-hydroxy-10-methoxy compounds and 10-hydroxy-9-methoxy compounds as those with the former substitution, exemplified by scoulerine[96], preferentially expel a methyl group from ion (c) yielding an ion at m/e 135. The 10-hydroxy-9-methoxy compounds, exemplified by thalifendine[97] and tetrahydrothalifendine[98], apparently lose a hydrogen giving rise to an ion at m/e 149.

The mass spectrum of the alkaloid is shown in Figure 10. The significant fragment ions appear at m/e 327 (molecular ion), 326, 178, 176, 150 and 135, and these can be

Phys. data	Aequaline	Scoulerine	Ref.
Base m.p.	232°	197°	96
Base R_f ($CHCl_3$-MeOH 19:1)	0.40	0.60	102
Base $(\alpha)_D$	-283°	-318°	96
B-HCl m.p.	280°	209°	102
B-picrate m.p.	155-6°	206°	102

TABLE II. Comparison of Aequaline with Scoulerine.

interpreted in terms of cleavages corresponding to the favoured fragmentation pathways of tetrahydroprotoberberine alkaloids.

The n.m.r. and mass spectroscopic evidence is compatible only with a tetrahydroprotoberberine structure in which the ring D has a 9-hydroxy-10-methoxy substitution pattern.

Complete O-methylation of the alkaloid by diazomethane gave L-(-)-tetrahydropalmatine (19) thereby establishing a 2,3,9,10- substitution pattern. As L-(-)-tetrahydropalmatine has S-configuration[99,100,101], the absolute configuration of aequaline is also S, i.e., the C14 hydrogen is alpha as shown in (18).

On the basis of its composition, spectroscopic and chemical evidence, the alkaloid can have the structure (18) or structure (20). Structure (20) represents the known alkaloid scoulerine, which is distinctly different from the Schefferomitra alkaloid as is evident from a comparison of their physical properties (table II).

The new alkaloid aequaline is thus represented by structure (18) as 3,9-dihydroxy-2,10-dimethoxy tetrahydro-protoberberine that has not been reported previously.

Schefferine (21) 9-Hydroxy-2,3,10-trimethoxy tetrahydro-protoberberine

The alkaloid schefferine crystallised from methanol as colourless prisms m.p. 176°, R_f 0.90, $(\alpha)_D$ -303°. Its molecular formula $C_{20}H_{23}O_4N$ was established by microanalyses and by the presence of a molecular ion peak at m/e 341 in the mass spectrum.

The compound showed maxima at 220 and 282 nm (log ε 4.18 and 3.84) in its ultra violet spectrum - consistent with its belonging to the tetrahydroprotoberberine type alkaloids.[94] The presence of a phenolic hydroxyl group was demonstrated by a bathochromic shift of the ultra violet absorption maxima on the addition of alkali.

A n.m.r. spectrum of the alkaloid is shown in Figure 11. The signals at δ 3.68 (6H, s, 2 x OCH_3) and δ 3.73 (3H, s, OCH_3) reveal the presence of three methoxyl groups. A one-proton peak at δ 9.02, which disappears after exchange with deuterium, can be assigned to a hydroxyl group. Four aromatic protons appear as two one-proton singlets at δ 6.67 and δ 6.86 and as one two-proton singlet at δ 6.71. The signals centred at δ 4.02 (J_{AB} = 16 Hz) can be interpreted as a half of an AB pair of doublets with the high-field half being obscured by the methoxyl signals, and are assigned to the C8 axial and equatorial protons which are magnetically non-equivalent when C9 is oxygenated.

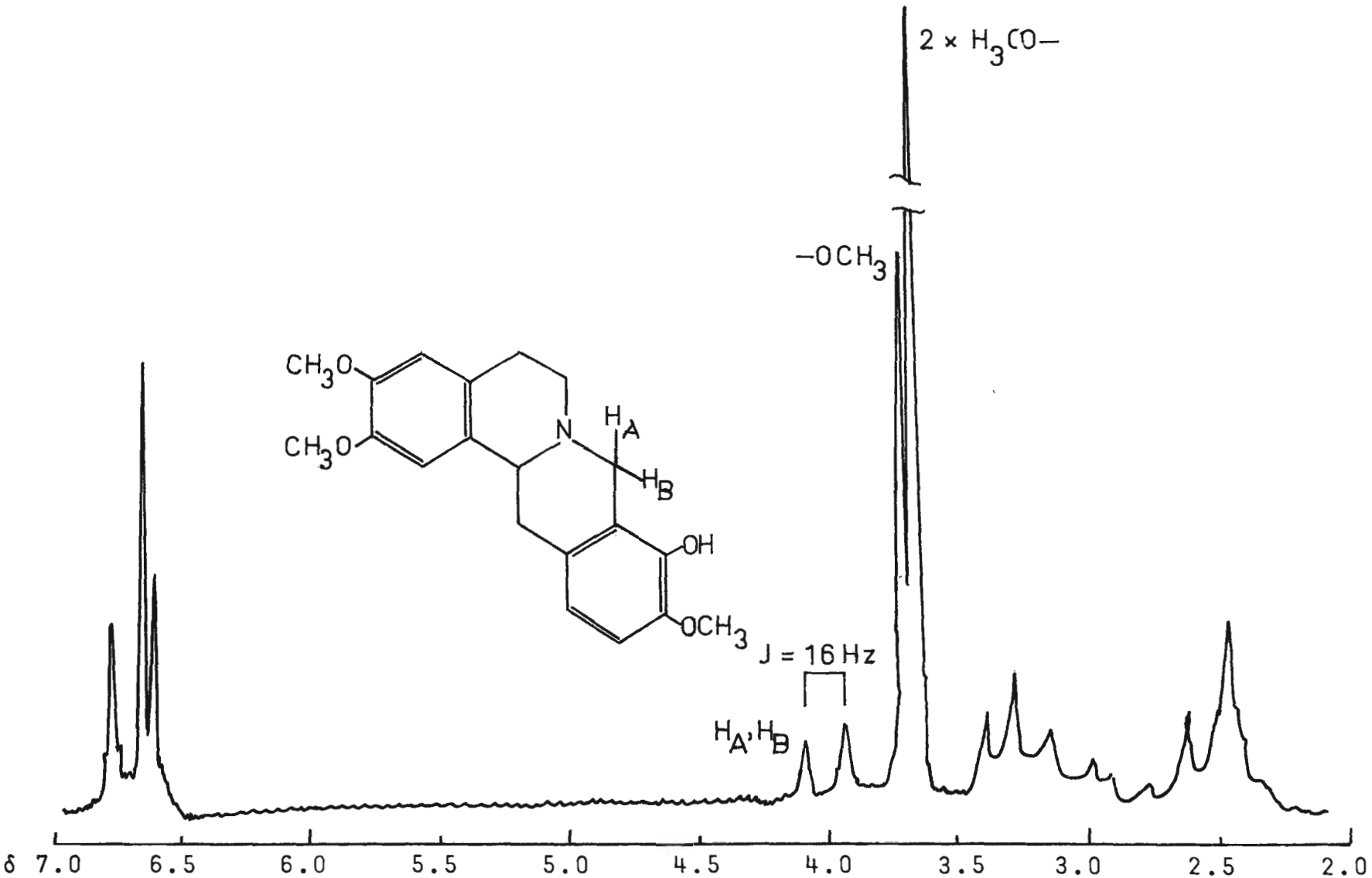


FIGURE 11. N.M.R. Spectrum of Schefferine (After deuterium exchange)

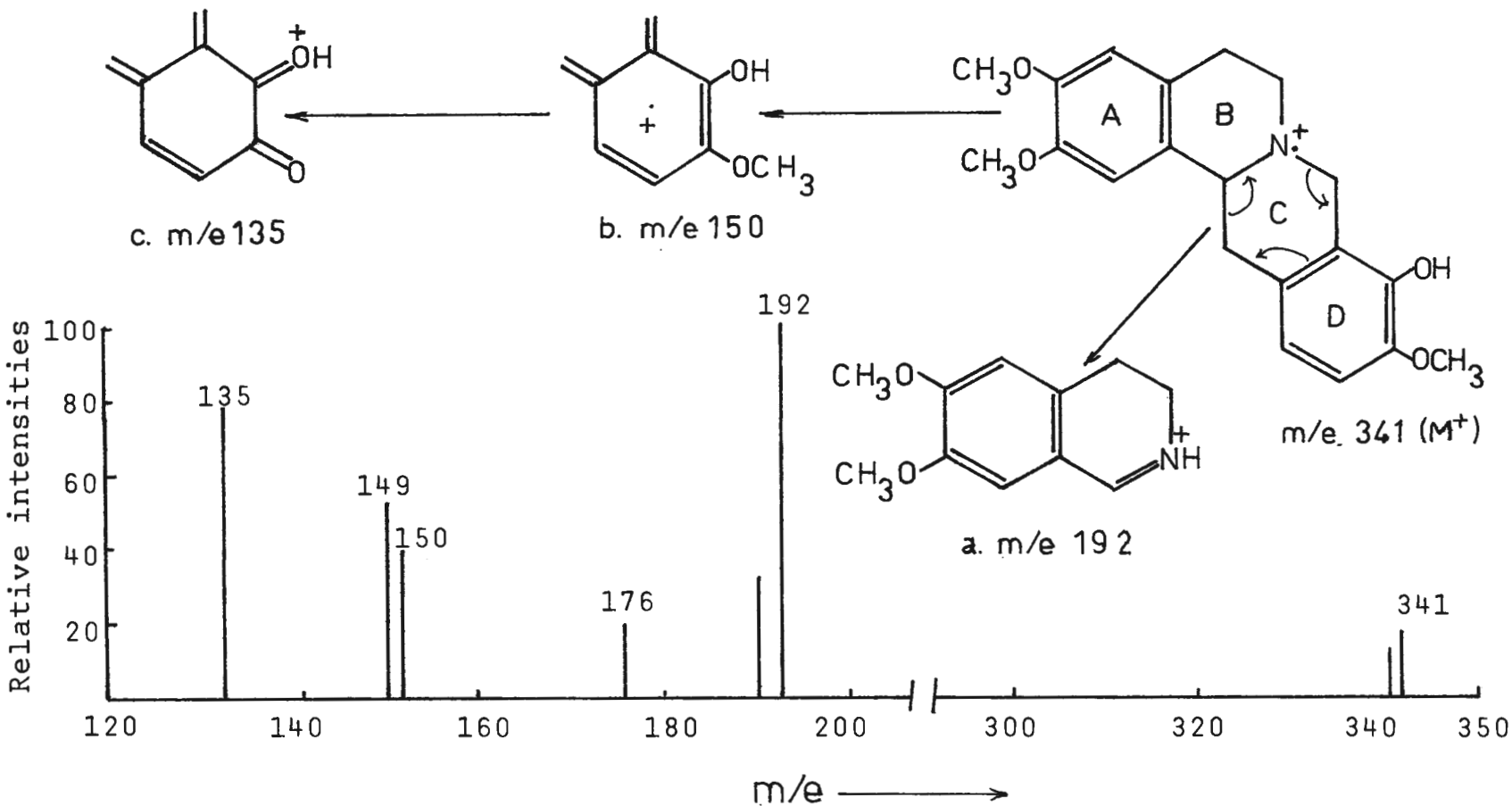


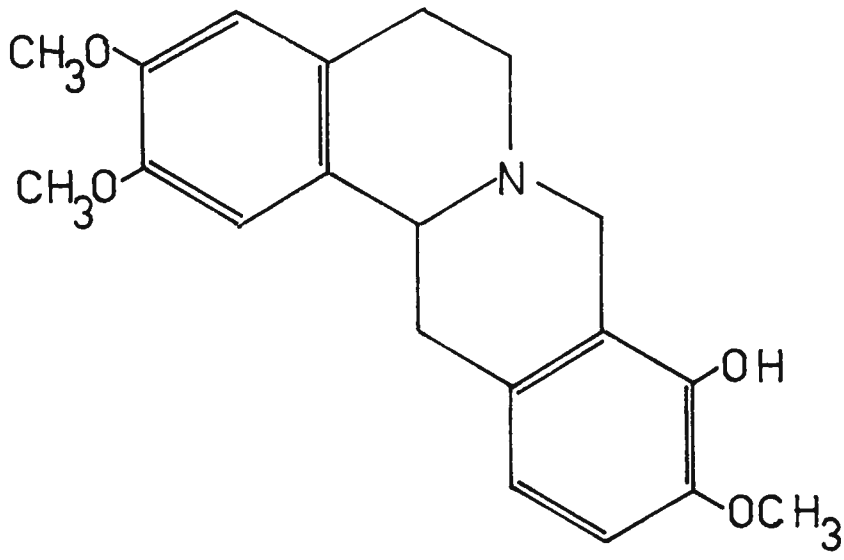
FIGURE 12. Mass Spectrum of Schefferine (Major Peaks)

The main fragment ions observed in the mass spectrum of the alkaloid are shown in Figure 12. The base peak at m/e 192 establishes the isoquinoline portion of the molecule as carrying two methoxyl groups as these are the only groups which can satisfy the mass requirement. The peaks at m/e 150 and m/e 135 can be assigned to ion fragments (b) and (c) respectively[95,103] showing that the hydroxyl group and the third methoxyl group are located in ring D. The intense peak at m/e 135 provides evidence that the hydroxyl group is located at C9.[95]

Partial O-methylation of aequaline and examination of the progress of reaction by t.l.c. showed that the alkaloid was identical with one of the mono-methylation products of aequaline. This provided additional evidence that the hydroxyl group in the alkaloid is located at C9.

Proof that the substituents are attached to the 2,3,9,10-positions of the tetrahydroprotoberberine skeleton was provided by the complete O-methylation of the alkaloid to give L-(-)-tetrahydropalmatine. This chemical correlation also establishes the absolute stereo-chemistry of the alkaloid as S. (cf. aequaline, p.49).

From this evidence the structure of the alkaloid is assigned as 9-hydroxy-2,3,10-trimethoxy tetrahydroprotoberberine.



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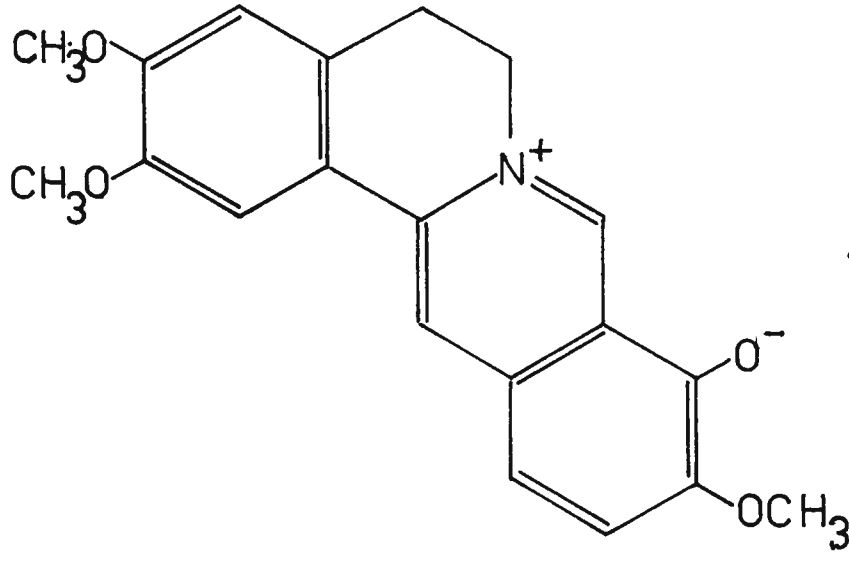

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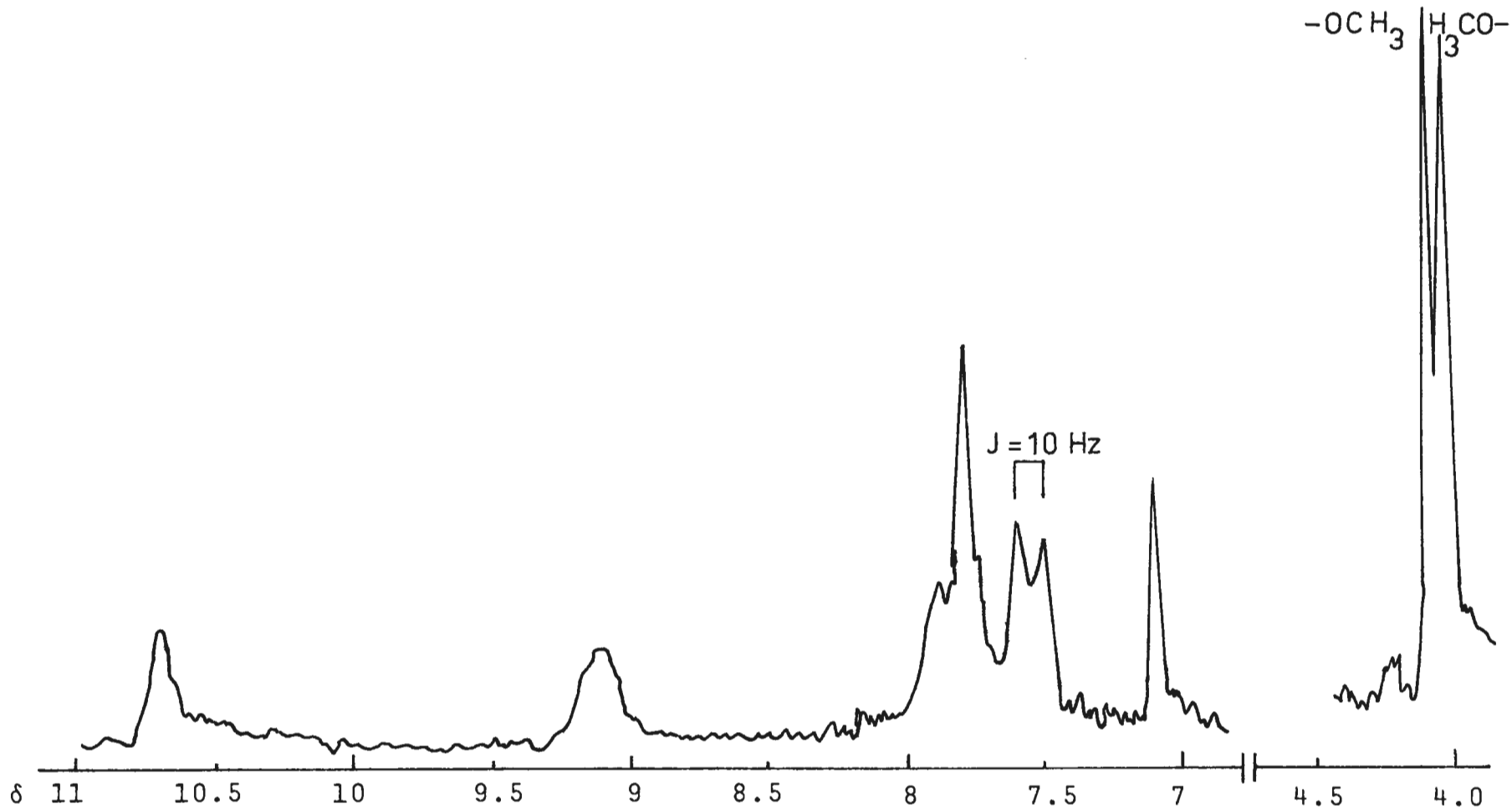


FIGURE 13. N.M.R. Spectrum of Alkaloid "Y"

The alkaloid is not known as a natural product, but Späth and Burger[104] have prepared its racemate by reducing palmatrubine (22).

Alkaloid "Y"

Alkaloid "Y" is present in Sch. subaequalis only in very small amounts, and was obtained as pale yellow crystals from methanol, m.p. 257-8°, R_f 0.86. Elementary micro-analyses and mass spectrum (molecular ion m/e 279) indicated that the compound has the molecular composition $C_{17}H_{13}O_3N$.

The u.v. spectrum of alkaloid "Y" is different from those of benzylisoquinolines, aporphines, and tetrahydroprotoberberines. The absorption maxima have high extinction coefficients at longer wavelengths and can well represent an extended aromatic conjugation pattern. A liriodenine type structure is excluded by the molecular formula as the n.m.r. spectrum (Fig. 13) shows the presence of two methoxyl groups (two three-proton peaks around δ 4.0) in the molecule. The presence of a broad one-proton peak at δ 10.7 - which moves towards higher field on elevation of temperature - indicates the presence of either an OH or an NH group. As the i.r. spectrum shows the presence of a carbonyl (amide) group, the low field proton must be assigned to an NH group. The low resolution mass spectrum, which does not show the metastable ions or matched peaks, cannot at this stage be properly interpreted. A dimethoxyphenanthrene type structure to which

an amide group (-CO-NH-) is linked to form a fourth ring which - depending on the positions - may be either a five or a six membered one could explain the low basicity and apparent optical inactivity of the alkaloid. However, until more material and better spectra become available the above thoughts must be considered only speculative and the elucidation of the structure of this minor constituent must remain pending.

Alkaloids of the Leaves

The leaves were extracted with methanol, and the extract was worked up by a procedure similar to that used for the bark to afford 0.03% yield of crude bases.

Analytical t.l.c. in several solvent systems indicated that the alkaloidal constituents of the crude mixture were very likely the same as those isolated from the bark with the exception that the yellow alkaloids liriodenine and alkaloid "Y" were absent. Hence the chromatographic fractionation and purification of the leaf alkaloids were carried out in essentially the same way as described under the isolation of the bark alkaloids.

All the leaf alkaloids were obtained in pure, crystalline state and were shown by direct comparison to be identical with the alkaloids isolated from the bark, namely:

anonaine, asimilobine, isoboldine, anolobine, aequaline, and schefferine.

The relative amounts of the alkaloids in the leaves bore a marked resemblance to those in the bark with anonaine and asimilobine constituting the bulk of the alkaloidal content while anolobine was present only in very small amounts.

B. THE CONSTITUENTS OF BOLETUS SPECIES

Allegedly hallucinogenic mushrooms, tentatively identified as Boletus, section Ixocomus, group Nudi[105] were collected at Mt. Hagen, New Guinea. The species grows - to our knowledge - exclusively in the Australian Territories of Papua and New Guinea and appears at the end of October, or later; sometimes as late as January.[82]

This mushroom is known to the natives as "namanama". It grows under acorn trees, has no ring or network on its stem and the cap is smooth. The pores bruise easily on handling causing the cream colour to change to ink blue. On cutting vertically, the yellow flesh turns ink blue for a short time then reverts to yellow.

"Namanama"

From a water colour painting.

The outward appearance of the mushroom is shown by the colour photograph on the opposite page.

The milled, air-dried mushrooms were extracted by percolation with methanol at room temperature, and the crude extract was fractionated between water and petroleum ether. The amino acids in the water soluble fraction were separated from associated carbohydrates, pigments and plant acids by adsorption upon and elution from an ion exchange resin (Zeo-Karb 225 in H^+ form) column.

The positions occupied by the amino acids on a two-dimensional paper chromatogram (solvents butanol-acetic acid-water followed by phenol-water) are shown diagramatically in Figure 14.

The amino acids were separated by the usual paper chromatographic technique, and the results are summarised in Figure 15.

No attempts were made to isolate the constituents of the fractions containing (a) leucine and isoleucine, (b) methionine and tyrosine, (c) threonine and glycine, and (d) arginine, lysine and histidine.

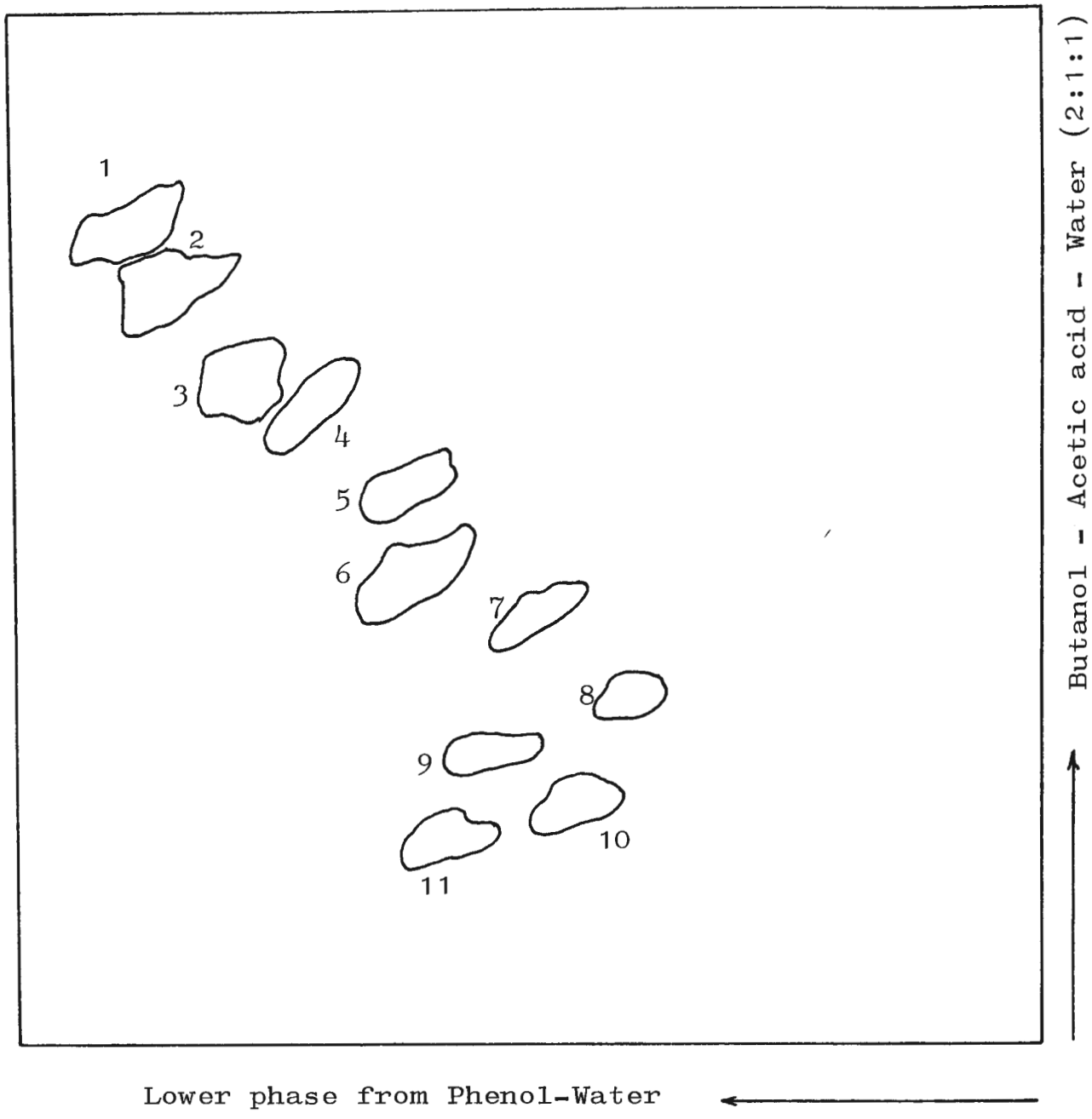


FIGURE 14. Two-dimensional Chromatogram of the Amino Acids present in the Boletus Species.

Key to chromatographic spots: 1, 2-amino-4-methyl-hex-5-enoic acid; 2, position of leucine and isoleucine; 3, valine; 4, methionine; 5, tyrosine; 6, alanine; 7, threonine; 8, glycine; 9, arginine; 10, lysine; 11, histidine.

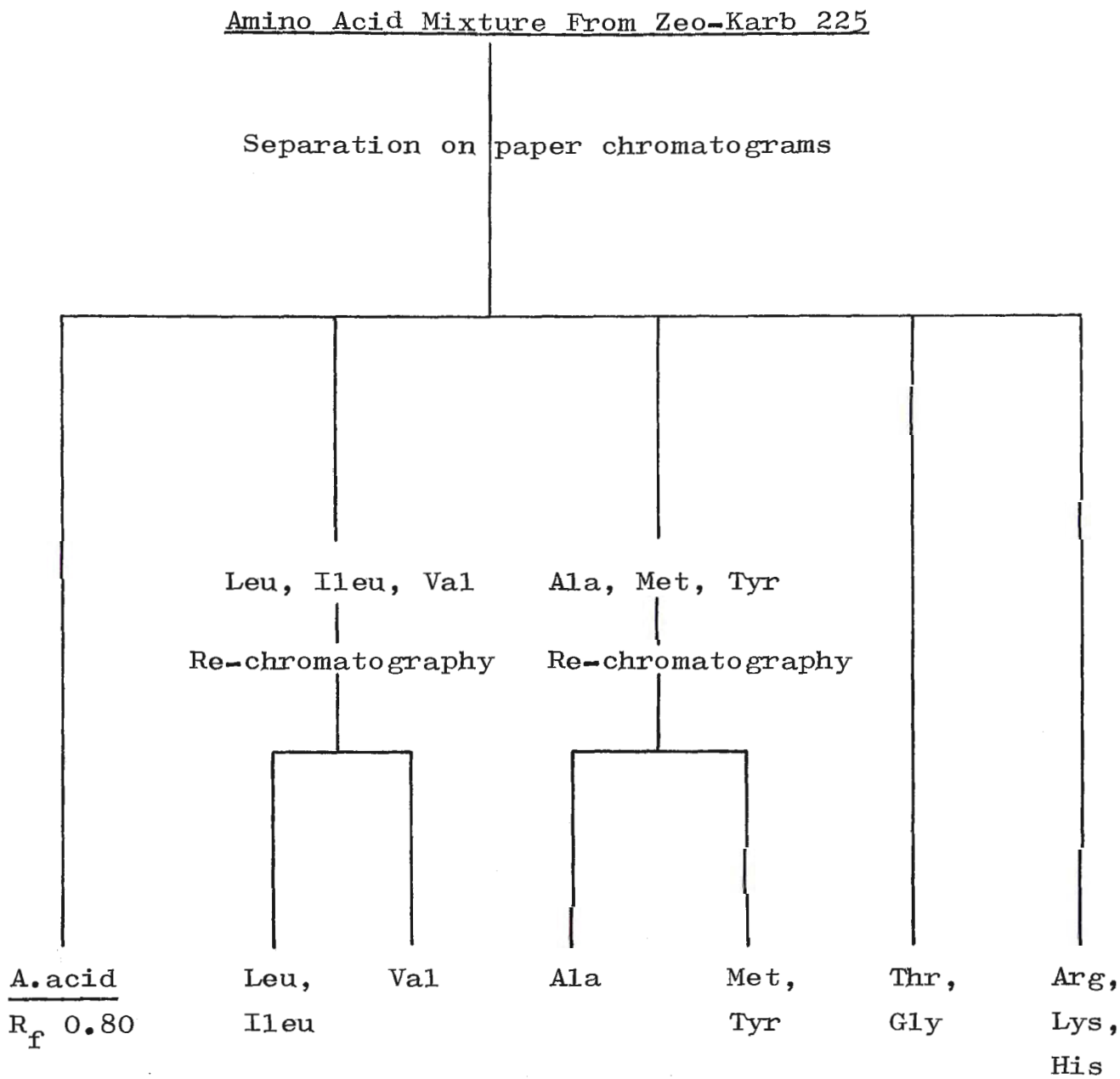


FIGURE 15. Paper Chromatographic Separation of the Amino Acids.

Identification of the Amino Acids

The amino acids were first tentatively identified by qualitative paper chromatography in the usual way, i.e., concurrent development of a sample of the amino acid isolated from the mushroom, a sample of the reference amino acid, and a mixed sample.

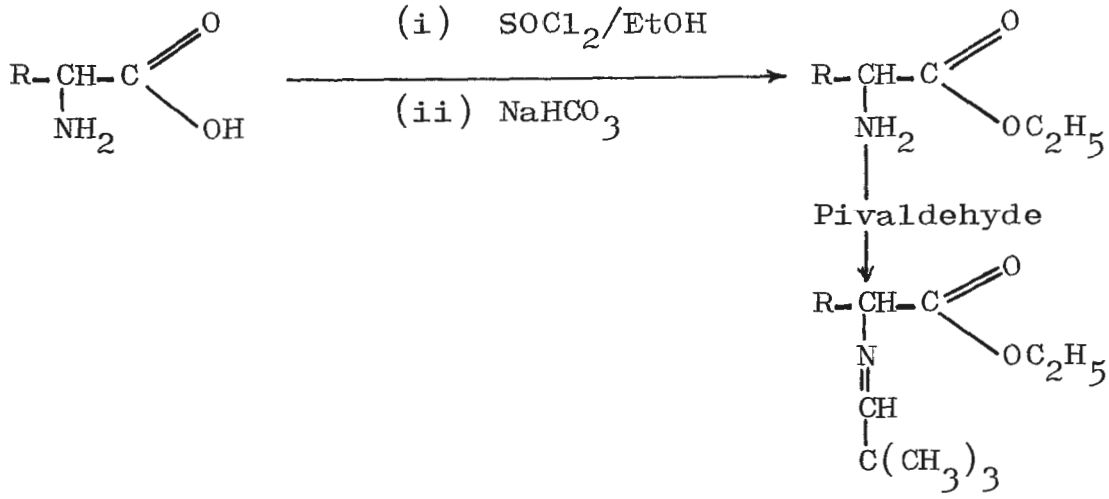
Final confirmation of the chemical identity of the amino acids was made by a method based on gas-liquid chromatography and mass spectral (GLC-MS) analysis.[106] This method is applicable to amino acid mixtures as well as to individual amino acids. The method, however, requires that the amino acids are converted to their more volatile esters prior to analysis, as the zwitterion nature of the free amino acids renders them rather nonvolatile, and thermal reactions can occur at the high temperatures required for vapourisation. It was found preferable to use the thionyl chloride - ethanol reagent recommended by Brenner[107] instead of the customary hydrochloric acid - ethanol reagent for esterification, because it is less sensitive to traces of moisture in the samples, and has the advantage that no water is formed during esterification. The reactive species is C_2H_5OSOCl which is formed as in (a) and reacts with the acids as in (b).

$$SOCl_2 + C_2H_5OH \longrightarrow C_2H_5OSOCl + HCl \quad \ldots (a)$$

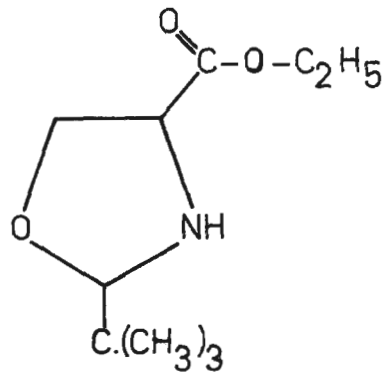
$$R\text{-}COOH + C_2H_5OSOCl \longrightarrow R\text{-}COOC_2H_5 + SO_2 \quad \ldots (b)$$

To improve the thermal stability of the amino acid

esters, the amino functions of the compound were protected by condensation with pivaldehyde (2,2-dimethylpropanal) to give the N-protected neopentylidene derivatives. These do not require isolation for the analysis. The reaction sequence is shown below.



For the β- hydroxy amino acid threonine an alternative structure (23) was postulated by Klyne.[108]



(23)

AMINO ACID	M^+	$M^+ - CH_3$	$M^+ - COOC_2H_5$	$M^+ - C.(CH_3)_3$
leucine isoleucine	-	212	154	170
valine	213	198	140	156
methionine	245	-	172	188
alanine	185	170	112	128
threonine	-	-	142	-
glycine	-	156	98	114

TABLE III Mass spectral fragments used for the identification of N-neopentylidene amino acid ethyl esters.

AMINO ACID	RETENTION TIME* (Minutes)	TEMPERATURE (°C)
leucine isoleucine	14.9	191
valine	13.2	178
methionine	20.8	238
alanine	11.0	160
threonine	15.8	198
glycine	11.6	165

TABLE IV GLC retention data for the N-neopentylidene derivatives of the amino acid ethyl esters.

* Retention times for temperature programmed separation from 80° at 8°/min.„ Packard-Becker 409 Gas Chromatograph Column 6' x $\frac{1}{8}$" stainless steel packed with 5% OV-17 on Chromasorb W (silanized). Nitrogen carrier gas - flow rate 20 ml/min.

The identification of the amino acids was made on the basis of retention time data in g.l.c. and of mass spectral fragmentation patterns. Most mass spectra of the N-neopentylidene derivatives of the amino acid ethyl esters show fragment ions corresponding to the loss of 15 mass units (loss of CH_3) and 57 mass units (loss of $-C.(CH_3)_3$) respectively. The compounds often display low intensity molecular ions and a fragment ion at $(M^+ - 73)$ corresponding to the loss of the ethoxy carbonyl function.

The characteristic mass spectral fragmentation patterns of the N-neopentylidene amino acid ethyl esters used for the identification of the amino acids are given in table III while the g.l.c. retention data for the N-neopentylidene amino acid ethyl esters are given in table IV.

Leucine and isoleucine cannot be separated on the column used. These isomers, however, can be separated if a very polar column is used. The amino acid tyrosine, and the basic amino acids arginine, lysine and histidine cannot be chromatographed on our column by the method employed. Tyrosine may be chromatographed if the phenolic hydroxyl is converted to the trimethylsilyl ether function in addition to the formation of the usual N-neopentylidene derivative.[109]

The presence of the amino acids tyrosine, arginine, lysine and histidine in the free amino acid pool of the

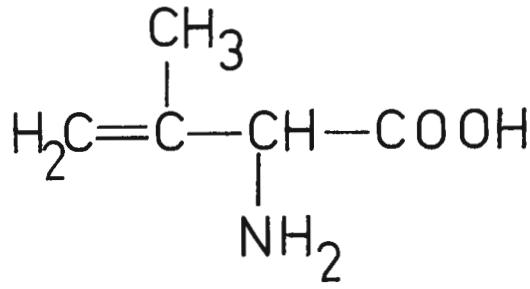
AMINO ACID	APPROX. % W/W
leucine, isoleucine	1.07
valine	0.30
methionine, tyrosine	0.15
alanine	0.56
threonine, glycine	0.25
arginine, lysine, histidine	0.10

TABLE V Amino acids isolated from Boletus, and their relative abundance in the species.

Boletus species was established from automatic amino acid analyser data, and by qualitative paper chromatography carried out concurrently with reference samples of known amino acids.

A list of the known amino acids isolated from the water soluble fraction of the Boletus species is given in table V. The relative abundance of the amino acids in the mushroom is expressed as the approximate percentage w/w of the dried mushroom.

In addition to the above known saturated amino acids a new unsaturated α-amino acid was also isolated from the free amino acid fraction of the mushroom. This finding is of special interest as the only other examples of occurrence of unsaturated amino acids in mushrooms reported so far are those of β-methylene-L-norvaline (24) from Lactarius helvus[110], L-2-amino-3-hydroxymethylpent-3-enoic acid (25) and L-2-amino-3-formylpent-3-enoic acid (26) from Bankera fuligineoalba[111], and 2(S)-aminohexa-4,5-dienoic acid (27) from Amanita solitaria.[112] The role, if any, these amino acids play in the metabolism of the mushrooms is not known.



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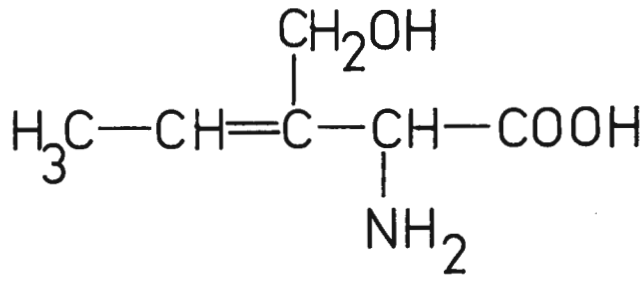

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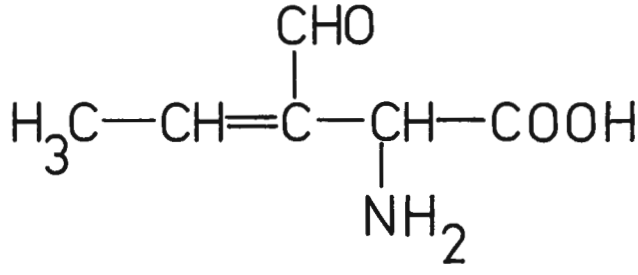

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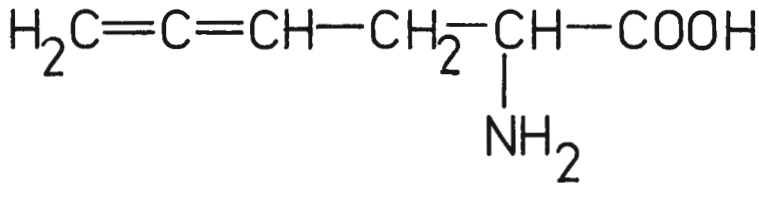

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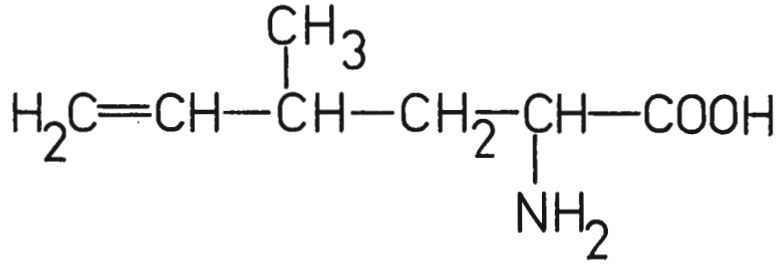


28

Elucidation of Structure of a New Unsaturated α- Amino Acid

The new unsaturated α-amino acid was obtained as colourless plates from aqueous ethanol and had a melting point 240-2° (dec.), and R_f 0.80 in butanol- acetic acid-water. The compound gave a blue-purple colour with ninhydrin.

The molecular formula $C_7H_{13}O_2N$ was established by microanalyses and by the presence of a molecular ion peak at m/e 143 in the mass spectrum of the compound.

The infrared absorption spectrum showed absorption bands at 1580 and 1405 cm^{-1}, characteristic for zwitterionic amino acids[113] and, in addition, contained peaks at 990 (medium intensity) and 920 cm^{-1} (strong intensity), which could be assigned to -CH- out of plane deformation of a terminal vinyl group.[114] The presence of a double bond was confirmed by catalytic hydrogenation of the amino acid, when one equivalent of hydrogen was taken up. Evidence favouring the presence of a terminal vinyl group was provided by the detection of formaldehyde as a product of ozonolysis of the amino acid. This was confirmed by the presence in the n.m.r. spectrum (Fig. 16) of a two-proton multiplet at δ 5.56 which is due to the terminal methylene group and a one-proton multiplet at

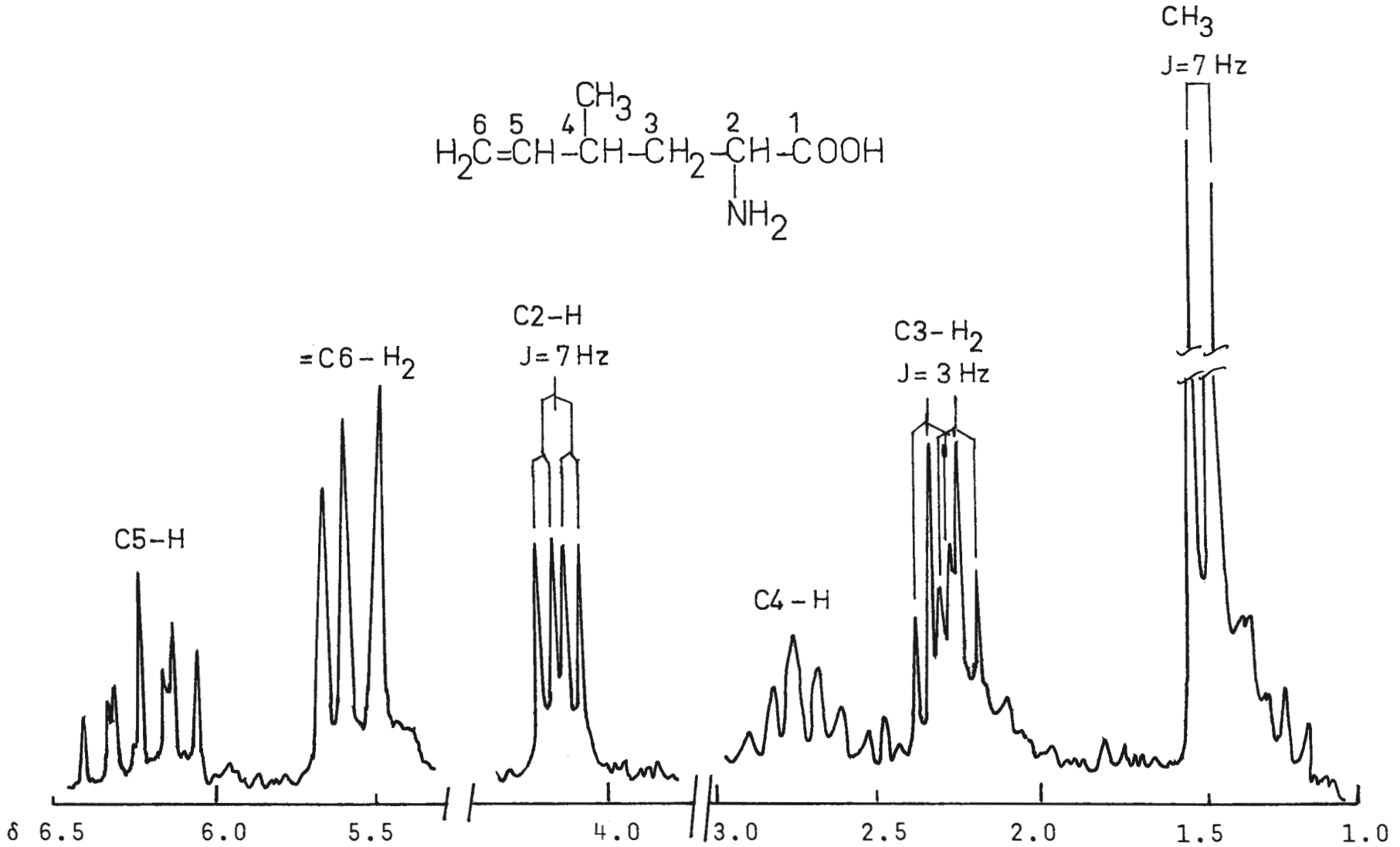


FIGURE 16. N.M.R. Spectrum of 2-Amino-4-methylhex-5-enoic Acid.

δ6.22 assigned to the vinylic proton which is deshielded by the other C5 substituents.[115] The n.m.r. spectrum also showed a three-proton doublet at δ 1.52 (J = 7 Hz) due to the protons of the methyl group coupling with the C4 proton; a two-proton multiplet at δ 2.30 (J = 3 Hz) due to the magnetically non-equivalent protons at C3 coupling with the C2 proton; a one-proton multiplet centred at δ 2.70 due to the C4 proton, and a one-proton pair of doublets (AB quartet) at δ 4.14 (J = 7 Hz) which can be assigned to the C2 proton signal split by the adjacent C3 protons.

The migration characteristics of the amino acid in paper electrophoresis at several pH values were typical of a neutral amino acid[116] and its ability to chelate with Cu^{++} established it to be an α- amino acid.[117]

Specific optical rotation values measured for the amino acid in water and in 1N hydrochloric acid showed a shift to more positive rotations in acid generally associated with an L configuration at the α- asymmetric centre in amino acids.[118] This assignment was confirmed independently by a gas liquid chromatographic method which relies on the consistency in the order of retention behaviour of diastereoisomeric N-trifluoro-acetyl-L-prolyl-DL-amino acid esters where the

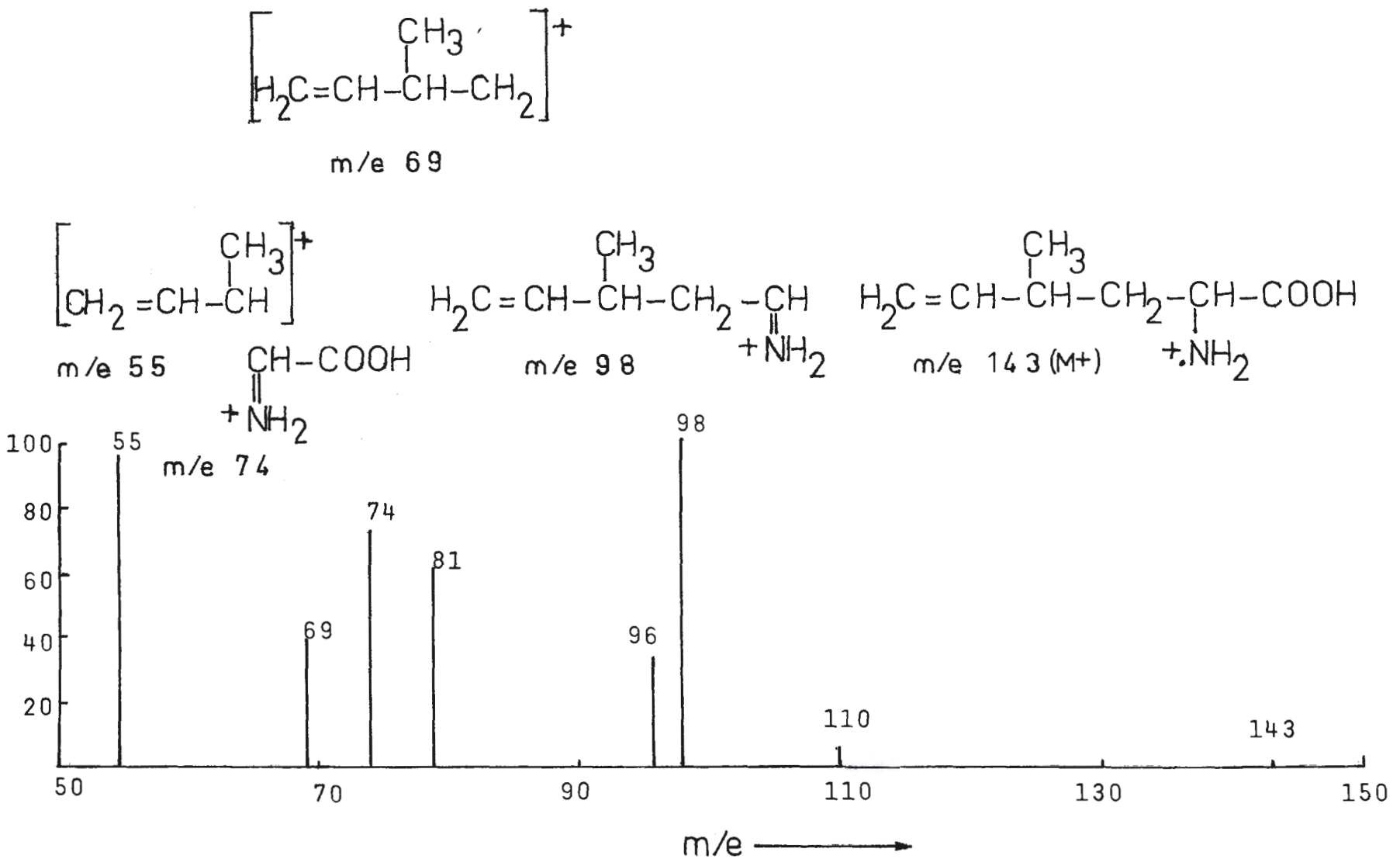


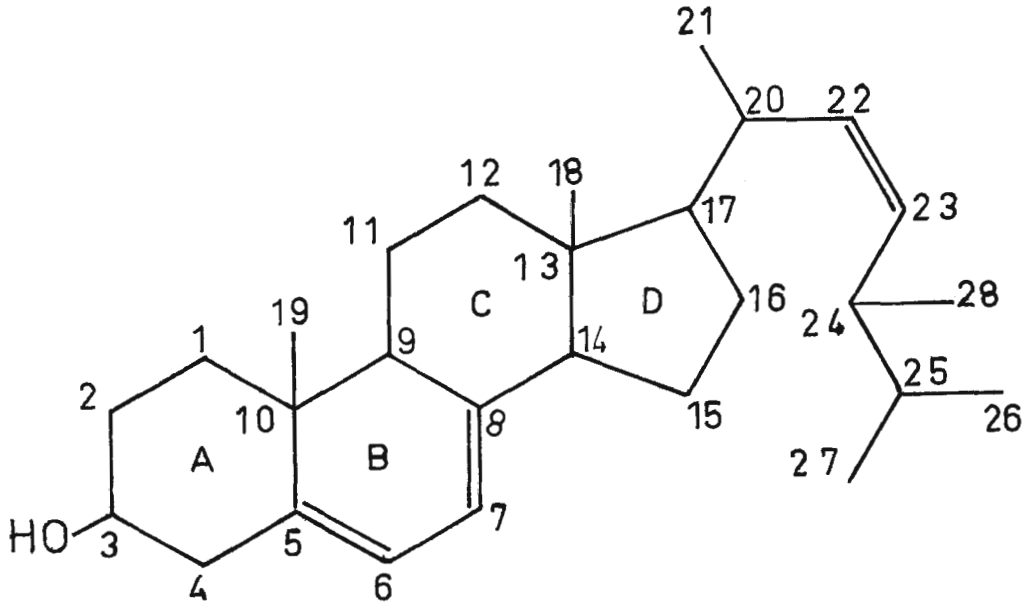
FIGURE 17. Mass Spectrum of 2-Amino-4-methyl-hex-5-enoic Acid (Characteristic Peaks).

LL - diastereoisomer always has a longer retention time than the LD - diastereoisomer.[119]

The gas chromatograph cannot distinguish between L-prolyl-L-amino acid and D-prolyl-D-amino acid because they are optical antipodes (enantiomers). It is, however, possible to determine the absolute configuration of an amino acid which is available only in one form by determining the respective retention times of the D-L and L-L diastereoisomers. As the L-L diastereoisomer always has a longer retention time than the D-L stereoisomer, the absolute configuration of the amino acid can be deduced from the g.l.c. retention times.

The analyses, spectral data and physical properties of the amino acid indicated that the compound is L-2-amino-4-methylhex-5-enoic acid (28). The absolute configuration at C4 is as yet not known.

Confirmation for this structural assignment was obtained from low resolution mass spectrometric analysis of the amino acid. This showed characteristic fragment ions at m/e 143 (molecular ion), 74, 69 and 55 which can be interpreted in terms of cleavages (Fig. 17) in accordance with the favoured fragmentation pathways of aliphatic α- amino acids.[120]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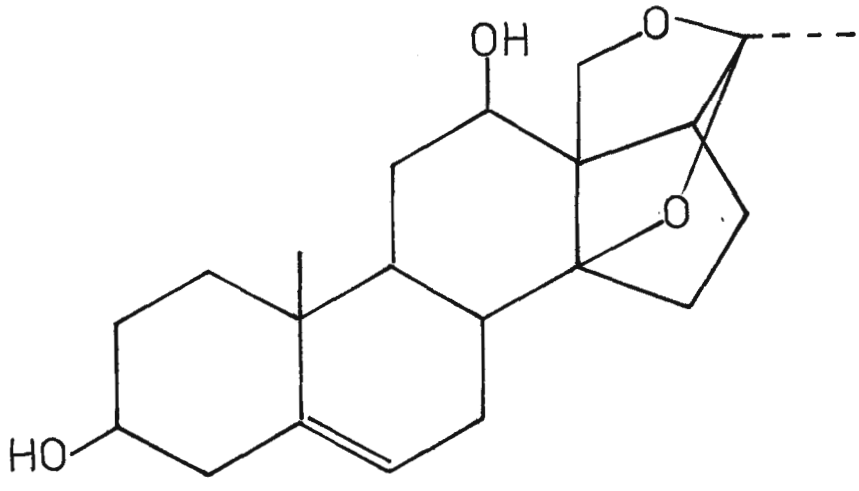
OH
O
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30

Isolation of Water-insoluble Compounds

Preliminary investigation of the dark-yellow gum originating from the fraction containing the water-insoluble compounds indicated that the mixture consisted predominantly of three components. One of these, later identified as ergosterol, constituted the bulk of the mixture.

Column chromatography of the material on silica developed with benzene-acetone mixtures afforded three constituents as chromatographically homogeneous compounds, with R_f values 0.96, 0.38 and 0.12 respectively.

Ergosterol (29)

The compound with R_f 0.38 crystallised from 95% ethanol as colourless plates, m.p. 158°, $(\alpha)_D$ -129.5°. Microanalyses, and the presence of molecular ion peak at m/e 396 in its mass spectrum established the molecular formula as $C_{28}H_{44}O$. The u.v. spectrum of the compound had one triple-peaked absorption band characteristic of ergosterol.[121] Its identity with ergosterol was confirmed by comparison with an authentic specimen, and by converting the compound to its acetyl derivative which was also shown to be identical with authentic ergosteryl acetate.

D-4

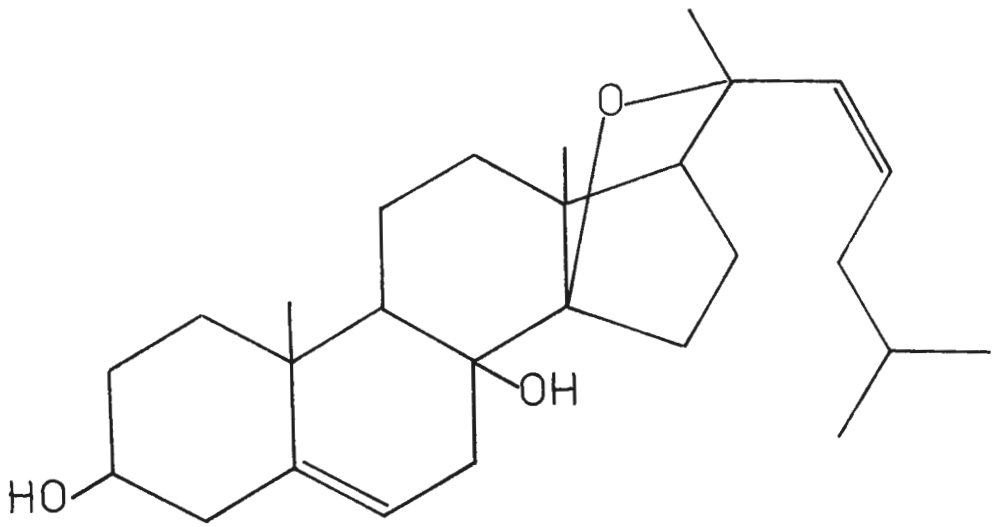
One of the minor components, R_f 0.96, crystallised from methanol-benzene, m.p. 109-10°. Its u.v. spectrum is superimposable on that of ergosterol establishing the same conjugated double bond system (ring B of the steroid molecule). This deduction was supported by the characteristic colour tests viz. Liebermann-Burchard[122], Salkowski[122] and Rosenheim[123], even though the Tortelli-Joffe test[122] gave a negative result. The lack of a hydroxyl group in the molecule was established by the absence of OH absorption in the infrared spectrum according to expectations in view of the high R_f value of this compound in benzene-acetone, while the presence of an i.r. absorption band at 1740 cm^{-1} indicated a ketone grouping. This, however, cannot be situated at C17 as suggested by the relevant steroidal ketone absorption bands[124] because the molecular weight of the compound (established by its mass spectrum as 380) indicates the presence of a side-chain at C17. The 970 cm^{-1} absorption band could be ascribed to a $\Delta 22$ double bond thereby retaining the ergosterol unsaturation pattern. As the mass spectrum shows that D-4 contains only 27 carbons (one less than in ergosterol) its structure may possibly be represented as desmethyl ergosterone. The elucidation of its structure must, however, await further investigation.

D-2

The other minor component, R_f 0.12, crystallised from methanol as colourless needles of m.p. 179-80°. It gave green colouration in the Liebermann-Burchard test for steroids. Its u.v. spectrum showed no absorption above 220 nm indicating the absence of conjugated double bonds. The Tortelli-Joffe test for ditertiary double bonds or bonds capable of migration to such a position was also negative. The absorption bands around 3400 cm^{-1} region in the infrared spectrum established the presence of hydroxyl groups in agreement of the low R_f value of the steroid in benzene-acetone solvent system. Other bands indicated the presence of e.g., 3-β-OH group (1045 cm^{-1}), Δ5 (803 and 838 cm^{-1}) and Δ22 (1667 and 970 cm^{-1}) double bonds.[121]

The low resolution mass spectrum of the compound was consistent with formula $C_{28}H_{44}O_3$ representing the addition of two oxygen atoms to ergosterol. As ergosterol was identified as the major constituent of this fraction it is reasonable to assume that the Δ7 double bond might be hydrated. The third oxygen atom could then be present as an ether function, i.e., part of a

tetrahydropyrane ring similar to that present e.g., in stapelogenin[125] (30). The structure of D-2 could therefore, tentatively and without actual proof, be represented as (31).



(31)

E X P E R I M E N T A L

G E N E R A L

Melting points are uncorrected and were determined on an electrically heated Reichert microscope hotstage.

All temperatures are expressed in degrees centigrade.

Microanalyses were carried out by the Australian Microanalytical Service, Melbourne and by Dr. E. Challen, Microanalytical Laboratory, School of Chemistry, University of New South Wales.

Micro ozonolysis was carried out by Dr. B. Moore, Division of Entomology, C.S.I.R.O.

Infrared absorption spectra were recorded on a Perkin - Elmer 237 Infrared Grating spectrophotometer.

Ultraviolet absorption spectra were recorded on a Perkin - Elmer 137 Ultraviolet - Visible spectrophotometer.

Nuclear magnetic resonance spectra were recorded on a Varian A60 spectrometer and on a J. E. O. L. 100 Mc. instrument by Mr. V. Pickles of the University of New South Wales and by Mr. B. Worth of the Australian National University, using tetramethylsilane as an internal reference. Abbreviations used to describe the n.m.r. spectra are: s, singlet; d, doublet; q, quartet and m. multiplet.

Mass spectra were recorded on a M.S. 902 instrument by the Mass Spectrometry Unit of the University of Sydney or on an E.A.I. Quad 300D mass spectrometer at Wollongong University College. The latter spectrometer is an electrically scanned instrument, which was interfaced with the gas chromatograph by means of a Biemann separator. The ion source temperature was 250°, the ionising current 150 μA and the ionising voltage either 20 eV or 70 eV.

Gas - liquid chromatographic separations were carried out with a Varian chromatograph Model 600C fitted with a hydrogen flame detector. The column used throughout was a 6' x $\frac{1}{8}$" stainless steel tube packed with 5% OV-17 on Chromosorb W (silanized). Nitrogen was used as a carrier gas at a flow rate of 20 ml/min. and the injector block temperature was 250°. Programmed separations were usually run at 6°/min. using a Varian aerograph linear temperature programmer, Model 330.

Paper electrophoresis was carried out in an "EEL" 6-strip electrophoresis bath operated by "EELite" power supply unit designed to deliver constant current of between 0.8 mA and 50 mA at voltages up to 600 V.

Unless otherwise stated the R_f data quoted for alkaloids refer to thin layer chromatograms on Kieselgel G (Merck) plates developed in the solvent system chloroform-methanol

(9:1). Spots were examined under ultraviolet light and developed with iodine.

Unless otherwise stated all qualitative and preparative paper chromatography of amino acids was performed on Whatman No. 3 chromatography grade paper using an ascending technique in the solvent system butan-1-ol - glacial acetic acid - water (2:1:1). Ninhydrin was used for the detection of the amino acids. The R_f values quoted refer to paper chromatograms developed in the above solvent system.

Unless otherwise stated the R_f values quoted for steroids refer to thin layer chromatograms on Kieselgel G (Merck) plates developed in benzene - acetone (95:5).

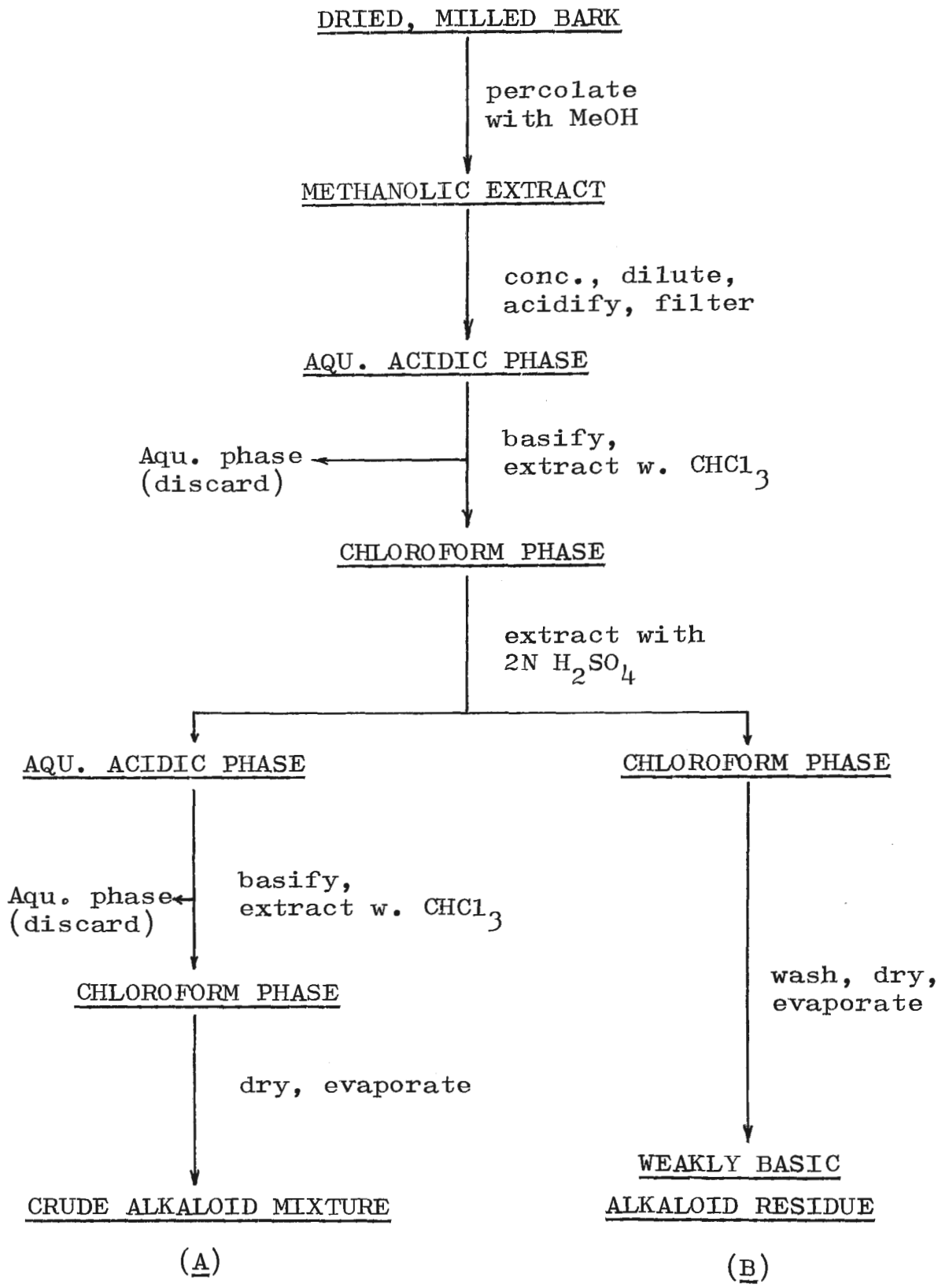
A. THE CONSTITUENTS OF SCHEFFEROMITRA SUBAEQUALIS

Extraction of the Bark

Finely milled dried bark (50 kg) was extracted by percolation with methanol at room temperature until the alkaloids were completely extracted as indicated by tests with Mayer's alkaloid reagent. The methanol extract was concentrated in a climbing-film evaporator as far as possible, and then further evaporated under reduced pressure to a consistency of a thin syrup. The concentrate was diluted with an equal volume of water and acidified with 1N sulphuric acid. The acid extract was allowed to stand for several days, and then filtered.

The clear reddish-brown filtrate was basified with conc. ammonia and exhaustively extracted with chloroform. A brown gum which separated during this process was homogenized with 5% sulphuric acid to remove occluded bases, and the residual gum was then discarded. The alkaloids were extracted from the chloroform solution with 2N sulphuric acid and the acid extract was combined with the above aqueous acid solution. The free bases were then recovered, after basification with ammonia, by chloroform extraction.

Evaporation under reduced pressure of the dried ($MgSO_4$) chloroform extract left 16.2 g of crude alkaloids



SCHEME I Extraction Procedure for Bark Alkaloids.

as a very dark gum (A).

The chloroform solution was, after extraction with acid, washed with water, then dried ($MgSO_4$), and evaporated under reduced pressure to give 19.3 g of a dark viscous residue (B), which gave a weak positive test for alkaloids with Mayer's reagent.

The procedure for the extraction of the bark alkaloids is summarised in scheme I.

Separation of the Individual Alkaloids

A portion (5.6 g) of the crude alkaloids (A) in chloroform solution was applied to a column of alumina activity grade II/III (Merck; 280 g) in chloroform. The column was initially developed with pure chloroform but, to speed elution of the slower moving constituents, the solvent was later changed to chloroform containing 5% methanol. The eluent was collected in 25 ml aliquots which were grouped into crude fractions according to their composition as determined by t.l.c.

Fraction	Solvent	Tube No	R_f values*
I	$CHCl_3$	1 - 3	<u>0.96</u>; 0.90
II	"	4 - 7	0.90; <u>0.49</u>; 0.26
III	"	8 - 12	0.49; <u>0.26</u>
IV	$CHCl_3$-MeOH (95:5)	13 - 23	0.26
V	"	24 - 36	<u>0.68</u>; 0.47
VI	"	37 - 52	0.21

*The R_f values of the most prominent spots are underlined.

An appreciable amount of very dark material was retained on the upper part of the column and could not be eluted. The alumina was therefore extruded from the column and extracted in a Soxhlet with several portions of methanol. The dark-brown extracts were combined, concentrated to a small volume, and filtered. The filtrate was evaporated to dryness under reduced pressure, and the black residue extracted with several portions of hot 2% hydrochloric acid. The insoluble residue (1.8 g) remaining after extraction with acid gave a negative Mayer's test, and was discarded. The aqueous acid extract was basified with ammonia and extracted with chloroform. The washed, and dried ($MgSO_4$) chloroform extract was filtered and added to fraction VI.

Each fraction was evaporated separately to dryness under reduced pressure. The residues obtained from fractions I and II were dark-brown and viscous while those from fractions III to VI were light-brown gums.

Fraction I was chromatographed on a column of silica gel in chloroform-methanol (98:2). Two main series of fractions were selected on the basis of examination by thin layer chromatography.

The rapidly eluted first series of fractions showed one main spot of R_f 0.96 and some weak, overlapping spots

at the solvent front. Removal of the solvent gave a yellow gum which was purified by preparative t.l.c. on silica developed in chloroform-methanol (95:5) to yield the constituent R_f 0.96 as an amorphous yellow solid.

The second main series of fractions consisted essentially of a single component of R_f 0.90.

Fraction II was chromatographed on a column of silica gel in chloroform-methanol (95:5), some of the sub-fractions requiring re-chromatography. The following sub-fractions were obtained:

sub-fraction (i): R_f 0.90; added to fraction R_f 0.90 from column chromatography of fraction I

sub-fraction (ii): R_f 0.49

sub-fraction (iii): R_f 0.49; 0.26; added to fraction III

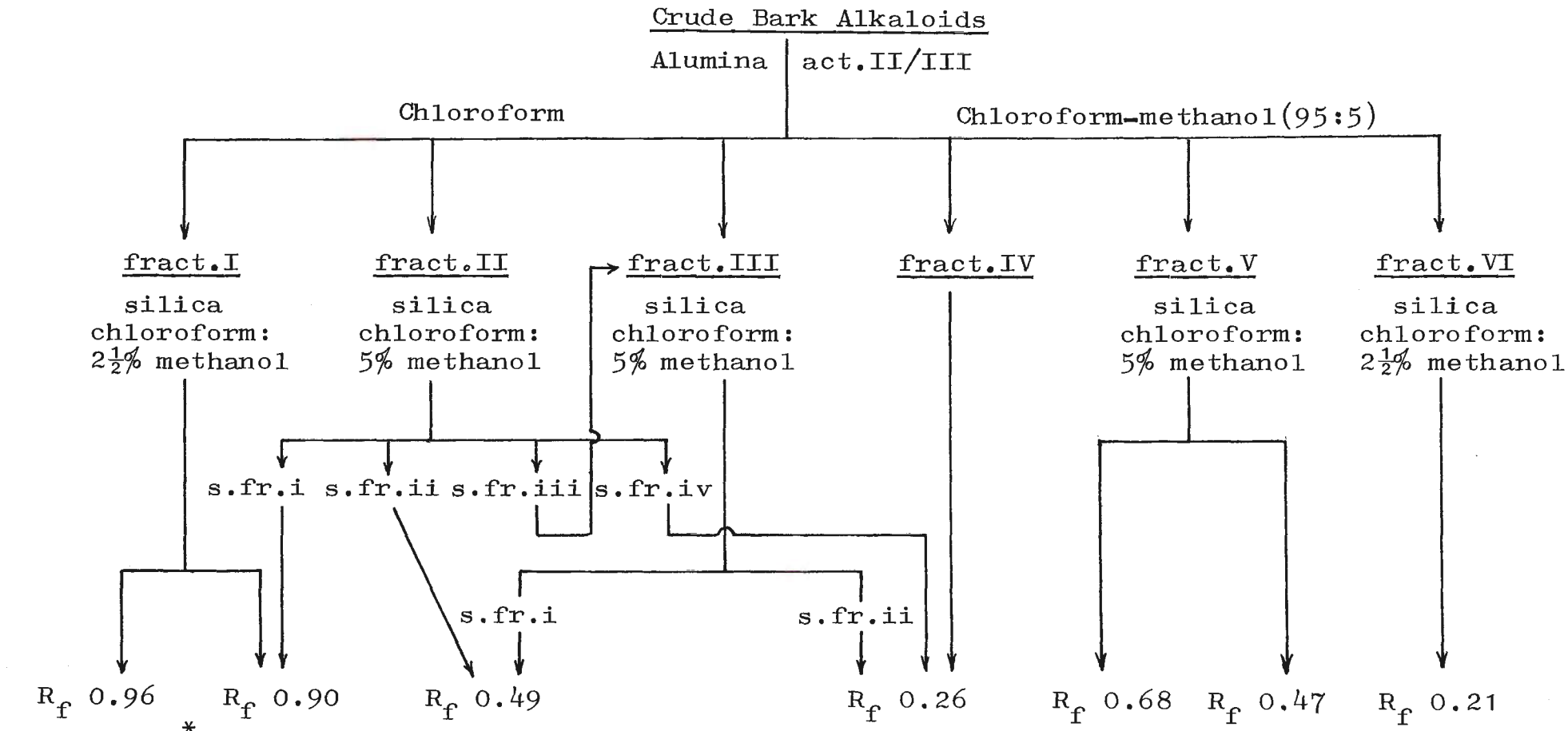
sub-fraction (iv): R_f 0.26; added to fraction IV.

Fraction III was chromatographed as the previous fraction. After re-chromatography of a small intermediate fraction two final sub-fractions were obtained:

sub-fraction (i): R_f 0.49

sub-fraction (ii): R_f 0.26; added to fraction IV.

Fraction IV was retained for further treatment.



*After prep. t.l.c.

Scheme II. Separation Sequence for Bark Alkaloids.

Fraction V: the components with R_f 0.68 and R_f 0.47 were separated by column chromatography on silica gel with chloroform - methanol (95:5), and by re-chromatography of an intermediate fraction.

sub-fraction (i): R_f 0.68

sub-fraction (ii): R_f 0.47.

Fraction VI was purified by chromatography on a column of silica gel. Elution with chloroform containing $2\frac{1}{2}\%$ methanol gave the component with R_f 0.21. Further elution with chloroform containing 10% methanol yielded a negligible amount of intractable dark gum which was discarded.

The chromatographic procedure for the separation of the bark alkaloids is summarised in scheme II.

Identification of the Bark Alkaloids

Liriodenine

The yellow solid obtained after preparative t.l.c. of the fraction consisting essentially of a single component with R_f 0.96 was recrystallised first from acetone then from a small volume of chloroform to give golden yellow needles. These, when dried *in vacuo* over P_2O_5, had m.p. 284-5° (lit.[26] m.p. 284-6°) which was not depressed on mixing with authentic liriodenine.

The respective ultraviolet and infrared absorption spectra of the alkaloid were identical with those of authentic liriodenine. The identity of the two compounds was also confirmed by their t.l.c. behaviour on silica, and on alumina in several solvent systems.

Anonaine

Sub-fraction (ii) of fraction II and sub-fraction (i) of fraction III were combined. Removal of the solvent gave a brown gum which could not be induced to crystallise. The gum was converted into the crystalline hydrochloride salt by treatment with a few drops of 5N hydrochloric acid followed by trituration with a small volume of methanol. Several recrystallisations from water or methanol gave the alkaloid hydrochloride as colourless needles, m.p. 269°

(dec.) (lit.[26] m.p. 267°). Regeneration of the free base from its hydrochloride salt by basification with ammonia, and recrystallisation from pentane gave colourless needles, m.p. 123-4° (lit.[126] m.p. 122-4°) undepressed when mixed with authentic anonaine.

The infrared spectrum of the alkaloid was identical with that of an authentic specimen of anonaine and the two alkaloids had the same R_f value (0.49) on silica in chloroform-methanol.

Asimilobine

Removal of the solvent from fraction IV gave a dark residue which did not solidify even after prolonged drying in a vacuum dessicator. Some purification of the crude material was achieved by dissolving it in chloroform and extracting the solution with 2N hydrochloric acid. The aqueous acidic phase was basified with ammonia and extracted exhaustively with chloroform. The dried ($MgSO_4$) chloroform extract left, on evaporation to dryness, a light-brown gum. The gum was treated with a few drops of 5N hydrochloric acid then triturated with a small volume of acetone. The hydrochloride salt crystallised spontaneously in large, colourless plates. Recrystallisation from methanol gave colourless, transparent prisms, m.p. 246-250° (dec.).

The free base was recovered by basification with ammonia followed by extraction with chloroform. Recrystallisation from benzene or acetone gave colourless transparent plates, R_f 0.26 m.p. 175-7° (dec.) after being dried in vacuo over P_2O_5 for 24 hours (lit.[16] m.p. 177-9°).

$(\alpha)_D^{27°}$ -206° (c, 0.40 in chloroform).

(Lit.[16] $(\alpha)_D^{14°}$ -213° (c, 0.64 in chloroform))

$\lambda_{max.}$ (Ethanol) 217 nm (log ε 4.28), 228 shoulder (4.22), 273 (4.21), 305 (3.48).

The n.m.r. spectrum in deuterated chloroform showed the following signals: δ 3.62 (3H, s, OCH_3), 6.74 (1H, s, C3-H), 7.16 - 7.42 (3H, m, C8-H, C9-H, C10-H), 8.2 - 8.4 (1H, m, C11-H).

The mass spectrum of the alkaloid showed peaks at m/e 267 (base peak and molecular ion), 266 (54%), 252 (47%), 251 (37%), 250 (23%), 238 (37%), 236 (27%), 223 (20%), 208 (11%) and 178 (14%).

The infrared absorption spectrum of the alkaloid was identical with that of an authentic sample of asimilobine. The t.l.c. behaviour of the two compounds was also identical and there was no depression of melting point in a mixed melting point determination.

Isoboldine

After removal of the solvent from sub-fraction (ii)

of fraction V the component with R_f 0.47 was obtained in the form of a dark-brown gum. Addition of a few drops of 5N hydrochloric acid and trituration with a little cold methanol afforded the crystalline hydrochloride salt. Recrystallisation of the salt from methanol gave colourless prisms, m.p. > 310° (lit.[127] m.p. > 300°).

The hydrochloride was converted to the free base and recrystallised from aqueous ethanol to give colourless plates, m.p. 122-4° (lit.[127] m.p. 120-24°), which turned light pink on prolonged exposure to air.
$\lambda_{max.}$ (Ethanol) 219 nm (log ε 4.58), 280 (4.04), 305 (4.12). The addition of methanolic KOH solution shifted the absorption maxima to longer wavelengths. The mass spectrum showed peaks at m/e 327 (molecular ion), 326 (base peak), 312, 284, 269, and 253.

The ultraviolet, infrared, and the mass spectra of the free base were identical with the respective spectra of an authentic sample of isoboldine. The two compounds showed no mixed melting point depression, and had the same R_f value (0.47) on silica in chloroform - methanol (9:1) (lit.[128] R_f 0.50).

Anolobine

Evaporation of fraction VI gave a brown gum which deposited a sparingly soluble hydrochloride on addition of

dilute aqueous hydrochloric acid solution. The hydrochloride separated as colourless needles from methanol, m.p. > 310°. The free base was liberated from the hydrochloride by treatment with ammonia, and crystallised from methanol as colourless needles, m.p. > 310°.

Found: C, 72.67; H, 5.34; N, 5.12%

Calculated for $C_{17}H_{15}O_3N$: C, 72.58; H, 5.37; N, 4.98%

$\lambda_{max.}$ (ethanol) 212 nm (log ε 4.23), 242 (4.08), 282 (4.24), 318 sh (3.68). Bathochromic shift in the presence of alkali.

$\nu_{max.}$ (Nujol) 3330 cm^{-1} (OH), 1500, 1418, 1345, 1110, 1050, and 940 cm^{-1}.

The 100 Mc/sec. n.m.r. spectrum in deuterated dimethylsulphoxide showed signals at δ 6.07 (2H, d; δ 6.15 and δ 6.0; J = 15 Hz, $-OCH_2O-$), δ 6.66 (1H, s, C3-H), δ 7.86 (1H, d; δ 7.91 and δ 7.82; J = 9 Hz, C11-H), a complex pattern at δ 2.75 - δ 3.2 (C4, C5, C6a and C7 protons), a broad peak at δ 4.30 which disappears after deuterium exchange (1H, -OH), and δ 6.81 (2H, triplet).

The mass spectrum of the alkaloid showed peaks at m/e 281 (molecular ion, 55%), 280 (base peak, 100%), 264 (2.5%), 252 (27.5%), 251 (12.5%), 222 (25%), 195 (6.3%) and 194 (17.5%).

<u>O-methylation of anolobine</u>. <u>Xylopine</u>.

A methanolic solution of anolobine (35 mg) was treated at 0° with an excess ethereal solution of diazomethane prepared from 1 g of <u>p</u>-tolylsulphonylmethylnitrosamide.[129]

After 48 hours the solvent was removed under reduced pressure to give a light-yellow solid residue. The crude product was purified by preparative t.l.c. on silica in chloroform-methanol (95:5), and was shown to be identical with authentic xylopine (O-methylanolobine) by direct comparison of their u.v. and mass spectra, and the t.l.c. behaviour of the two compounds.

N-methylation of O-methylanolobine. N-methylxylopine.

To O-methylanolobine (12 mg) was added 3 drops of a mixture of formic acid and aqueous formaldehyde (40%) (1:1). The solution was heated on a boiling water bath for 4 hours, then cooled and diluted to a convenient volume with water. The aqueous solution was basified with ammonia and extracted with chloroform. The dried ($MgSO_4$) chloroform extract was evaporated to dryness under reduced pressure to give a light-brown solid which was purified by preparative t.l.c. on silica in chloroform-methanol (95:5).

The O,N-dimethylanolobine described above was shown to be identical with N-methylxylopine (prepared from xylopine in the same manner) by comparison of their u.v., i.r. and mass spectra and t.l.c. behaviour.

Elucidation of the Structures of New Alkaloids

Aequaline. 3,9-Dihydroxy-2,10-dimethoxy tetrahydroprotoberberine

The sub-fraction (i) of fraction V, consisting essentially of the component with R_f 0.68, was evaporated to dryness under reduced pressure to give a light-brown gum which could not be induced to crystallise. The gum was treated with a few drops of 5N hydrochloric acid followed by the addition of a small quantity of ether, and allowed to stand in a few drops of methanol when the hydrochloride salt of the alkaloid crystallised in large, greyish plates. Recrystallisation from methanol afforded the alkaloid hydrochloride as colourless, transparent plates, m.p. > 280°.

Conversion of the salt to the free base, and recrystallisation from methanol gave the alkaloid as colourless, transparent prisms, m.p. 232° (dec.).

Found: C, 69.80; H, 6.43; N, 4.52%

$C_{19}H_{21}O_4N$ requires: C, 69.72; H, 6.47; N, 4.28%

$(\alpha)_D^{24}$ -283° (c, 0.24 in methanol)

$\lambda_{max.}^{EtOH}$ (neutral) 212 nm (log ε4.5), 225 shoulder (4.10), 284 (3.79).

$\lambda_{max.}^{EtOH}$ (base) 212, 246, 295 nm.

$\nu_{max.}$ (KBr disc) 3,400 cm^{-1} (OH), 3020, 2960, 2840, 1455, 1340, 1240, 1190, 1125 and 1025 cm^{-1}.

Inlet temp. 250°

M/E	R.I.
328	7.0
327	28.0
326	20.0
312	5.0
297	7.0
179	17.0
178	100.0
177	25.0
176	45.0
163	18.0

Electron Voltage 70 e.v.

M/E	R.I.
151	39.0
150	73.0
149	25.0
136	12.0
135	42.0
134	11.0
133	20.0
132	27.0
131	37.0
130	28.0
107	45.0

TABLE VIII Mass Spectrum of Aequaline.

The 100 Mc/sec. n.m.r. spectrum in deuterated dimethylsulphoxide showed signals at δ 3.74 (3H, s, OCH_3), δ 3.77 (3H, s, OCH_3) and a broad two-proton peak at δ 8.80, which disappears after deuterium exchange (2 x OH). Four aromatic protons appear at δ 6.48 (1H, s), δ 6.80 (1H, s), and at δ 6.68 (2H, s). There is half of a pair of doublets (AB quartet) centred at δ 4.02 (J = 16 Hz) (the high-field half being obscured by the methoxyl signals) which was assigned to the C8 protons. The other aliphatic protons appear as a complex pattern centred around δ 3.2.

The mass spectrum (table VIII) showed a strong molecular ion signal at m/e 327 (28%) and strong signals at m/e 326 (20%), 178 (base peak 100%), 176 (45%), 150 (73%) and 135 (42%).

Base picrate.

The alkaloid picrate was formed in ethanol as usual and was recrystallised from the same solvent to give yellow crystals, m.p. 155-6° (decomp.).

O-methylation of the alkaloid.

A methanolic solution (25 ml) of the alkaloid (60 mg) was methylated at 0° with an excess of ethereal diazomethane prepared from 2.14 g of p-tolylsulphonylmethylnitrosamide. The reaction mixture was kept in a refrigerator for seven days during which period more of the

diazomethane solution was added until tests of the reaction mixture on t.l.c. showed the presence of only one spot on exposure to iodine vapour. The solution was evaporated to dryness under reduced pressure and the light-yellow residue was recrystallised from a small volume of ethanol to give colourless needles, m.p. 141° undepressed on admixture with an authentic sample of (-)-tetrahydropalmatine.
$(\alpha)_D^{22}$ -286° (c, 1.38 in ethanol). Lit.[101] $(\alpha)_D$ -290° (c, 1.0 in ethanol) .

The ultraviolet, infrared and the mass spectra of the methylation product were identical with the respective spectra of authentic (-)-tetrahydropalmatine and the two alkaloids had the same R_f value (0.95) on silica t.l.c. in chloroform containing 2½% methanol.

Partial O-methylation of the alkaloid.

A few mls of an ethereal solution of diazomethane, prepared from p-tolysulphonylmethylnitrosamide, were added to an ice cold solution containing few mg of the alkaloid in a small volume of methanol. The reaction mixture was spotted at one-hourly intervals on silica, and the t.l. plates developed concurrently with samples of schefferine isolated from Sch. subaequalis. One of the mono-methylation products of the alkaloid had the same R_f values as schefferine (0.40 in chloroform/2% methanol and 0.75 in chloroform/5% methanol).

Schefferine. 9-Hydroxy-2,3,10-trimethoxy tetrahydroprotoberberine

The second series of fractions obtained from column chromatography of fraction I were combined and evaporated to dryness under reduced pressure. The alkaloid was purified as its crystalline hydrochloride salt by recrystallisation from methanol to give colourless needles, m.p. 193-5° (dec.).

The hydrochloride salt was converted to the free base and recrystallised several times from methanol or aqueous ethanol to give the new alkaloid as colourless prisms, m.p. 176°, R_f 0.90.

Found: C, 70.15; H, 7.04; N, 4.29%

$C_{20}H_{23}O_4N$ requires C, 70.35; H, 6.79; N, 4.10%

$[\alpha]_D^{22}$ -303 (c, 1.14 in ethanol)

$\lambda_{max.}^{EtOH}$(neutral) 220 nm (log ε 4.18), 282 (3.84)

$\lambda_{max.}^{EtOH}$(base) 235, 287 nm.

$\nu_{max.}$(Nujol) 3550 cm^{-1} (OH), 1610, 1590, 1340, 1260, 1190, 1130, 1040, 1026, 780 cm^{-1}.

The n.m.r. spectrum in deuterated dimethylsulphoxide showed signals at δ 3.68 (6H, s, 2 x OCH_3), δ 3.73 (3H, s, OCH_3), δ 6.67, δ 6.71, δ 6.85, and δ 9.02 (1H, s, OH).

The mass spectrum (table IX) showed peaks at m/e 341

Inlet temp. 250°

M/E	R.I.
341	11.0
340	9.3
193	11.9
192	100.0
191	9.5
190	31.0
176	19.0
151	19.2
150	40.0
149	50.0
135	78.0

Electron Voltage 70 e.v.

M/E	R.I.
120	14.3
121	26.2
107	46.4
105	13.1
104	15.5
103	13.1
91	35.7
89	11.9
79	29.7
78	23.8
77	73.8

TABLE IX Mass Spectrum of Schefferine.

(molecular ion, 11%), m/e 340 (9.3%), 192 (base peak, 100%), 190 (31%), 176 (19%), 150 (40%), 149 (50%) and 135 (78%).

O-methylation of the alkaloid.

To an ice-cold methanolic solution of the alkaloid (74 mg in 10 ml) was added ethereal diazomethane (from 2.14 g of p-tolylsulphonylmethylnitrosamide) and the solution was set aside in a refrigerator. A further addition of diazomethane was made after 24 hours and the solution was allowed to stand overnight in a refrigerator. Evaporation of the solution to dryness left a crystalline residue which on recrystallisation from a small volume of ethanol afforded the methylated base as colourless needles, m.p. 141° alone or in admixture with an authentic sample of (-)-tetrahydropalmatine. The ultraviolet, infrared and the mass spectra of the methylation product were identical with the respective spectra of authentic (-)-tetrahydropalmatine. The two compounds had the same R_f value on silica t.l.c.

Alkaloid "Y"

The dark viscous residue (B) which contained the material not extracted from chloroform solution by 2N sulphuric acid (cf. p.87) was introduced on a column of B.D.H. alumina prepared in chloroform. The fraction eluted by chloroform was collected, and the solvent removed under reduced pressure to give a small amount of a colourless semicrystalline neutral compound which was not further investigated.

The second fraction eluted by chloroform containing 5% methanol was shown to be homogeneous by the appearance of a single spot of R_f 0.86 in t.l.c.

Further elution yielded an appreciable quantity of intractable gum which gave a negative Mayer's test.

The fraction containing the compound with R_f 0.86 was evaporated to dryness, and the residue recrystallised several times from methanol to give alkaloid "Y" as pale yellow needles, m.p. 257-8° after drying for 24 hours in vacuo over P_2O_5.

Found: C, 73.26; H, 4.82; N, 5.13%

$C_{17}H_{13}O_3N$ requires C, 73.11; H, 4.66; N, 5.03%

λ_{max} (ethanol) 210 nm (log ε 4.41), 232 (4.52), 264 (4.46), 277 (4.51), 320 infl. (3.93), 388 (3.88).

$\nu_{max.}$ (Nujol) 3450 (broad), 1720, 1665 cm^{-1}.

The 100 Mc/sec. n.m.r. spectrum in deuterated chloroform showed signals at δ 4.05 (3H, s, OCH_3), δ 4.10 (3H, s, OCH_3), δ 7.06 (1H, s), δ 7.55 (2H, d, J = 10 Hz), δ 7.80 (2H, s), δ 9.10 (1H, s) and δ 10.73 (1 x OH or NH). The mass spectrum (70 e.v.) showed peaks at m/e 279 (parent, base peak), 264 (43%), 236 (43%), 221 (46%), 218 (58%), 209 (75%), 208 (50%) and 181 (70%).

Extraction of the Leaves

Dried, crushed leaves (14 kg) were extracted by continuous percolation with methanol at room temperature. The methanol extract was concentrated to a syrupy consistency, diluted with water (3 x volume) and acidified with 2N sulphuric acid. The aqueous acidic solution was allowed to stand for several days. The dark resinous sediment which formed on standing was filtered off. The clear filtrate was shaken with several portions of petroleum ether 60-80°/ether mixture then basified with ammonia and exhaustively extracted with chloroform. The washed chloroform extract was concentrated to a smaller volume and the bases extracted into dilute sulphuric acid.

The aqueous acidic extract was basified with ammonia and extracted with chloroform. The chloroform extract was washed, dried ($MgSO_4$), and the solvent removed under reduced pressure to give a black syrupy residue.

The crude residue was taken up in chloroform, and the solution extracted with 2N hydrochloric acid. The aqueous acidic phase was shaken with several portions of ether, then basified with ammonia and re-extracted with chloroform. The chloroform extract was washed with several portions of water, and dried ($MgSO_4$).

Evaporation of the chloroform extract to dryness

afforded the crude alkaloid mixture as a light brown foam (4.4 g).

Separation of the Individual Alkaloids

The crude alkaloids (4.4 g) were chromatographed on neutral alumina activity grade II/III (200 g) in chloroform followed by chloroform containing 5% methanol. The eluant was collected in 25 ml aliquots which were examined by t.l.c. and combined according to their composition to give five final fractions.

Fraction	Solvent	Tube No	R_f values*
I	$CHCl_3$	1 - 5	0.90; 0.49 (trace)
II	$CHCl_3$-MeOH (95:5)	6 - 15	0.49; 0.26; 0.90 (trace)
III	"	16 - 19	0.26; 0.49
IV	"	20 - 25	0.68; 0.47
V	"	26 - 60	0.47; 0.21

* The R_f values of the most prominent spots are underlined.

Identification of the Leaf Alkaloids

Schefferine

Fraction I was chromatographed on a column of silica in chloroform - methanol (95:5). A series of rapidly eluted fractions were shown to consist essentially of schefferine (yellow spot, R_f 0.90). These fractions were combined, evaporated to dryness and the residue crystallised from aqueous ethanol to give colourless prisms of the alkaloid, m.p. 176° identical with schefferine isolated from the bark (mixed m.p., i.r. spectra).

Anonaine

Fraction II was chromatographed on a column of silica. Chloroform - methanol (95:5) eluted a component which on t.l.c. showed a single yellow spot (R_f 0.49) gradually fading to green on exposure to atmosphere. The gum obtained on removal of the solvent was purified as its crystalline hydrochloride, m.p. 267° (dec.), identical with the hydrochloride salt of anonaine from the bark (t.l.c., i.r.). The hydrochloride salt was converted to the free base, and identified by mixed melting point determination and by comparison of its i.r. spectrum with that of anonaine isolated from the bark.

Asimilobine

Fraction III was chromatographed as fraction II. The first component eluted was asimilobine as shown by t.l.c. (brown spot changing to purple, R_f 0.26). Evaporation of the eluate to dryness gave the crude alkaloid as a foam which was purified through its crystalline hydrochloride. The recovered free base was recrystallised from acetone to give colourless plates, m.p. 175-6° (dec.) identical with asimilobine isolated from the bark (mixed m.p., u-v., i.r.).

Aequaline

Fraction IV was chromatographed on a silica column with chloroform - methanol (95:5) as eluant. After rechromatography of an intermediate fraction, two final fractions containing the components with R_f 0.68 and R_f 0.47 respectively were obtained.

The fraction containing the component with R_f 0.68 was taken to dryness and the residue crystallised from methanol to give colourless prisms, m.p. 230-32° (dec.) identical with aequaline isolated from the bark (mixed m.p., u.v., i.r.).

Isoboldine

Removal of solvent from the fraction containing the component with R_f 0.47 yielded the alkaloid as a pink,

impure solid. It was purified *via* its crystalline hydrochloride salt, reconverted to the free base and recrystallised from aqueous ethanol to give colourless plates, m.p. 122-4° undepressed on admixture with isoboldine isolated from the bark. The identification was confirmed by the identity of their i.r. and mass spectra.

Anolobine

Fraction V was chromatographed on a column of silica with chloroform containing 2½% methanol. The first small fractions contained isoboldine. After isoboldine had been eluted from the column, the next series of fractions consisted of a single component with R_f 0.21. Removal of solvent from the combined fractions gave only a very small amount of dark, impure residue. Addition of a few drops of 5N hydrochloric acid followed by trituration with a little ether gave a discoloured semicrystalline hydrochloride salt which was removed by filtration. After recrystallisation from methanol the hydrochloride salt was decomposed by the addition of ammonia and the free base recovered by extraction with chloroform. The purified base then crystallised readily from methanol to give the alkaloid as colourless needles, m.p. > 310°. Its i.r. spectrum was identical with that of anolobine isolated from the bark.

B. THE CONSTITUENTS OF BOLETUS SPECIES

Extraction of the Mushrooms

Air-dried mushrooms (1,276 g) were finely milled, and percolated with methanol at room temperature. The light-brown percolate (54 litres) was concentrated below 40° in a climbing-film evaporator to ca 2 litres. A colourless solid that separated from the concentrate was collected by filtration. Another crop of this solid was obtained by allowing the methanolic concentrate to cool overnight in a refrigerator.

The filtered methanolic mushroom concentrate was evaporated under reduced pressure, the syrupy residue taken up in water (1 litre), and exhaustively extracted with petroleum ether (b.p. 40-60°).

The aqueous phase was evaporated under reduced pressure as far as possible to give a viscous syrup (163 g) which did not solidify even after prolonged drying in a vacuum dessicator (A).

The petroleum ether extract was washed with several small portions of water, then dried. Removal of the solvent gave a dark-yellow gum (6.5 g) (B).

Fractionation of the Amino Acids

25 G of crude extract (A) was dissolved in the minimum amount of water, and the pH of the solution adjusted to approx. 5 by careful addition of dilute hydrochloric acid. Some black, amorphous material suspended in the solution was removed by filtration, and the filtrate introduced on a column (4.5 x 32 cm) of ion exchange resin "Zeo-Karb 225" in the hydrogen form. Water was passed through the column until the effluent was neutral to litmus, and gave a negative test for the presence of chloride ions. Evaporation of the effluent gave a yellow-brown syrup (14 g) which showed on paper chromatograms only a few, very weak ninhydrin-positive spots.

The material remaining adsorbed on the column was eluted with 2N ammonia until the eluent no longer showed presence of ninhydrin-positive compounds. The eluate was evaporated to dryness, and the solid residue dried in a vacuum dessicator. Yield 7.2 g. Two-dimensional paper chromatogram of the material indicated the presence of at least 12 ninhydrin-positive components.

A concentrated solution of the amino acid mixture was streaked under the flow of warm air onto 36 sheets of Whatman No 3MM chromatographic paper (46 x 57 cm). The papers were developed for 24 hours, then dried in air. The

amino acids were located by the use of narrow test strips cut from the main sheets, and developed with ninhydrin reagent. Five, more or less overlapping, zones were marked off and numbered from 1 to 5 starting from the top of the papers. These zones were cut from the papers, and the corresponding strips of each zone were extracted thoroughly with warm water. The aqueous extracts were separately concentrated to volumes of ca 10 ml each, and their compositions examined by analytical paper chromatography.

Zone No.	R_f values of the components
1.	0.80; 0.76 (trace)
2.	0.80 (trace); 0.76; 0.66; 0.64 (v. weak)
3.	0.64; 0.60 (v. weak); 0.55; 0.47
4.	0.47 (trace);0.43; 0.37
5.	0.31; 0.26; 0.22

Zones 2 and 3 were separately re-chromatographed, and the fractions with similar composition were combined to give seven final fractions. After concentrating, each fraction was decolourised with charcoal and evaporated to dryness to yield nearly colourless solid residues.

Fraction	Weight (g)	R_f values of the components
I	1.8	0.80; 0.76 (trace)
II	2.1	0.76; 0.66 (trace)
III	0.6	0.66
IV	0.3	0.64; 0.60 (trace); 0.55
V	1.1	0.47
VI	0.5	0.47 (trace); 0.43; 0.37
VII	0.2	0.31; 0.26; 0.22

Identification of the Amino Acids

Leucine and Isoleucine

Fraction II was purified by recrystallisation from aqueous ethanol until it showed only a single spot (R_f 0.76) on paper chromatograms. Literature[130] gives R_f values of 0.76 for isoleucine, and 0.78 for leucine.

The material (10 mg) was esterified with thionyl chloride - ethanol, and the excess reagent and solvent removed. The residue was redissolved in ethanol (50 µl), and the solution buffered to pH 8 by the addition of ion exchange beads (Biorad: AG1-X8 in bicarbonate form). Pivaldehyde (10 µl) was then added, and the reaction mixture allowed to stand for 30 min. over molecular sieve(3A).

An aliquot (3 µl) of the solution was injected into the GLC-MS system programmed from 80° at the rate of 8°/min.

The derivatised material showed only one elution peak in the g.l.c. with retention time of 14.9 minutes.

The mass spectrum showed characteristic peaks at m/e 212 (M - 15), 170 (M - 57), and (M - 73).

Analysis of a sample of fraction II on an automatic amino acid analyser showed two distinct elution peaks, which occurred in the positions characteristic of leucine and isoleucine respectively.

Valine

Several recrystallisations of fraction III from small volumes of aqueous ethanol gave the amino acid as colourless plates. Paper chromatograms of the amino acid separately or in admixture with authentic valine showed a single spot R_f 0.66 (lit.[130] valine R_f 0.67).

N-pivaldehyde derivative of the amino acid ethyl ester was prepared and injected into the GLC-MS system as described earlier. The retention time for the derivatised amino acid was 13.2 minutes.

The mass spectrum of the derivative showed characteristic peaks at m/e 198 (M - 15), 156 (M - 57), and m/e 140 (M - 73).

The mass spectrum of the free amino acid was identical

with that of valine, and showed characteristic peaks at m/e 118 (M + 1), 74, 72, 45, 43 and m/e 29.

Methionine and Tyrosine

Fraction IV was dissolved in hot water, the solution decolourised with charcoal, and filtered. The filtrate was concentrated to a small volume, and cooled in a refrigerator. Ethanol was added to the concentrate to the point of precipitation and the solution was allowed to stand at room temperature. The amorphous solid which precipitated from the solution was collected by filtration, and dried in a vacuum dessicator.

Paper chromatography of the material showed two spots with R_f 0.64 and R_f 0.55 respectively. Literature[130] gives R_f 0.65 for methionine and R_f 0.55 for tyrosine.

The amino acid mixture was derivatised and injected into the GLC-MS system as described earlier. Only one component of the mixture was eluted from the column. The retention time of this component was 20.8 minutes, and its mass spectrum showed characteristic peaks at m/e 245 (M^+), 188 and m/e 172.

A sample of the material from fraction IV was analysed on an automatic amino acid analyser. Two elution peaks were obtained, and their positions in the chromatogram were

characteristic of the positions of methionine and tyrosine respectively. The relative heights of the two elution peaks indicated that methionine was themajor component in fraction IV.

Alanine

Fraction V was recrystallised from aqueous ethanol to give the amino acid as colourless needles, R_f 0.47 (lit.[130] alanine R_f 0.49).

Paper chromatography of the amino acid separately or in admixture with authentic alanine showed in eachcase a single spot (R_f 0.47).

The amino acid was derivatised and injected into the GLC-MS system as described earlier. The retention time of the compound was 11.0 min., and its mass spectrum showed characteristic peaks at m/e 185 (M^+), 170, 128 and m/e 112.

Threonine and Glycine

A solution of fraction VI in hot water was decolourised with charcoal and then concentrated to a small volume. The concentrate was treated with ethanol until cloudiness occurred, then allowed to stand in a refrigerator overnight. The colourless semi-crystalline precipitate was collected by filtration, and dried in a vacuum dessicator.

Paper chromatography of the material showed two spots

with R_f 0.43 and R_f 0.37 respectively. Literature[130] gives R_f 0.44 for threonine and R_f 0.39 for glycine.

A sample of fraction VI was derivatised and injected into the GLC-MS system as described earlier. The retention times of the compounds responsible for the peaks were 11.6 min., and 15.8 min. respectively.

The mass spectrum of the component with the retention time 11.6 min. showed characteristic peaks at m/e 156, 114, and 98.

The component with the retention time 15.8 min. showed a characteristic peak at m/e 142 in its mass spectrum.

Arginine, Lysine, Histidine

Fraction VII was dissolved in hot water, the solution treated with a small amount of charcoal, and filtered. The filtrate was evaporated to dryness and the nearly colourless solid residue dried in a vacuum dessicator.

One-dimensional paper chromatography of the material showed three spots with R_f values 0.31, 0.26, and 0.22 respectively. Literature[130] gives R_f values 0.37 for arginine, 0.29 for lysine, and 0.28 for histidine.

Two dimensional paper chromatography using the solvent system butanol - acetic acid - water (2:1:1) for the first

development, and the lower phase from a mixture of phenol and water for the second development was carried out with:

(a) a sample from fraction VII

(b) a reference sample made up of equimolar amounts of arginine, lysine, and histidine

(c) a mixture of (a) and (b).

The chromatograms showed identical distribution patterns of spots:

in first dimension: R_f 0.31 (arginine); R_f 0.26 (lysine); R_f 0.22 (histidine)

in second dimension: R_f 0.56 (histidine); R_f 0.52 (arginine); R_f 0.43 (lysine).

Analysis of a sample of fraction VII on an automatic amino acid analyser showed three elution peaks. The positions of the peaks in the chromatogram were characteristic of the positions of peaks shown by authentic samples of arginine, lysine, and histidine respectively. The relative heights of the peaks indicated that arginine was the major constituent of the mixture. Lysine and histidine were present only in small amounts.

Elucidation of the Structure of a New Unsaturated α - Amino Acid

L-2-Amino-4-methylhex-5-enoic Acid

Fraction I, which consisted essentially of a single component (R_f 0.80), and giving a blue-purple colour with ninhydrin, was crystallised from a concentrated aqueous solution by the addition of ethanol in the cold. Two more recrystallisations in the same manner gave 58 mg amino acid as shiny, colourless plates, m.p. 240-2° (dec.).

$(\alpha)_D^{28}$ - 9.6° (c, 1.777 in water)

$(\alpha)_D^{28}$ +5.7° (c, 0.7 in 1N HCl)

Found: C, 58.49; H, 9.22; N, 9.58%

$C_7H_{13}O_2N$ requires C, 58.71; H, 9.15; N, 9.78%

Catalytic hydrogenation: found 15.35 ml

Calculated for one double bond 15.65 ml

Ozonolysis of a suspension of the amino acid in CS_2 gave formaldehyde.

ν_{max} (Nujol, weak mull), 2920, 2850, 1605, 1580, 1515, 1460, 1380, 1360, 1335, 995, 920, 860, 835 and 775 cm^{-1}.

The 100 Mc/sec n.m.r. spectrum in deuterium oxide showed signals at δ 1.52 (3H, d, CH_3), δ 2.3 (2H, m), a complex pattern centred around δ 2.75 (1H), δ 4.14 (1H, pair of doublets), δ 5.56 (2H, m) and δ 6.22 (1H, m).

The mass spectrum of the amino acid showed peaks at m/e 143 (M^+, 0.8%), 110 (4.3%), 98 (100%, base peak), 96 (33.3%), 81 (53.3%), 74 (73.3%), 69 (43.3%) and 55 (96%).

Paper electrophoresis

Samples (0.5 µl) of the amino acid solution were spotted on Whatman No. 3MM paper strips (3 x 34 cm) impregnated with the respective electrolyte, and equilibrated for 15 min. by enclosure within the electrophoresis chamber. Electrolysis was allowed to proceed at about 16 V/cm for periods of 2 to 3 hours, depending on the electrolyte used. The paper strips were subsequently dried in the oven at 100° and the amino acid located with ninhydrin reagent.

Electrolyte	pH	duration	migration	direction
5N acetic acid	1.7	2 hours	44 mm	to cathode
KH-phthalate[131]	5.9	3 hours	6 mm	to cathode
$NaHCO_3/Na_2CO_3$[132]	9.2	2 hours	32 mm	to anode

The Cu^{++} complex of the amino acid.

To a solution of 2 mg of the amino acid in 2 ml of water a few drops of copper sulphate solution buffered with ammonium acetate were added. After slight warming, and allowing the solution to stand for several minutes, a deep blue crystalline precipitate was formed.

N-neopentylidene-L-2-amino-4-methylhex-5-enoic acid ethyl ester.

The amino acid (10 mg) was esterified with thionyl chloride - ethanol (Brenner and Huber)[107], and the excess reagent and solvent removed. The residue was redissolved in ethanol (50 µl), and the solution buffered to pH 8 by the addition of ion exchange beads (Biorad: AG1-X8 in bicarbonate form). Pivaldehyde (2,2-dimethylpropanal) (10 µl) was then added, and the reaction mixture allowed to stand over a few pieces of molecular sieve 3A for 30 minutes.

A sample of the solution (3 µl) was injected into the GLC-MS system programmed from 100° at a rate of 6°/min., The retention time of the amino acid derivative was 7.5 min. The mass spectrum showed peaks at m/e 240 ($M^+ + 1$), 224, 210, 196, 182, 166, 156, 126, 112, 101, 98 and m/e 81.

Stereochemical analysis by g.l.c.

N-Trifluoroacetyl -(DL)-prolyl chloride reagent containing 1 mmole of N-TFA-(DL)-prolyl chloride in 10 ml was prepared according to the method described by Pereira and Halpern[133] from a mixture containing 9 parts of L-proline and 1 part of D-proline.

The amino acid (14.3 mg; 0.1 mmole) was esterified with

thionyl chloride - methanol, and the excess reagent and solvent removed.

An excess of N-TFA-(DL)-prolyl chloride reagent (1.2 ml; 0.12 mmole) was added to the amino acid methyl ester hydrochloride, and the mixture adjusted to pH 9 with triethylamine. The reaction mixture was allowed to stand for 15 min., then diluted with dichloromethane to a convenient volume. The solution was washed successively with water, 20% citric acid solution, water, saturated sodium bicarbonate solution, and water. The dried ($MgSO_4$) organic layer was evaporated to dryness, the residue taken up in ethyl acetate (1 ml), and the solution injected into the gas chromatograph.

The chromatogram showed two distinct elution peaks with peak heights in the ratio 9:1. The retention time for the larger peak was longer than that for the smaller peak. As the larger peak must be due to L-prolyl-amino acid and the fact that it has a longer retention time than the smaller peak, which must be due to the D-prolyl-amino acid, indicates that the amino acid must have the L configuration at the alpha asymmetric centre.

Isolation and Identification of the Water-insoluble Compounds

Examination of the petroleum ether soluble fraction (B) by t.l.c. on silica showed that it was a complex mixture. One component, later identified as ergosterol, constituted the bulk of the material. Three constituents of the mixture gave well defined colours on t.l.c. plates sprayed with conc. sulphuric acid followed by heating for several minutes at 120°.

The crude material (6.5 g) was chromatographed on a column of silica (300 g) in benzene containing 5% acetone, and the eluent collected in 25 ml fractions. Some very dark material retained at the top of the column was not eluted.

Eluent fractions which showed identical t.l.c. spot patterns were combined to give five final chromatographic fractions. These were labelled from I to V in their order of emergence from the column.

Fraction	Ml collected	Composition according to t.l.c.
I	200	Intractable; yellow colouration at solvent front; pronounced streaking
II	250	one component; orange-brown spot R_f 0.96
III	300	one component; blue spot R_f 0.38
IV	575	two components; blue spot R_f 0.38 and blue spot R_f 0.12
V	900	one major component; blue spot R_f 0.12, traces of several components with fluorescent spots of very low R_f values.

Fraction IV was re-chromatographed on a silica column with benzene - acetone (9:1), and the individual components with R_f 0.38 and R_f 0.12 were added to fractions III and V respectively. Each fraction was then evaporated separately under reduced pressure, and the residues were dried in a vacuum dessicator.

Fraction I (ca 400 mg) was obtained as a brown gum which was extremely soluble in alcohol, chloroform, benzene, and petroleum ether. Attempts to resolve this fraction into individual components by chromatography in several solvent systems failed, and the material was not further investigated.

Compound with R_f 0.96

Fraction II after several recrystallisations from methanol-benzene gave the compound as colourless plates

(120 mg), m.p. 109-10°.

The ultraviolet spectrum of the compound was superimposable on that of ergosterol.

$\nu_{max.}$ (Nujol) 1740 (>C=O), 1220, and 1175 cm^{-1}

The mass spectrum showed peaks at m/e 380 (M^+), 378, 363, 253, 237, 213, 199, 185, 171, 157, 143, 129, 98, 73, 69, 60, 57, 55, 44 and 43.

Liebermann-Burchard test[122]: positive; strong green colour.

Salkowski test[122]: positive; yellow to yellowish-green colour.

Rosenheim test[123]: positive; red colour turning to blue.

Tortelli-Joffe test[122]: negative, no colour development.

Ergosterol

Fraction III was purified by chromatography on silica column in benzene - acetone (95:5) to give a colourless semi-crystalline compound, R_f 0.38. Several recrystallisations from 95% ethanol yielded colourless plates (2.44 g), m.p. 158° (lit.[134] m.p. 160-3°).

$(\alpha)_D^{24}$ -129.3° (c, 0.99 in chloroform). (Lit.[124] $(\alpha)_D$ -130°).

Found: C, 81.28; H, 10.89; O, 7.1%

Calculated for $C_{28}H_{44}O.H_2O$: C, 81.11; H, 11.19; O, 7.73%

$\lambda_{max.}$ (Ethanol) 262 nm (shoulder), 270, 282 and 293 nm.

$\nu_{max.}$ (Nujol) 3,400 (OH), 2950, 2870, 1660 (Δ^{22} C=C stretch), 1610, 1160, 1060, (C-O stretch in Δ^5 C=C, C3 equatorial OH in steroid alcohols), 1040 (OH, A/B trans C3 equatorial), 970

(Δ^{22} CH bending), 835 and 800 (Δ^{5} C=C bending) cm^{-1}. The mass spectrum showed a molecular ion peak at m/e 396 and peaks at m/e 363, 261 and 253.

The ultraviolet, infrared, and the mass spectra of the compound were identical with the respective spectra of authentic ergosterol. The two compounds showed no mixed m.p. depression and had the same R_f value on silica in benzene containing 5% acetone (R_f 0.38), and on alumina in benzene containing 10% acetone (R_f 0.47).

Ergosteryl acetate

The compound (58 mg) was dissolved in dry pyridine (1 ml), and freshly distilled acetic anhydride (1 ml) was added. The solution was warmed on a water bath for 2 hours and then left to stand overnight at room temperature. The reaction mixture was taken to dryness under reduced pressure, and the colourless crystalline residue was re-crystallised from ethanol to give colourless plates (47 mg), m.p. 163-5° undepressed by mixing with a sample similarly prepared from authentic ergosterol.

Found: C, 82.44; H, 10.84; O, 7.6%

Calculated for $C_{30}H_{46}O_2$: C, 82.14; H, 10.57; O, 7.3%

Their identity was confirmed by i.r., u.v., mass spectra comparison, and by t.l.c.

Compound with R_f 0.12

Fraction V was purified by preparative t.l.c. on silica developed with benzene - acetone (9:1). Extraction of the appropriate zone yielded a colourless compound (84 mg), which was recrystallised from methanol as colourless needles, m.p. 179-80°.

Found: C, 78.39; H, 10.34; O, 11.8%

Calculated for $C_{28}H_{44}O_3$: C, 78.45; H, 10.35; O, 11.20%

$\lambda_{max.}$ (Ethanol) 220 nm.

$\nu_{max.}$ (Nujol) 3540, 3410 (OH), 1660 (Δ^{22} C=C stretch), 1045 (3β -OH), 970 (Δ^{22} CH bending) cm^{-1}. Also absorption bands at 1155, 1080, 940, and 860 cm^{-1}.

The mass spectrum (70 e.v.) of the compound showed a weak molecular ion peak at m/e 428 and peaks at m/e 410, 392, 305, 304, 303, 251, 81, 69, 57, 55, 43.

Liebermann-Burchard test: positive; strong green colour.

Tortelli-Joffe test: negative; no colour development.

Identification of Mannitol

The colourless solid obtained on concentrating the methanolic mushroom extract was recrystallised several times from aqueous ethanol to give fine, colourless needles, m.p. 166° not depressed by mixing with a sample of authentic

mannitol. Paper chromatography in butanol-acetic acid-water (2:1:1) of the compound alone, or in admixture with authentic mannitol gave a single spot with aniline hydrogen phthalate reagent[135], R_f 0.36 (lit.[130] mannitol R_f 0.39).

Acetylation

A mixture of the compound (50 mg), dry pyridine (1 ml), and acetic anhydride was refluxed on a water bath for 5 minutes. The solution was cooled to room temperature, and 3-4 ml of ice water was added. The semi-crystalline acetyl derivative was filtered off, washed with cold 2% hydrochloric acid, then washed with water. Recrystallisation from aqueous methanol yielded colourless needles, m.p. 120° undepressed by mixing with a sample of mannitol hexaacetate (m.p. 119-20°)[136] prepared similarly from authentic mannitol.

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