(21) Snyder, H. R., *et al.*, *J. Am. Chem. Soc.*, **75**, 4672 (1953).
(22) Üfer, H., Ger. pat. 702,064(Jan. 2, 1941); through *Chem. Abstr.*, **36**, 98(1942).
(23) Pomerantz, A., and Connor, R., *J. Am. Chem. Soc.*, **61**, 3386(1939).
(24) Mannich, C., and Lammering, D., *Ber.*, **55**, 3510 (1922).
(25) Barney, A. L., U. S. pat. 2,641,594(June 1953); through *Chem. Abstr.*, **47**, 11805(1953).
(26) Uyeda, Y., *J. Chem. Soc. Japan*, **52**, 410(1931); through *Chem. Abstr.*, **26**, 5082(1932).
(27) Backlund, D., *Arkiv. Kemi, Mineral. Geol.*, **1A** (No. 1), (1940); through *Chem. Abstr.*, **34**, 7860(1940).
(28) Tröger, J., and Beck, O., *J. Prakt. Chem.*, **87**, 289 (1913).
(29) Fromm, A., *Ann.*, **253**, 161(1889).
(30) Otto, R., and Tröger, J., *Ber.*, **25**, 3428(1892).
(31) Bernstein, J., *et al.*, *J. Am. Chem. Soc.*, **73**, 906(1951).

Tumor Inhibitors VI

Cissampareine, New Cytotoxic Alkaloid from *Cissampelos pareira*. Cytotoxicity of Bisbenzylisoquinoline Alkaloids

By S. MORRIS KUPCHAN, A. C. PATEL, and EIICHI FUJITA

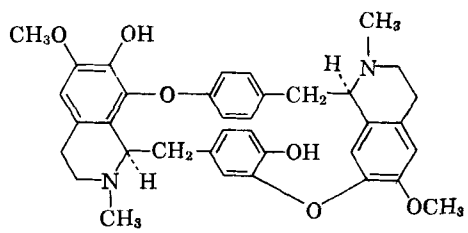
A preliminary study of *Cissampelos pareira* Linn. from Peru yielded a new alkaloid, cissampareine. Evidence is presented for assignment to cissampareine of the empirical formula, $C_{37}H_{38}N_2O_6$. Cissampareine and four other bisbenzylisoquinoline alkaloids isolated from menispermaceous plants were found to show significant and reproducible inhibitory activity against human carcinoma of the nasopharynx carried in cell culture (KB).

CISSAMPELOS PAREIRA Linn. is a climbing shrub distributed throughout warm parts of Asia, East Africa, and America. The roots are reported to have found use as a diuretic, febrifuge, remedy for heart trouble, and against dysentery and sores (1).

In 1840, Wiggers (2) isolated an amorphous alkaloid from the roots of a South American *C. pareira* sample, and the name pelosine was assigned to the alkaloid. Scholtz (3, 4) showed that pelosine is identical to *l*-curine (I). Bhattacharji *et al.* reported in 1956 (5) that *C. pareira* Linn. from Kashmir yielded two new alkaloids, hayatine and hayatinine, and that the same species from Pilibhit yielded hayatine and *l*-curine but no hayatinine. The methiodide of hayatine was shown to possess powerful neuromuscular blocking activity comparable to that of *d*-tubocurarine chloride (6, 7). Structural studies of hayatine (8) and hayatinine (9) indicate that both are alkaloids of the bisbenzylisoquinoline type.

DISCUSSION

An earlier study led to isolation from the roots and vines of *C. pareira* Linn. from Madras, India, of *l*-curine (I),[1] *d*-isochondrodendrine (II),[1] and hayatine (10). Preliminary pharmacological evaluation of the methanol-extractable alkaloids, of the methiodide prepared from the latter mixture, and



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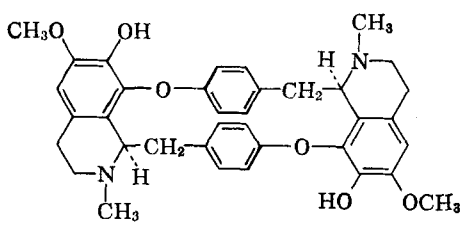

II

of the quaternary alkaloids, showed that all had curarelike activity (10).

The present report describes a preliminary study of the alkaloids of a sample of *C. pareira* Linn. from Department Huanuco, Peru,[2] and the isolation and characterization of cissampareine, a new cytotoxic alkaloid. Coarsely ground whole plant was extracted successively with petroleum ether, methanol, and 1.5% hydrochloric acid solution. Each extract was processed for alkaloid

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[1] Absolute configurations indicated for I–IV are those assigned by Tomita, M., and Kunimoto, J., *J. Pharm. Soc. Japan*, **82**, 734, 741(1962).

[2] The plant sample (whole plant) was procured by the Ciba Pharmaceutical Co., Summit, N. J. (acquisition number C-974), and identified by the late Professor Robert E. Woodson, Jr., Department of Botany, Washington University, St. Louis, Mo. The authors thank Dr. E. Schlittler and Dr. H. B. MacPhillamy for the plant material.

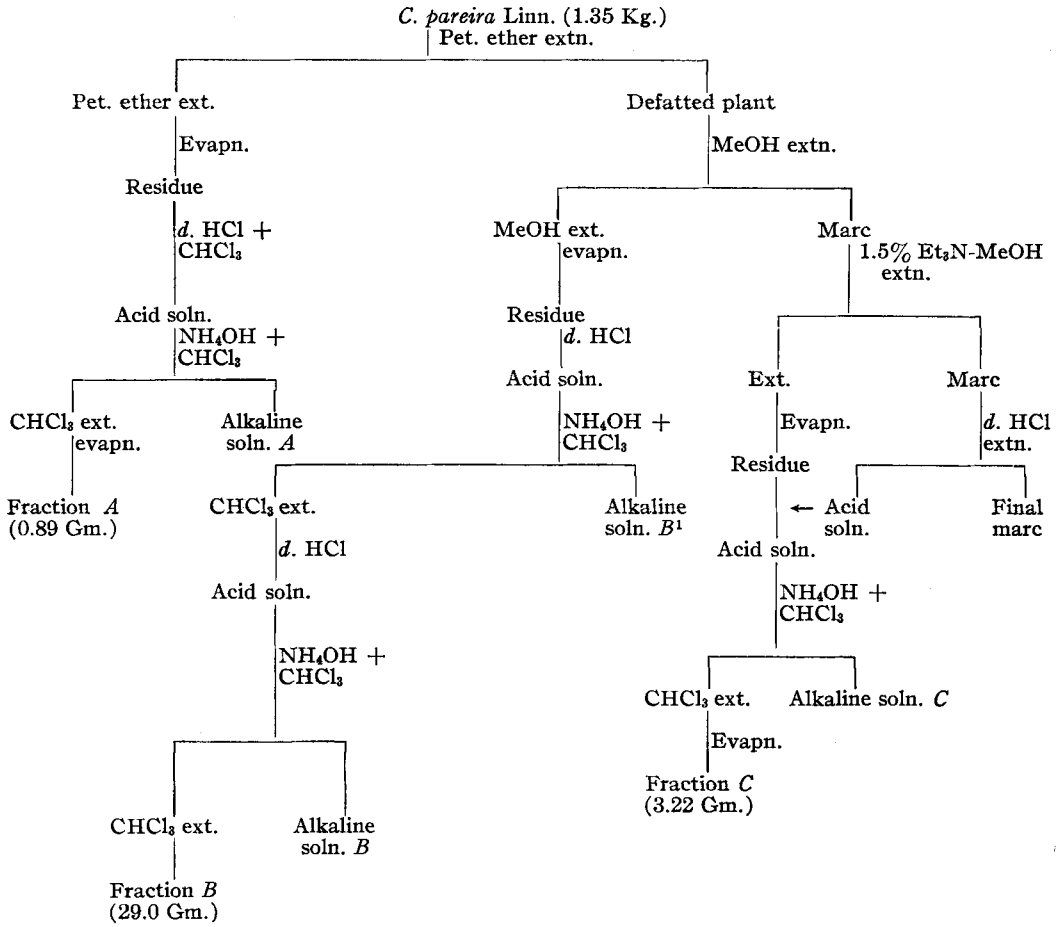


Fig. 1.—Flow sheet for separation of alkaloids of *C. pareira* Linn.

content by the procedure summarized in Fig. 1, whereby a 2.5% yield of crude alkaloids was obtained. The principal fraction (*B*) was purified by reprecipitation from dilute acid solution with ammonium hydroxide solution and extraction into ether. The residue from the ethereal extract was crystallized from benzene to yield the principal alkaloid (1.2%). Recrystallization from acetone gave colorless rods, m.p. 239–240° dec., $[\alpha]_D^{26}$ −111° (chloroform), and ultraviolet, infrared, and NMR spectral characteristics which indicated that the material is a new alkaloid of the bisbenzylisoquinoline type. The name cissampareine, reflecting the botanical origin, is proposed for the alkaloid.

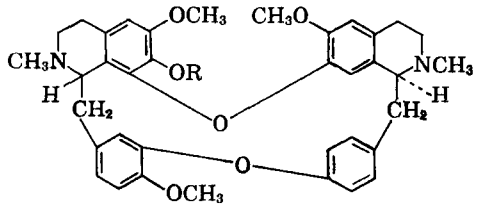
The molecular formula, $C_{37}H_{38}N_2O_6$, was assigned for cissampareine on the basis of elemental analysis and molecular weight determination by mass spectrometry.[3] Analysis showed the presence of three *O*-methyl groups and one *N*-methyl group. The NMR spectrum in deuterated chloroform supports the formula, showing nine *O*-methyl protons and three *N*-methyl protons. The infrared spectrum indicates the presence of aromatic rings and aromatic *O*-methyl groups, a free hydroxyl group, and a double bond.

Two derivatives were prepared to seek confirmation of the empirical formula and to yield information useful in characterizing functional groups. Although cissampareine was found to be sparingly soluble in 5% sodium hydroxide solution, its infrared absorption spectrum showed a band at 2.88 μ, indicative of the presence of a free phenolic hydroxyl group. Methylation with diazomethane yielded *O*-methylcissampareine, m.p. 192–194°, which showed no hydroxyl absorption in the infrared. Analysis afforded results which support a $C_{38}H_{40}N_2O_6$ formula, with four *O*-methyl groups. Reduction of cissampareine with sodium borohydride afforded dihydrocissampareine, $C_{37}H_{40}N_2O_6$, m.p. 208–212°, which showed ultraviolet absorption characteristic of bisbenzylisoquinoline alkaloids of the isochondrodendrine series (10, 11) and greatly diminished absorption at 6.22 μ in the infrared. The sensitivity of cissampareine toward reduction with sodium borohydride is suggestive of the presence of a C=N double bond, perhaps in a 3,4-dihydroisoquinoline environment (*cf. Reference 12*).

Cissampareine was evaluated for activity against human carcinoma of the nasopharynx carried in cell culture (KB) and was found to have significant and reproducible inhibitory activity. To evaluate the potential generality of this activity among alkaloids of the bisbenzylisoquinoline group, *l*-

[3] The authors thank Professor K. Biemann and Dr. B. C. Das, Massachusetts Institute of Technology, for the mass spectral data.

curine (I), *d*-isochondrodendrine (II), fangchinoline (III)[1] (13, 14), and *d*-tetrandrine (IV)[1] (13, 14) also were tested, and all four alkaloids showed reproducible activity against KB. The evaluation



III, R = H
IV, R = CH_3

of the KB assay results by the Cancer Chemotherapy National Service Center in sequential testing is such that a purified compound is considered active if the average ED_{50} of two tests $\leqq$ 4 mcg./ml. and if this result is reproducible when repeated by a second screener (15). In the event that a compound has an ED_{50} of <1 in the first test, the second sequential test is omitted, and it is submitted directly to a second screener for confirmation. In assays performed by the CCNSC, the following results were obtained: cissampareine, ED_{50} 1.1, 2.5, and 3.8 mcg./ml.; *l*-curine (I), ED_{50} 0.14 and 2.9 mcg./ml.; *d*-isochondrodendrine (II), ED_{50} 0.17 and 2.6 mcg./ml.; fangchinoline (III), ED_{50} 0.12 and 0.93 mcg./ml.; *d*-tetrandrine (IV), ED_{50} 0.091 and 0.17 mcg./ml. The cytotoxicity of the aforementioned alkaloids is sufficiently high to warrant scheduling the compounds for testing in a variety of *in vivo* tumor systems.

EXPERIMENTAL

Melting points have been corrected for stem exposure. Values of $[\alpha]_D$ have been approximated to the nearest degree. Infrared spectra were determined on a Beckman IR-5A infrared spectrophotometer. Ultraviolet spectra were determined in methanol on a Cary 11MS recording spectrophotometer.

Extraction of Alkaloids from *C. pareira*

Separation into Main Fractions.—Coarsely ground *C. pareira* Linn. (whole plant 1.35 Kg., from Department Huanuco, Peru) was extracted continuously for 60 hr. with petroleum ether (b.p. 60–80°, 6 L.) in a Soxhlet-type extractor. The solvent was evaporated under reduced pressure to give a semisolid residue (60 Gm.), which was dissolved in chloroform (150 ml.). The chloroform solution was extracted with 2.5% hydrochloric acid (four 50-ml. portions). The combined acid extract was washed with ether and made alkaline with ammonium hydroxide (to pH 10). The precipitated base was extracted with chloroform (2 L.), and the extract was concentrated to about 300 ml. The extraction with acid and reconversion to free base was repeated. The chloroform extract was dried over anhydrous sodium sulfate and evaporated to dryness under reduced pressure to yield a semisolid alkaloidal residue. [Fraction *A*, 0.89 Gm. (see Fig. 1).]

The dried marc after extraction with petroleum ether was extracted continuously with methanol (6 L.) for 6 days, with a fresh charge of methanol at the end of 2 days. When the extraction was stopped after 6 days, the extract returning to the pot yielded a residue which did not give a positive test with Mayer's reagent. The methanol extract was concentrated under reduced pressure to give a semisolid residue, which was triturated with 2.5% hydrochloric acid. The acid extract was washed with ether, made alkaline with ammonium hydroxide solution, and repeatedly extracted with chloroform (10 L.). The chloroform extract was concentrated under reduced pressure (to about 500 ml.) and extracted with 2.5% hydrochloric acid solution. The acid extract was made alkaline with ammonium hydroxide and extracted with chloroform. The chloroform extract was dried over anhydrous sodium sulfate and evaporated to dryness under reduced pressure to yield a crude solid alkaloidal residue (fraction *B*, 29.0 Gm.).

The marc remaining after methanol extraction was extracted continuously with 1.5% triethylamine in methanol for 4 days. The marc remaining after the latter extraction was percolated with 1.5% hydrochloric acid solution. The triethylamine–methanol extract was evaporated to dryness under reduced pressure, and the residue was triturated with the dilute hydrochloric acid extract of the marc. The acid extract was washed with chloroform, made alkaline with ammonium hydroxide, and repeatedly extracted with chloroform. The chloroform solution was dried over anhydrous sodium sulfate and evaporated to dryness under reduced pressure to give a brownish-black residue (fraction *C*, 3.22 Gm.).

The alkaline aqueous layers (*A*, *B*, *B*[1], and *C*) were combined, acidified with dilute hydrochloric acid (to pH 3), and treated with a saturated Reinecke salt solution to yield 26.4 Gm. of reineckate salts of the quaternary alkaloids.

Isolation of Cissampareine

Fraction A.—Crystallization of the crude alkaloid fraction (0.89 Gm.) from benzene yielded needles (70 mg.), m.p. 150–170°. Paper chromatographic[4] and infrared spectral examination indicated this alkaloid to be cissampareine (see below).

Fraction B.—A sample of the crude alkaloid fraction (2.12 Gm.) was dissolved in 2% sulfuric acid. The acid solution was washed with ether, then made alkaline with ammonium hydroxide, whereupon a yellow precipitate separated. The suspension was extracted with ether, and the ether extract was washed with 5% sodium hydroxide solution. The ethereal solution was washed with water, dried over anhydrous sodium sulfate, and evaporated to dryness to yield a yellow amorphous residue (1.85 Gm.). The latter material was shown to be essentially homogeneous upon paper chromatographic examination. Crystallization from benzene yielded needles (1.20 Gm.), m.p. 155–156°. Two recrystallizations from acetone afforded colorless rods, m.p. 239–240° dec.; $[\alpha]_D^{26}$ −111° (c 1.05, chloroform); $\lambda_{max.}$ 282 mμ (ε 10,000); λ_{sh} 320 (ε 4,000);

[4] Paper chromatography was conducted by the descending technique employing Whatman No. 4 paper. The procedure employed was similar to the one described by Levine and Fischbach (16) for the ascending technique. This method involves the use of prewetted pH 3.5 buffered paper and the detection of alkaloids by spraying with a chloroform solution of bromophenol blue. The solvent system used was the organic layer of a mixture of *n*-butanol : *n*-butyl acetate: pyridine: water (30:15:10:50 by volume), prepared by shaking and allowing to stand at room temperature.

mass spectral molecular ion peak, 606. The NMR spectrum ($CDCl_3$) showed τ = 6.08, 6.15, 6.25 (9H, *O*-methyl), 8.01 (3H, *N*-methyl). The infrared spectrum showed a band at 2.88 μ, indicative of the presence of a phenolic hydroxyl group, and a band at 6.22 μ, indicative of the presence of a double bond.

Anal.—Calcd. for $C_{37}H_{38}N_2O_6$: C, 73.24; H, 6.31; N, 4.62; 3(OCH_3), 15.33; 1(NCH_3), 4.77. Found: C, 72.92; H, 6.26; N, 4.56; (OCH_3), 16.15; (NCH_3), 4.07.

O-Methylcissampareine.—Cissampareine (1.093 Gm.) in methanol was treated with an excess of an ethereal solution of diazomethane, and the solution was allowed to stand at room temperature for 48 hr. The solution was concentrated partially, then treated with a second charge of excess diazomethane in ether for 24 hr. The solvent was evaporated to yield a syrupy residue, shown to be homogeneous upon paper chromatography. The residue was dissolved in 2% sulfuric acid, washed with ether, made alkaline with 5% sodium hydroxide solution, and extracted with ether. The ethereal solution was dried over anhydrous potassium carbonate and evaporated to give a yellowish residue (1.02 Gm.), which was crystallized easily from acetone to yield colorless rods (0.62 Gm.), m.p. 192–194°; $[\alpha]_D^{26}$ −121° (c 1.36, chloroform); $\lambda_{max.}$ 279 mμ (ϵ 13,500). The NMR spectrum ($CDCl_3$) showed τ = 6.00, 6.14, 6.24 (12 H, *O*-methyl) and 7.64 (3H, *N*-methyl). The infrared spectrum showed no absorption in the 2.7–3.0-μ region.

Anal.—Calcd. for $C_{38}H_{40}N_2O_6$: C, 73.52; H, 6.50; N, 4.51; 4(OCH_3), 19.97. Found: C, 73.29; H, 6.52; N, 4.50; (OCH_3), 18.97.

Dihydrocissampareine.—A solution of cissampareine (35 mg.) in methanol (5 ml.) was treated portionwise with sodium borohydride (40 mg.), and the mixture was allowed to stand at room temperature for 3 hrs. Evaporation yielded a colorless residue, which was suspended in water and extracted with ether. The ethereal solution was dried over anhydrous sodium sulfate and evaporated to yield a colorless residue. Crystallization from methanol afforded colorless prisms (17 mg.), m.p. 208–212°; $[\alpha]_D^{27}$ −157° (c 0.7, chloroform); $\lambda_{max.}$ 274 mμ (ϵ 3,800), 284 mμ (ϵ 3,400).

Anal.—Calcd. for $C_{37}H_{40}N_2O_6$: C, 73.00; H, 6.62; N, 4.60. Found: C, 72.62; H, 6.94; N, 4.83.

REFERENCES

(1) Chopra, R. N., *et al.*, "Indigenous Drugs of India," 2nd ed., V. N. Dhur and Sons, Calcutta, India, 1958.
(2) Wiggers, A., *Ann.*, **33**, 81(1840).
(3) Scholtz, M., *Ber.*, **29**, 2054(1896).
(4) Scholtz, M., *Arch. Pharm.*, **237**, 199(1899).
(5) Bhattacharji, S., Sharma, V. N., and Dhar, M. L., *J. Sci. Ind. Res. India*, **15B**, 363(1956).
(6) Pradhan, S. N., and De, N. N., *Brit. J. Pharmacol.*, **8**, 399(1953).
(7) Mukerji, B., and Bhandari, P. R., *Planta Med.*, **7**, 250(1959).
(8) Agarwal, K. P., *et al.*, *J. Sci. Ind. Res. India*, **19B**, 479 (1960).
(9) Bhattacharji, S., Roy, A. C., and Dhar, M. L., *ibid.*, **21B**, 428(1962).
(10) Kupchan, S. M., Yokoyama, N., and Beal, J. L., THIS JOURNAL, **49**, 727(1960).
(11) Kikuchi, T., and Bessho, K., *J. Pharm. Soc. Japan*, **78**, 1408(1958).
(12) Ban, Y., Yonemitsu, O., and Terashima, M., *Chem. Pharm. Bull. Tokyo*, **8**, 194(1960).
(13) Kupchan, S. M., Yokoyama, N., and Thyagarajan, B. S., THIS JOURNAL, **50**, 164(1961).
(14) Kupchan, S. M., Asbun, W. L., and Thyagarajan, B. S., *ibid.*, **50**, 819(1961).
(15) *Cancer Chemotherapy Rept.*, **25**, 1(1962).
(16) Levine, J., and Fischbach, H., THIS JOURNAL, **44**, 713(1955); **46**, 191(1957).

Inhibition of Acetylcholinesterase by Chelates II

By SANFORD BOLTON

Inhibition of acetylcholinesterase by 1–1 cupric chelates of ethylenediamine and glycine has been analyzed and shown to be essentially of a noncompetitive type. Inhibition by the 1–1 nickel chelates is weaker than the corresponding cupric chelates; but in contrast to the lack of activity of 2–1 cupric chelates, 2–1 nickel chelates exert significant inhibition. This suggests that in these chelates the metal is binding to the enzyme and that the availability of coordination sites in the metal is more important than chelate charge. This fact plus the noncompetitive nature of the inhibition suggests that the binding does not occur at the active site of the enzyme. Increased inhibition at higher pH in the nickel systems is further evidence that the chelate is interacting with an ionizing group, as previously reported.

IN THE FIRST paper of this series it was shown that inhibition of acetylcholinesterase by cupric chelates of ethylenediamine (en) and glycine could be attributed to the 1–1[1] chelate species and, under certain conditions, free cupric ion (1). The 2–1 chelate species did not inhibit the enzyme noticeably. This investigation has been extended to include nickel chelates of these compounds and to elucidate further the nature of the cupric chelate inhibition.

Results of this study show that acetylcholinesterase inhibition by nickel chelates of ethylenediamine and glycine at pH's 8.0 and 9.0 can be described by simultaneous interaction of both 1–1 and 2–1 chelate species. A more detailed analysis of the 1–1 cupric chelate inhibition indicates that the inhibition is essentially noncompetitive.

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[1] The 1–1 and 2–1 chelate species refer to (ligand–metal) and (ligand$_2$–metal) complexes, respectively.