Plant Anticancer Agents V: New Bisindole Alkaloids from *Tabernaemontana johnstonii* Stem Bark

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Abstract □ The isolation and structure elucidation of the three new bisindole alkaloids, gabunamine, tabernamine, and 19,20-epoxyconoduramine, from *Tabernaemontana johnstonii* stem bark are described. The isolation of the seven known alkaloids, conodurine, conoduramine, gabunine, isovoacangine, ibogamine, pericyclivine, and perivine, from the same source also is noted. The alkaloids gabunamine, gabunine, and tabernamine showed significant cytotoxicity against the P-388 cell culture system.

Keyphrases □ Alkaloids, various—isolated from *Tabernaemontana johnstonii* stem bark, structures elucidated, cytotoxic activity evaluated □ *Tabernaemontana johnstonii*—stem bark, various alkaloids isolated, structures elucidated, cytotoxic activity evaluated □ Cytotoxic activity—various alkaloids isolated from *Tabernaemontana johnstonii* stem bark evaluated

As part of a systematic survey of botanical sources for anticancer activity, an aqueous alcoholic extract of the stems and bark of *Tabernaemontana johnstonii* Pichon (Apocynaceae) was examined and gave reproducible activity against the cell culture (KB) of human carcinoma of the nasopharynx and against lymphocytic leukemia of the mouse (P-388). No previous studies on the constituents of this plant have been reported, but plants classified in the Tabernaemontaneae tribe are a rich source of indole alkaloids (1).

The tribe is almost the sole plant source of the iboga-type alkaloids, the related voacamine group of bisalkaloids, and the vobtusine group of bisalkaloids; it is also an important source of the 2-acylindole alkaloids and has afforded other types of indole alkaloids (1–3). The plant *T. holstii*, a close relative of *T. johnstonii*, was shown to contain the bisindole alkaloids conodurine, conoduramine, and gabunine and the indole alkaloids coronaridine, 19-oxocoronaridine, pericyclivine, perivine, and vobasine (4).

The purpose of the present work was to isolate the components of *T. johnstonii* responsible for the observed cytotoxicity and/or antileukemic activity.

EXPERIMENTAL

Materials and methods were generally the same as those previously described (4).

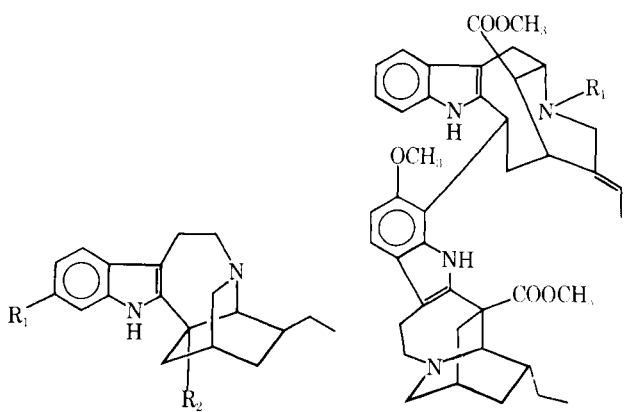
Plant Material—The air-dried stems and bark[1] of *T. johnstonii* Pichon (Apocynaceae) were collected in Kenya during 1971.

Extraction and Initial Separation of Alkaloids—plant material (10.3 kg) was extracted, and the crude alkaloid fraction (232 g) was subjected to chromatography on alumina as previously described (4). It yielded a benzene eluate, A (130 g), which was used in all subsequent work. Column chromatography of Fraction A on TLC grade silica gel[2,3] with elution by a chloroform–methanol gradient, yielded 14 fractions, B–O.

Isovoacangine (I)—Chromatographic separation of the combined Fractions C and D (14 g) on a silica gel column, with elution by chloroform, yielded a brown oil (3 g) in one group of fractions. Crystallization from methanol gave isovoacangine (I) as colorless crystals, mp 156–157°, undepressed in admixture with authentic material (5). The spectroscopic properties (IR, UV, and NMR) of the isolated material were consistent with this structural assignment.

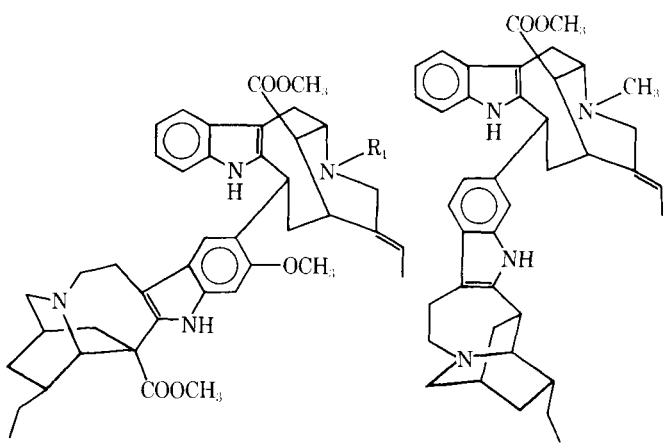
Conodurine (II) and Conoduramine (III)—Fraction G (13.5 g) deposited crystals on standing in methanol at 0°. Purification of the crystalline material by preparative TLC, with development by ether–hexane–methanol (4:5:1) followed by final crystallization from methanol, yielded II. Fraction I (19 g) was subjected to column chromatography on TLC grade silica gel, with elution by ether–hexane–methanol (4:5:1), to yield a major fraction; this fraction gave pure III on crystallization from methanol. Both alkaloids were identical with the samples previously described (4).

Ibogamine (IV), Pericyclivine, and 19,20-Epoxyconoduramine (V)—Chromatographic separation of Fraction J on silica gel, with elution by benzene–dichloromethane–ether (10:8:5), yielded 14 major fractions. Purification of fraction 3 (1.2 g) by preparative TLC, with development



I: $R_1 = OCH_3$, $R_2 = COOCH_3$
IV: $R_1 = R_2 = H$

II: $R_1 = CH_3$
VI: $R_1 = H$



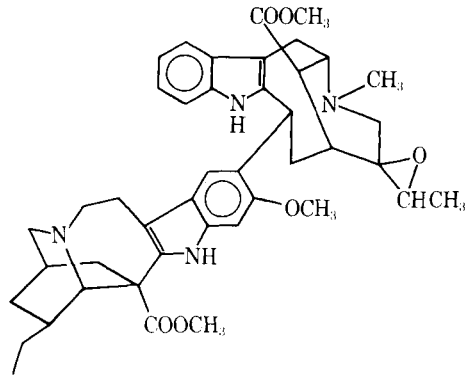
III: $R_1 = CH_3$
VII: $R_1 = H$

VIII

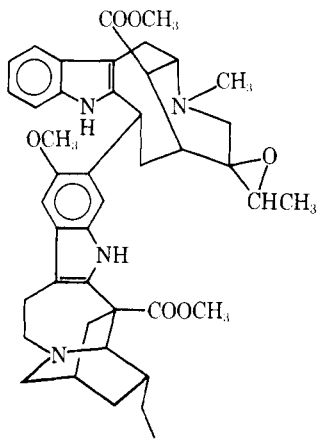
[1] Voucher specimens are on deposit at the U.S. Department of Agriculture, Beltsville, Md.

[2] Silica gel PF-254, EM Laboratories, Inc.

[3] R. L. Lyon, H. H. S. Fong, and N. R. Farnsworth, Department of Pharmacognosy and Pharmacology, University of Illinois at the Medical Center, Chicago, IL 60612, personal communication.



V



IX

by benzene–ether (5:1), yielded ibogamine (IV) as the major product, mp 159–161°, undepressed in admixture with authentic material (6, 7). Its IR spectrum was superimposable on that of authentic material. Fraction 7 (0.22 g) was purified by preparative TLC, with development by ether, to yield pericyclivine as the major component, mp 222–223° (after crystallization from methanol), undepressed in admixture with authentic material (4).

Fraction 10 (0.96 g) was subjected to preparative TLC in benzene–dichloromethane–ether–methanol (25:20:25:7). The center band was eluted and subjected to repeated preparative TLC in benzene–dichloromethane–ether–methanol (20:16:20:3) to yield 19,20-epoxyconoduramine (V) as colorless crystals (10 mg). The material had an R_f of 0.75 in the second solvent system and gave a brown color with the ceric sulfate spray reagent. The UV spectrum of the isolate showed λ_{max} 228 (log ϵ 4.72), 288 (4.12), and 295 (4.16) nm; its IR spectrum showed a strong band at λ_{max} 1730 cm^{-1}.

Perivine, Gabunine (VI), and Gabunamine (VII)—Fractions L and M were combined (10 g) and subjected to chromatography on silica gel, with elution by benzene–dichloromethane–ether (5:4:3) containing between 1 and 10% methanol, to yield 25 fractions. Fraction 5 (600 mg) was purified by preparative TLC in chloroform–methanol (90:10) to give perivine (280 mg) and gabunine (VI, 90 mg), identical with the samples previously described.

Fraction 8 (680 mg) was purified by preparative TLC in chloroform–methanol (95:5) to give gabunamine (VII, 84 mg). The isolated material was homogeneous on TLC, with R_f 0.35 in benzene–dichloromethane–ether–methanol (25:20:15:6), and gave an orange-brown color with the ceric sulfate spray reagent. Its IR spectrum showed several similarities to that of gabunine, with major absorptions at λ_{max} 3430, 1715, and 740 cm^{-1}. Its mass spectrum showed principal ions at m/e 704 (4), 690 (M^+, 2), 646 (6), 525 (3), 510 (4), 451 (4), 394 (4), 352 (10), 338 (21), 336 (20), 310 (12), 225 (43), 194 (60), 183 (22), 182 (36), 181 (26), 180 (40), 172 (40), 166 (100), 158 (47), 136 (35), and 122 (50). Its NMR spectrum showed signals at δ 0.90 (3H, triplet), 1.70 (3H, doublet), 2.48 (3H, singlet), 3.70 (3H, singlet), 3.96 (3H, broad singlet), 6.80 (1H, singlet), and 7.08 (4H, broad peak) ppm.

Chemical Modifications of VII—Compound VII (5 mg) was heated under reflux with 10% HCl–methanol (1:1) (5 ml) for 12 hr (8). Standard workup gave a crude product containing I (identified by TLC and its color with ceric sulfate) as the major component. Methylation of VII by a literature method (9) gave a compound identified as III by its TLC characteristics.

Synthesis of VI and VII—Compound I (60 mg) was heated under reflux in a nitrogen atmosphere with 1.5% methanolic hydrogen chloride; perivinol (120 mg) (10) was added in small portions over 1 hr. Reflux was continued for an additional 1.5 hr, and the mixture was cooled, made basic with sodium carbonate, and extracted with dichloromethane. The extracts were washed, dried, and evaporated to yield a crude product (165 mg), which was purified by preparative TLC in benzene–dichloromethane–ether–methanol (25:20:15:6). Elution of the band with R_f 0.35 yielded VII, identical with the sample isolated; elution of the band with R_f 0.50 yielded VI.

Isolation of Tabernamine (VIII)—Fraction N (9.7 g) was subjected to chromatography on TLC grade silica gel, with elution by ethyl acetate containing increasing amounts of ethanol, to give 11 fractions. The fifth fraction, which showed the greatest cytotoxicity, was subjected to preparative TLC in dichloromethane–methanol (85:15). The major component was eluted and purified further by high-pressure liquid chromatography (HPLC) on a silica gel column, 0.7 × 30 cm, with elution by ethyl acetate–hexane (9:1). The major fraction from this procedure was obtained as a white amorphous solid, homogeneous on TLC in two solvent systems and on HPLC, and was named tabernamine. The physical properties of tabernamine were described previously (11).

DISCUSSION

Tabernamine (VIII)—The major evidence for the structure of this new bisindole alkaloid was presented previously (11). It is of some importance, however, to assign unambiguously the position of attachment of the vobasane ring system to the ibogamine moiety. Therefore, the original arguments are amplified as follows.

The basic question concerns the assignment of the signals of the indole protons in the NMR spectrum of tabernamine. In deuterochloroform, tabernamine shows a doublet at δ 7.45 (1H) ppm and what appears to be a portion of another doublet at 7.03 (1H) ppm. One part of this second signal is obscured, however, by a large complex signal centered at δ 7.1 ppm because of the aromatic protons of the vobasane indole ring. In methanol, an *AB* quartet is clearly visible as two doublets at δ 6.88 and 7.28 ppm.

In simple indoles, the 4-proton (corresponding to the 11′-proton of tabernamine) absorbs downfield of the 5-, 6-, and 7-protons; for indole itself in acetone, for example, these resonances occur at δ 7.55, 7.00, 7.08, and 7.40 ppm, respectively (12). In various methyl indoles, the 4-proton absorbs downfield from the remaining protons at about δ 7.4–7.5 (chloroform) or 7.2 (acetone) ppm (13). On this basis, it is possible to assign the doublet observed at δ 7.45 (chloroform) or 7.28 (methanol) ppm to the 11′-proton in tabernamine, coupled with a coupling constant of 8 Hz to the 12′-proton whose resonance is observed at δ 7.03 (chloroform) or 6.88 (methanol) ppm. Since the 12′-proton shows no further coupling, the 13′-position must be occupied by the vobasane moiety, as indicated in Structure VIII.

Furthermore, it is known that the 7-proton of indole frequently shifts downfield when a more polar solvent is employed (14), while the 4-proton frequently shifts upfield under the same conditions (13). The observed upfield shift of the signal at δ 7.45 ppm in chloroform to 7.28 ppm in methanol is consistent only with its assignment to the 11′-proton, corresponding to the 4-proton of indole.

A further argument in favor of Structure VIII for tabernamine comes from studies of the position of electrophilic substitution in indole. Although electrophilic substitution on the benzene ring of indoles occurs in both the 5- and 6-positions, the 6-position is usually the major site of attack (15). In particular, electrophilic substitution on indoles containing a basic side chain occurs almost exclusively in the 6-position; tryptophan nitrate, for example, undergoes nitration in the 6-position to yield 6-nitrotryptophan (16). Therefore, it is most likely that ibogamine, containing a basic side chain, undergoes substitution in the 13′-position, corresponding to the 6-position of indole, to yield tabernamine of Structure VIII.

19,20-Epoxyconoduramine (V)—The evidence for the structure of this compound, isolated in very small yield from one specific fraction only, is entirely spectroscopic. The structural assignment should be regarded as tentative unless or until a larger sample can be made available for degradative studies.

The UV spectrum of the compound showed typical indole absorption, and its mass spectrum showed peaks typical of bisindole alkaloids of the conoduramine type. The parent ion was 16 mass units higher than that

Table I—Cytotoxicity of Certain Fractions and Pure Alkaloids from *T. johnstonii*[a]

Fraction or Alkaloid	ED_{50}, μg/ml P-388	KB
C	5.7	
D	16	
Isovoacangine (I)	18	59
E	18	
F	27	
G	19	
Conodurine (II)	26	31
Conoduramine (III)	20	19
H	13	
I	2.6	
J	3.0	
Ibogamine (IV)	–	>100
Pericyclivine	13	>100
K	2.6	
L	2.6	
M	2.7	
Perivine	20	70
Gabunine (VI)	3.2	–
Gabunamine (VIII)	1.3	5.8
N	2.3	
Tabernamine (VIII)	2.1	–
O	2.4	

[a] Bioassays were performed by the A. D. Little Co., Cambridge, Mass., using established protocols.

of conoduramine, requiring the placement of an additional oxygen in the molecule. A careful analysis of the mass spectrum indicated that the additional oxygen atom must be situated on the vobasane moiety of the bisalkaloid. Thus, the normal peaks derived from the alicyclic portion of the iboga moiety were observed at *m/e* 122, 136, and 148 (17). The peaks at *m/e* 180, 182, and 194, however, which would be expected from the alicyclic portion of the vobasane moiety (17), were shifted largely to *m/e* 196, 198, and 210; the small intensity peaks remaining at *m/e* 180, 182, and 194 presumably arise from the $(M - 16)^{+\cdot}$ ion discussed later.

Two important ions indicate that the additional oxygen atom is located as an epoxide function in the 19,20-position of the vobasane moiety. An intense peak at *m/e* 676 is due to loss of 44 mass units from the parent ion; the fragment lost in this process can also bear the charge and, in this case, appears as an ion at *m/e* 44. Loss of 44 mass units is a relatively uncommon process in mass spectrometry and is not observed from normal bisindole alkaloids of this type. It can be explained adequately, however, by the 19,20-epoxide structure proposed, since epoxides undergo transannular cleavage, leading in this case to the loss of a C_2H_4O fragment (18). The observation of a fragment ion at *m/e* 704 (M − 16) is also consistent with the epoxide formulation, since loss of oxygen has been observed from other alkaloidal epoxides (17).

The NMR spectrum of the new compound fully supports the structure proposed. In particular, the three-proton doublet at δ 1.34 ppm may be assigned to the 18-methyl group (19), and three-proton singlets at δ 2.42, 2.60, and 3.66 ppm occur at essentially the same chemical shift as the corresponding signals in conoduramine. The observation of a broadened three-proton signal at δ 3.92 ppm is also significant, because a similarly broadened signal occurs in the NMR spectrum of conoduramine and may be assigned to the hydrogen-bonded methoxyl group of the isovoacangine ring. Finally, the aromatic region of the spectrum of the new compound shows a similar pattern of peaks to conoduramine, establishing that the vobasane unit is linked to the 12′-position of the isovoacangine moiety.

The preceding evidence establishes the major features of the structure of the new compound as being those of 19,20-epoxyconoduramine (V) or 19,20-epoxyvoacamine (IX). Since no voacangine-derived bisalkaloids have been detected in *T. johnstonii*, Structure V probably may be assigned to the new compound. Study of the details of the stereochemistry of the epoxy substituent and definitive proof of this structural assignment await the isolation of a further quantity of material.

This is the first report of the isolation of any alkaloid of the vobasane skeleton containing a 19,20-epoxy function, although a recent report (20) described such a function in an alkaloid with the dihydroakuammiline skeleton.

Gabunamine (VII)—The structure of this new compound is established by its degradation to isovoacangine, by its synthesis from isovoacangine and perivinol, and by its conversion to conoduramine. The spectroscopic data for the compound are entirely consistent with the assigned structure. In particular, the mass spectrum indicated its relationship to conoduramine and related molecules, while the NMR spectrum indicated that it differed from conoduramine only in the lack of an *N*-methyl group. The name gabunamine is assigned to the compound since it is related to conoduramine in the same way that gabunine is related to conodurine.

Cytotoxic Activities—The isolation of the alkaloids described was undertaken on the basis of the cytotoxicity of the various fractions investigated; the cytotoxicities of Fractions C–O, and of the alkaloids isolated therefrom, are given in Table I. The cytotoxicity of 19,20-epoxyconodurine could not be determined because of the scarcity of material.

The P-388 *in vitro* assay system appears to be more sensitive to most indole alkaloids tested than does the KB assay, and it thus might prove to be a generally useful screen for compounds of this type. The alkaloids isolated also largely account for the cytotoxicity of the fractions from which they were isolated, although the possibility cannot be excluded that other, unknown compounds also contribute to the cytotoxicities of the crude fractions.

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