Catharanthus Alkaloids XXXI: Isolation of Ajmalicine, Pericalline, Tetrahydroalstonine, Vindolinine, and Ursolic Acid from *Catharanthus trichophyllus* Roots

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Abstract □ Certain active cytotoxic alkaloid fractions prepared from *Catharanthus trichophyllus* roots were investigated. This work resulted in the isolation of ajmalicine, pericalline, tetrahydroalstonine, vindolinine, and ursolic acid. These compounds were not responsible for the observed cytotoxic activity. Ajmalicine, tetrahydroalstonine, and ursolic acid were previously isolated from this plant, whereas vindolinine and pericalline are now reported for the first time from *C. trichophyllus*.

Keyphrases □ *Catharanthus* alkaloids—isolation of ajmalicine, pericalline, tetrahydroalstonine, vindolinine, and ursolic acid from *C. trichophyllus* roots, cytotoxic activity □ Vindolinine—isolation and identification from *C. trichophyllus* roots, cytotoxic activity □ Pericalline—isolation and identification from *C. trichophyllus* roots, cytotoxic activity

It has been reported that certain alkaloid fractions prepared from *Catharanthus trichophyllus* exhibit a significant inhibitory activity against the P-1534 leukemia in DBA/2 mice, as well as a cytotoxic activity against Eagle's 9KB carcinoma of the nasopharynx in cell culture (1). Prior to the present study, phytochemical investigations were limited to the aboveground parts of this plant and led to the isolation of ajmalicine (2), tetrahydroalstonine (1), vindorosine (1), and ursolic acid (1). The present phytochemical study dealt with the roots of *C. trichophyllus* and resulted in the isolation of ajmalicine, tetrahydroalstonine, vindolinine, pericalline, and ursolic acid.

EXPERIMENTAL[1]

Plant Material—The coarsely milled and air-dried roots[2] of *C. trichophyllus* (Bak.) Pich. (Apocynaceae) used in this study were collected in Madagascar during 1969.

Petroleum Ether Extraction and Fractionation (Scheme I)—A total of 35 kg of plant material was exhaustively extracted with petroleum ether (bp 35–60°) in a Lloyd extractor to furnish a total of 144 liters of combined concentrated extract (Extract A). On standing at room temperature, Extract A deposited 13 g of amorphous material (Extract A′) which was collected by filtration. The filtrate was concentrated *in vacuo* to a thick syrup (Extract B) and treated with 1 liter of methanol, and the solid material that separated was collected by filtration (Extract B′). The filtrate (Extract C) was evaporated *in vacuo* to give a residue which was redissolved in 500 ml of benzene. This benzene solution was mixed with 500 ml of warm 0.2 *M* citric acid solution, the benzene was removed *in vacuo* on a steam bath, and the aqueous mixture was cooled and filtered. The filtrate was reserved and the residue was redissolved in benzene and treated with citric acid, followed by removal of the benzene and filtration as before. This procedure was repeated three times to give 2.5 liters of acidic filtrate (Extract D) and 92 g of nonbasic, insoluble residue (Extract C′). Extract D was made alkaline with 58% NH_4OH and extracted with benzene (5 × 2.5 liters). The combined benzene extracts were washed with water, dried (anhydrous sodium sulfate), and taken to dryness *in vacuo* to give 2.42 g of crude, weakly basic alkaloids (Extract D′).

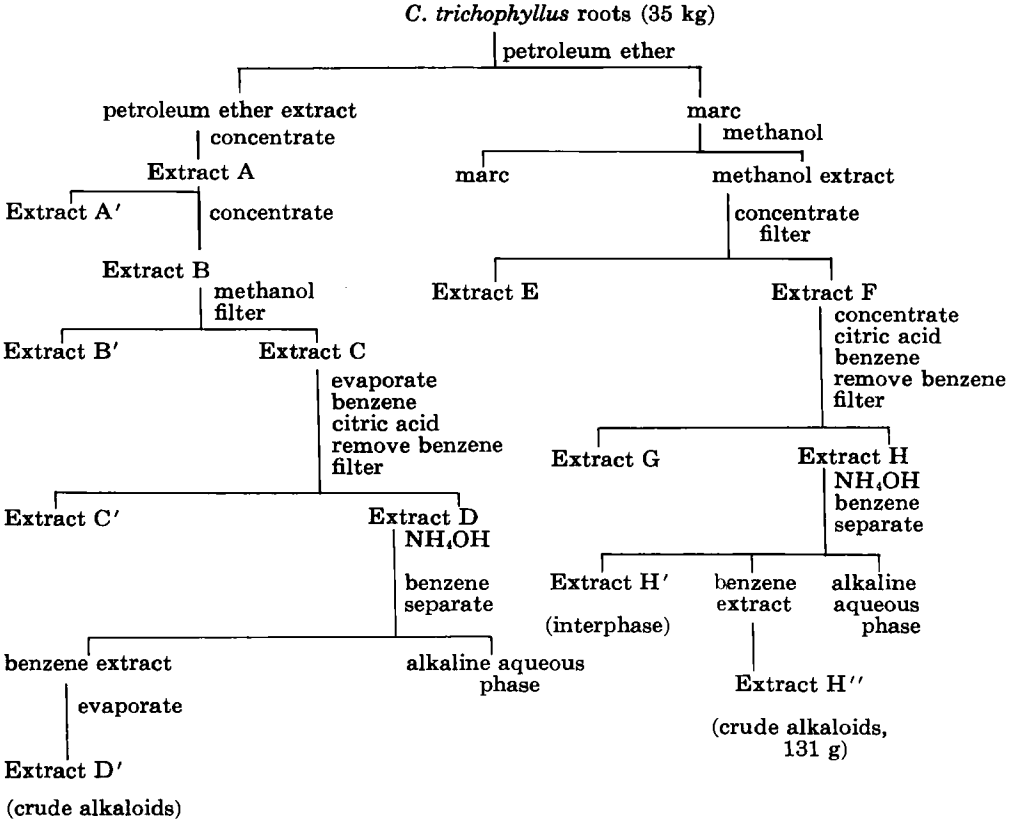
Isolation of Ursolic Acid—A sample (0.5 g) of Extract A′ was repeatedly crystallized from 95% ethanol to give colorless needles (0.018 g), mp 296–298°, $[\alpha]D^{28.5}$ + 55° (c 0.47 in pyridine). This isolate was homogeneous by TLC and gave R_f values of 0.70, 0.58, and 0.28 when analyzed in Solvent Systems A, D, and E, respectively. The UV spectrum was transparent over the 210–400-nm range. The mass spectrum showed the apparent molecular ion at *m/e* 456 ($C_{30}H_{48}O_3$), as well as additional fragments considered diagnostic for Δ^{12}-unsaturated oleananes and ursene-type triterpenes (5, 6). The IR, specific rotation, TLC, and mass spectroscopy data of the isolate were identical with those derived from authentic ursolic acid, and a mixed melting-point determination showed no depression. The isolate was further characterized by preparation of the acetate derivative, which proved to be identical (TLC and mass spectroscopy) with authentic acetylursolic acid.

Preparation of Total Crude Alkaloid Fraction (Scheme I)—The air-dried petroleum ether-extracted plant material was exhaustively extracted by repeated cold maceration and percolation, using a total of 1600 liters of anhydrous methanol. After the percolates were combined, they were concentrated *in vacuo* to 40 liters (Extract F), which resulted in the separation of 263 g of nonalkaloid material (Extract E) which was collected by filtration. The filtrate was further concentrated *in vacuo* to afford about 6 liters of a viscous syrup which was mixed with 6 liters of 0.2 *M* citric acid solution. This mixture was heated *in vacuo* on a steam bath to remove residual methanol and finally the mixture was cooled and filtered. This filtrate was set aside and the residue was redissolved in methanol and treated with citric acid solution, followed by removal of the methanol and filtration as before. Extract F was thus extracted a total of three times with citric acid solution to give 320 g of insoluble residue (Extract G) and 20 liters of combined acidic alkaloid filtrate (Extract H). Extract H (20 liters) was made alkaline (pH 9.0) with 58% NH_4OH and extracted with benzene (6 × 20 liters). Nonalkaloid interphase solids, which formed during the benzene extractions, were removed by filtration to give 320 g of Extract H′. The combined benzene extracts were washed with water, dried (anhydrous sodium sulfate), and taken to dryness *in vacuo* to give 131 g of crude alkaloid residue (Extract H″).

Dialysis Purification of Fraction H″ Alkaloids (Scheme II)—The following procedure is a modified version of the method described by Klasek (7). Fraction H″ (131 g) was worked up per batch as follows. Fraction H″ (24 g) was dissolved in 90 ml of citric

[1] Melting points were determined in open capillary tubes using a Thomas-Hoover apparatus and are uncorrected. Specific rotations were measured using a Rudolph model 70 precision polarimeter. UV spectra were taken in ethanol or methanol using a Perkin-Elmer model 202 spectrophotometer. IR spectra were measured in KBr pellets *versus* air with a Beckman model IR-8 spectrophotometer. Mass spectra were recorded at 70 ev using a LKB-9000 mass spectrometer (LKB Produkter, Stockholm, Sweden), operated in the direct probe mode. NMR spectra were determined in deuterochloroform, containing tetramethylsilane as the interior standard, using a Varian model A-60 spectrometer or HR-100 spectrometer. All concentrations and evaporations were carried out with water pump vacuum at less than 40°. Routine TLC was carried out using silica gel G (Merck) plates. The solvent systems used were: A, ethyl acetate–absolute ethanol (3:1); B, 1-butanol–acetic acid–distilled water (4:1:1); C, anhydrous methanol (100%); D, chloroform–acetone (5:4); E, benzene–ethyl acetate (7:2); and F, benzene–ethyl acetate–diethylamine (7:2:1). Nonalkaloid substances were detected on TLC plates by spraying with sulfuric acid followed by charring at 120°. Ceric ammonium sulfate reagent (3) and modified Dragendorff (4) reagent were used as spray reagents to visualize resolved alkaloids on TLC plates. Preparative thick-layer chromatography was carried out on silica gel PF_{254} (Merck) 1-mm layers, resolved components being detected by quenching under 254-nm UV light. Appropriate zones were scraped from the plates and pure components were recovered from the removed zones by elution with ethanol, followed by filtration and removal of the solvent.

[2] Obtained from the Curran Corp., South Hackensack, N.J. Voucher specimens from the collection are on deposit at the Department of Pharmacognosy and Pharmacology, College of Pharmacy, University of Illinois at the Medical Center, Chicago, Ill. The identification was confirmed by N. R. Farnsworth.



Scheme I—Fractionation of C. trichophyllus *Roots*

acid solution (10 g of citric acid and 10 ml of distilled water), diluted to 180 ml with distilled water, and divided into six cellophane dialysis tubes[3]. These dialysis tubes, each containing 30 ml of acidic alkaloid solution, were suspended in a 2-liter beaker containing 1 liter of distilled water. The dialysate was stirred using a magnetic stirring bar, and the tubes were dialyzed against distilled water at 4°. At intervals of 8 hr, the dialysate was removed and reserved, fresh distilled water was added to the beaker, and the dialysis process was resumed. This protocol was repeated until each 24-g batch of alkaloids had been dialyzed for a total of four 8-hr periods. The combined acidic (pH 2.2) dialysates were made alkaline (pH 9.0) with 58% NH_4OH and exhaustively extracted with benzene to remove alkaloids. These combined benzene extracts were washed with water, dried (anhydrous sodium sulfate), and taken to dryness *in vacuo* to give the dialyzed alkaloid fraction (Extract I). In this manner, Extract H″ (131 g) yielded a total of 107.5 g of Extract I.

Chromatographic Separation of Extract I Alkaloids (Scheme II)—Extract I (107 g) was chromatographed batchwise as follows. Extract I (20 g) was dissolved in 30 ml of methanol–ethylene dichloride (7:3) and chromatographed on a 5 × 55-cm column[4] previously slurry-packed in methanol–ethylene dichloride (7:3). Elution was carried out using the same solvent at a flow rate of 75 ml/hr, and 5-ml fractions were collected. Individual fractions were monitored by TLC for the presence of alkaloids (Solvent System A), and consecutive fractions were combined on the basis of similar patterns of resolved alkaloids. A total of 570 fractions was collected from the column for each 20-g batch; these fractions were analyzed and combined into three groups. Removal of the solvent *in vacuo* for each group gave three fractions: J, K, and L.

Separation of Extract K Alkaloid Salts (Scheme II)—Treatment of a solution of Extract K (96 g) in 300 ml of cold absolute ethanol with dry hydrogen chloride gas resulted in the formation of a crystalline product, which was removed by filtration and dried over sodium hydroxide *in vacuo* to afford 22.9 g of Extract M. The filtrate was evaporated to dryness *in vacuo*, and the residue was dried over sodium hydroxide *in vacuo* to give 87.6 g of Extract N.

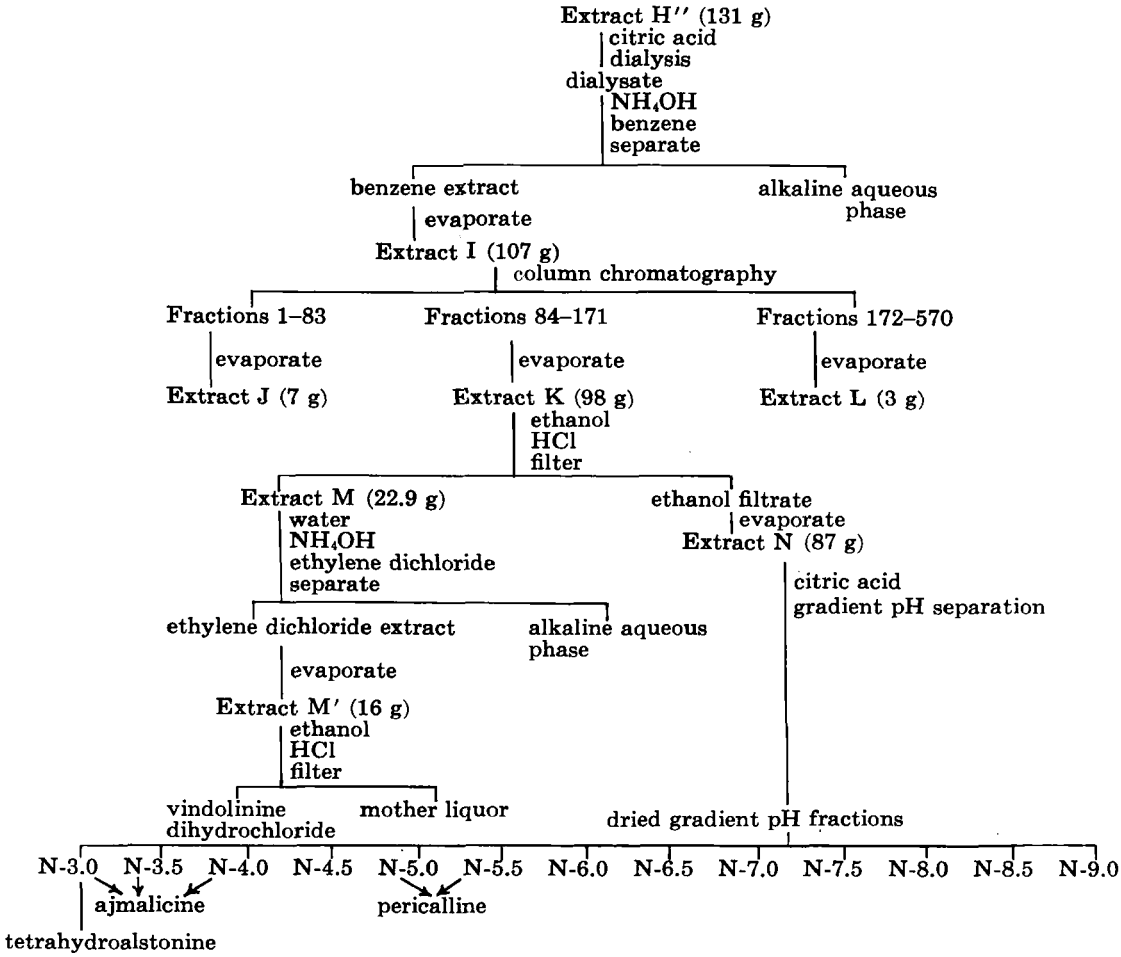
Isolation of Vindolinine Dihydrochloride—A solution of Extract M (22.9 g) in 1 liter of distilled water was decolorized with activated charcoal, filtered, alkalinized (pH 9.0) with 58% NH_4OH, and exhaustively extracted with ethylene dichloride. The combined ethylene dichloride extracts were washed with water, dried (anhydrous sodium sulfate), and evaporated *in vacuo* to give 16 g of Extract M′. A sample (0.25 g) of Extract M′ dissolved in 5 ml of absolute ethanol was treated with 1.5 ml of concentrated hydrochloric acid, and the solution was chilled. Crystals that formed were harvested and recrystallized from benzene–ethanol (1:1) to give 0.13 g of colorless needles, mp 218–220°, $[\alpha]D^{29.5}$ − 8.0° (c 0.5 in water). This isolate was homogeneous by TLC in Solvent System B (R_f 0.43) and displayed a red-orange chromogenic response with the ceric ammonium sulfate reagent. The UV absorption spectrum showed λ_{max} (C_2H_5OH) 206 (log ε 4.48), 246 (3.94), and 310 (3.44) nm, typical of dihydroindole alkaloids unsubstituted on both the indole nitrogen and the aromatic portion of the molecule (8). The IR, specific rotation, UV, NMR, TLC, and mass spectroscopy data were identical with those derived from authentic vindolinine dihydrochloride, and a mixed melting-point determination showed no depression.

Gradient pH Separation of Extract N Alkaloids (Scheme II)—A solution of Extract N (87 g) in 4.2 liters of 0.2 *M* citric acid was adjusted to pH 3.0 with 10% NH_4OH and extracted with benzene (2 × 4 liters). Following removal and pooling of the benzene extracts, the aqueous phase was adjusted to pH 3.5 with ammonium hydroxide and reextracted with benzene (2 × 4 liters) as before. This procedure was repeated, the pH of the aqueous phase being increased by 0.5 pH unit increments prior to each extraction with benzene, until a final pH of 9.0 was reached. The pooled benzene extracts from each pH level were washed with water, dried (anhydrous sodium sulfate), and taken to dryness *in vacuo* to give a total of 13 alkaloid fractions. Each of these 13 fractions was subjected to direct crystallization attempts using common solvents and solvent mixtures.

Isolation of Ajmalicine—Crystals harvested from the chilled acetone solutions of Extract N-3.5 and Extract N-4.0 were combined on the basis of similar TLC patterns. Further concentration

[3] Catalog number 8-667D, Fisher Scientific Co., Pittsburgh, Pa. Each tube was prepared from a 50-cm length of tubing.

[4] 250 g Sephadex LH-20, Pharmacia Fine Chemicals, Inc., Piscataway, N.J.



Scheme II—Fractionation of Extract H'' Alkaloids

and chilling of the mother liquors gave additional crystals, which were combined with the first crop to afford a total of 0.71 g of crude pale-yellow crystals. Repeated crystallization of this material from hot acetone gave 0.33 g of pale-yellow stout rosettes, mp 257° dec., $[\alpha]D^{29.5}$ −85.4° (c 0.55 in chloroform). This isolate was homogeneous by TLC, displayed a yellow-green chromogenic response with the ceric ammonium sulfate reagent, and gave R_f values of 0.70, 0.53, 0.68, 0.67, 0.24, and 0.67 when analyzed in Solvent Systems A–F, respectively. The UV absorption spectrum showed λ_{max} (CH_3OH) 225 (log ϵ 4.60), 288 (3.85), and 290 (3.75) nm, typical of alkaloids containing a tetrahydro-β-carboline moiety (8). The IR, specific rotation, UV, NMR, TLC, and mass spectroscopy data were identical with those derived from authentic ajmalicine, and a mixed melting-point determination showed no depression.

Isolation of Pericalline—Crystals harvested from the chilled benzene solutions of Extract N-5.0 and Extract N-5.5 were combined on the basis of similar TLC patterns. Further concentration and chilling of the mother liquors gave additional crystals, which were combined with the first crop to afford a total of 0.6 g of crude yellow crystals. Recrystallization of this crude material from hot acetone gave 0.27 g of fine colorless needles, mp 195.5–196° dec., $[\alpha]D^{29.5}$ −182° (c 0.50 in chloroform). This isolate was homogeneous by TLC, displayed an initial purple chromogenic response with the ceric ammonium sulfate reagent, and gave R_f values of 0.18, 0.56, 0.25, 0.12, 0.03, and 0.54 when analyzed in Solvent Systems A–F, respectively. The UV absorption spectrum showed λ_{max} (C_2H_5OH) 208 (log ϵ 4.35) and 305 (4.18) nm with a shoulder at 230 nm. The IR, specific rotation, UV, NMR, TLC, and mass spectroscopy data were identical with those derived from authentic pericalline, and a mixed melting-point determination showed no depression.

Isolation of Tetrahydroalstonine—Crude brown crystals (0.1 g) harvested from a chilled benzene solution of Extract N-3.0 were shown to be a mixture of two alkaloids by TLC. A solution of these crude crystals (0.1 g) in 0.2 *M* citric acid was made alkaline with ammonium hydroxide solution, and the liberated alkaloids were extracted by repeated shaking with ethylene dichloride. The organic extracts were combined, washed with water, dried (anhydrous sodium sulfate), and evaporated *in vacuo* to give a pale-yellow oil, which was further separated into two components by preparative thick-layer chromatography (Solvent System E). The first component (R_f 0.24) crystallized from benzene to give a product identical in all respects with a reference sample of ajmalicine. The second component (R_f 0.51) crystallized from benzene–hexane (1:1) to give fine colorless needles (0.016 g), mp 227–228° dec., $[\alpha]D^{29.5}$ −103° (c 0.47 in chloroform). This isolate was homogeneous by TLC, displayed a yellow-green chromogenic response with the cerric ammonium sulfate reagent, and gave R_f values of 0.77, 0.55, 0.74, 0.76, 0.52, and 0.69 when analyzed in Solvent Systems A–F, respectively. The UV absorption spectrum showed λ_{max} (CH_3OH) 226 (log ϵ 4.71), 284 (3.99), and 291 (3.92) nm, typical of alkaloids containing a tetrahydro-β-carboline moiety (8). The IR, specific rotation, UV, TLC, and mass spectroscopy data were identical with those derived from authentic tetrahydroalstonine, and a mixed melting-point determination showed no depression.

Cytotoxicity and Antitumor Activities—Certain crude fractions and extracts were submitted to the Drug Research and Development Branch, National Cancer Institute, for bioassay using established protocols (9) in the P-388 leukemia (PS) and Eagle's carcinoma of the nasopharynx in cell culture (KB) systems. The results of these tests are presented in Table I. The alkaloids isolated in this study were previously shown to be devoid of antitumor and cytotoxic activity (10).

SUMMARY

The total crude alkaloid fraction derived from *C. trichophyllus* roots was found to exhibit a moderate degree of cytotoxicity

Table I—Cytotoxicity and Antitumor Test Results from Selected Fractions of *C. trichophyllus*

Extract	KB Results[a], ED_{50}, μg/ml	PS Results[b], mg/kg, T/C
A′	—[c]	—
B′	17.7	—
C′	100.0	—
C	—[c]	—
D′	27.7	50/90
E	100.0	50/95
G	100.0	50/95
H′	100.0	50/104
H″	2.66	25/104
I	2.77	25/113
J	0.56	12.5/104
K	5.99	12.5/140
L	Not tested	
M	100.0	25/95
N	3.33	12.5/131
N-3.0	25.5	50/90
N-3.5	2.33	50/118
N-4.0	3.99	25/118
N-4.5	2.22	50/131
N-5.0	3.22	12.5/125
N-5.5	2.77	12.5/127
N-6.0	4.77	25/90
N-6.5	24.4	25/90
N-7.0	22.2	25/90
N-7.5	25.5	100/104
N-8.0	100.0	—
N-8.5	200.0	—
N-9.0	27.7	—

[a] A fraction is considered active if it has an $ED_{50} \leq 10$ μg/ml. [b] Dose, mg/kg/prolongation of life (percent). A fraction is considered as active if it has a T/C of ≥125. [c] Not tested in KB; inactive in PS.

against the KB culture *in vitro*, and certain fractions were active to a moderate degree in the PS *in vivo* system. The major active fraction was separated and purified using a combination of dialysis, gel permeation chromatography, and gradient pH fractionation techniques. Further purification led to the isolation of ajmalicine, pericalline, vindolinine (dihydrochloride), and tetrahydroalstonine. The common triterpene ursolic acid was isolated from a nonalkaloid fraction. Since the alkaloids isolated in this study were previously shown to be devoid of cytotoxic and/or antitumor activity, work is being continued to elucidate the constituent(s) responsible for the observed activities. Ajmalicine and tetrahydroalstonine have been previously isolated from *C. trichophyllus* aerial parts, but this is the first record of their isolation from the roots of this plant. Vindolinine (dihydrochloride) and pericalline are being reported for the first time from this apocynaceous species.

REFERENCES

(1) H.-K. Kim, R. N. Blomster, H. H. S. Fong, and N. R. Farnsworth, *Econ. Bot.*, **24**, 42(1970).

(2) M. Gabbai, "Les Alcaloides des Pervenches *Vinca* et *Catharanthus* (Apocynacees)," thesis, Universite de Paris, Faculte de Pharmacie, Serie U, No. 291, Paris, France, 1958.

(3) I. M. Jakovljevic, L. D. Seay, and R. W. Shaffer, *J. Pharm. Sci.*, **53**, 553(1964).

(4) R. Munier and M. Macheboeuf, *Bull. Soc. Chim. Biol.*, **33**, 846(1951).

(5) C. Djerassi, H. Budzikiewicz, and J. M. Wilson, *Tetrahedron Lett.*, **1962**, 263.

(6) H. Budzikiewicz, J. M. Wilson, and C. Djerassi, *J. Amer. Chem. Soc.*, **85**, 3688(1963).

(7) A. Klasek, *Sep. Sci.*, **3**, 319(1968).

(8) A. W. Sangster and K. L. Stuart, *Chem. Rev.*, **65**, 69(1965).

(9) Anon., *Cancer Chemother. Rep.*, **25**, 1(1962).

(10) N. R. Farnsworth, R. N. Blomster, and J. P. Buckley, *J. Pharm. Sci.*, **56**, 23(1967).

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