Catharanthus Alkaloids XXI. Isolation of Lochnerinine, One of the Cytotoxic Principles of *C. pusillus*

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Catharanthus pusillus **alkaloid fractions were found to be active against Eagle's 9 KB carcinoma of the nasopharynx in cell culture. Chromatographic separation of one of the active fractions resulted in the isolation of lochnerinine, and the activity of this alkaloid against the 9 KB carcinoma has been confirmed. Gradient pH fractionation of another active alkaloid fraction yielded ajmalicine and vindorosine, which were not cytotoxic.**

An apocynaceous plant indigenous to India is *Catharanthus pusillus*. Prior phytochemical investigations in these laboratories have yielded the alkaloids ajmalicine and vindorosine (1). Majumdar and Paul have reported the isolation of two amorphous alkaloids having cardiotoxic properties, which they named pusiline and pusilinine, in addition to three unidentified sterols (2). Battersby and Kapil isolated a neutral substance from a weakly basic alkaloid fraction of this plant in a yield of $1 \times 10^{-4}\%$, which was identified as *N*-benzoyl-L-phenylalaninol (3). No other phytochemical studies on *C. pusillus* have been reported in the literature.

In a continuing study of *Catharanthus* species for new antitumor agents, the authors have examined the alkaloids of *C. pusillus* leaves, utilizing different procedures than in previous studies from their laboratories (4). This study has led to the confirmation of ajmalicine and vindorosine in this plant, and reports for the first time the isolation of the cytotoxic alkaloid lochnerinine from *C. pusillus*.

EXPERIMENTAL

Plant Material—The *Catharanthus pusillus* (Murr.) G. Don (*Apocynaceae*) leaves used in this study were obtained from Drugland Corporation, Delhi, India, and a voucher specimen has been identified by one of the authors (N.R.F.), and has been deposited in the herbarium of the Department of Pharmacognosy, School of Pharmacy, University of Pittsburgh.

Preparation of Alkaloid Fractions—A sample of coarsely milled leaves (19.75 kg.) was extracted continuously for 24 hr. in a Lloyd extractor with skellysolve B. This extract was concentrated and removed from the extractor and a fresh charge of solvent was added. The procedure was repeated four times. Pooling of the four concentrates was followed by reduction of the volume to 2.0 l. To this concentrate was added 1.6 l. of 2 *N* HCl, and the mixture was distilled *in vacuo* to remove the skellysolve B, cooled, and filtered. A residue on the filter pad was dissolved in benzene and re-extracted into 2 *N* HCl as described above, and the two acid filtrates were combined (*ca.* 3 l.). This extract was decolorized with charcoal and filtered through a diatomaceous earth[1] filter pad. Ice was added to the clear filtrate and ammonium hydroxide solution was added in small portions with continuous stirring until the solution was alkaline to litmus paper. In order to extract the alkaloids from this basic solution, it was shaken several times with ethylene dichloride, and the ethylene dichloride extracts were dried over anhydrous sodium sulfate, filtered, and taken to dryness *in vacuo* to yield 6.7 g. of crude, weakly basic alkaloids (Fraction I).

The skellysolve B-extracted *C. pusillus* leaves were air dried, macerated with methanol, and exhaustively extracted by repeated maceration and percolation. The combined percolates (*ca.* 400 l.) were concentrated to 20 l. and then extracted into 20 l. of a 2% tartaric acid solution, residual methanol being removed *in vacuo*, and the acid solution filtered. Three 20-l. volumes of benzene were used to extract the combined filtrates, and the benzene extracts were pooled, dried over anhydrous sodium sulfate, filtered, and concentrated to give 43 g. of Fraction II (acid benzene solubles).

Residual benzene was removed from the aqueous acid layer of post-Fraction II *in vacuo*, and the resulting solution was chilled and rendered alkaline with ammonium hydroxide solution. This alkaline solution was then extracted with three 20-l. volumes of ethylene dichloride. The ethylene dichloride extracts were pooled, dried over anhydrous sodium sulfate, filtered, and were taken to dryness to yield 50 g. of crude tertiary alkaloids. This fraction was further divided into the nonphenolic part (Fraction III), and the phenolic part (Fraction IV) by passing it through a column of ion-exchange resin (IRA-400) (500 g.), which was pretreated with 5% sodium hydroxide solution, and washed with distilled water until the effluent was neutral to litmus paper. Fraction III (37 g.) was obtained by eluting the resin column containing the crude tertiary alkaloids with methanol (*ca.* 16 l.), and Fraction IV (6 g.) was removed from the column by elution with 0.1% HCl in methanol (*ca.* 4 l.).

The quaternary alkaloids (Fraction V) were obtained as chlorides from the aqueous alkaline solution after the removal of the total tertiary bases. This was accomplished according to the method described by Hogg *et al.* (5), to yield 29 g. of Fraction V. The mother liquor remaining after removal of Fraction V was termed Fraction VI. A scheme showing the method of preparation of the alkaloid fractions is presented in Scheme I.

Cytotoxicity Evaluation of Alkaloid Fractions—Fractions I–VI were evaluated for cytotoxicity against Eagle's 9 KB carcinoma of the nasopharynx in cell culture according to established protocols,[2]

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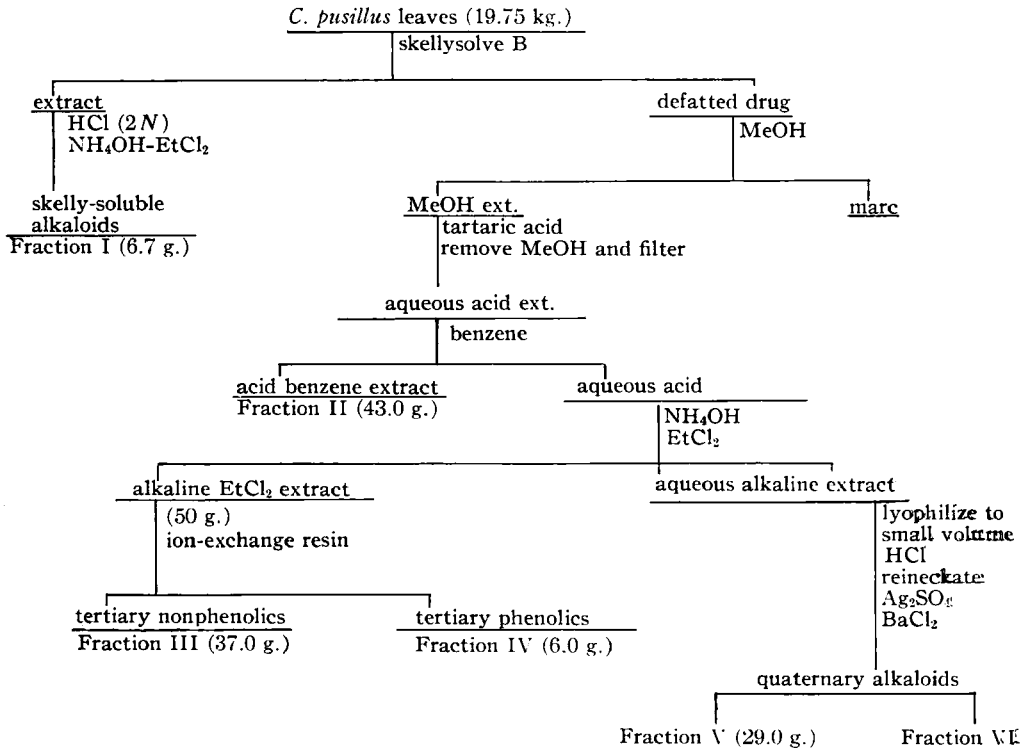
[1] Celite, Johns-Manville, New York, N. Y.

[2] For a description of the methods employed see *Cancer Chemotherapy Rept.*, **25**, 1(1962).



Scheme I

through the auspices of the Cancer Chemotherapy National Service Center, Bethesda, Md. The results are presented in Table I. Phytochemical work-up was made on the basis of active fractions being given first priority.

Chromatographic Separation of Fraction I—A glass chromatographic column (3 × 38 cm.) was slurry packed with 170 g. of alumina (Alcoa F-20) and benzene as previously described (4). The fraction (6.0 g.) was dissolved in a small volume of benzene and this solution was adsorbed to the top of the column, followed by the addition of benzene. Elution of the alkaloids was accomplished with benzene, followed by mixtures of benzene and chloroform, and finally with chloroform. Twenty-milliliter fractions were collected from the column, analyzed by means of TLC on silica gel G plates (6), and combined on the basis of the pattern of resolved alkaloids and chromogenic reactions after spraying each plate with the ceric ammonium sulfate reagent (6). The results of this chromatographic separation are presented in Table II.

TABLE I—CYTOTOXICITY OF *Catharanthus pusillus* FRACTIONS

Fraction	ED_{50} (mcg./ml.)[a]
I	2.6×10^{0}[b]
II	1.7×10^{1}
III	1.4×10^{0}[b]
IV	6.1×10^{0}[b]
V	1.0×10^{2}
VI	1.0×10^{2}

[a] An active fraction is one that exhibits $ED_{50} \leq 15.0$ mcg./ml. [b] Active cytotoxic fraction.

Isolation of Lochnerinine—Combined Fractions V–VI from the column appeared to contain only one alkaloid as evidenced by TLC. This alkaloid was suspected to be lochnerinine on the basis of its R_f value in three different solvent systems (6). The combined fractions were freed from solvent to yield a thick oil, which was dissolved in a small volume of methanol and hydriodic acid solution was added dropwise until an acidic solution resulted (pH *ca.* 3.0). Refrigeration of the solution for about 2 hr. resulted in the formation of fine yellow needles, which were removed by filtration. Recrystallization from methanol yielded 24.5 mg. of alkaloid, which when dried *in vacuo* at 100° for 24 hr., gave m.p. 207°. The UV and IR spectra, as well as TLC

TABLE II—CHROMATOGRAPHIC SEPARATION OF *C. pusillus* FRACTION I ALKALOIDS

Fraction No.[a]	Eluent
1–4	Benzene
5–6	Benzene
7–13	Benzene
14–48	Benzene
49–70	Benzene
71–154	Benzene
155–200	Benzene
201–240	Benzene–chloroform (3:1)
241–350	Benzene–chloroform (3:1)
351–380	Benzene–chloroform (1:1)
381–400	Benzene–chloroform (1:1)
401–449	Chloroform
450–480	Chloroform

[a] Each fraction was 20 ml. and all fractions contained alkaloids.

results (6), showed that the isolated alkaloid was identical with a reference sample of lochnerinine hydriodide which was prepared from authentic lochnerinine, isolated in these laboratories from *C. lanceus* (7).

Gradient pH Separation of Fraction III—Fraction III was subjected to a gradient pH separation according to the method of Svoboda (8), with only minor modifications. The fraction (32.0 g.) was dissolved in a minimum volume of ethylene dichloride and extracted into 1.5 l. of 0.2 *M* citric acid solution by heating on a steam bath and removal of the ethylene dichloride *in vacuo*. The resulting aqueous phase was decolorized with charcoal, and filtered. Extraction of this acidic filtrate (pH 2.7) was effected with three separate volumes of benzene and the aqueous layer was adjusted to pH 3.2 with ammonium hydroxide solution, followed by three benzene extractions. This procedure was continued with increases in pH by 0.5 units, with ammonium hydroxide solution, until a final pH of 9.2 was attained. Benzene extracts from each pH level were combined, taken to dryness, and adjusted to *ca.* 100 ml. with benzene. TLC (6) of each of the pH fractions served to point out those pH fractions to be combined prior to crystallization attempts. In this manner, the following groupings were made:

Group	pH	Wt., g.
III-A	2.7–3.2	3.42
III-B	3.7	1.84
III-C	4.2–5.2	3.55
III-D	5.7–7.2	4.73
III-E	7.9–9.2	1.25

These five groups were then each subjected to crystallization attempts using benzene, chloroform, acetone, ethylene dichloride, ethanol, or methanol.

Isolation of Vindorosine—Fine needles (250 mg.) were obtained from a benzene solution of Group II-A. An analytical sample was obtained following several recrystallizations from benzene. This sample was identical (m.p., UV, IR, TLC) with a reference sample of vindorosine.

Isolation of Ajmalicine—Colorless crystals (50 mg.) were obtained from an ethanol solution of Group III-B. Several crystallizations from ethanol afforded an analytical sample which was shown to be identical with reference ajmalicine.

Cytotoxicity of Lochnerinine—Ajmalicine and vindorosine were found to be inactive in the cytotoxicity test of the Cancer Chemotherapy National Service Center screening laboratories.[2] Lochnerinine, however, exhibited $ED_{50} < 1.0 \times 10^{-2}$ mcg./ml. aginst the 9 KB cell culture. A compound is considered an active cytotoxic agent if it has $ED_{50} \leq 1.0$ mcg./ml.[2] All three alkaloids were inactive when evaluated against the P-1534 leukemia in DBA/2 mice.[2]

DISCUSSION AND SUMMARY

Catharanthus pusillus leaves of Indian origin were studied in this investigation. The total crude alkaloids (0.6%) were separated into six fractions, and three of these were found to be cytotoxic against Eagle's 9 KB carcinoma of the nasopharynx in cell culture. Lochnerinine, an α-methyleneindoline base previously reported isolated only from the related *C. roseus* and *C. lanceus*, was isolated from the weakly basic alkaloid Fraction I by column chromatography, as the hydriodide salt, and was shown to be responsible for at least a part of the cytotoxicity of this fraction. Additional alkaloids could not be isolated from other chromatographic cuts of this fraction, each of which was extremely complex.

Fraction III, representing the nonphenolic tertiary bases, was subjected to a gradient pH separation and vindorosine was isolated in a yield of 0.2% of the total crude alkaloids, from the pH 2.7–3.2 fraction. Vindorosine has been isolated previously only from the related *C. roseus*. From the pH 3.7 fraction was isolated ajmalicine in a yield of 0.04% of the total crude alkaloids. This base also occurs in *C. roseus*, *C. lanceus*, and *C. trichophyllus*, as well as in many other apocynaceous plants.

The remaining pH fractions of Fraction III were complex and did not yield alkaloids by conventional crystallizing techniques. Neither vindorosine nor ajmalicine were found to be cytotoxic, and the active entity in Fraction III remains unknown.

As yet no attempt has been made to separate alkaloids from Fractions II, IV, or V.

REFERENCES

(1) Fylypiw, W. M., Farnsworth, N. R., Blomster, R. N., Buckley, J. P., and Abraham, D. J., *Lloydia*, **28**, 354(1965).
(2) Majumdar, D. N., and Paul, B., *Indian J. Pharm.*, **21**, 255(1959).
(3) Battersby, A. R., and Kapil, R. S., *Tetrahedron Letters*, **1965**, 3529.
(4) Loub, W. D., Farnsworth, N. R., Blomster, R. N., and Brown, W. W., *Lloydia*, **27**, 470(1964).
(5) Hogg, R., Beal, J. L., and Cava, M. P., *ibid.*, **24**, 45(1961).
(6) Farnsworth, N. R., Blomster, R. N., Damratoski, D., Meer, W. A., and Cammarato, L. V., *ibid.*, **27**, 302(1964).
(7) Maloney, E. M., Farnsworth, N. R., Blomster, R. N., Abraham, D. J., and Sharkey, A. G., Jr., *J. Pharm. Sci.*, **54**, 1166(1965).
(8) Svoboda, G. H., *Lloydia*, **24**, 173(1961).

Keyphrases

Catharanthus alkaloids
Lochnerinine, *C. pusillus*—isolation, identification
Column chromatography—separation
TLC—separation, identification
Cytotoxicity—lochnerinine