Alkaloids of *Peumus boldus*. Isolation of (+) Reticuline and Isoboldine

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Chromatographic investigation of alkaloid fractions from the leaves of *Peumus boldus* Molina revealed the presence of 17 alkaloidal components. Four of these were correlated with previously isolated aporphine constituents and two others were isolated and identified as (+) reticuline and isoboldine.

THE LEAVES of the Chilean evergreen shrub *Peumus boldus* Molina (boldo leaves) have long been used as a choleretic, diuretic, stomachic, sedative, and anthelmintic (1). Preparations from the leaf have not been official in the NF since 1936, but the drug is still used, especially in "over-the-counter" remedies. Some 40 preparations containing the drug are currently available in Canada. The activity of the leaves has been variously ascribed to the alkaloid, the glycoside, or the essential oil content (1).

Boldine (I, $R_1 = R_3 = H$, $R_2 = CH_3$) was isolated from *P. boldus* by Merck in 1922 (2) and its structure determined 10 years later (3). By paper chromatography Rüegger, in 1959 (4), detected the presence of 11 alkaloids and he isolated and characterized three of these: isocorydine (II, $R = CH_3$), norisocorydine (II, R = H), and *N*-methyllaurotetanine (I, $R_3 = H$, $R_1 = R_2 = CH_3$).

This paper describes preliminary separation schemes for the isolation of some of the unknown alkaloidal constituents of boldo leaves and the isolation and identification of (+)-reticuline (III, (R = H) and isoboldine (I, $R_2 = R_3 = H$, $R_1 = CH_3$).

EXPERIMENTAL AND RESULTS

TLC Procedures—TLC plates of Silica Gel G (Merck) (layer thickness 0.25 mm.) were prepared according to standard techniques[1] using either water (for basic solvent systems) or 0.1 *N* sodium hydroxide (for nonbasic solvent systems).

The most useful solvent systems were: *A*, chloroform–methanol (85:15); *B*, ethyl acetate–acetone–methanol (50:20:30); *C*, benzene–methanol (80:20); *D*, ethyl acetate–acetone–methanol–diethylamine (45:30:20:5). The most commonly used spray reagents were: A, potassium iodoplatinate (5); B, Gibb's (6).

Extraction—Boldo leaves[2] were ground to a coarse powder in a Wiley mill and defatted by portionwise extraction in a Soxhlet apparatus with petroleum ether (b.p. 30–60°) for 6 hr. No alkaloids could be detected in this extract by TLC after separation of any bases by acid extraction. One-kilogram portions of the defatted powder were moistened with 5% ammonium hydroxide (1 L. and exhaustively extracted with chloroform in a percolator. The combined extracts from 5 Kg. of boldo leaves were reduced to 1.5 L. in a flash evaporator and extracted four times with 1 *N* hydrochloric acid (500 ml.) and once with 4 *N* hydrochloric acid (500 ml.). The acid extracts were neutralized with ammonia (pH 8–9) and extracted with chloroform until alkaloids could no longer be detected in the extracts by TLC. The combined chloroform extracts were reduced to 200 ml. in a flash evaporator (extract *a*). TLC examination (system *A*, spray reagent A) revealed the presence of 5 main alkaloid-positive areas at R_f 0.7 (isocorydine),[3] R_f 0.6 (*N*-methyllaurotetanine and norisocorydine), R_f 0.5 (boldine), R_f 0.3, and R_f 0.2.

Countercurrent Distribution—Extract *a* formed the lower phase of the first tube of a seven-tube counter-current distribution (in separators) between chloroform and an aqueous solution of 1% tartaric acid and 10% sodium chloride (200 ml. of each phase in each tube). After distribution was completed, the pH of each aqueous phase was adjusted to 10 by addition of ammonia and the chloroform layers were separated. Each aqueous phase was then extracted three times with 100 ml. chloroform and the combined chloroform extracts from each tube washed (water) and reduced to 50 ml. in a flash evaporator. The contents of tubes 2–4 and 5–7 were combined and the two resulting solutions again subjected to the countercurrent distribution. The contents of tube 1 were distributed (7 tubes) between chloroform and an aqueous solution of pH 6 (McIlvaine buffer). All tubes from these distributions having similar groups of alkaloids were combined to give four solutions (*1*, *2*, *3*, *4*). TLC examination (solvent system *A*; spray reagents A and B) showed that boldine (R_f 0.50) was present mainly in solutions *2* and *3* and isocorydine (R_f 0.73), *N*-methyllaurotetanine (R_f 0.60), and norisocorydine (R_f 0.58) were mainly in solution *4*. Other alkaloid-positive spots were located at R_f 0.16, 0.17, and 0.30 in solution *1*, R_f 0.32 in solutions *2*, *3*, and *4*, R_f 0.55 and 0.65 in solution *3*, and R_f 0.18 in solution *4*. After further resolution of these four solutions by column chromatography and preparative TLC, 17 alkaloids could be detected by TLC to be present in the extract of boldo leaves. They were numbered 1–17 according to increasing R_f value in solvent system *A*.

Isolation of (+)-Reticuline—A slurry of silicic acid (100 mesh) and 0.1 *N* sodium hydroxide was filtered and the solid dried by suction and then at 100°. Solution *2* was evaporated on alkali-treated silicic acid (12 Gm.) and the dried and pulverized powder packed on top of a column of the same silicic acid (25 × 2.5 cm.). The column was developed first with chloroform and then with chloroform–methanol mixtures containing increasing concentrations of methanol. Forty-five fractions (15 ml.)

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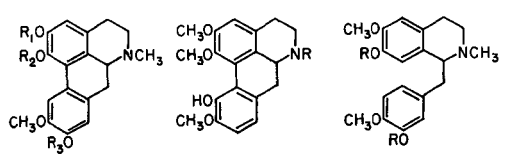
[1] Unoplan, Shandon Scientific Co. Ltd.

[2] Purchased from Dominion Herb Distributors, Montreal, Quebec, Canada.

[3] The authors are grateful to Dr. A. Rüegger, Sandoz Ltd. Basle, Switzerland, for authentic samples of isocorydine, norisocorydine, and *N*-methyllaurotetanine. Boldine was obtained from E. Merck (Darmstadt), Germany.

were collected and each fraction examined by TLC. Fractions 24–33 contained boldine (R_f 0.58; system *B*) in admixture with other alkaloids, mainly one (alkaloid 7) of R_f 0.50 (system *B*), characterized by giving a bright blue coloration with Gibb's reagent. Alkaloid 7 was concentrated by further column chromatography and a fraction was obtained which contained only boldine and alkaloid 7, in approximately equal amounts. After preparative TLC (system *A*) of this fraction, alkaloid 7 was obtained chromatographically pure. Further small amounts were obtained by preparative TLC of fractions obtained by chromatography of solutions *3* and *4*. The alkaloid was precipitated as an amorphous solid by *n*-hexane from ether solution, but attempts to crystallize it were unsuccessful. Yield 47 mg. (0.001%), m.p. 65–70°, $[\alpha]^{22}$ + 108°. The TLC (10 solvent systems, 11 spray reagents) and spectral (ultraviolet and infrared) properties of the alkaloid proved to be identical with those of an authentic sample of (±)-reticuline and the NMR spectrum ($CDCl_3$) was comparable with a published spectrum for (±)-reticuline taken in an unspecified solvent (7). It had a six-proton peak at 3.8 δ (two methoxyl groups), a three-proton peak at 2.44 δ (one *N*-methyl group), and a two-proton peak at 5.54 δ (two hydroxyl groups) which disappeared on addition of D_2O.

Alkaloid 7 was methylated with diazomethane in ethanol–ether solution for 20 hr., and the solution extracted with 1 *N* hydrochloric acid. This extract was made alkaline with ammonia, extracted in turn with chloroform, and the chloroform solution dried ($MgSO_4$), and evaporated. The product was amorphous and attempts to crystallize it were unsuccessful. It was shown to be homogeneous and identical with laudanosine[4] (III, R = CH_3) by TLC and infrared spectroscopy.



I II III

Isolation of Isoboldine—TLC comparison (system *A;* spray reagents A and B) of a sample of isoboldine[5] with solutions *1*, *2*, *3*, and *4* showed that alkaloid 11 (R_f 0.55) corresponded with isoboldine both in R_f and color reactions. The alkaloid was detected only in solution *3* and was concentrated by column chromatography and countercurrent distribution (chloroform *versus* 1% sodium carbonate, to separate it from boldine; chloroform *versus* 1% tartaric acid + 10% sodium chloride, to separate it from norisocorydine and *N*-methyllaurotetanine). Alkaloid 11 was obtained homogeneous only after preparative TLC and was shown to be identical with a sample of isoboldine by TLC (solvent systems *A–D*, 6 spray reagents) and by ultraviolet and infrared spectroscopy. Insufficient material was obtained for crystallization.

[4] Laudanosine was obtained from E. Merck (Darmstadt), Germany.

[5] The authors are grateful to Professor R. Tschesche, University of Bonn, West Germany, for an authentic sample of isoboldine and to Dr. R. H. F. Manske, Dominion Rubber Research Laboratory, Guelph, Ontario, Canada, for a sample of glaucine.

Alkaloid 11 was methylated with diazomethane in ethanol–ether solution for 72 hr. TLC examination of the product (3 solvent systems, 6 spray reagents) showed it to be identical with glaucine[5] (I, $R_1 = R_2 = R_3 = CH_3$).

DISCUSSION

Although the initial objective of this study was to characterize the alkaloids of boldo leaves by TLC alone, it soon became clear that, because of the large number of alkaloids present, preliminary resolution by countercurrent and chromatographic techniques was essential. After many purification steps it was possible to record unambiguously both color reactions and R_f values in various solvent systems for 17 alkaloids. Positive reactions with several alkaloidal spray reagents, in combination with the method of extraction, indicate that all 17 compounds are alkaloids. Furthermore, alkaloids 1–15 and 17 appear to be phenolic on the basis of positive reactions observed with other reagents. This was confirmed by repeated extraction of an ether solution of the alkaloids with 5 *N* sodium hydroxide, when only alkaloid 16 remained in the ether phase.

Considering the method of extraction used, the alkaloids detected in this study are probably nonquaternary. Preliminary work showed that acidification of the extracted boldo leaves, followed by further extraction with ethanol, yielded a fraction containing small amounts of quaternary bases. Alkaloids 8, 9, 14, 15, and 16 appear to be present in amounts too small to permit their isolation and identification, but work is in progress on the isolation and characterization of some of the other alkaloids, based on the purification processes described here.

Reticuline was originally isolated from *Cocculus laurifolius* and named coclanoline (8) but the name reticuline is now accepted by most workers and it has since been found in a number of plant species [*e.g.*, opium (*Papaver somniferum*) (7, 9), *Anona reticulata* (10), and *Romneyi coulteri* (11)]. Its isolation from *Peumus boldus* Molina is of interest since it is the first nonaporphine alkaloid to be isolated from this source and since it has been shown to be a key intermediate in the biogenesis of both the morphine (12) and berberine-protopine (13) series of alkaloids. It has also been postulated to be a biogenetic precursor of certain aporphines (14) and, although there is not yet direct *in vivo* evidence to support this hypothesis, reticuline has been converted *in vitro* in low yield into the aporphine isoboldine by oxidation with specific reagents (15). The two alkaloids have also been recently reported to occur together in opium (16). This prompted us, having isolated and characterized reticuline, to search for evidence for the presence of isoboldine in boldo leaves. The alkaloid was easily detected by TLC of solution *3* from the countercurrent distribution because of a characteristic green color given by it with potassium iodoplatinate reagent, all the other alkaloids giving the usual purple coloration. Separation of the pure alkaloid proved to be very difficult because of the presence of many interfering substances and because of the instability of the alkaloid. Enough was obtained, however, for comparison with an authentic sample of isoboldine by TLC, ultraviolet, and infrared spectroscopy. Furthermore, glaucine was shown by TLC identification to be the product of methylation with diazomethane.

The specific rotation of reticuline has been found to vary according to its origin between −55° and +132° and this is probably due to different rates of consumption of the two enantiomorphs in the biosynthetic pathways of the various species. Comparison with the value (+132°) found by Kusuda (8) for (+)-reticuline indicates that reticuline from *P. boldus* contains, at most, 10% (−)-reticuline.

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Keyphrases

Alkaloids-*Peumus boldus*
(+)-Reticuline—isolation
Isoboldine—isolation
Countercurrent distribution
Column chromatography—separation
TLC—analysis
IR spectrophotometry—structure
UV spectrophotometry—structure
NMR spectrometry

Michaelis Constants for Isolated Cholinesterase Systems

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The enzymatic hydrolysis of acetylcholine chloride by human plasma cholinesterase has been studied in detail. Results suggest that at substrate concentrations below 0.01 *M*, Michaelis-Menten kinetics are followed and enzyme hydrolysis rates are due to a single enzyme component. At higher substrate concentrations, a second enzyme component appears to contribute significantly to the total velocity of the reaction and a Lineweaver-Burk plot yields a hyperbolic-type curve. Apparent K_m values were calculated for the two components. The autohydrolysis rate for acetylcholine chloride at 26° was determined.

EVALUATION OF accurate Michaelis constants (K_m) has long been considered by the enzymologist to be practical only with highly purified isolated enzyme preparations. Nevertheless, enzyme kinetic constants from preparations of varying degrees of purity by investigators with divergent disciplinary backgrounds has led to the publication of a wide range of K_m values for most enzymes. This is particularly true in the case of human plasma (pseudo) cholinesterase (acylcholine acylhydrolase; E.C. 3.1.1.8) where many *apparent* K_m values (1–7) have been reported for the hydrolysis of butyrylcholine and benzoylcholine although comparatively little attention has been given to the hydrolysis of acetylcholine halides by the same enzyme. Acetylcholine is generally the substrate utilized in cholinesterase inhibitor evaluation.

In considering the variety of enzyme preparations (*e.g.*, commercial preparations,[1] Harvard fractions, IV-6-3 through IV-6-4 (8), Kabi fraction IV-6-3 (9), and completely unfractionated human serum (10), used for kinetic and inhibitor studies, it became of interest to ascertain the validity of expressing the Michaelis constants obtained with these preparations over wide ranges of substrate concentrations as valid, reproducible expressions of the true Michaelis constant.

METHOD

Acetylcholine chloride (Sigma Chemical Co.) was used as substrate in these studies; 17 different substrate concentrations covering a range from $3.00 \times 10^{-3}M$ to $1.50 \times 10^{-1}M$ were utilized. Enzyme initial velocity measurements were determined at 26.0° (±0.05°) with a continuous titration method (11) in a 0.15 *M* sodium chloride solution

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[1] Sigma Chemical Co., Worthington Biochemical Corp., Cutter Laboratories, Inc., and A. B. Kabi (Stockholm, Sweden), among others.