Natural and Synthetic Bis-indole Alkaloids as Cytotoxic Agents

by

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INTRODUCTION

Cancer has increased in its affliction of the human population since the turn of the century. As a consequence there has occurred in the past decade a tremendous surge of work in the scientific field to detect and combat the disease. The result of this has been the development of several medical techniques of which surgery, immunotherapy, and chemotherapy have seen increased study. However, what compounds the problem of research in this field is that cancer is not just one disease, but a collection of over 100 forms and each must be studied separately to find the optimum therapeutic treatment. Of these, the chemotherapy approach has attracted a major portion of interest since 1941 when the first anti-neoplastic compound, estrone, was used in treatment of prostate cancer in men.[1]

The aim of chemotherapy is to destroy or inhibit selectively all the rapidly multiplying malignant cells wherever they occur. Since total inhibition would lead to destruction of normal cells and eventual death of the organism, selective inhibition is an essential criterion in this work. Thus, the range of specificity is a narrow one and the struggle for a cure becomes increasingly more difficult.

To date, over 450,000 compounds have been tested for

their anti-tumor activity by the National Cancer Institute.[2] As a direct result of this testing about 45 anticancer drugs are in medical use and 40 are in preclinical trials. Compounds for testing originate from various sources depending on the method used to find an active structure. Two successfully used methods include using structure - activity correlations and isolation of compounds from natural sources.

The structure - activity[3,4] approach has seen wide attention because of the relative ease of creating a range of compounds of structural similarity, testing them for activity, and attempting to make some correlation between structure and degree of anti-tumor effect. Eventually it is hoped that all information gained from this approach would lead to compounds of superior activity. The success of this method depends on a good guide or rationale for planning the structure of an effective cytotoxic agent.

Alternatively, in the field of natural products, active anti-tumor compounds are isolated from terrestrial and marine plants and animals.[5] Much of the success in this method must be attributed to the National Cancer Institute in conjunction with the U.S. Department of Agriculture who discovered that numerous plants possess anti-tumor activity.[6] They initiated research in the procurement of plant samples, fractionation, and isolation of active plant constituents and eventual structure

determination.

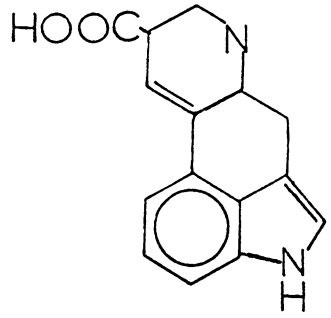
The research contributed and described here concerns, for the most part, the isolation and identification of new alkaloidal compounds from the plant Tabernaemontana johnstonii. This plant, which originates from Kenya, Africa, was initially screened by the National Cancer Institute and was found to possess some anticancer activity. This investigation was directed toward determining the area of highest activity and the eventual isolation of an active compound. In conjunction with the results of this study, additional work was done using a specific structure - activity approach to, hopefully, improve upon the activity of the natural material.

HISTORICAL

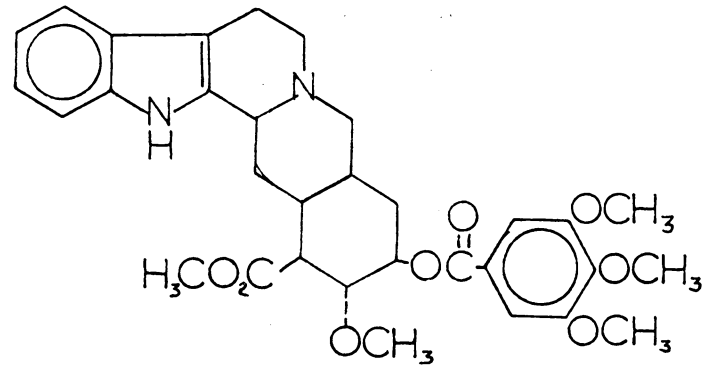
A. Origin of Indole Alkaloids

The name alkaloid is usually applied to basic, nitrogen containing heterocyclic ring compounds of natural origin. Usually they have complex molecular structures and often manifest significant pharmacological activity. Morphine, in 1805, was the first alkaloid isolated, and today a little over two thousand alkaloids are known and it is estimated they are present in 10 - 15% of vascular plants. Their function in plants, if any, is largely unknown. They have been regarded for the most part as by-products of plant metabolism, possibly used as protective agents against attack by insects or disease and maybe even reservoirs for protein synthesis.

Among the alkaloids there exist many types, classified according to particular structural similarities. The indole alkaloids are one type out of 15 - 20 groups that have been isolated and whose pharmacological properties have been studied. Probably the largest impetus to the study of indole alkaloids evolved from the discovery of lysergic acid amides[7] (1) (isolated from *Claviceps* Spp.) and reserpine[8] (2) (from *Rauwolfia serpentina*) both of which showed remarkable pharmacological activity.

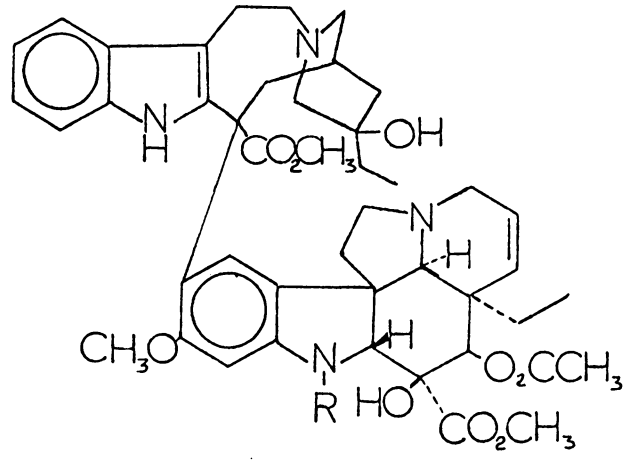


1



2

With regard to cytotoxic and anti-tumor activity, the two most significant isolations were the vinca alkaloids vinblastine (3) and vincristine[9] (4) which are now used in the treatment of Hodgkin's disease and acute leukemia, respectively.

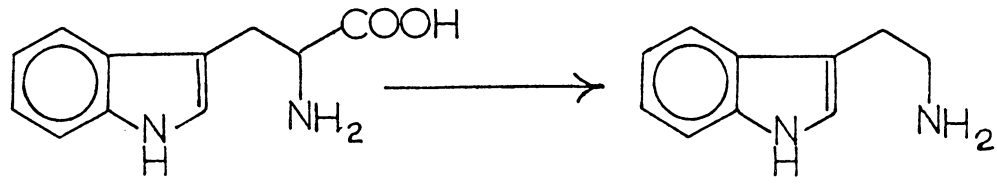


3 R= CH_3

4 R= CHO

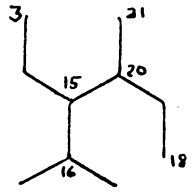
These compounds belong to the class of indole-indoline dimeric alkaloids and have been shown through subsequent isolation work to carry the highest cytotoxic activity of indole alkaloids so far. They represent what are generally called bis-indole alkaloids, one of the newer groups to be isolated and characterized, and which have received paramount interest because of their specific pharmacological properties.

Before discussing the types of alkaloids that have been found it is important to briefly review the biosynthetic pathways linking the formation of each alkaloid type and show how they are interrelated. In most indole alkaloids, the formation and origin of the indole portion has not been of major concern. It has been traced to the amino acid tryptophan (17) which is converted to tryptamine (18) and then condensed with some terpenoid or non-tryptamine portion.[10] The origin of the terpenoid portions, containing nine or ten skeletal atoms, has, on the contrary, been given much more attention.

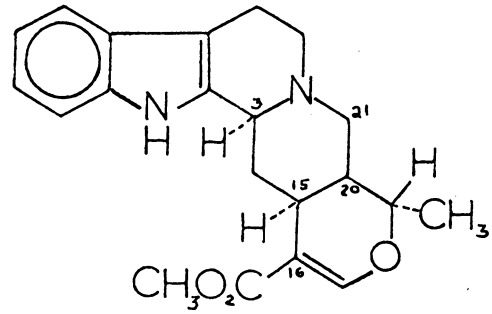


17 18

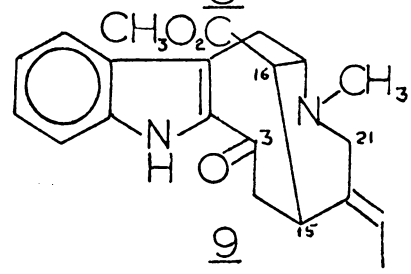
Despite the bewildering variety of different structures, there are three types of C_9-C_{10} units that are of primary concern because of their abundance in plants. The corynanthe-strychnos type (5) is exemplified by ajmalicine (8) or vobasine (9); the iboga type is isolated frequently in Tabernaemontana, voacangine (10) being an example; and the aspidosperma type (7) is represented by vindoline (11). With the use of ^{14}C and ^{3}H labeling it was shown that all three types of C_9-C_{10} units (5), (6), and (7) are monoterpenoid in origin. Two mevalonate residues (12) are joined head to tail to produce the common geraniol intermediate (13).[11] Thomas and Wenkert[12,13] proposed that some type of intermediate cyclopentane monoterpene was formed, which by cleavage of the indicated single bond generated (5) (Scheme I). It was further recognized that unit (5) is structurally related to (6) and (7), and can be mentally transferred into these other types. The aspidosperma (7) and iboga (6) families are thus often referred to as rearranged systems.[14] This proposal was later verified by experiment with the discovery that the cyclopentane monoterpene was loganin (14).[15] When fed to Vinca rosea shoots with ^{14}C and ^{3}H labels, four common indole alkaloids were isolated and through degradation found to have the radioactivity at the expected sites (Scheme II).[16,17] These experiments rigorously confirmed that loganin is a specific precursor of all three types


3
21
15
20
18
16

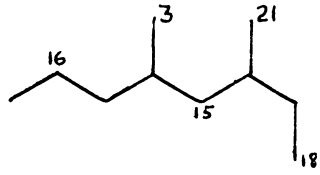
5


N
H
3
N
21
H
H
15
20
H
CH3
H
CH3O2C
16
O

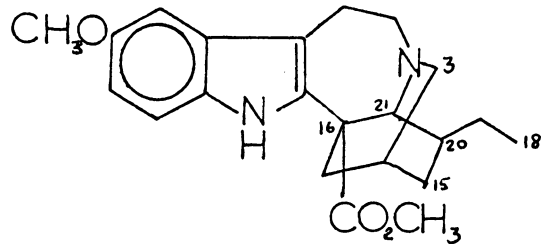
8


CH3O2C
16
CH3
N
N
H
3
O
21
15

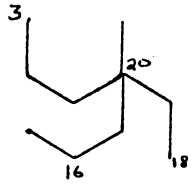
9


3
21
16
15
18

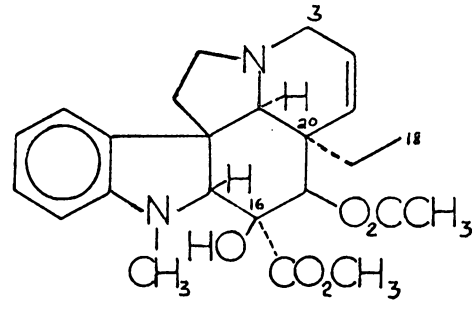
6


CH3O
N
3
N
H
21
16
20
18
15
CO2CH3

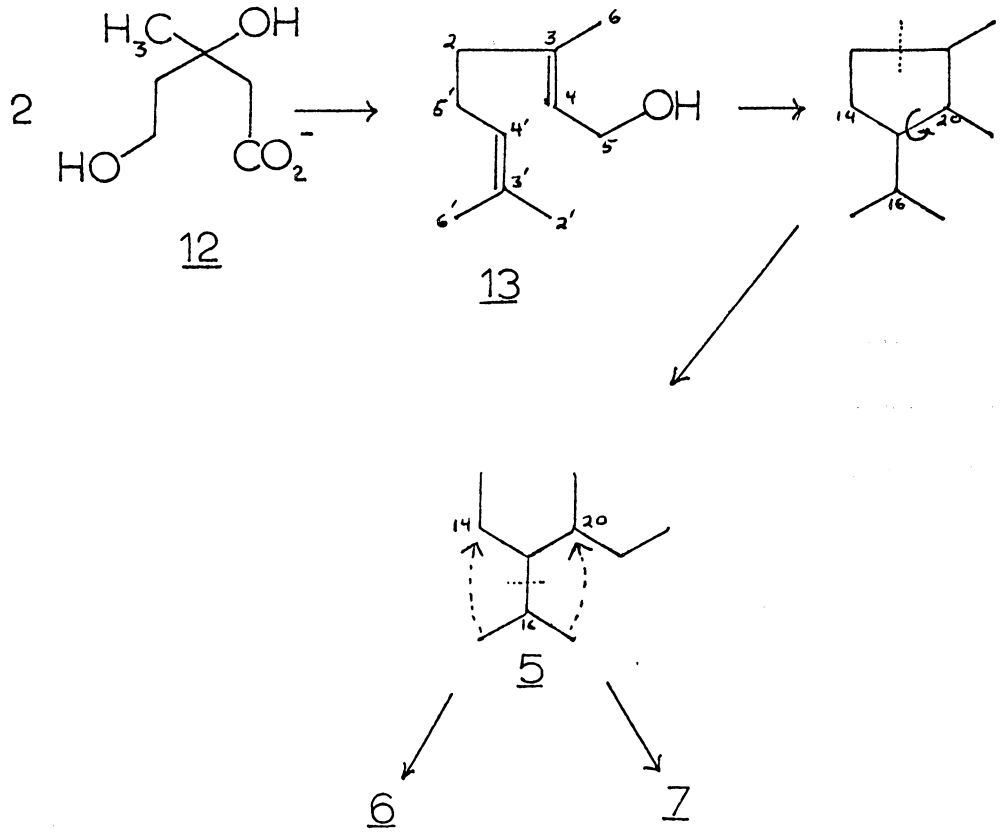
10


3
20
18
16

7


3
N
H
20
18
H
N
16
O2CCH3
HO
CH3
CO2CH3

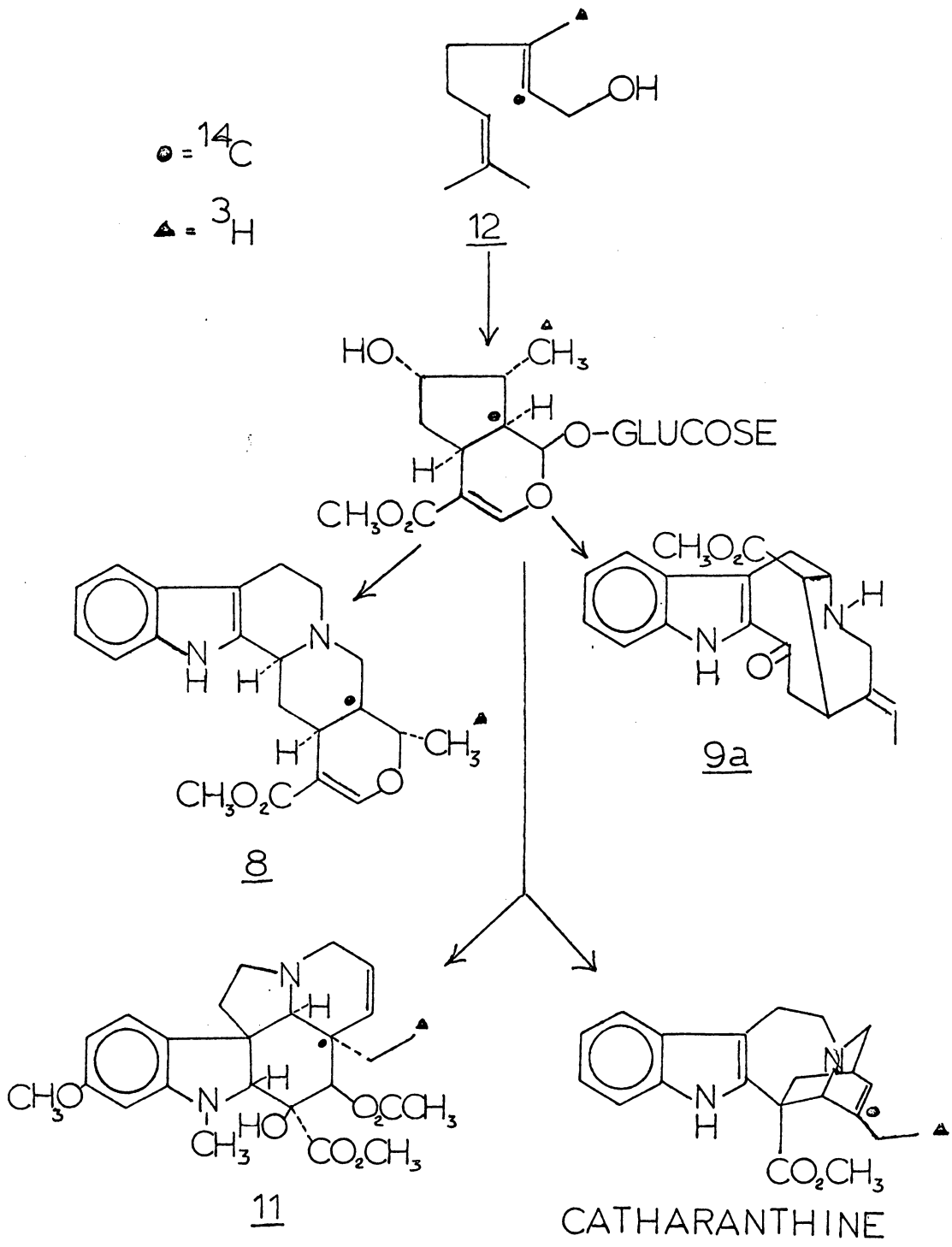
11


2
H3C
OH
HO
CO2-
12
2
3
6
6'
4
OH
4'
5
3'
6'
2'
13
14
20
16
14
20
16
5
6
7
HO
CH3
H
O-GLUCOSE
H
CH3O2C
O
14

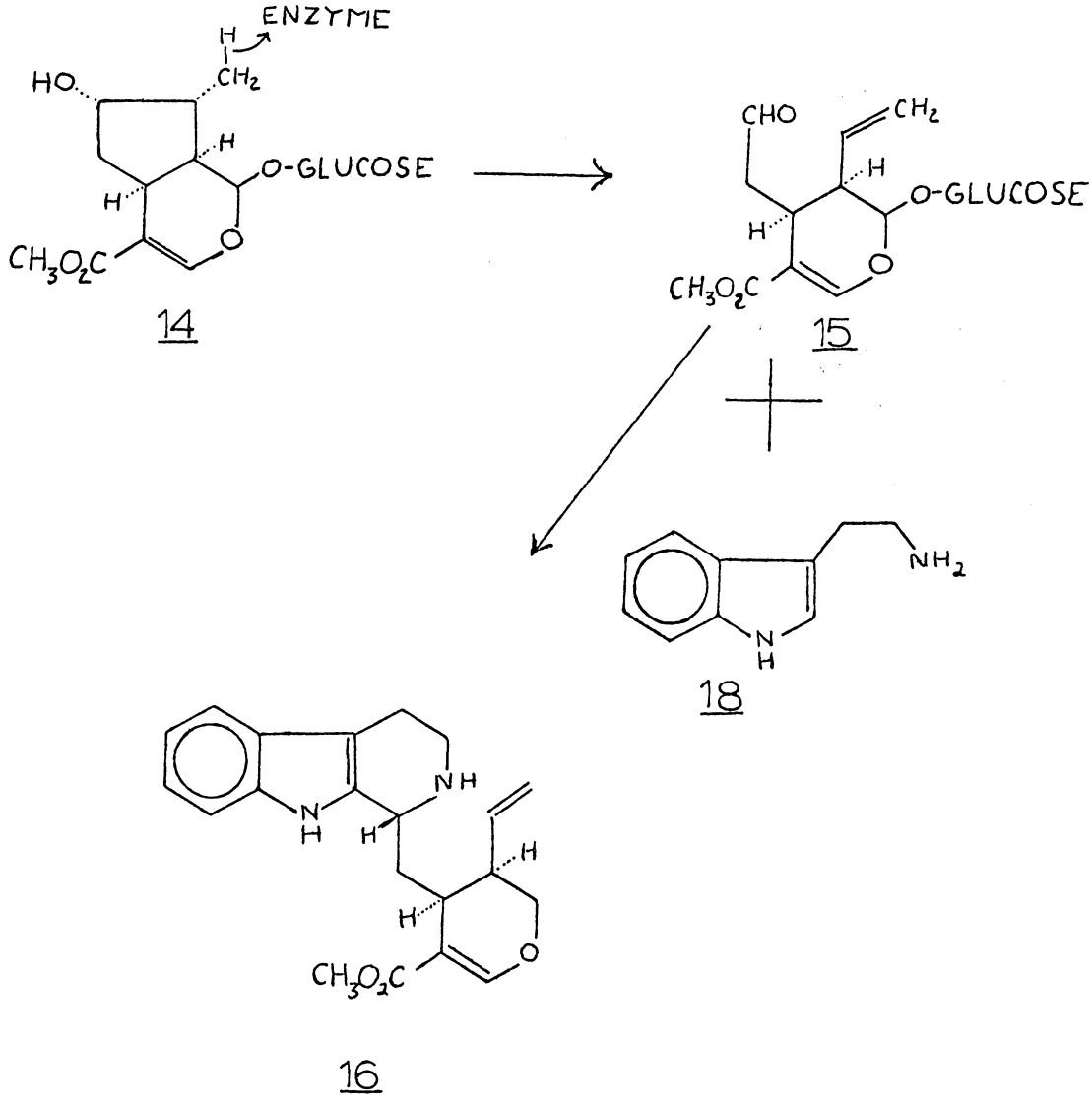
Scheme I

of indole alkaloids.

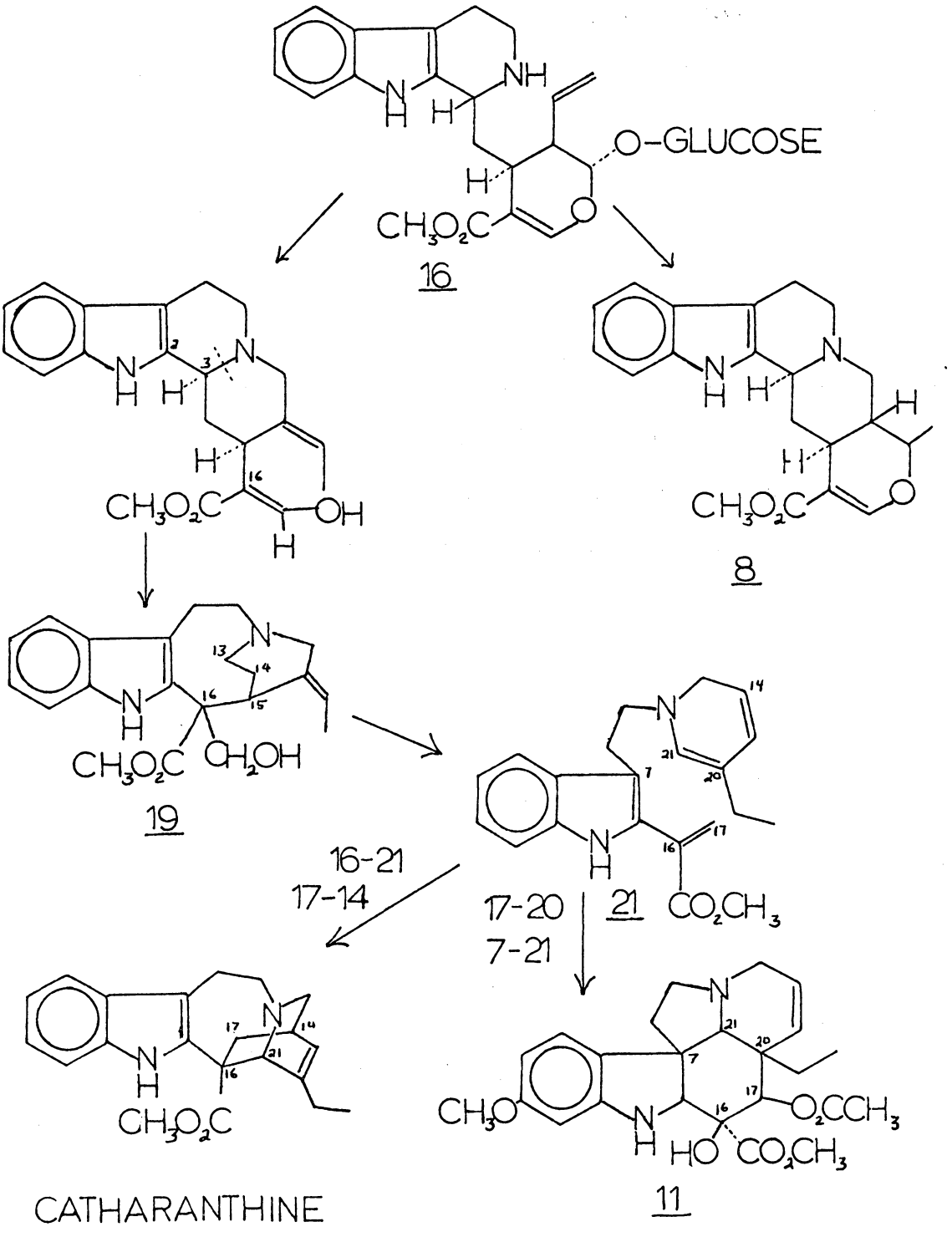
Additional work in this area revealed the more detailed steps taken toward the formation of the alkaloids. Loganin (14) was found to undergo bond breaking to form secologanin (15) which then condensed in a Mannich type reaction with a molecule of tryptamine (18) to form vincoside (16) as the first terpenoid indole alkaloid [18,11,19] (Scheme III). From vincoside to the other types of indole alkaloids the pathways rapidly increase in complexity. The best study that has been done concerns the sequential isolation of complex alkaloids at regular intervals after feeding the radioactive precursor vincoside (16) to the plant. In this way one can asses the order of biosynthesis of the molecules, thus allowing conjectures to be made on specific rearrangement, cleavage, or condensation reactions. Such sequence studies have revealed that the glucose moiety of vincoside (16) is hydrolyzed to form ajmalicine (8) (corynanthe´ type) after appropriate cyclization, but another pathway leads through an intermediate to stemmadenine (19) by bonding of the 16 carbon to the 2 carbon of the indole and cleavage of the indicated nitrogen-carbon bond. Stemmadenine (19) then forms an acrylic ester (21) which is believed to be the fork in the pathway to the formation of two classes of terpenoid indole alkaloids (Scheme IV).[20,21,22]


● = 14C
▲ = 3H
OH
12
HO
CH3
H
O-GLUCOSE
H
CH3O2C
O
N
H
H
CH3
CH3O2C
8
CH3O2C
N
H
O
N
H
9a
N
H
CH3O
N
CH3
H
HO
O2CCH3
CO2CH3
11
N
H
N
CO2CH3
CATHARANTHINE

Scheme II

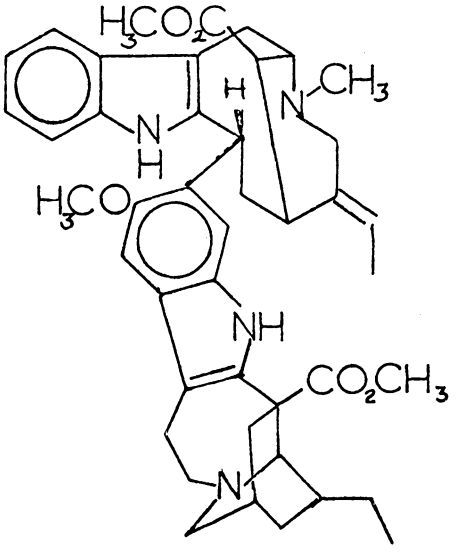

ENZYME
H
HO
CH2
H
O-GLUCOSE
H
CH3O2C
O
14
CHO
CH2
H
O-GLUCOSE
H
CH3O2C
O
15
NH2
N
H
18
NH
N
H
H
H
H
CH3O2C
O
16

Scheme III



Scheme IV

As mentioned before, the past ten years have seen an increased study of the bis-indole alkaloids, which are compounds composed of two individual indole alkaloid units. Those of the voacamine type [voacamine being a prototype (20)] are of particular interest in this study because of their wide appearance in the Tabernaemontana species as well as Callidilia, Conopharyngia, Gabunia, Stemmadenia, and Voacanga. All these alkaloids are composed of a corynanthe type [vobasine (9)] and an iboga type [voacangine (10)] unit.[23] In all cases the nature of the linkage is the same and consequently the chemical behavior very similar.



20

The biosynthetic pathways leading to the formation of those materials common to Tabernaemontana johnstonii have only briefly been outlined here. Not all points in their synthesis have been fully elucidated to date. However, the essential information to be gained are the types of alkaloids to be expected from the plant and how they are interrelated. Also, any new compounds found will have a high probability of fitting into the general biosynthetic scheme, and thus be merely precursors or derivatives of some known compound.

B. Bioassay Test Procedure

The purpose of the bioassay tests conducted by NIH and carried out at biomedical laboratories under NIH contract is for the screening of plant extracts to determine the particular fractions that have the highest potential of containing active anti-cancer agents. The ultimate goal is to isolate a pure compound that shows high activity in the tumor systems tested and may therefore eventually be used as a drug.[24]

There are three test systems used in this investigation. One is a "survival tumor" system conducted with rats and the other two are *in vitro* cell cultures.

In the "survival tumor" system, the plant extract is evaluated according to its ability to increase the lifetime of test animals implanted with a tumor as opposed to control animals that have the same tumor but are left untreated. The control animals have a well defined life span after tumor implantation. The tumor in this case is the P388 lymphocytic leukemia and the system is given the code letters "PS". Results are reported as the ratio of survival time of treated animals (T) to control animals (C) expressed as a percentage (T/C %). The T/C must be greater than or equal to 125% to be of interest as a source of anti-tumor agents or in the case of a pure compound to be eligible for further testing.

The two *in vitro* cell cultures are designated "KB" and "PS". The former is the human epidermoid carcinoma of the nasopharynx and the latter is the P388 lymphocytic leukemia. Treated cultures are compared with control cultures as in the *in vivo* test. The results, however, are reported as the ED_{50} — the dose that inhibits growth to 50% of control growth. The ED_{50} is expressed in micrograms per milliliter since the extracts are tested by weight. An ED_{50} of less than 20 micrograms per milliliter has a high probability of containing an active compound in the *in vivo* system.[24]

The initial fractionation of plant material is usually monitored for activity in the *in vivo* "PS" system since large amounts are usually available. As fractionation continues however, the "KB" and "PS" *in vitro* system must be used in order to conserve material. Once a pure compound is obtained which shows favorable *in vitro* activity it is subjected to *in vivo* testing whenever larger amounts become available.

RESULTS AND DISCUSSION

A. Isolation of Tabernamine (F262)

A normal size tree of the species Tabernaemontana johnstonii was collected in Kenya, Africa by the U.S. Department of Agriculture and supplied to our laboratory by the National Institute of Health cancer research program. The dried roots, stems, and bark were ground into fine particles, then subjected to initial extraction with 95% ethanol. All the organic soluble material was then partitioned between concentrated base (NH_4OH) and ethyl acetate to remove acidic and phenolic material. This ethyl acetate fraction was then back extracted into an acidic aqueous media (5% H_2SO_4). The acid soluble fraction was neutralized and re-extracted with organic solvent to recover all bases. This final extract was composed of crude alkaloidal materials as tested by Mayer's reagent[48] and assigned the fraction number F201. The "F" number refers to fractions which are mixtures of components and each is assigned in sequence as a new fraction is obtained.

Examining the two dimensional thin layer chromatography of F201 in two solvent systems revealed that it was an extremely crude fraction containing many components of widely varying Rf values. It was obvious additional fractionation was necessary in order to focus attention

on a single active component. The fraction was subjected to an open column step gradient development with Woelm Alumina and various solvents ranging from low to high polarity. It was felt that this procedure would help to separate the alkaloid constituents mainly by polarity. Table I is a schematic of this column showing the fractions eluted with the particular solvents. It is essential to note that elution of all material was not possible due to irreversible retention of some plant material, thus a loss of 5.2% by weight was incurred. All fractions were submitted for PS *in vivo* and KB *in vitro* testing and the results are shown in table II.

Attention was first given to the benzene fraction (F202) from the Alumina column because of its higher activity and greater weight. Two dimensional thin layer chromatography again showed an abundance of compounds of varying Rf values in all solvent systems used. Additional open column chromatography of this fraction was initiated with the hope that careful monitoring by thin layer chromatography would concentrate the activity in two or three fractions containing only a few components. A large open column, slurry packed with a very fine silica gel support was used with a solvent system of increasing methanol - chloroform ratio to fractionate F202. Each step of the development was followed by thin layer chromatography and fractions were combined according to

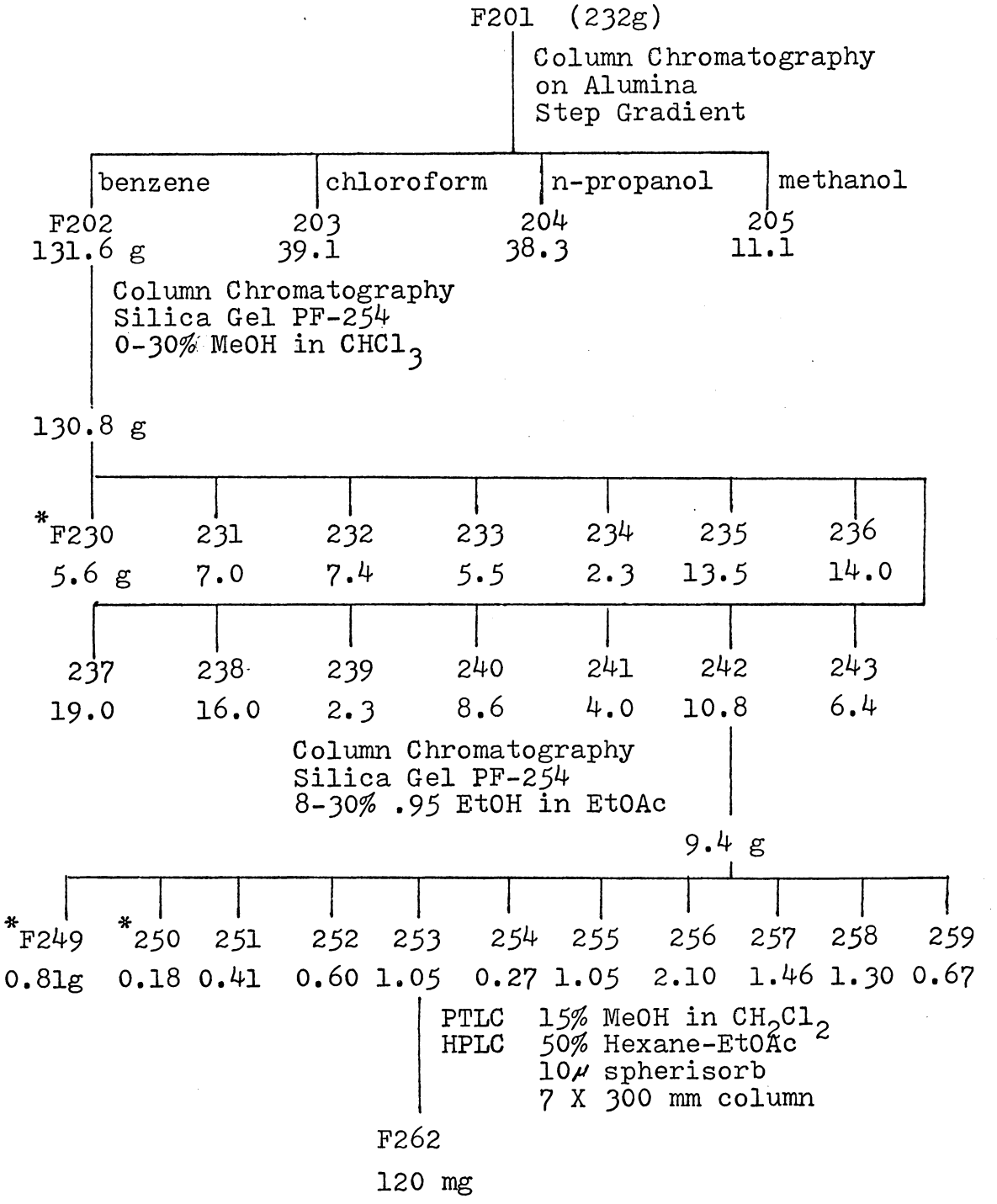
their similarity. In all, fourteen fractions were collected and each was sent for PS *in vivo*, at several doses, and KB and PS *in vitro* testing. Table I gives a schematic of the fractionation and table III shows the bioassay results of each.

It was clear that several fractions were of high enough activity in all test systems to be considered for further study and fractionation. One fraction stood out as having by far the greatest activity at a relatively low dose. This was F242 and thin layer chromatography along with reverse phase H.P.L.C. showed it to be composed of 10 - 15 components, many of relatively small quantity and possibly two or three major components. This was a rather polar fraction as compared to the others obtained from the column. At first, preparative thin layer chromatography was attempted, but the fraction proved to be too complex for this procedure. Since 10 g of material were available, it was decided that another open column chromatographic separation of F242 would be the best course to take in order to concentrate the activity and possibly obtain a pure compound in high yield. The same type of slurry packed silica gel column used for F202 was used to fractionate F242 except the solvent system was changed to one of 95% ethanol - ethyl acetate. Eleven fractions resulted from the column (Table I) with one in particular having primarily one component. The bioassay results

later showed that the fraction with the highest activity was F253, the one with the purest composition. It was further purified by preparative thin layer chromatography and high pressure liquid chromatography to give a colorless, amorphous, pure sample of F262. All attempts to crystallize this material from the usual solvents failed, but T.L.C. and H.P.L.C. showed it to be one compound under the conditions employed for chromatography. The preliminary PS *in vitro* bioassay results (Table IV) showed the compound to be highly promising as a cytotoxic agent. When more material was obtained it was tested for its PS *in vivo* activity. Unfortunately this test, which is more critical in terms of potential use as a drug, gave a low activity reading (Table IV). Further tests are being conducted at this time to verify this result. In total, approximately 120 mg of F262, later named tabernamine, were isolated from this plant, giving 0.0012% yield of this constituent based on dry plant weight.

TABLE I

FRACTIONATION TREE OF TABERNAEMONTANA JOHNSTONII



* Contaminated by solvent

TABLE II

BIOASSAY RESULTS OF F201 - F205

TEST	DOSE	F201	F202	F203	F204	F205
ED_{50} KB	g/ml	1.2	.029	11.0	8.6	15
PS *in vivo*	200 mg/kg	--	—	60	137	153
(T/C %)	100	--	66	87	133	133
	50	--	146	153	83	142
	25	--	151	135	126	99

(—) toxic at this dose

(--) not tested at this dose

TABLE III A

BIOASSAY RESULTS FOR F230 - F236

TEST	DOSE	F230	F231	F232	F233	F234	F235	F236
ED_{50} KB	μg/ml	100	100	54	24	24	10	2.7
ED_{50}PS	μg/ml	—	5.7	16	18	27	19	13
PS in vivo	200 mg/kg	114	134	—	—	49	66	—
(T/C %)	100	105	123	130	148	140	157	68
	50	105	107	107	138	173	123	130
	25	132	123	107	138	147	97	115
	12.5	--	--	--	--	--	--	110
	6.25	--	--	--	--	--	--	112
	3.12	--	--	--	--	--	--	91

(—) toxic at this dose

(--) not tested at this dose

TABLE III B

BIOASSAY RESULTS FOR F237 - F243

TEST	DOSE	F237	F238	F239	F240	F241	F242	F243
ED_{50} KB	μg/ml	1.7	1.5	1.0	2.4	2.6	2.3	2.4
ED_{50} PS	μg/ml	2.6	3.0	2.6	2.6	2.7	2.3	2.4
PS <u>in vivo</u>	200 mg/kg	—	—	52	66	—	58	—
(T/C %)	100	72	58	72	148	85	75	126
	50	148	76	142	140	157	142	130
	25	130	132	165	138	147	247	109
	12.5	151	173	122	163	171	156	--
	6.25	142	135	142	105	142	115	--
	3.12	122	122	112	69	151	94	--

(—) toxic at this dose

(--) not tested at this dose

TABLE IV

BIOASSAY RESULTS OF F249 - F259 AND F262

TEST	DOSE	F249	F250	F251	F252	F253	F254
ED_{50} KB	μg/ml	80	19.5	23	23.5	2.2	3.7
ED_{50} PS	μg/ml	17	8.2	8.2	8.3	0.38	0.70

TEST	DOSE	F255	F256	F257	F258	F259	F262
ED_{50} KB	μg/ml	8.0	21.5	22.5	3.2	18.5	--
ED_{50} PS	μg/ml	0.91	0.52	2.3	1.4	1.9	1.9
PS *in vivo* (T/C %)	25 mg/kg	--	--	--	--	--	125

(--) not tested in this system

B. Structure Elucidation of F262 (Tabernamine)

Before spectral data and other qualitative analytical tools could be used to deduce the structure of F262, it was important to ensure that the compound be pure. Since crystallization did not occur high pressure liquid chromatography along with preparative thin layer chromatography had to be relied upon to give F262 in its purest form. It was homogeneous on T.L.C. in two solvent systems and also on H.P.L.C. (Partisil - 10 with elution by 10% hexane in ethyl acetate). A particular problem, however, that became quite apparent when these methods were used, was the instability of the compound in solution and during chromatographic development. Allowing the compound to sit in organic solvents for any length of time produced break - down products. In view of this, the material had to be stored dry and preferably in an inert atmosphere.

The first spectral data to be obtained for F262 was the mass spectrum (fig. 1). It was hoped this would give an indication of the molecular weight and the compound class. The highest mass peak was seen at m/e 630(04) and some abundant peaks were 616(40), 182(94), 136(84), and 122(100). With this seemingly high molecular weight and the fact that the U.V. spectrum had a characteristic indole alkaloid absorption pattern, the first indication of a structure for F262 was a bis-indole alkaloid. Also

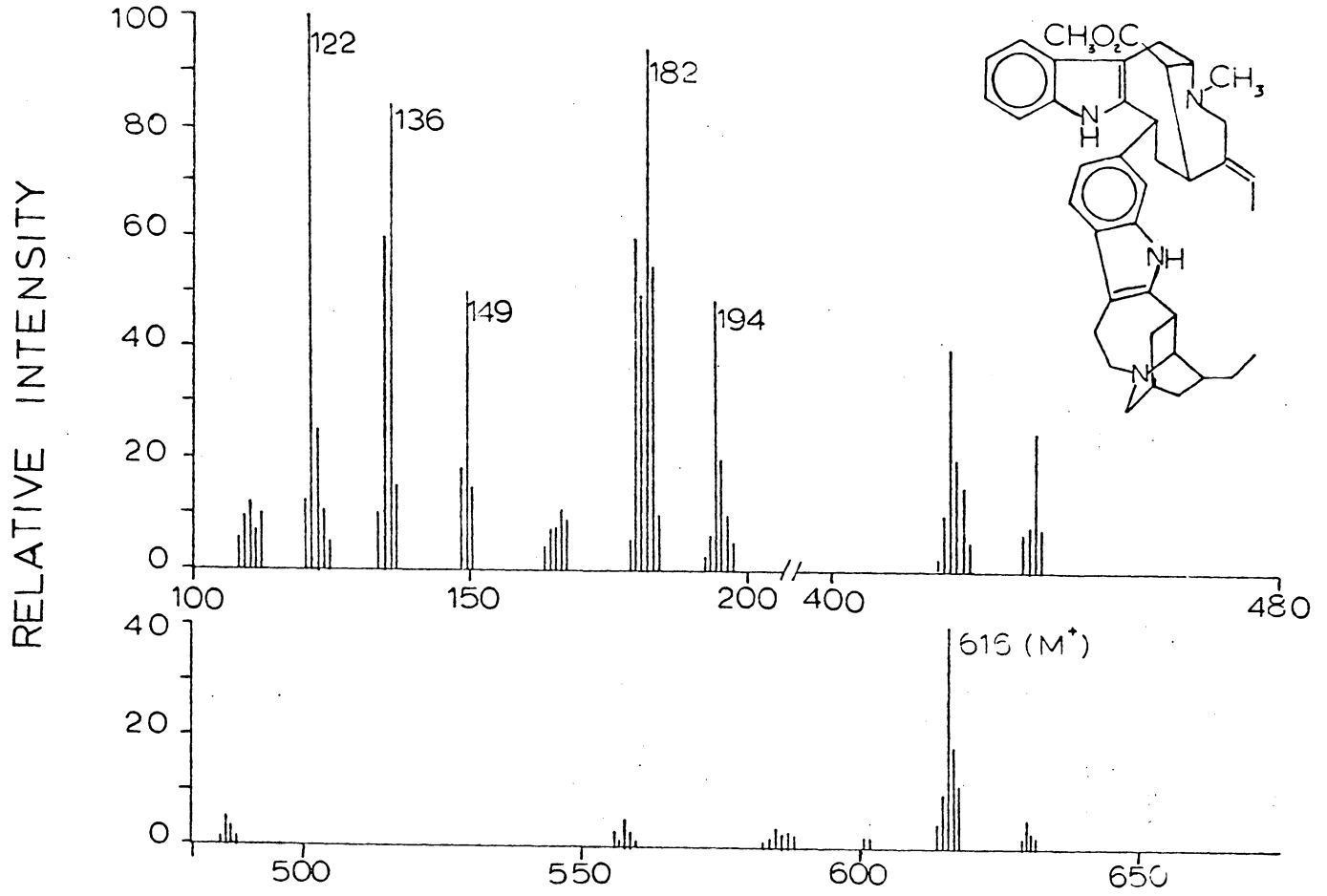
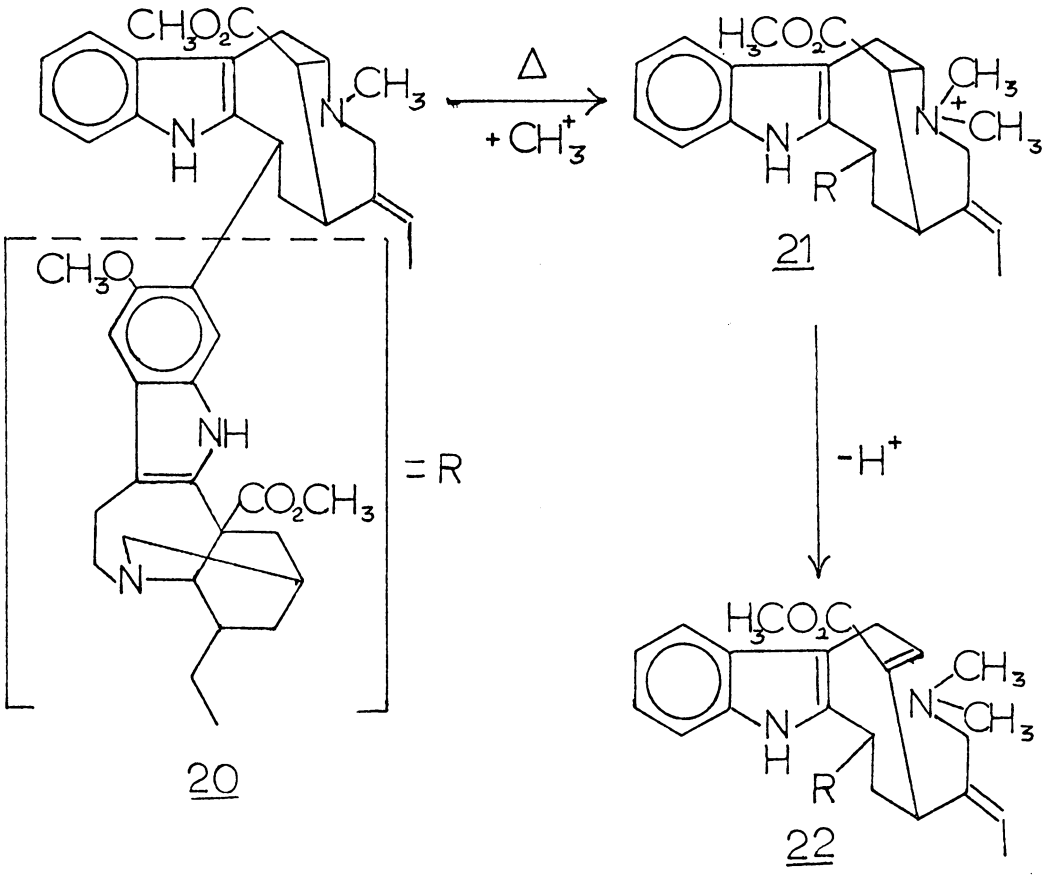


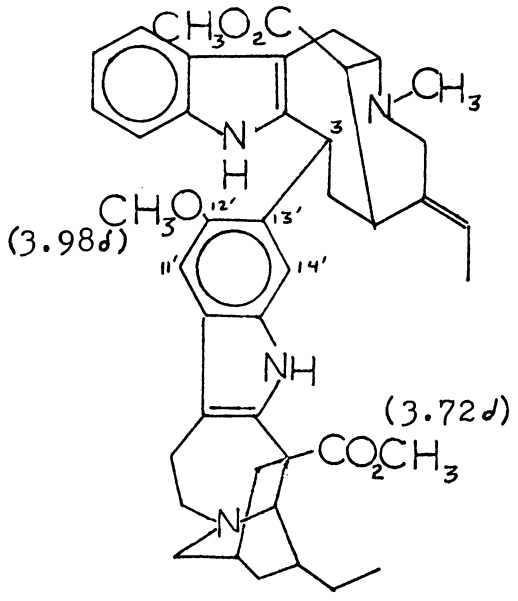
Figure 1 Mass Spectrum of F262 (Tabernamine).

it was known that bis-indoles of close molecular weight were found in the same plant and similar species. Continuing with this line of thought, the question of the exact molecular weight had to be settled first. Although an m/e of 630 may at first appear to be the parent ion, a loss of 14 mass units to an intense m/e 616 placed this first suggestion in doubt. The literature on bis-indole alkaloids contained information on the somewhat novel mass spectra of these compounds.[25] In the case of voacamine (20) for example, it was shown by Biemann and Thomas[26] that thermal decomposition in the mass spectrometer of one molecule liberated a methyl group which served to quarternize the basic nitrogen atom of a second voacamine molecule (21), this being followed by Hofmann elimination. The resulting product (22) would then possess a molecular weight 14 mass units higher than voacamine (20) (Scheme V).

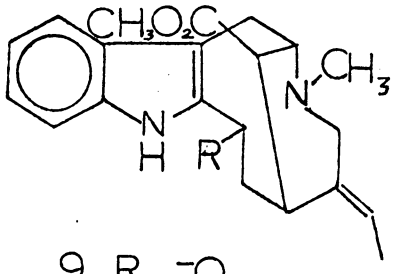

CH3O2C
N
H
N
CH3
Δ
+ CH3+
H3CO2C
CH3
N+
CH3
R
21
CH3O
NH
CO2CH3
N
= R
20
-H+
H3CO2C
CH3
N
CH3
N
H
R
22

Scheme V

Suspecting that this may also be the case with F262, the molecular weight was assumed to be 616 with an M+14 peak of 630. Thus, the idea that F262 was a voacamine type compound led to the mental combination of known indole alkaloids in the hope of finding one with a mass of 616 while at the same time retaining the intermolecular methyl transfer. Voacamine (20) itself is composed of vobasan, derived from the 2-acyl indole vobasine (9), and voacangine (10), an iboga alkaloid. The units are bonded between C-3 of vobasan and C-13 of voacangine.[23] One possible combination for F262 was vobasan bonded to ibogamine (23). This alkaloid is the lowest oxidized state of all iboga alkaloids and unlike voacangine (10) it is devoid of a methoxy at C-12 and a carbomethoxy at C-18, making it 88 mass units lower, at a molecular weight of 616. This combination would still be able to exhibit an M+14 peak by intermolecular methyl transfer. In addition, the low intensity of the peak at m/e 630 (M+14) (fig. 1) is consistent with the absence of the carbomethoxy group in the ibogamine portion of the molecule.

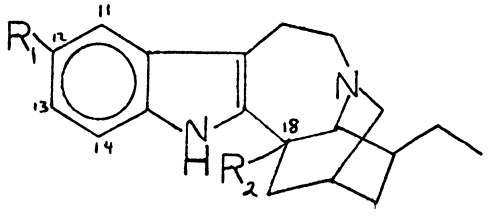


20



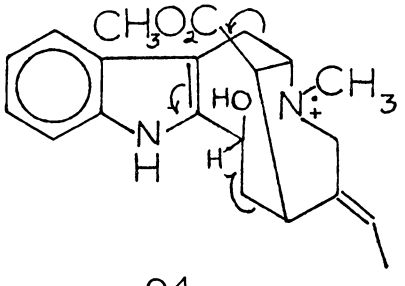
9 R =O

24 R -OH

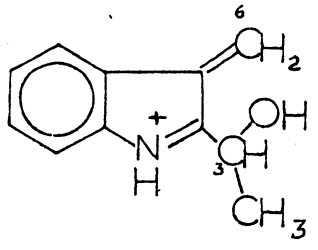


10 R_1= OCH_3, R_2= CO_2CH_3

23 R_1= H, R_2= H



24



25

m/e 174

A more detailed look at the mass spectrum reveals the presence of the characteristic spectra of the two presumed monomers, vobasan and ibogamine, which can be postulated to have fragmented independently. The ibogamine spectrum has intense fragment ions at 122(38), 136(100), and 149(35),[28] all of which are found in the mass spectrum of F262. Also, the vobasinol spectrum which would be very similar to that of vobasan is characterized by peaks at 122(75), 174(20), 180(60), and 194(20).[27] They are all found in the spectrum of F262 except for the m/e 174 fragment. Its absence is particurlarly significant since in vobasinol (24) this fragmentation includes carbon atoms 3 and 6 as well as the indole nucleus (25). These findings suggest that a vobasan moiety is linked through carbon atoms 3 or 6 to the ibogamine portion.

Turning to the PMR spectrum in both deuterated methanol (fig. 2) and chloroform (fig. 3), one finds signals due to the methyl portion of an ethyl group, to an N-methyl, a high field carbomethoxy group, and to the methyl and methine portions of a $=CH-CH_3$ group. Of all signals the one most characteristic of a vobasan type moiety is the high field carbomethoxy signal at 2.56 δ in CD_3OD which is shielded by the diamagnetic anisotropy of the indole nucleus.[29] The presence of only two methyl singlets in contrast to voacamine,[23] which has four, gives additional support to the idea that the loss of 88 mass units

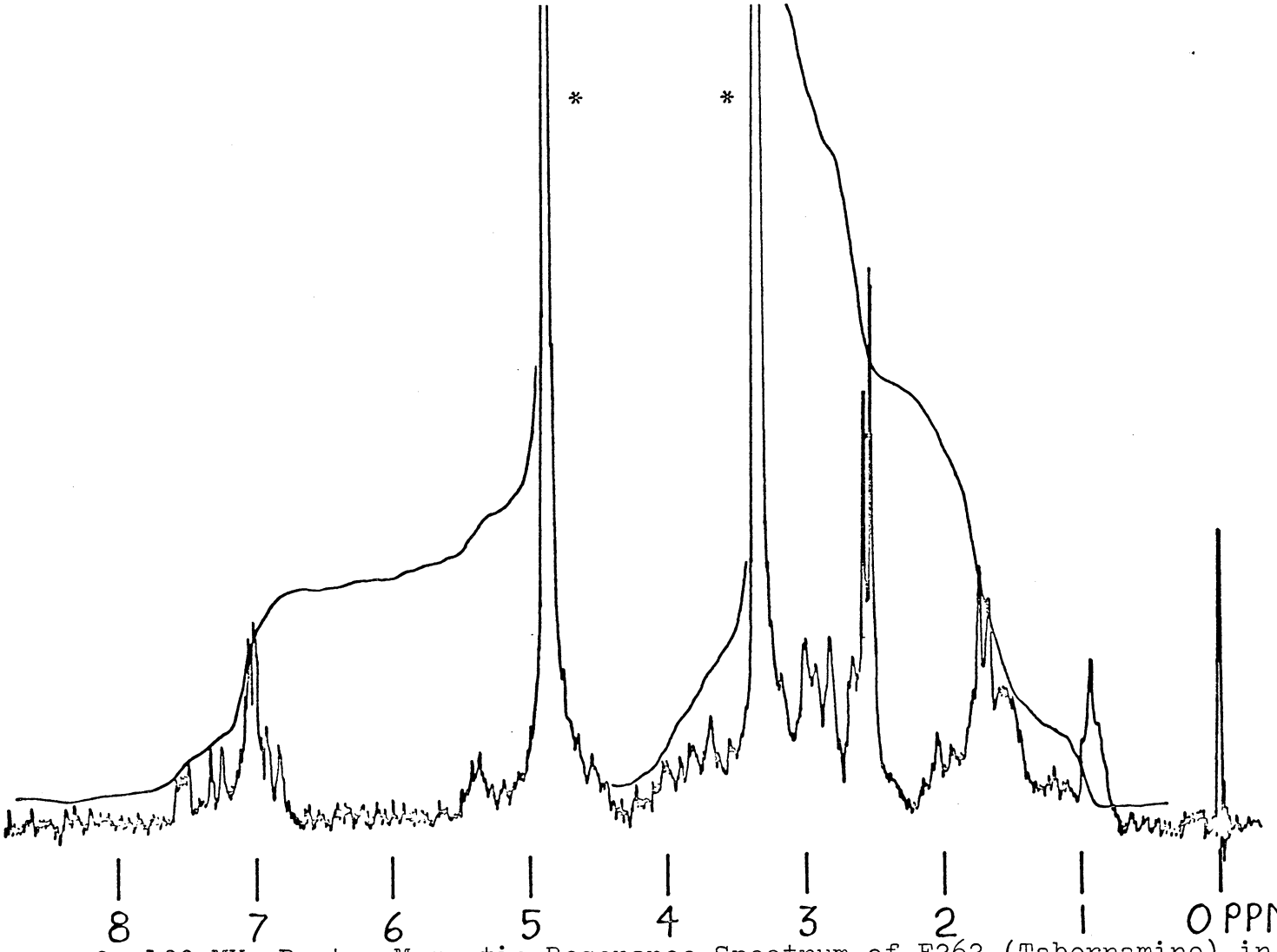


Figure 2 100 MHz Proton Magnetic Resonance Spectrum of F262 (Tabernamine) in CD_3OD.

* Solvent Peak

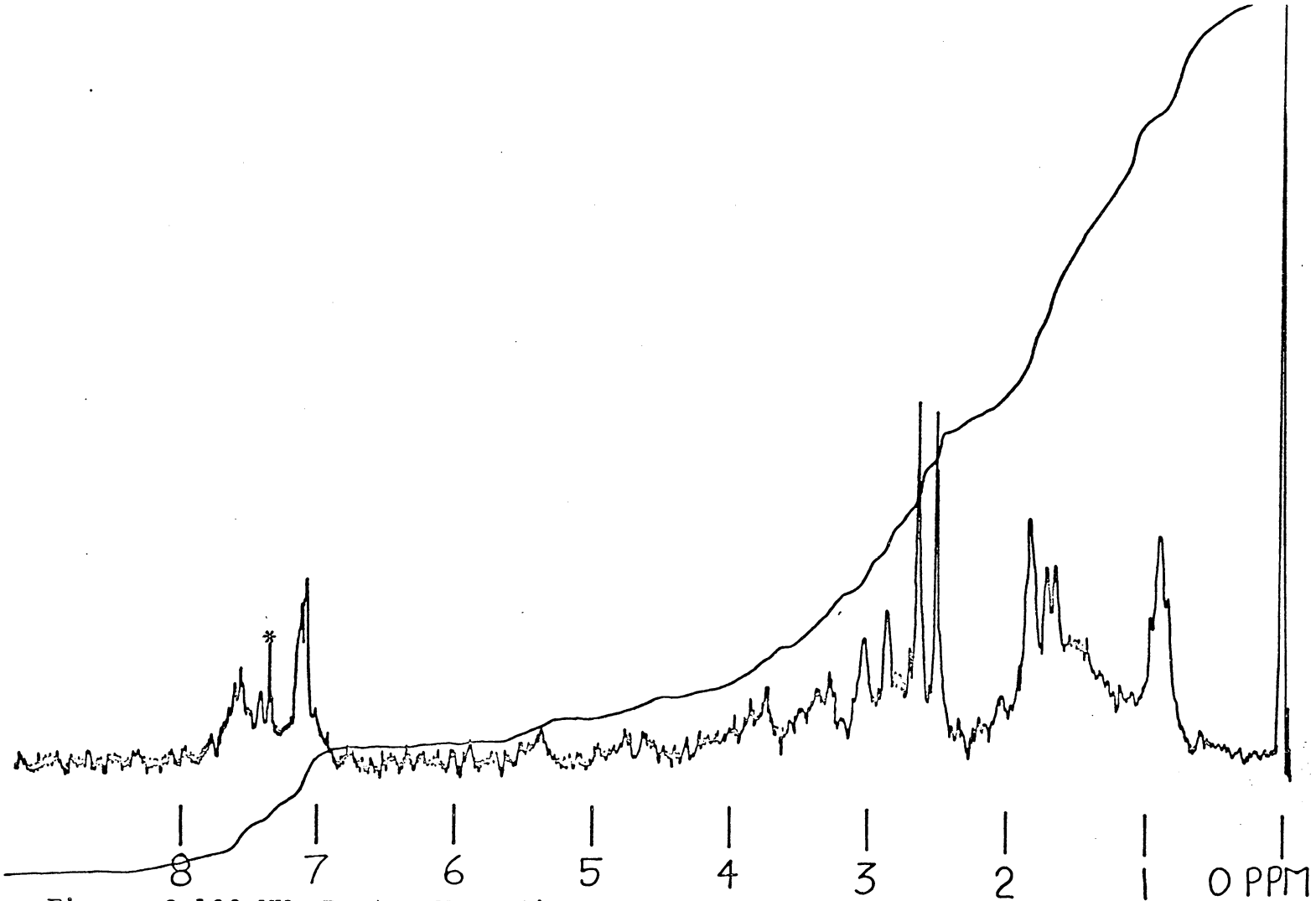
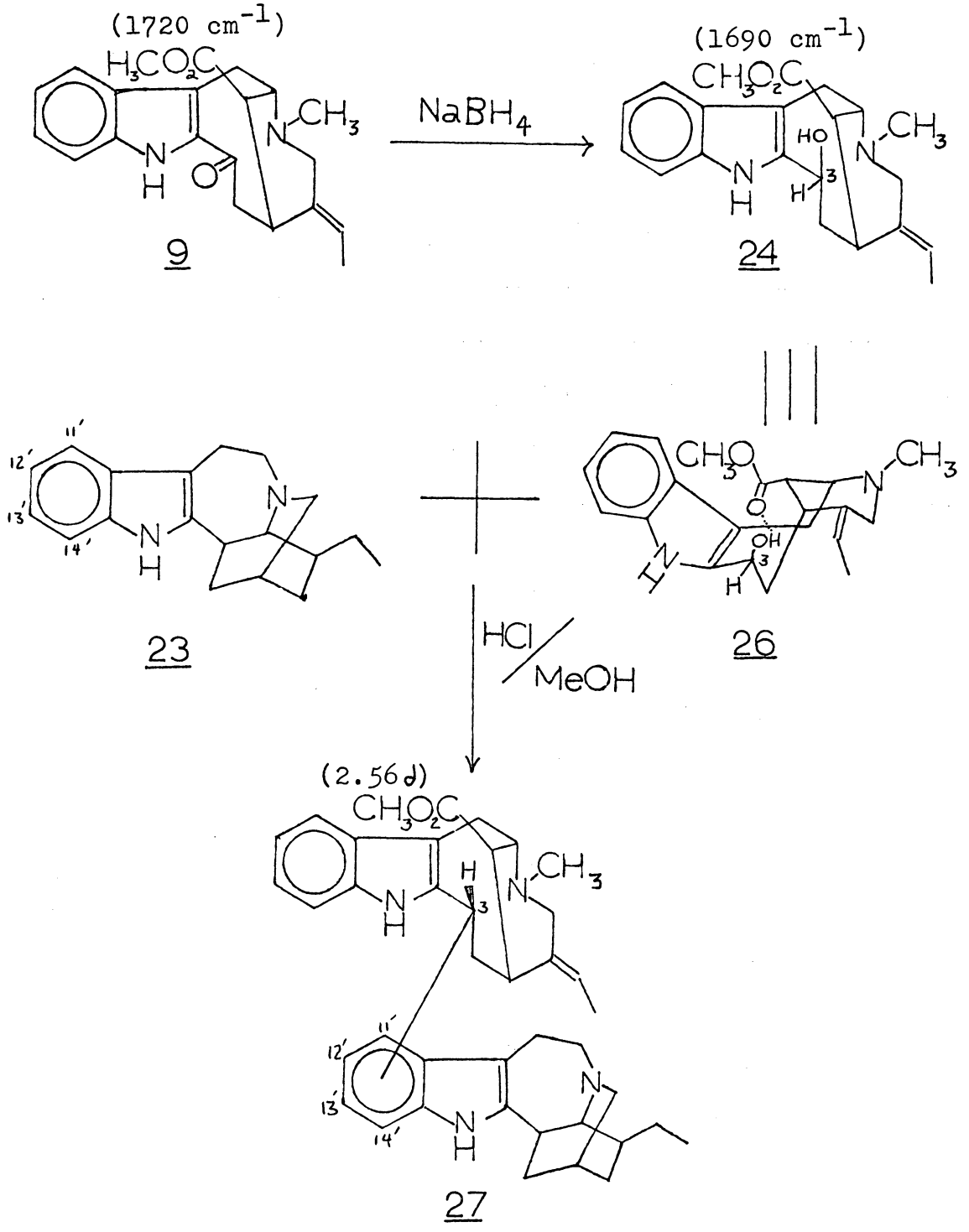


Figure 3 100 MHz Proton Magnetic Resonance Spectrum of Tabernamine in $CDCl_3$.
* Solvent peak

corresponds to the absence of methoxy and carbomethoxy groups. The two methyl singlets in voacamine at 3.98δ and 3.72δ, due to the iboga ring methoxy and carbomethoxy groups, are completely absent in the PMR spectra of F262, which confirms that these groups are absent in tabernamine.

Verification for the presence of ibogamine (23) within the bis-alkaloid was made by acid cleavage of F262,[23] yielding ibogamine which was identified by T.L.C. and H.P.L.C. and comparison of its mass spectrum with that of an authentic sample. In addition, the nature of both monomers was finally confirmed in the partial synthesis of F262 by the condensation of vobasinol and ibogamine (Scheme VI). It was identical by T.L.C., H.P.L.C., PMR MS, and optical rotation with that isolated from Tabernaemontana johnstonii.

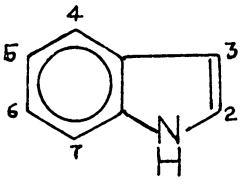
Vobasinol was synthesized from vobasine by $NaBH_4$ reduction and it was assumed that the reagent attacked the carbonyl group of vobasine from the sterically less crowded side. This would place the OH on the β side of the molecule where it is in the right proximity for hydrogen bonding to the carbonyl oxygen atom (26).[23] This was confirmed by the infrared spectrum which showed a shift of the ester carbonyl frequency from 1720 cm^{-1} in vobasine to 1690 cm^{-1} in vobasinol. It was thus assumed that attack of the nucleophilic aromatic ring of the ibogamine molecule would occur only from the less hindered α side,


(1720 cm-1)
H3CO2C
CH3
N
H
O
9
NaBH4
(1690 cm-1)
CH3O2C
HO
3
H
24
11'
12'
13'
14'
23
CH3O
OH
26
HCl
MeOH
(2.56 d)
27

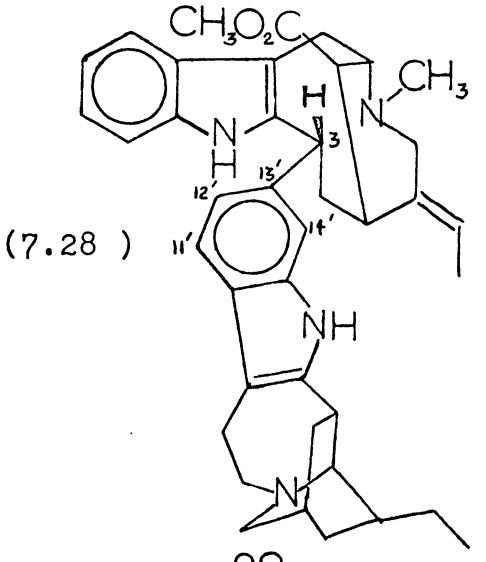
Scheme VI

thus the stereochemistry at C-3 is proposed to be that shown in (27).

The remaining point of consideration is the position of attachment of the vobasan moiety to the ibogamine molecule. Electrophilic substitution on the benzene ring of indoles occurs most readily at the 6 position (28),[30] thus the vobasan unit is most likely linked at the 13′ position of the ibogamine molecule (29). Support for this conclusion originates from the CD_3OD PMR spectrum of F262 (fig. 2) which shows a doublet at 7.28 δ. Since the C-4 proton of the indole nucleus (28) absorbs downfield from the remaining aromatic protons,[31] this doublet is assigned to the proton in the 11′ position, being coupled (8 Hz) to the 12′ proton. All this indicates that the point of attachment is at the 13′ position, thus F262 can be represented by structure (29).



28



29

Compound F262 is the first alkaloid of the voacamine type to lack a methoxy group in the iboga portion of the dimer. Being derived from the Tabernaemontana species and resembling voacamine, it was given the name tabernamine. It is believed that the compound is a natural product and not an artifact since the conditions required for the synthesis of tabernamine were rather vigorous.

C. Structure - Activity Studies

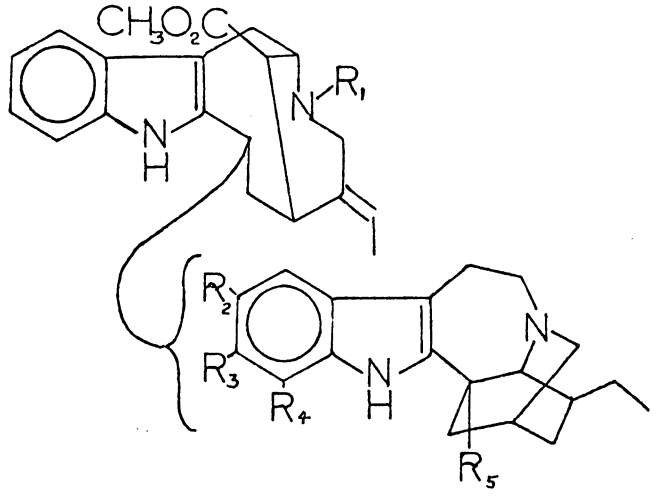
1. Synthesis of Peribogamine

The isolation of active constituents from plants in many cases can provide a basis for the development of more powerful anti-cancer agents by synthesis of structural analogs. Such was the case after the isolation and identification of tabernamine, which, by comparison with known bis-indole alkaloids and their activities, provided the possibility of synthesizing a more active compound from other alkaloids. Previously isolated from Tabernaemontana johnstonii were three alkaloids of similar structure to tabernamine. These were conodurine (30), conoduramine (31), and gabunine (32).[32,33] Table V shows the activity measurements of these bis-indole alkaloids and reveals the structural differences that may possibly account for the observed changes in activity. Conodurine (30) and conoduramine (31) are structural isomers differing only in their place of attachment on the isovoacangine moiety.[34] Their measured activity indicates that they are not very effective anti-tumor agents though they do border on consideration for further in vivo testing. Next in line is gabunine (32),[35] which shows a striking increase in activity merely by changing the vobasine unit to perivine, i.e. by the replacement of an N-methyl group by a hydrogen. The range of activity of these bis-alkaloids

TABLE V

ACTIVITY AND COMPOSITION OF SOME BIS-INDOLE ALKALOIDS

DIMER	COMPOSITION	ED_{50}"PS"
CONODURINE	VOBASAN + ISOVOACANGINE	29 g/ml
CONODURAMINE	VOBASAN + ISOVOACANGINE	20
GABUNINE	PERIVAN + ISOVOACANGINE	3.2
TABERNAMINE	VOBASAN + IBOGAMINE	1.9
PERIBOGAMINE	PERIVAN + IBOGAMINE	?



30 CONODURINE: $R_1=CH_3$, $R_2=H$, $R_3=OCH_3$, R_4=VOBASAN, $R_5=CO_2CH_3$

31 CONODURAMINE: $R_1=CH_3$, R_2=VOBASAN, $R_3=OCH_3$, $R_4=H$, $R_5=CO_2CH_3$

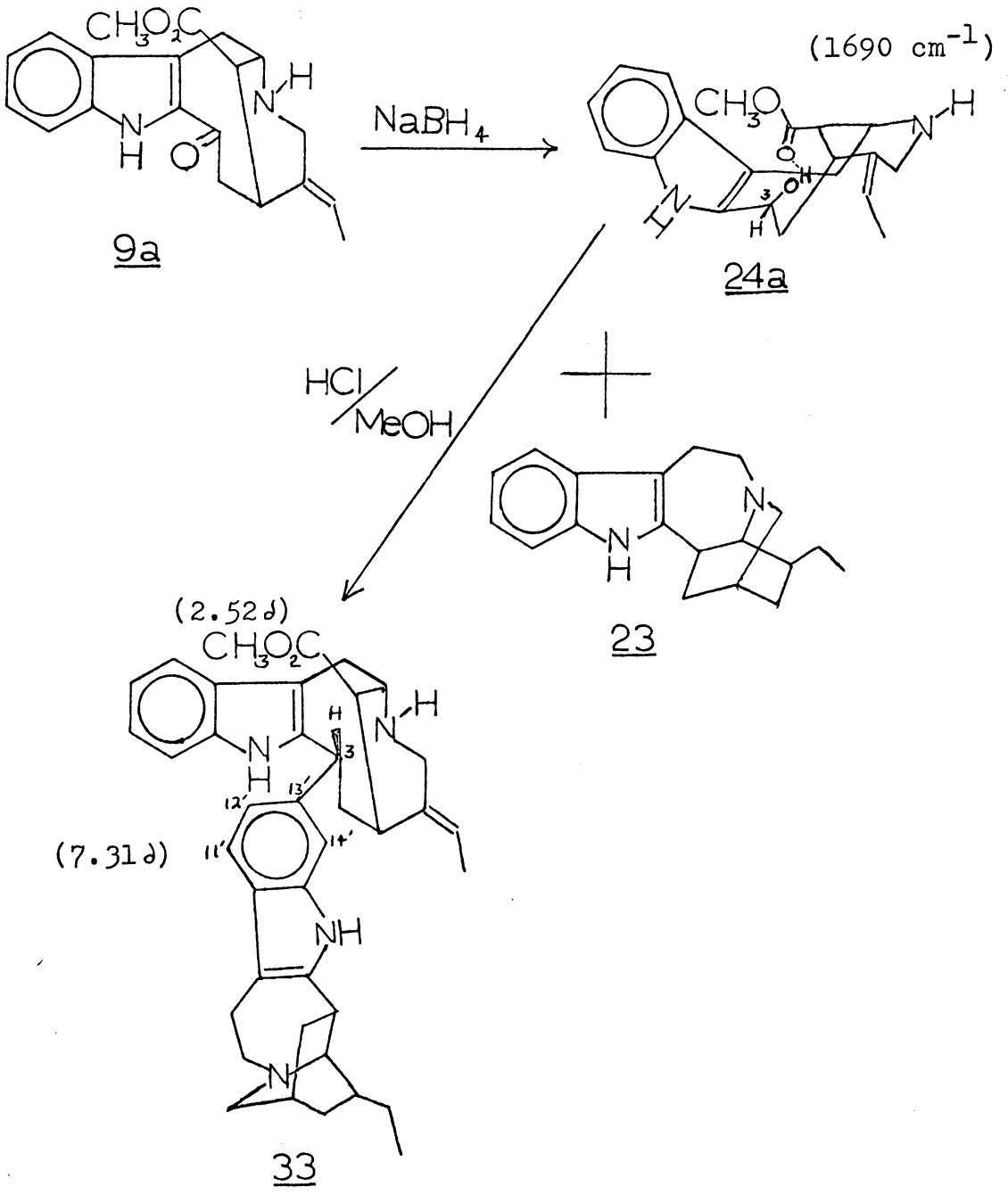
32 GABUNINE: $R_1=H$, $R_2=H$, $R_3=OCH_3$, R_4=PERIVAN, $R_5=CO_2CH_3$

29 TABERNAMINE: $R_1=CH_3$, $R_2=H$, R_3=VOBASAN, $R_4=H$, $R_5=H$

33 PERIBOGAMINE: $R_1=H$, $R_2=H$, R_3=PERIVAN, $R_4=H$, $R_5=H$

was increased even more through the isolation of tabernamine. It has the vobasan moiety, but differs in the iboga portion where it lacks a methoxy and carbomethoxy group. It was thought that it may be possible to improve further the activity of this class of alkaloids by combining the seemingly good characteristics of both gabunine (32) and tabernamine (29) in a new bis-alkaloid. Assuming that the removal of the vobasan N-methyl group was the cause for the dramatic increase of activity in going from conoduramine to gabunine and the removal of the iboga methoxy and carbomethoxy groups likewise in going from gabunine to tabernamine, it was thought that a new bis-alkaloid derived from perivine and ibogamine may result in an additive or multiplicative increase in activity.

The approach to the partial synthesis of peribogamine (perivine plus ibogamine) was essentially the same as for the synthesis of tabernamine (29).[23] It was a two step process where perivine (9a) in crystalline form was reduced to perivinol (24a) which was then condensed with ibogamine (23). Similarly it was assumed that perivinol was in the 3β- OH form where the hydroxy group was hydrogen bonded with the carbonyl oxygen as seen in (24a). The IR spectrum supported this with a carbonyl absorption at 1690 cm^{-1} in $CHCl_3$. Electrophilic attack of perivinol on to ibogamine would most likely occur from the less hindered α side (Scheme VII).



Scheme VII

The major product from the condensation was obtained in approximately 50% yield. The mass spectrum (fig. 4) revealed a parent ion at m/e 602 and a low intensity peak representing a methyl transfer at m/e 616. As in tabernamine, the low intensity of the M+14 peak may be due to the lack of a carbomethoxy in the iboga portion of the alkaloid. Carbon, hydrogen, and nitrogen analysis indicated the molecular composition of $C_{39}H_{46}N_4O_2$, in agreement with the predicted value for a perivan - ibogamine dimer. Looking again at the mass spectrum, peaks corresponding to the ibogamine portion of the alkaloid were present at m/e 136(100), 122(42), 149(40), and 182(23). The fragments arising from the perivan portion of the molecule are of much lower intensity. The most important fragment is m/e 166, which is the base peak in perivine,[33] but has a relative intensity of only 28 in peribogamine. Other perivine peaks at m/e 130, 165, and 184 are present in the dimer, but again are greatly overshadowed by the much more intense ibogamine fragments.

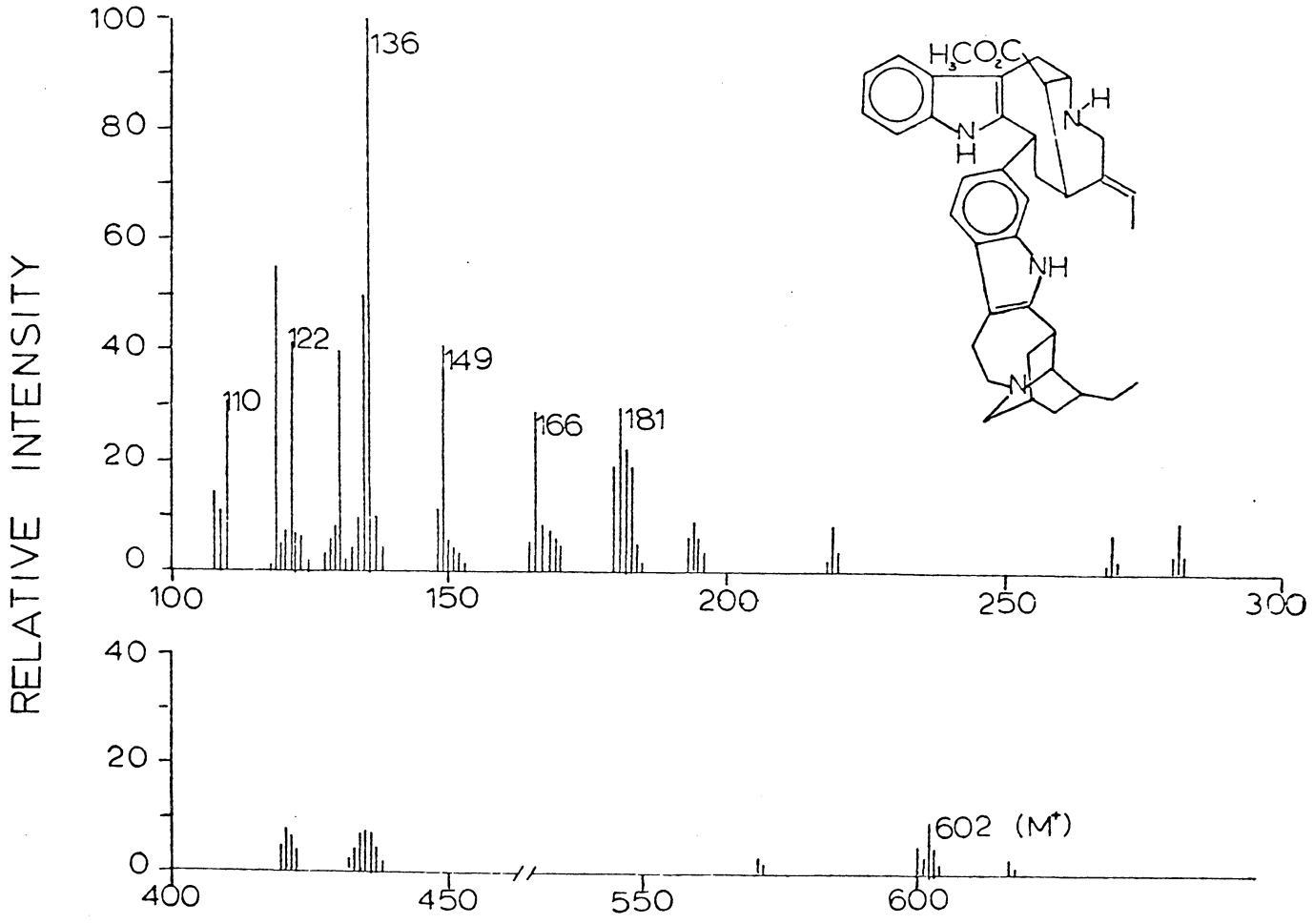


Figure 4 Mass Spectrum of Peribogamine

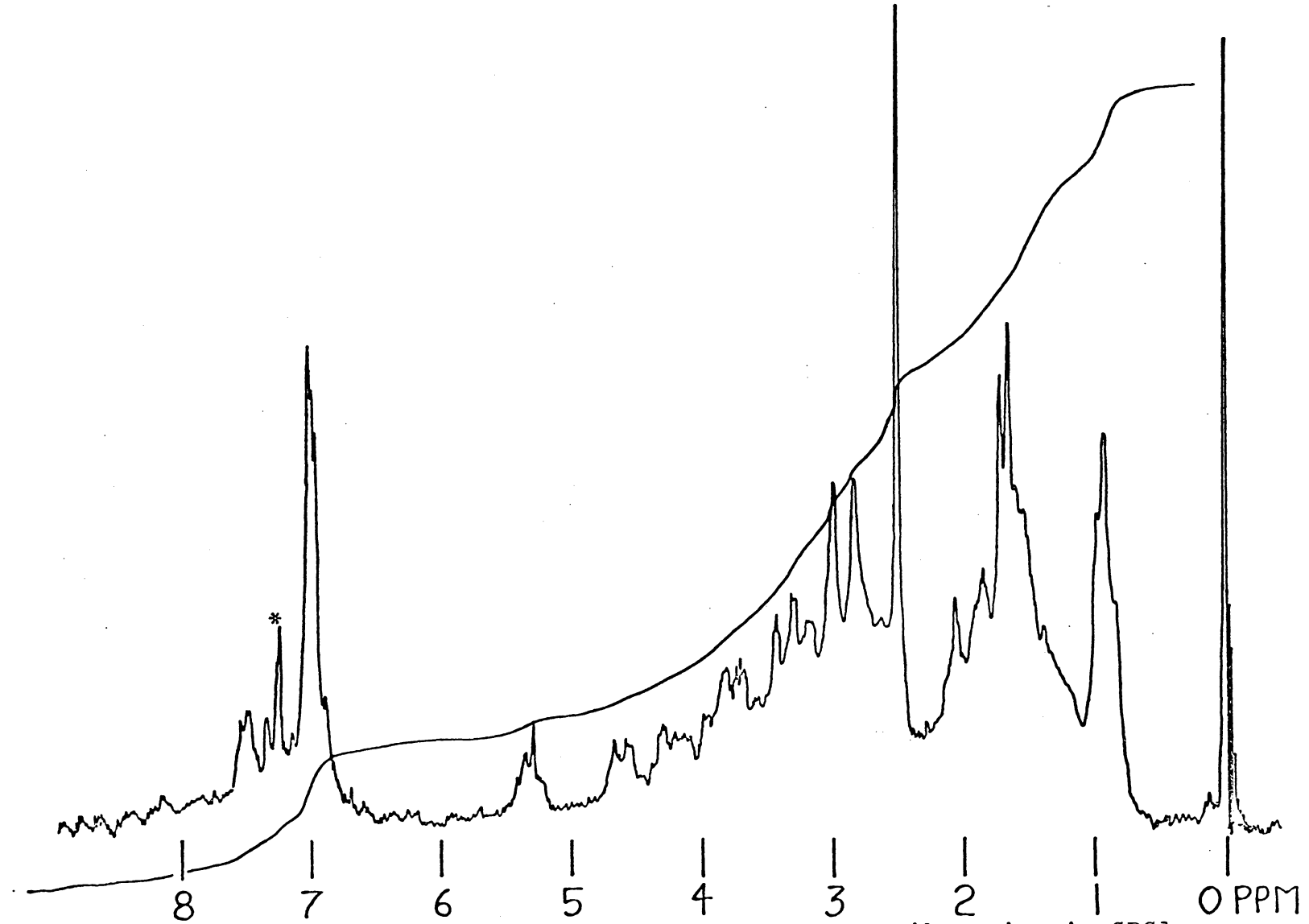


Figure 5 100MHz Proton Magnetic Resonance Spectrum of Peribogamine in $CDCl_3$.

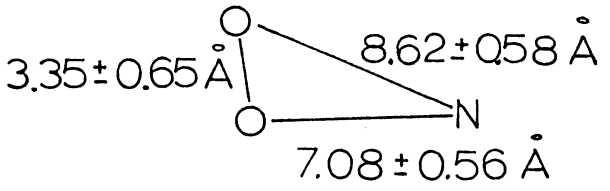
* Solvent Peak

The $CDCl_3$ PMR of the coupled product (fig. 4) supports the proposed structure well. Two critical points were the presence of only one methyl singlet located at 2.52 δ representing the carbomethoxy of perivan and the doublet at 7.31 δ which, as in tabernamine, represents the 11′ proton ortho coupled (8 Hz) to the 12′ proton. This indicates that the point of attachment between perivan and ibogamine is at the 13′ carbon, the same result obtained for tabernamine. The IR spectrum and UV spectrum were very much in accord with the expected structure and again differed very little from that of natural tabernamine.

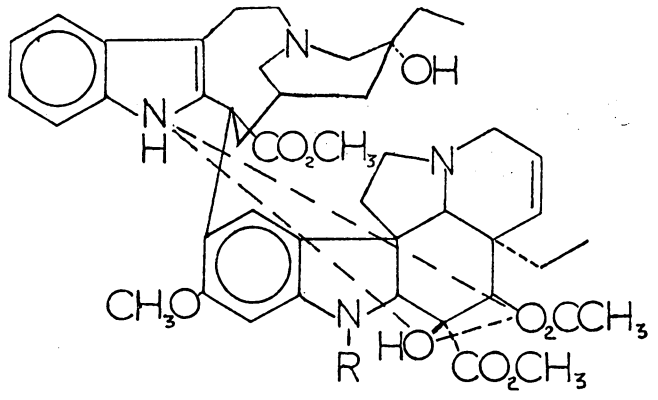
Peribogamine (33) was tested in the PS *in vitro* cell culture to compare its activity with the other dimers of this class. It is possible that peribogamine is a natural product in this or some other closely related plant which has so far not been isolated. Only one naturally occurring bis-alkaloid containing perivine has been isolated, namely gabunine (32). The bioassay results of peribogamine are not known at the time of this writing yet they may have a large bearing on the direction of this particular structure - activity approach.

2. Synthesis of Vobasivindoline

Of the numerous structure - activity studies that have been conducted on various oncolytic agents, a common feature was noted among a number of anti-leukemic agents that has a very close tie with the type of indole dimers obtained from the Tabernaemontana species. This structural feature is the O-N-O triangulation (34) observed in some non-alkylating anti-leukemic agents.[36] The presence of this particular arrangement does not necessarily mean a compound will have anti-leukemic activity, but it can be found in several classes of anti-leukemic drugs. A well known example is seen in the indole-indoline dimers of vinblastine (3) and vincristine (4).[36,37] These two compounds were discovered in the species Vinca rosea Linn,[38,39] and were found to have excellent activity against the leukemic P-1534 tumor; they are now employed in chemotherapy for the inhibition of this tumor in man.[40] It is hypothesized that the O-N-O triangular structure arrangement might assist the in vivo bonding of the drug to one of the pertinent biological receptor sites involved in leukemic genesis.[36]



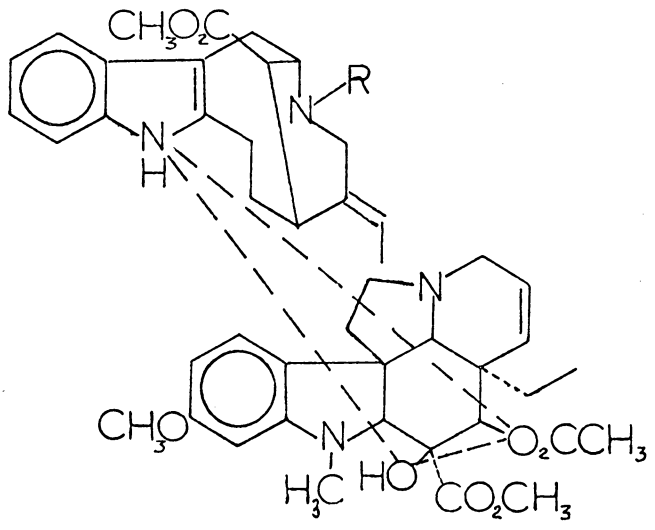
34



3 R= CH_3

4 R= CHO

Vinblastine (3) and vincristine (4) consist of a velbanamine type alkaloid, similar to the iboga alkaloids, bonded to a 2,3-dihydro indole alkaloid of the aspidosperma type.[37] These dimers form the O-N-O triangle as shown. Gorman and Sweeny[41] synthesized a new bis-alkaloid from perivine and vindoline and named it perivindoline (36). This alkaloid, although it contains the O-N-O pharmacophore, was reported as being inactive in the P-1534 leukemia



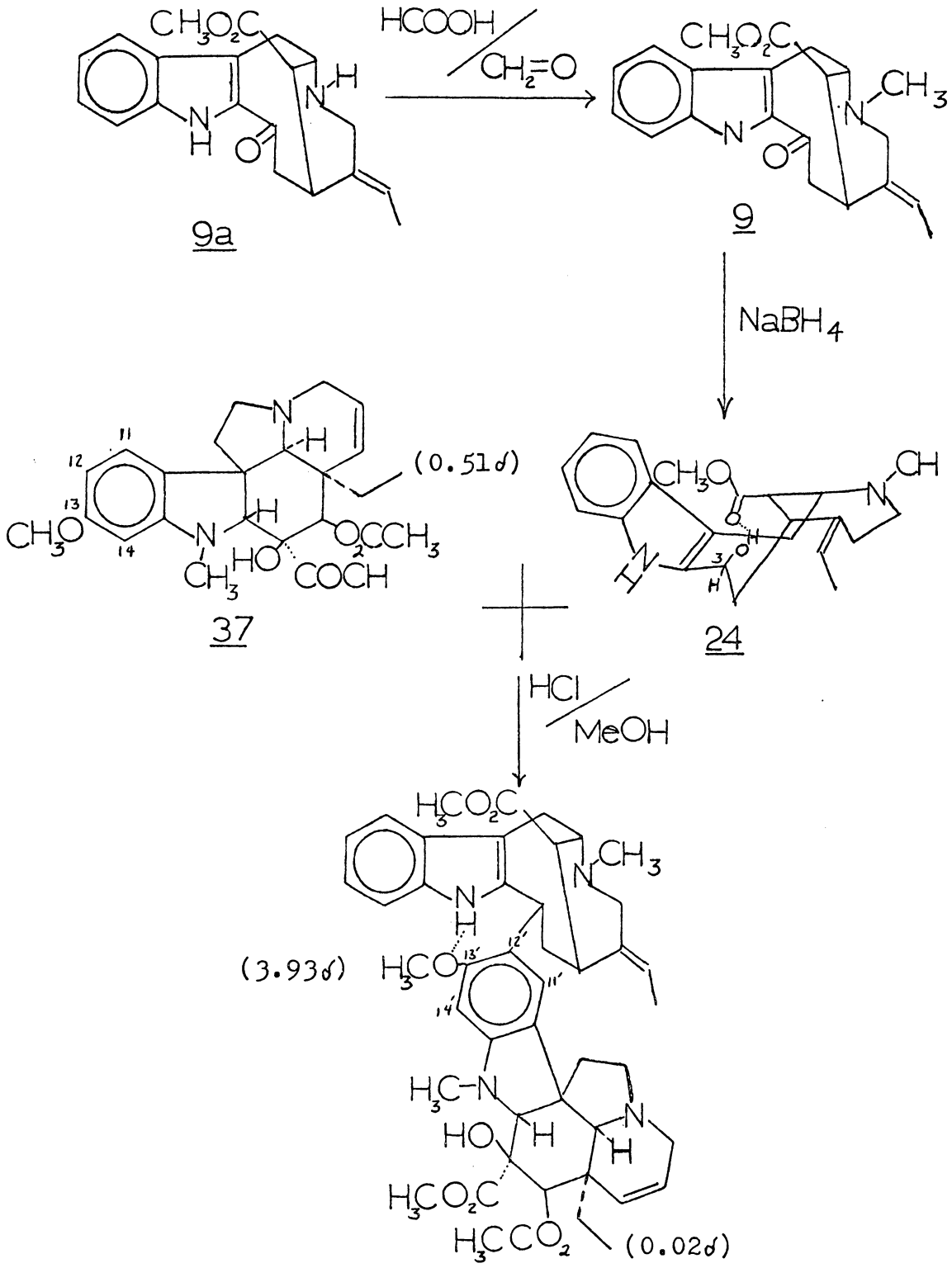
35 R= H

36 R= CH_3

assay, a test very sensitive to the biological properties of vinblastine and vincristine.[42] This is the only bis-indole alkaloid tested to date not showing anti-leukemic activity yet still possessing the specified O-N-O triangle dimensions. Consequently, this initiated in our laboratory the desire to synthesize a vobasine - vindoline dimer of similar structure and test it in the PS cell culture system to determine quantitatively the degree of activity and to compare it against the natural indole dimers found in the Tabernaemontana species. The difference between vobasivindoline (36) and perivindoline (35) is merely the presence of an N-methyl group in place of a hydrogen. It was hoped that this slight difference along with the existence of the O-N-O triangle structure

would improve the dimer's activity.

The partial synthesis of vobasivindoline (36) was conducted in three steps (Scheme VIII), in a similar fashion to the synthesis of tabernamine. Perivine (9a) was first subjected to reductive methylation to form vobasine (9), which was then reduced to the alcohol vobasinol (24) and this was condensed with vindoline (37), obtained from the Eli-Lilly Co.. The major reaction product obtained in 70% yield was purified by P.T.L.C. but it did not crystallize. The carbon, hydrogen, and nitrogen analysis indicated a composition of $C_{46}H_{56}N_4O_8$ based on a molecular weight of 792, obtained from the mass spectrum (fig. 6). The spectrum also showed intense fragment ions corresponding to the individual fragmentation



9a 9 37 24 36

Scheme VIII

of vobasine and vindoline. The base peak of m/e 122 is characteristic of both vobasinol and vindoline, while the ions at m/e 180 and 182 probably originate from vobasinol alone since they are present at high intensity in its spectrum. The vindoline portion is readily apparent in the fragment ions at m/e 144, 135, and 107, which in general characterize the aspidospermine skeleton.

The PMR spectrum ($CDCl_3$) (fig. 7) clearly indicated the formation of the vobasine - vindoline bis-alkaloid especially when compared to the spectra of the starting products. Five methyl singlets could quite plainly be seen. The sixth at 3.93δ was considerably broadened due to hydrogen bonding. This methyl is the carbomethoxy group on vindoline and, as seen in (36), is in the correct proximity to the indole nitrogen of vobasine to form a hydrogen bond. Another interesting point of the spectrum is the shielded methyl and methylene signals of the ethyl side chain in vindoline. The PMR spectrum of vindoline alone gives a methyl triplet at 0.51δ and two multiplets of the non-equivalent methylene protons at 1.70 and 1.18δ. Due to the fact that this ethyl side chain is anisotropically shielded by the vobasine indole ring system in the bis-alkaloid, the methyl triplet and pair of multiplets occur at 0.02δ and 1.42 and 0.98δ respectively.

The final essential point of the PMR spectrum to be

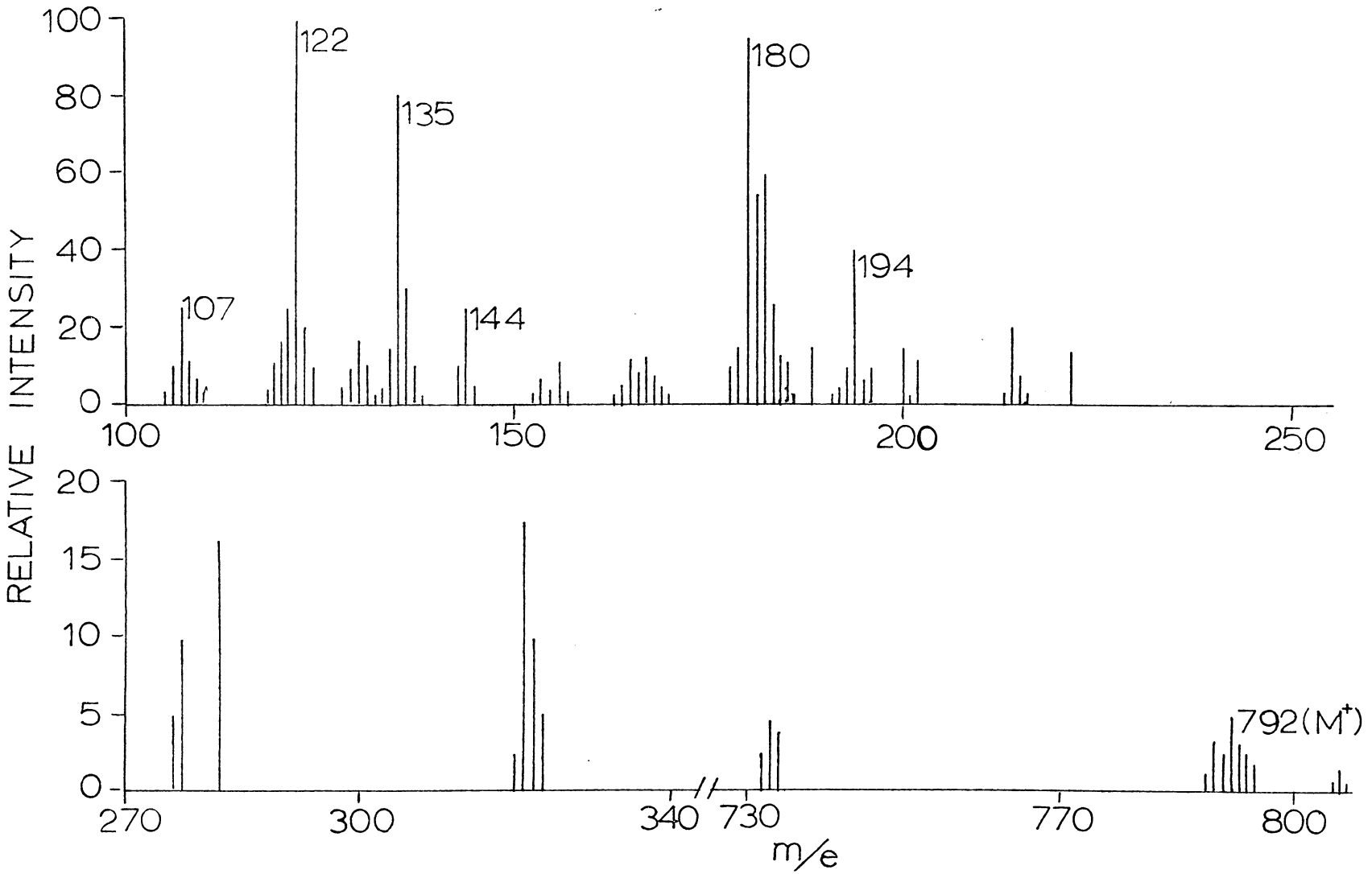


Figure 6 Mass Spectrum of Vobasivindoline

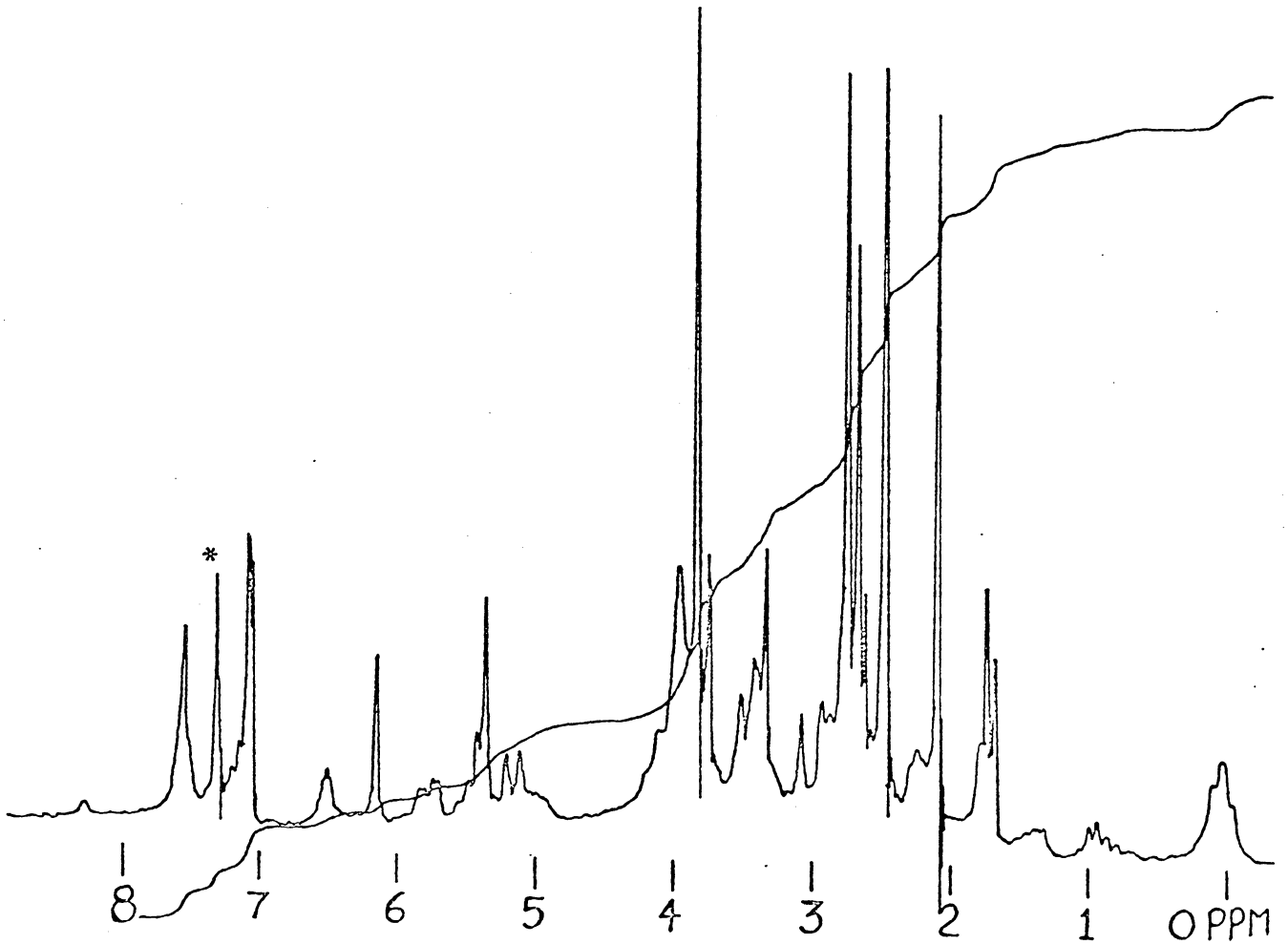


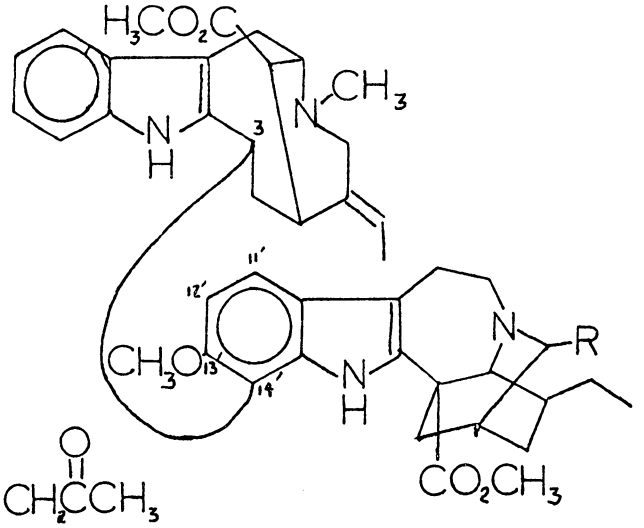
Figure 7 100 Hz Proton Magnetic Resonance Spectrum of Vobasivindoline in $CDCl_3$.
* Solvent peak

noted is the aromatic region which provides information on the point of attachment of vobasine to vindoline. This point seems to be at the 12′ carbon since in the PMR of vindoline itself the 12′ proton is a doublet of doublets at 6.30 δ yet it is completely absent in the dimer. Also, the 6.95 δ doublet representing the 11′ proton and the 6.08 δ doublet of the 14′ proton have coalesced to singlets (para splitting not detectable). Thus, the final structure of vobasivindoline is that shown in (36). The IR and UV spectra were not essential in verifying the absolute structure, yet they corresponded well with the structure proposed by evidence given in the PMR and mass spectra.

Vobasivindoline is the second of two compounds comprising a new class of dimeric indole alkaloids not isolated from natural sources. It is being tested for PS *in vitro* activity at the time of this writing. If it shows activity less than 20 μg/ml then testing on the PS *in vivo* scale will proceed.

D. Attempted Synthesis of 19-Acetonyl Voacamine

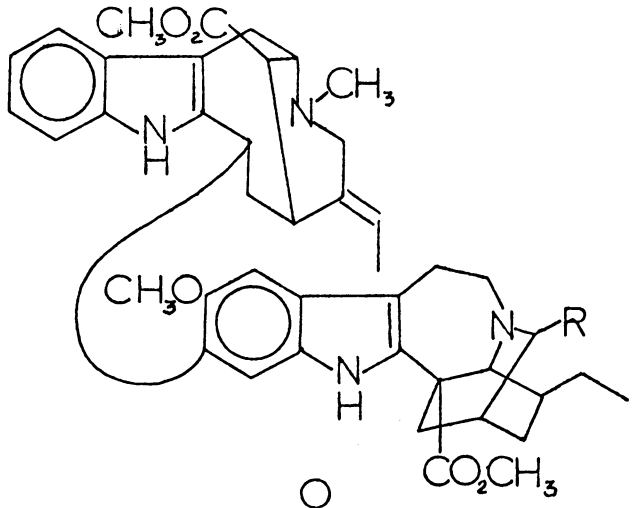
Recently a new type of indole dimer was isolated from *Tabernaemontana holstii* by Li,[32] who proposed a structure for it very similar to a voacamine type bis-alkaloid, but possessing an acetonyl group bonded at the C-19 position. From all available evidence it was assigned the structure 19-acetonyl conodurine (37).[32] Unfortunately very little of the compound was isolated (20 mg), and the structure could not be confirmed unambiguously from the spectroscopic evidence alone. The material had an ED_{50} activity of 2.4 μg/ml in the PS system. Being a new bis-indole alkaloid, coupled with the possibility that it may have accounted for the activity in the fraction from which it was isolated, it was of interest to us to attempt a synthesis of it.



37 R= $CH_2C(=O)CH_3$

30 R= H

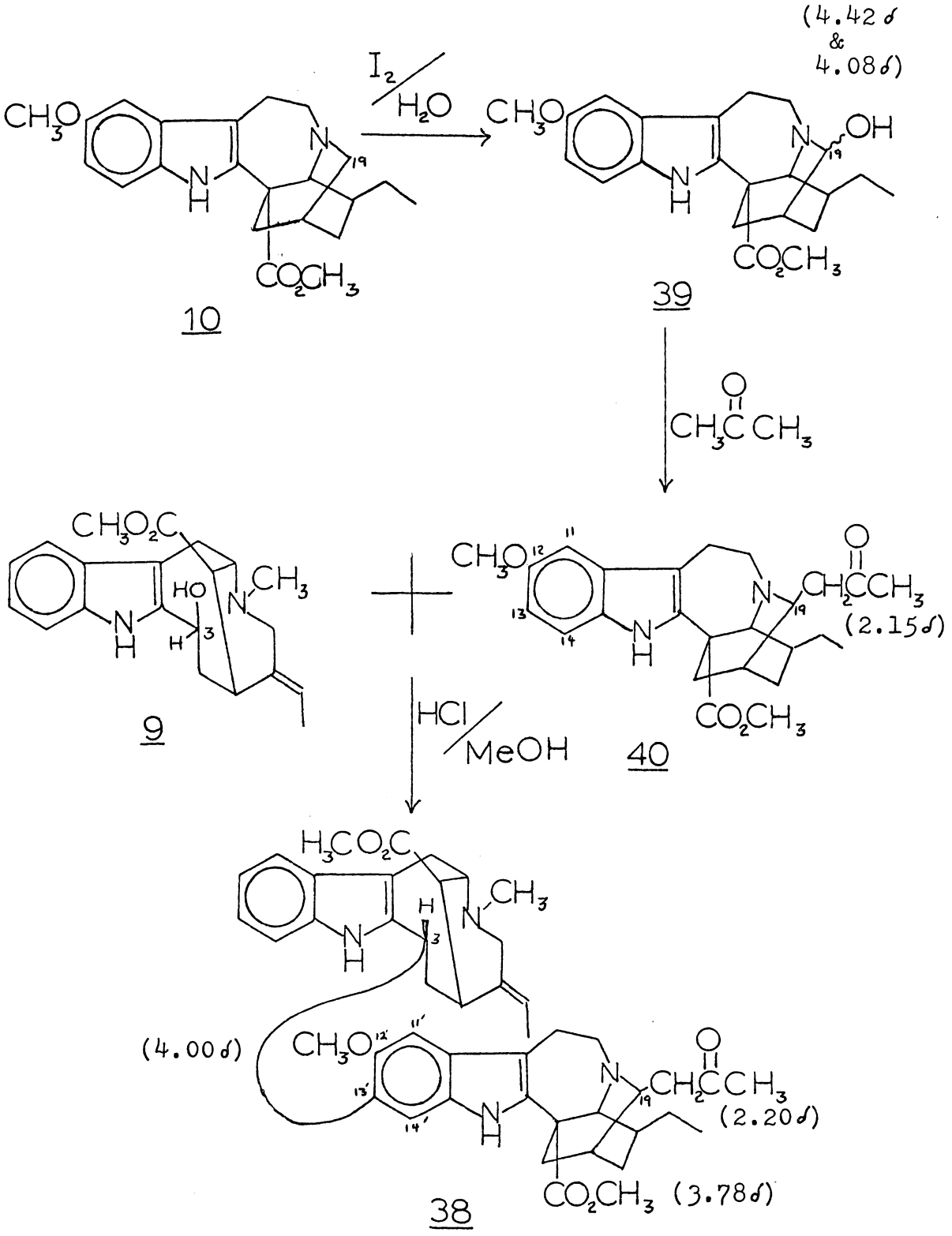
Conodurine itself is composed of a vobasine and isovoacangine moiety bonded between the C-3 of vobasine and C-14 of isovoacangine (30). The newly isolated compound is believed to have an acetonyl group on C-19 of the isovoacangine unit (37), but the stereochemistry at this point is uncertain. Although isovoacangine was isolated from the plant previously, it could not be obtained again. Thus, it was decided instead to use the isomer voacangine, isolated from Tabernaemontana arborea, to make the C-19 acetonyl derivative. The plan was then to condense this unit with vobasinol in the usual way to make 19-acetonyl voacamine (38), an isomer of 19-acetonyl conodurine (37).



38 R= $CH_2C(=O)CH_3$

41 R= OH

The approach to the synthesis of this bis-alkaloid was derived from previous work done by Agwada,et.al.,[43] who made 19-hydroxy isovoacangine from isovoacangine and then by condensation with acetone produced the 19-acetonyl derivative. It was our intention to do the same with voacangine (10) and then condense it with vobasinol (9) to form the 19-acetonyl isomer (Scheme IX). In total, this was a three step synthesis and it was expected that isomers would result from the hydroxylation and condensation reactions. Hopefully, they could be separated and normal spectral means could be used to determine one from the other. Even so, obtaining the 19-acetonyl dimer with the ketopropyl group in either or both configurations would be satisfactory, since this would, in effect, provide the correct structure for an evaluation of its general *in vitro* activity. If it proved to be an effective cytotoxic agent then attempts would be made to synthesize and purify both stereoisomers for a more precise activity reading.



Scheme IX

The first step in the synthesis was a selective oxidation reaction of voacangine on C-19, one of three carbons adjacent to the terpenoid nitrogen atom. The appearance of the hydroxylated voacangine (39) was detected quite readily in the PMR spectrum of one of the products, which showed two one proton doublets at 4.42 and 4.08δ. These signals resulted from the C-19 proton adjacent to the hydroxy group and signified the presence of isomers.[43] Some stereoselectivity was occurring however, since the 4.08δ doublet was four times the area of the 4.42δ doublet. This ratio of isomers was subjected to condensation with acetone, where only one isomer of 19-acetonyl voacangine (40) was isolated and characterized. It was not possible however, to determine from the available spectral data the exact conformation of the acetonyl group. The PMR spectrum clearly showed the methyl singlet of the acetonyl group at 2.15δ and the mass spectrum (fig. 8) revealed the correct molecular weight (424) along with significant fragments at m/e 368(100), 264(60), 184(56), 136(50), 122(54), and 43(69). Evidently, the loss of the acetonyl group (M-57) to m/e 368 occurred quite easily. Also m/e 43 is probably the $\oplus O{\equiv}CCH_3$ group, fragmented from the acetonyl side chain.

The IR spectrum in $CHCl_3$ was unusual in that it had a strong absorption at 1720 cm^{-1} for the carbomethoxy and only a small shoulder at 1705 cm^{-1} that was assigned

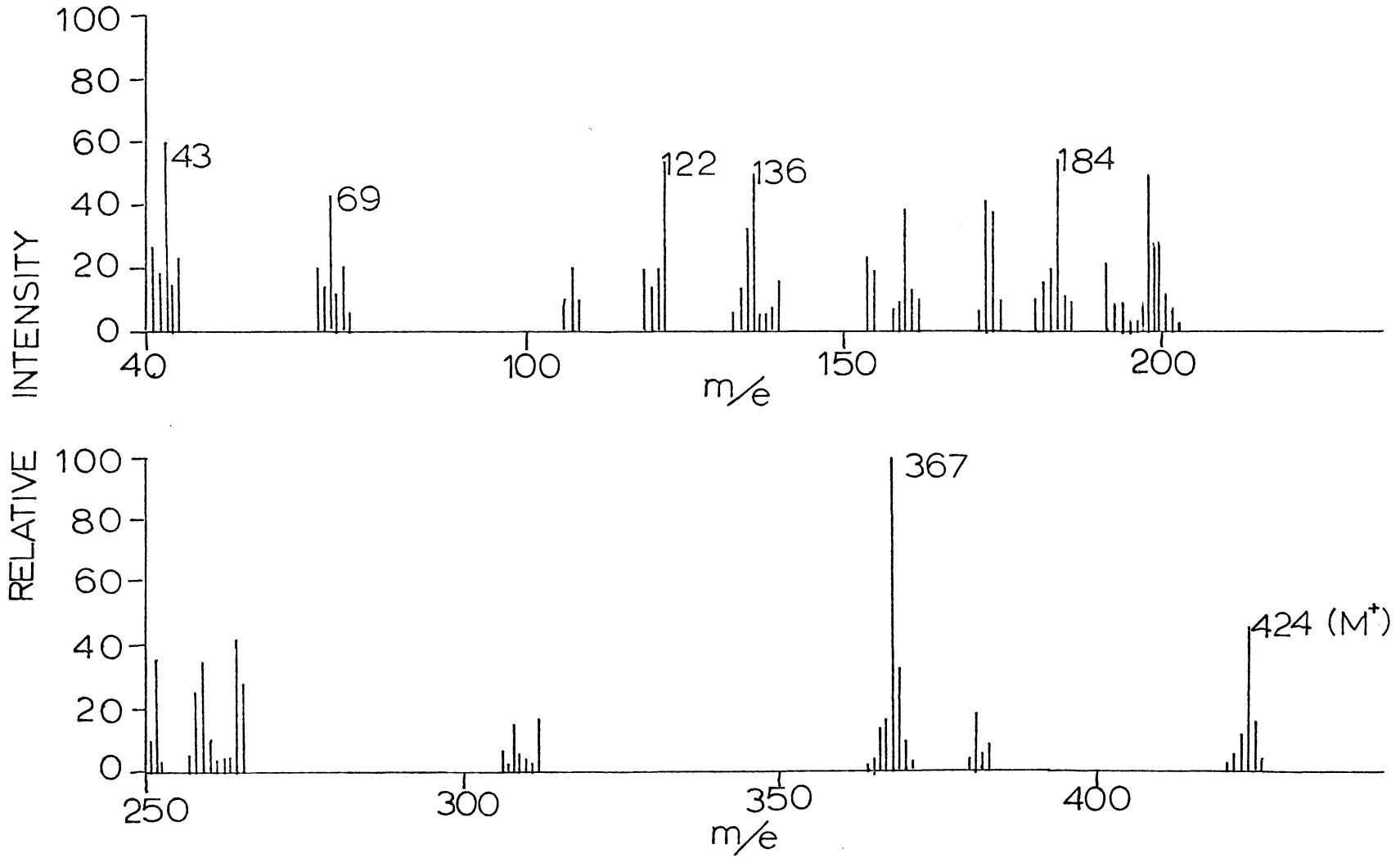


Figure 8 Mass Spectrum of 19-Acetonyl Voacangine

to the carbonyl absorption of the acetonyl group. This same observation however, was made by Li for 19-acetonyl conodurine,[32] and by Agwada for 19-acetonyl isovoacangine,[43] and evidently is characteristic of these species.

The next step in the synthetic scheme was the condensation of 19-acetonyl voacangine (40) with vobasinol (9). This was accomplished using the same procedure as in the synthesis of tabernamine and peribogamine.[23] Many products were obtained from the reaction, with the most abundant being isolated and purified by P.T.L.C.. It was pure on T.L.C., but H.P.L.C. showed the appearance of two major components in a 2:1 ratio (fig. 9). In spite of this, some spectral data were obtained to determine whether the product of interest had been formed. The mass spectrum, indicating a mass of 760 and a M+14 peak, is consistent with the expected structure. The fragmentation pattern was very similar to that of the isomer 19-acetonyl conodurine,[32] but the relative intensities differed quite dramatically. The only definite information that could be gained from the spectrum was the presence of 19-acetonyl voacamine (38), yet it was quite possible that voacamine (20) or 19-hydroxy voacamine (41) were present as contaminents. Support for this idea comes from the PMR spectrum (fig. 10), which clearly indicated more than one compound. The acetonyl methyl singlet appeared at 2.20 δ and quite evident were the

Column	Partisil-10 (2mm o.d.x25cm)
Eluant	10% Hexane 90% Ethyl Acetate
Flow Rate	2 ml/min.
Pressure	1100 psi
Detector	uv (280 nm)
Sensitivity	0.16 aufs
Chart Speed	0.14 in./min.

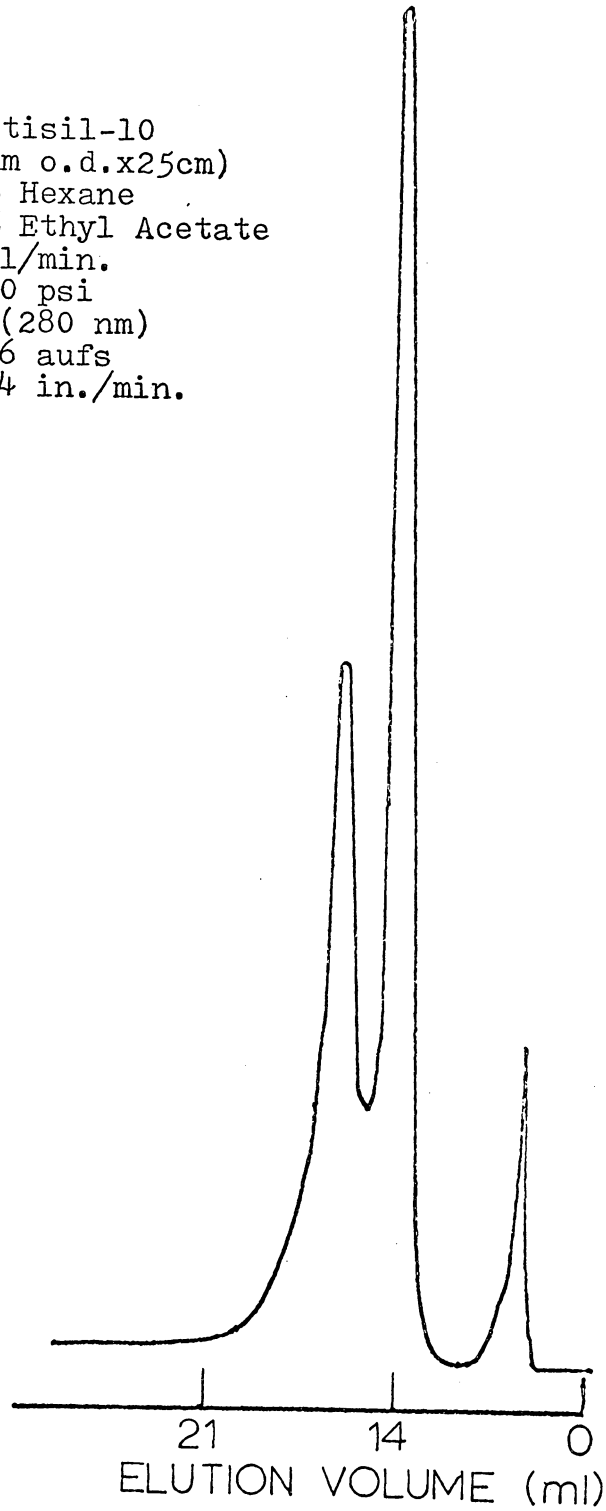


Figure 9 Separation of Products from Synthesis of 19-Acetonyl Voacamine

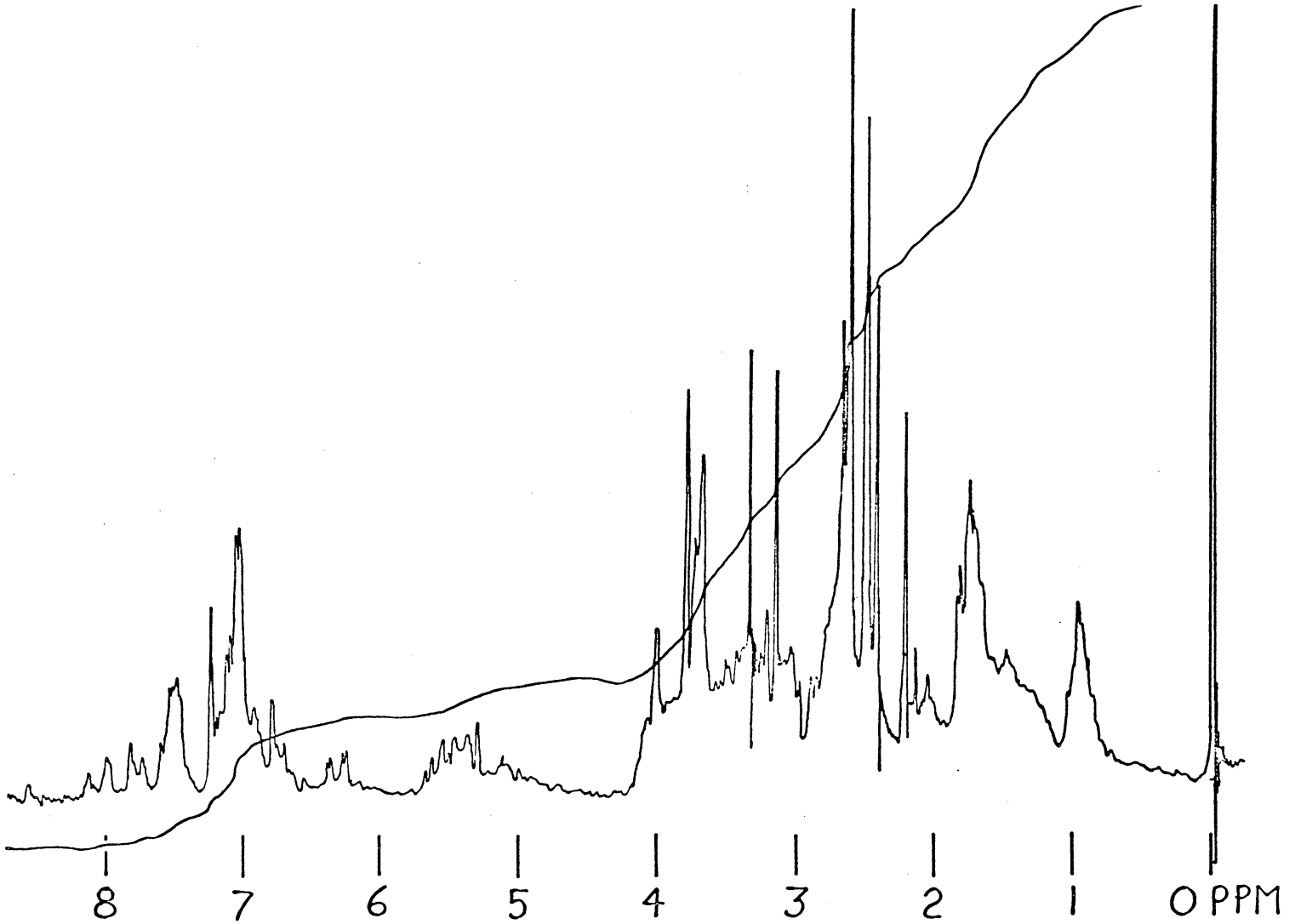
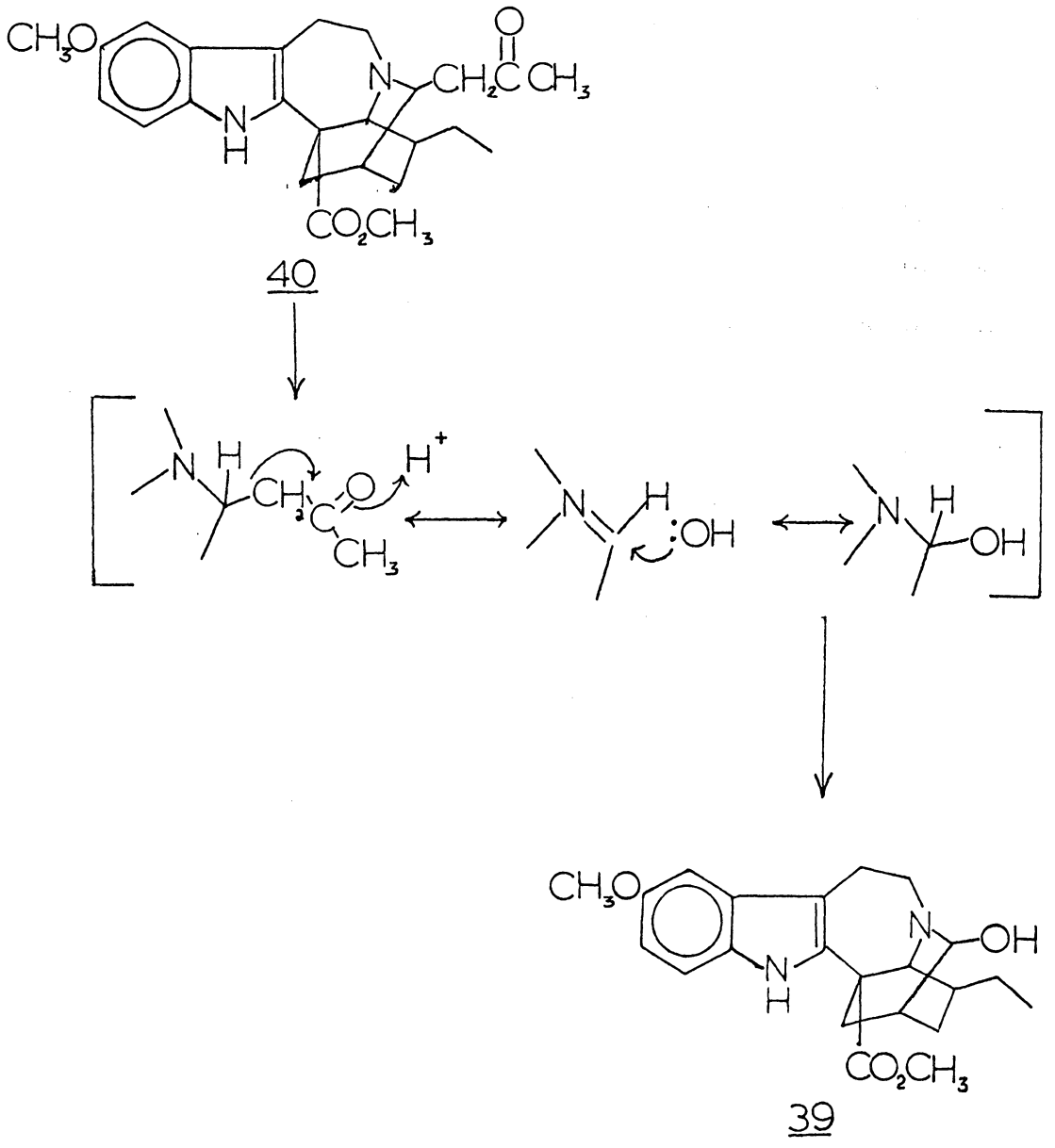


Figure 10 100 MHz Proton Magnetic Resonance Spectrum of 19-Acetonyl Voacamine isomers in $CDCl_3$.

singlets for the methoxy (4.00δ) and carbomethoxy (3.78δ) of the voacangine moiety. One unusual aspect of the spectrum was the methyl singlets of the vobasan portion between 2.40 and 2.65 δ. There were four methyl singlets where normally there should be two, representing the $N\text{-}CH_3$ and anisotropically shielded carbomethoxy groups of vobasan. Evidently, from this evidence it is possible to assume the presence of 19-acetonyl voacamine, yet there are obviously additional structures present, possibly 19-hydroxy voacamine (41) or voacamine itself (20).

During the rather vigorous refluxing in acidic methanol it is quite possible the acetonyl group was hydrolyzed to form the hydroxy derivative (Scheme X). This might then have condensed with vobasinol to form 19-hydroxy voacamine (41). In any case, considering the numerous products obtained and the difficulty met in isolating one product, a different synthetic scheme may be warranted. One alternative route is to make hydroxy voacamine first by condensing vobasinol (9) and 19-hydroxy voacangine (39) and then reacting this bis-indole with acetone in the usual fashion. The yield from this method may be higher, and an additional benefit may be the ease of purification as a result of fewer by-products and side reactions.



Scheme X

SUMMARY

This work has been concerned with the phytochemical investigation of the plant species Tabernaemontana johnstonii and the synthesis of new alkaloids based on structure - activity correlations. The purpose of both studies was to obtain a pure anti-carcinogenic compound effective against the tumor systems established by the National Cancer Institute.

The phytochemical work involved the location in the plant of the highest area of anti-tumor activity by means of bioassay monitoring at each stage of fractionation. This process led to the concentration of highest activity among the alkaloid constituents and resulted in the isolation of a new bis-indole alkaloid. Testing of this compound by the National Cancer Institute showed that it was very effective in the in vitro system (1.9 μg/ml), but failed in the in vivo system (125% at 25 mg/kg) to meet the required level of activity considered for further testing. By way of spectral, degradative, and synthetic means, the structure of this compound was established as a new bis-indole alkaloid belonging in the voacamine series. It was named tabernamine and is composed of two monomeric species, vobasan and ibogamine, bonded together in a similar fashion as voacamine.

The isolation of tabernamine extended the list of

alkaloids in the voacamine series that have been isolated from this plant and other Tabernaemontana species. Previously isolated were the structural isomers conodurine and conoduramine and the closely related alkaloid gabunine. It was recognized that listing these four bis-indole alkaloids, including tabernamine, in the increasing order of their in vitro activities revealed a definite correlation between structure, in terms of the presence or absence of particular functional groups, and activity. This structure - activity correlation led to the postulation that a new bis-indole alkaloid of the voacamine series consisting of the monomers perivan and ibogamine may possibly show an increase in activity over the other related alkaloids. This new bis-indole alkaloid, named peribogamine, was synthesized from the monomers perivinol and ibogamine and was found to have a structure identical to tabernamine except for the presence of a hydrogen in place of an N-methyl group.

Additional structure - activity investigations involved the application of the O-N-O triangular pharmacophore found in many anti-leukemic compounds. This particular arrangement of two oxygen atoms and a nitrogen atom separated by specified distances was postulated to exist in a new type of bis-indole alkaloid composed of vobasan and vindoline. This compound, named vobasivindoline, was synthesized from the monomers vobasinol

and vindoline to test for any anti-carcinogenic activity that might appear as a result of the O-N-O triangular structure. Vobasivindoline also represents a new "synthetic" class of bis-indole alkaloids comprising a corynanthe´ skeleton (vobasan) bonded to the aromatic region of an aspidosperma alkaloid (vindoline). It is the second member in this series to be synthesized, perivindoline (perivan plus vindoline) being the first.[41]

The final area of synthetic work directed toward finding an effective cytotoxic agent concerned the synthesis of a somewhat novel bis-indole alkaloid that was presumed from spectral evidence to be the bis-alkaloid conodurine containing an acetonyl group on the C-19 carbon of the iboga moiety (isovoacangine). This new compound, 19-acetonyl conodurine, elicited a high activity in the in vitro test (2.4 μg/ml), which in all probable cases was due to the acetonyl group since conodurine itself had a low activity level of 29 μg/ml. Due to the availability of only a limited amount of the required monomeric alkaloids it was not possible to synthesize this structure, but synthesis of the isomer, 19-acetonyl voacamine was possible with the available indole monomers voacangine and vobasine. It was hoped that the slight differences between the two structures would not critically affect the cytotoxic activity. Along the pathway to this synthesis the new monomeric indole alkaloids of 19-hydroxy

voacangine and 19-acetonyl voacangine were synthesized and characterized, however the condensation of vobasinol with 19-acetonyl voacangine to produce the sought after structure was not fully isolated and characterized. It was present, however, as detected through available spectral evidence and then submitted in its' impure form for *in vitro* testing.

Only the natural product bis-indole alkaloid tabernamine has so far been tested in the tumor systems established by the National Cancer Institute. The other synthetic structures were submitted, but as of present there are no results to report. A sign of high activity among these new alkaloids will lend support to the particular structure - activity correlations that resulted in their synthesis and will most probably be impetus for further investigations along this line.

EXPERIMENTAL

A. General Information

Infrared spectra were either recorded in KBr or $CHCl_3$ on a Beckman IR-20AX infrared spectrometer.

Ultraviolet spectra were run in 95% ethanol solution on a Cary 14 U.V.-visible spectrometer.

Proton Magnetic Resonance spectra were obtained on a JEOLCO JNM-PS-100 instrument using deuteriochloroform as the solvent unless otherwise noted. Tetramethylsilane (TMS) was used as the internal standard.

The mass spectra were obtained on a Varian MAT-112 double focussing spectrometer. Perfluorokerosene was used as the reference in counting the spectra.

The data from carbon - hydrogen - nitrogen analysis was obtained on a Perkin-Elmer 240 Elemental Analyzer.

Solvents employed were reagent grade and were redistilled in glass.

The thin layer chromatography plates were made in the laboratory. Analytical plates were 0.30 mm of silica gel GF-254 spread from an aqueous slurry. The preparative plates were silica gel PF-254, from 1-1.5 mm thick, spread also from an aqueous slurry. Visualization of T.L.C. plates was performed using a Chromato-Vue CC-20 long and short wave viewing apparatus manufactured by Ultra-violet Products, Inc.. Further visualization was

accomplished by spraying with ceric sulphate- sulphuric acid solution (saturated solution of ceric sulphate in 65% sulphuric acid).

High pressure liquid chromatography separation and analysis were performed with a Waters M-6000A high pressure pump and a Glenco 5480 U.V. Monitor. Columns used were either laboratory packed spherisorb (10μ particle) or prepacked normal phase and ODS columns from Reeve Angel. Other specifications are mentioned in specific applications.

B. Extraction and Fractionation

A total of 10.3877 kg of dried plant material was put through the initial extraction process in 4 parts with each containing approximately 2.5 kg. The dried plant was placed into 4 liter beakers, stirred with cold 95% ethanol for 24 hours, decanted, and stirred again with fresh ethanol for 4 hours. These ethanol extracts were decanted again and fresh ethanol was added followed by warming on a hot plate with stirring for 4 additional hours. Dilute NH_4OH was added to each beaker and stirred for another 4 hours.

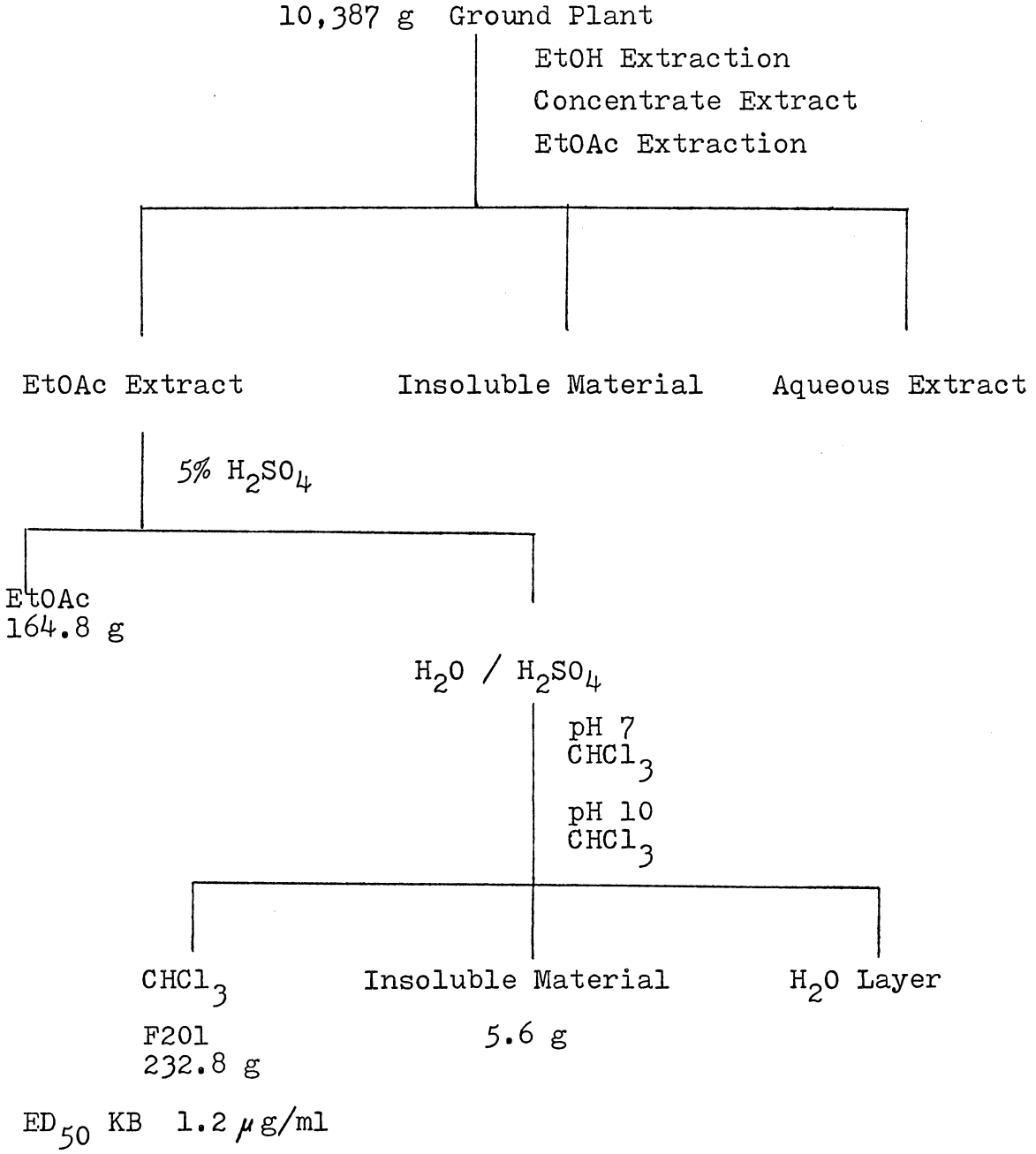
The remaining solid plant material was filtered off and discarded, and all the ethanol extracts were combined and evaporated to 1 liter. This extract was added to a separatory funnel with 300 ml of water and shaken 3 times with 500 ml portions of ethyl acetate. The ethyl acetate fractions were combined, while the remaining aqueous layer was made basic with NH_4OH and put in a liquid-liquid extractor. Exhaustive extraction with ethyl acetate was continued for three days with the solvent being changed each day. The final ethyl acetate extract was practically colorless.

All of the ethyl acetate extracts were combined, dried with anhydrous $MgSO_4$, and evaporated to 1 liter. The ethyl acetate was then extracted with six 500 ml

portions of 5% H_2SO_4 and washed with 1 liter of water. The H_2O - H_2SO_4 was partitioned with 2 liters of fresh ethyl acetate. The H_2O - H_2SO_4 extract was adjusted to pH 7 with NH_4OH and extracted with chloroform until the chloroform layer was clear. This was repeated with the water layer at pH 10. In all, about 10 liters of chloroform were used. The chloroform layer was dried with anhydrous $MgSO_4$, filtered, and evaporated to dryness yielding 232.8 g of a crude alkaloid fraction designated F201 (Table VI).

TABLE VI

EXTRACTION OF TABERNAEMONTANA JOHNSTONII



1. Column Chromatography of F201

Three open columns were run of fraction F201. The columns were 5 X 120 cm and each was dry packed with 300 g of Woelm Alumina (Grade III). A "free flowing solid" was prepared by mixing 80 g of the sample with 100 g of alumina support and benzene and then evaporating to dryness on a rotary evaporator. The sample was then poured on top of the packed bed and elution started with benzene, then chloroform, n-propanol, and finally methanol. Fractions were collected until the eluant became clear, then the next solvent was used (Table I).

2. Column Chromatography of F202

The column packing procedure was as follows:[44] 2,530 g of silica gel PF-254 were dried in an oven at 120 for 24 hours. It was deactivated by vigorously shaking with 10% water in 3 separate 4 liter flasks. A 25% w/v slurry (10.1 liters) was made with the eluting solvent, chloroform. This slurry was vigorously shaken and allowed to equilibrate for 12 hours. Glass wool and sand were placed at the bottom of a 10.5 X 140 cm glass column and the stopcock was left slightly open to assure only a small initial flow. All the slurry was added in one continuous pouring which filled the column to capacity. The flow rate was adjusted to approximately 20 ml per minute for the first hour and 2.25 ml per minute for 24 hours. The final flow rate became 1.5 ml per minute with the stopcock fully open and the column full of solvent. In total 131.6 g of F202 were divided into 3 equal parts and each part was developed separately on the same column packed bed. The sample was dissolved into the eluting solvent, chloroform, to make a 15% solution (44 g sample/290 ml solvent). This was then gently applied by a pipet to the top of the column bed. The column was developed by progressively increasing the percent of methanol in chloroform and was monitored by T.L.C. and weight of fractions. After the first sample

was completed the column was washed with 30% methanol in chloroform for 24 hours and then re-equilibrated with 100% chloroform for 48 hours. The second and third samples were developed in the same way, with solvent polarity being increased according to the elution volume. Thin layer chromatography was then used as a guide to combine the three separate runs into fourteen fractions (Table I).

3. Column Chromatography of F242

A 5.5 X 100 cm glass column was packed with 516 g of silica gel PF-254 made up in a 25% w/v slurry of 8% 0.95 ethanol in ethyl acetate. Essentially the same procedure was used as in the column packing of F202. 9.745 g of F242 were dissolved in 65 ml of solvent and applied to the column bed which had a height of 50.5 cm. The final flow rate with the column filled with solvent was 1.2 ml per minute. The column was developed starting with 8% 0.95 ethanol in ethyl acetate and increasing in increments of 2% to 16% ethanol in ethyl acetate. Finally the column was completely eluted with pure 0.95 ethanol. The development was monitored by weight of material eluted, thin layer chromatography, and reverse phase high pressure liquid chromatography. The high pressure column used in this case was a 0.2 X 25 cm Partisil 10μ ODS column prepacked by Whatman Inc. (Table I).

C. Isolation of Tabernamine (29)

100 mg of F253 from column chromatography of F242 were subjected to preparative thin layer chromatography on a 1.5 mm silica gel PF-254 plate developed with 15% methanol in methylene chloride. Three full developments of the plate brought the desired U.V. absorbing component to 0.60 Rf. The plate was allowed to dry fully and the band containing the desired component was scraped off and washed with 50 ml methanol : methylene chloride 25:75. This slurry was then filtered and the clear filtrate evaporated down to dryness. T.L.C. of the component revealed some impurities, but mainly one spot that appeared very dark when sprayed with cerric sulphate spray and heated to 110°C. Streaking of the component on the T.L.C. plate was a common occurance and difficult to control. Final isolation or clean up of the single component was accomplished with high pressure liquid chromatography. A Partisil 10μ 4.6 mm X 25 cm column prepacked by Reeve Angel was used in the analytical run. Preparative isolation of Tabernamine was accomplished with a 7 mm X 30 cm 10μ spherisorb column packed in a slurry. The packing material was from Phase Separations Inc. and is composed of perfectly spherical silica gel particles.

The component collected from H.P.L.C. was a white amorphous solid, chromatographically pure on T.L.C.

in two solvent systems, but crystallization did not occur in any of the common solvents. It had $[\alpha]_D^{22}$ -51 (c=0.18, CH_3OH); mass spectrum m/e 630(4), 616(M^+,40), 585(20), 182(60), 136(84), 122(100); λ_{max}^{EtOH} (log ϵ) 235(4.53), 287(4.02), and 295(4.00) nm. Its IR spectrum showed ν_{max} 1720 cm^{-1}, and its PMR spectrum (CD_3OD) showed peaks at 0.95δ(3H, triplet), 1.68δ(3H, doublet), 2.50δ(3H, singlet), 2.56δ(3H, singlet), 5.38δ(1H, m), 6.88δ(1H, doublet, J = 8Hz), 7.04δ(4H, complex), 7.28δ(1H, doublet, J = 8Hz), and 7.54δ(1H, complex). It is important to note that the PMR spectrum of Tabernamine had to be recorded under an inert atmosphere. Decomposition in the solvent prevented the recording of the spectrum while exposed to oxygen in the air.

D. Acid Cleavage of Tabernamine (F262) (29)[23]

26 mg of F262 were dissolved in 18 ml of 50% HCl - methanol solution. This was heated under reflux for 12 hours. The solution was treated with saturated Na_2CO_3 solution until neutral to pH. It was extracted with methylene chloride, dried with magnesium sulphate, and evaporated down to dryness. Preparative thin layer chromatography in ethyl ether yielded ibogamine, identified by T.L.C. in 3 systems (10% methanol in benzene, 10% methanol in chloroform, and ethyl ether) and H.P.L.C. in one (Partisil 10 , 2 mm X 25 cm column with 100% ethyl acetate), and comparison of its mass spectrum with that of the authentic sample.

E. Reductive Methylation of Perivine (9a) to Vobasine (9)[41]

1.00 g of Perivine was dissolved in 20 ml dioxane and 20 ml formalin and to that 100 mg of palladium over charcoal catalyst (10%) were added. The mixture was stirred in a hydrogen atmosphere and the volume uptake of hydrogen was recorded as shown below.

time	volume hydrogen uptake
0:00	0 ml
1:20	21 ml
3:00	48 ml
7:00	80 ml

The reaction was completed in approximately seven hours. The entire mixture was filtered to remove carbon, extracted with ether once and methylene chloride three times. It was dried with $MgSO_4$, evaporated and weighed 1.055 g.. Preparative thin layer chromatography with 8% methanol in methylene chloride yielded 496 mg of vobasine (9). This was obtained as a white amorphous powder identical to the known alkaloid in the IR and PMR spectra. Its IR spectrum showed ν_{max} 1720 cm^{-1}, 1640 cm^{-1}, and 3420 cm^{-1}, and its PMR spectrum ($CDCl_3$) showed peaks at 2.58δ(3H, singlet), 2.62δ(3H, singlet), 5.40δ(1H, multiplet), and 1.72δ(3H, doublet).

F. Alternative Method for the Reductive Methylation of Perivine (9a) to Vobasine (9)[45]

111 mg of perivine were added to 3 ml of a 1:1 solution of 91% formic acid (aq) and 37% formaldehyde in a 15 ml round bottom flask. The mixture was stirred and heated lightly (50°C) for 2½ hours. It was then poured into 50 ml of a 1% HCl solution. After washing with ether the aqueous layer was made basic with two NaOH pellets and extracted three times each with 20 ml of ethyl acetate. The ethyl acetate layer was washed once with salt solution, dried with $MgSO_4$, and evaporated. The yield of crude product was 130 mg. Purification of Vobasine was accomplished by P.T.L.C. in 6% methanol in methylene chloride. This proved to be a better method in terms of yield and steps to purification of the product than the catalytic reduction described in part H. Final yield was 86 mg vobasine.

G. Reduction of Vobasine (9) to Vobasinol (24)

286 mg of vobasine were dissolved in 10 ml methanol in a 3 necked round bottom flask. CO_2 gas was continuously bubbled through the solution through a pipet while the reaction mixture was vigorously being stirred. 2.5 g of $NaBH_4$ was added in small increments over a 5 hour period. The reaction was monitored by removing a capillary amount of solution, treating it with a drop of base, and shaking this with ethyl ether. T.L.C. of the ether portion in 5% methanol in methylene chloride over several hours showed the gradual disappearence of vobasine. The reaction solution was diluted with 20 ml water and extracted three times with methylene chloride. It was dried with $MgSO_4$ and evaporated down to dryness. P.T.L.C. in 10% methanol in methylene chloride yielded 154 mg of vobasinol identical to the literature IR spectrum in $CHCl_3$,[46] ν_{max} 1690 cm^{-1} and broad 3440 cm^{-1}.

H. Synthesis of Tabernamine (F262) (29)[23]

120 mg of vobasinol and 138 mg of ibogamine were dissolved in 10 ml of a 1.5% HCl - methanol solution in a 25 ml 3 necked round bottom flask. A nitrogen atmosphere was maintained throughout the reaction by passing the gas through a three way glass joint, one lead immersed in water, one originating from the N_2 tank, and the third passing into the round bottom flask. A condenser was placed on top, all other openings were sealed, and the solution was refluxed. The reaction was monitored by T.L.C. and after 12 hours it appeared that no vobasinol was left. The solution was neutralized with Na_2CO_3 saturated solution and extracted three times with methylene chloride. This was dried and evaporated to dryness to yield 288 mg of product. P.T.L.C. in 12% methanol - methylene chloride yielded 41 mg of pure dimerized product identified as tabernamine (29) by comparison of T.L.C., IR, PMR, and mass spectrum.

I. Reduction of Perivine (9a) to Perivinol (24a)[23]

500 mg of perivine were dissolved in 20 ml of methanol. While CO_2 was continuously bubbled through the mixture, 3 g of $NaBH_4$ were added over an 8 hour period. The reaction mixture was diluted with 50 ml water and extracted 3 times with 15 ml methylene chloride to yield 418 mg of product. P.T.L.C. in 12% methanol - methylene chloride yielded 93 mg of perivinol identified by the literature IR spectra in chloroform,[46] ν_{max} 1690 cm^{-1} (CH_3OCO-). The apparently low ν_{max} is due to hydrogen bonding of the C-3 hydroxy group to the carbonyl oxygen atom (24a).[23]

J. Synthesis of Peribogamine (33)

164 mg of perivinol and 179 mg of ibogamine were dissolved in 20 ml of 1.5% HCl - methanol solution. The reaction mixture was refluxed for 12 hours under a nitrogen atmosphere. The solution was diluted with 20 ml of water and neutralized with 0.5 ml saturated Na_2CO_3 solution. Extracting three times with methylene chloride, drying with $MgSO_4$, and evaporating to dryness yielded 287 mg of crude product. P.T.L.C. in 12% methanol - methylene chloride yielded 57 mg of peribogamine (33), an amorphous white powder homogeneous on T.L.C. in three systems and H.P.L.C. in one. It had a mass spectrum m/e 602(10), 616(2), 181(30), 169(45), 149(42), 122(42), 136(100), 122(42), 119(55); $\lambda_{max}^{EtOH}(\log\epsilon)$ 235(4.55), 287(4.08), and 295(4.03) nm. Its IR spectrum showed ν_{max} 1705 cm^{-1}, and its PMR spectrum (CD_3OD) showed peaks at 0.98δ(3H, triplet), 1.75δ(3H, soublet), 2.52δ (3H, singlet), 5.45δ(1H, multiplet), 6.88δ(1H, doublet, J = 8Hz), 7.31δ(1H, doublet , J = 8Hz). Analytical Calculated for $C_{39}H_{46}N_4O_2$: C, 77.74; H, 7.64; N, 9.30; found: C, 78.31; H, 7.74; N, 9.53.

K. Synthesis of Vobasivindoline (36)

77 mg of vobasinol and 88 mg of vindoline were dissolved in 20 ml of 1.5% HCl - methanol in a 50 ml 3 necked round bottom flask. The solution was refluxed for five hours under nitrogen atmosphere. After cooling, 20 ml of water were added along with enough saturated Na_2CO_3 to neutralize the solution. This was extracted 3 times with 15 ml of methylene chloride, dried with $MgSO_4$, and evaporated to dryness. The yield was 166 mg of crude product. The vobasivindoline which appeared 0.1 Rf units below vobasinol, was isolated and purified by P.T.L.C. in 6% methanol in methylene chloride. The yield of pure product was 56 mg. It did not crystallize, but was homogeneous in two T.L.C. systems. It had a mass spectrum of m/e 792(M^+(08)), 733(05), 282(25), 194(40), 183(25), 182(60), 181(55), 180(95), 144(25), 136(15), 135(80), 122(100), and 107(20); λ_{max}^{EtOH}(logϵ) 204(4.65), 286(4.04), and 294(4.64) nm. Its IR spectrum ($CHCl_3$) showed ν_{max} 3420 cm^{-1}, 1730 cm^{-1}, 1710 cm^{-1}, 1605 cm^{-1} and its PMR spectrum ($CDCl_3$) showed peaks 0.02δ(3H, triplet), 1.68δ(3H, doublet), 2.08δ(3H, singlet), 2.45δ(3H, singlet), 2.64δ(3H, singlet), 2.73δ(3H, singlet), 3.80δ(3H, singlet), 3.93δ(3H, broad singlet). Analytical Calculated for $C_{46}H_{56}N_4O_8$: C,69.70; H, 7.07; N, 7.07; found: C, 70.01; H, 7.00; N, 7.12.

L. Oxidation of Voacangine (10) to 19-hydroxy Voacangine (39)[43]

130 ml benzene, 130 ml water, and 600 mg voacangine were placed in a 1 liter round bottom flask and stirred vigorously. 600 mg I_2 dissolved in 120 ml benzene were added over a 2 hour period at room temperature. The solution was then stirred for 48 hours at room temperature. The benzene phase was separated, washed with 50 ml 10% $NaHCO_3$ solution and 40 ml 10% $Na_2S_2O_3$ solution. Added to the aqueous phase was 10 ml 10% $NaHCO_3$ solution and 10 ml 10% $Na_2S_2O_3$ solution. The aqueous phase was extracted with methylene chloride and this was washed with water. The organic phases were combined and dried with $MgSO_4$. Evaporation yielded 700 mg crude product. P.T.L.C. in ethyl ether:hexane, 3:2, showed that considerable starting material was still present (approximately 20%). 220 mg of the isomers were obtained and shown to be homogeneous on T.L.C.. It could not be crystallized. The PMR spectrum of this compound showed doublets for H-C(19) at 4.10 δ and 4.45 δ providing evidence for two isomers with the hydroxy group in either of two positions at carbon 19 (39).

M. Synthesis of 19-Acetonyl Voacangine (40) from 19-Hydroxy Voacangine (39)[43]

74 mg of partially pure 19-hydroxy voacangine were placed in 10 ml of pure acetone. The mixture was heated at 40°C for 24 hours. T.L.C. of the product in ethyl ether:hexane, 3:2, showed the appearance of a spot at 0.7 Rf, just 0.1 Rf below voacangine. This component turned deep blue when sprayed with cerric sulphate spray and heated to 110°C. The material was shown to be pure in two T.L.C. systems and H.P.L.C., but only a thin, clear film would form when dried; no amorphous or crystalline material could be obtained. It appeared from the PMR spectrum that the product was not a mixture of isomers. It had a mass spectrum of m/e 424(M^+(46)), 368(100), 264(40), 259(34), 258(28), 184(56), 174(37), 173(41), 166(39), 136(50), 122(54), 69(44), 43(62); λ_{max}^{EtOH} (logϵ) 225(4.45), 286(3.95) nm. Its IR spectrum showed ν_{max} 1720 cm^{-1}, 3460 cm^{-1}, and 1490 cm^{-1}. And its PMR spectrum ($CDCl_3$) showed peaks at 0.90δ(3H, triplet), 2.15δ(3H, singlet), 3.75δ(3H, singlet), and 3.84δ(3H, singlet).

N. Synthesis of 19-Acetonyl Voacamine

130 mg of vobasinol (24) and 100 mg of 19-acetonyl voacangine (40) were combined in 15 ml 1.5% HCl - methanol solution. This was refluxed gently overnight under a nitrogen atmosphere. The product solution was diluted with 20 ml water and neutralized with saturated Na_2CO_3 solution. Extraction with methylene chloride four times, drying with $MgSO_4$, and evaporating off solvent left 223 mg of crude product. T.L.C. in 6% methanol - methylene chloride showed many products. One component was isolated by P.T.L.C. in 6% methanol - methylene chloride with a Rf value of 0.4 and below the starting materials. This was found to be two components as seen by H.P.L.C. on a Partisil, 10 μ, 2 X 250 mm prepacked column from Reeve Angel Inc. with 10% hexane in ethyl acetate as the eluting solvent. The mass spectrum of this mixture had peaks at m/e 760(M^+, 05), 774(M+14, 03), 718(05), 704(09), 552(10), 510(08), 368(10), 336(50), 277(70), 221(45), 181(65), 180(90), 168(95), 167(100), 122(90).

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NATURAL AND SYNTHETIC BIS-INDOLE ALKALOIDS AS CYTOTOXIC AGENTS

by

Bruce B. Gerhart

(ABSTRACT)

The National Cancer Institute, in conjunction with the U.S. Department of Agriculture, procured the African tree species Tabernaemontana johnstonii and found that the plant possessed anti-tumor activity in two bioassay tests. Consequently, extraction and systematic fractionation of the plant in conjunction with bioassay monitoring revealed that most of this activity was concentrated in the alkaloidal portion.

Purification of active compounds was attempted by open column liquid chromatography with both alumina and silica gel supports and by thin layer and high pressure liquid chromatography on silica gel. A compound was eventually isolated and purified and it proved to be a new bis-indole alkaloid. It was given the name tabernamine and showed an activity of 1.9 μg/ml (ED_{50}) in the PS in vitro system and a T/C % of 125 at a dose of 25 mg/kg in the PS in vivo system.

In conjunction with these results and the knowledge of the structure and activity levels of similar alkaloids a new bis-indole alkaloid called peribogamine was synthesized. It was conjectured that this compound would elicit a

greater cytotoxic activity since it combined the apparently beneficial qualities of the others. Vobasivindoline was also synthesized since it contained the specified O-N-O triangular dimensions, a pharmacophore feature prevalent in many anti-leukemic drugs. Finally, a synthesis of 19-acetonyl voacamine was attempted as a result of the high activity of the previously isolated isomer 19-acetonyl conodurine.