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Alkaloids of *Veratrum eschscholtzii* Gray. II.[1] The Ester Alkaloids

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RECEIVED OCTOBER 19, 1953

Escholerine ($C_{41}H_{61}O_{13}N$), a new tetraester of protoverine, as well as neogermitrine and veratroylzygadenine, have been isolated from *Veratrum eschscholtzii* Gray. On hydrolysis, escholerine yielded the alkamine protoverine, two moles of acetic acid and a mole each of (*levo*)-α-methylbutyric acid and angelic acid.

Escholerine ($C_{41}H_{61}O_{13}N$), a new tetraester of protoverine, and neogermitrine have been found to be the main hypotensive principles of *Veratrum eschscholtzii* Gray.[2] Veratroylzygadenine, previously known as a constituent of *Zygadenus venenosus* Wats[3] and *Veratrum fimbriatum* Gray[4] was also isolated from this species in trace amounts.

The preliminary fractionation of the basic chloroform extract of the roots and rhizomes of *Veratrum eschscholtzii*, as described in the first paper of this series,[1] was carried out in close conjunction with bioassays[5] for hypotensive activity. This procedure yielded an amorphous resin (fraction V) which accounted for the bulk of the hypotensive activity in the crude extract. On subjecting this fraction to a series of countercurrent distributions, the new hypotensively active alkaloid, *escholerine*, was obtained as well as neogermitrine and veratroylzygadenine.

The infrared spectrum showed distinct similarities to the other veratrum ester alkaloids with strong absorption being evidenced at 5.7 and 8.1 μ which can be attributed to the C=O and C–O–C vibrations of an ester linkage. Escholerine had previously exhibited its ester character by a positive hydroxamic acid[6] ester test. This simple test was found to be a convenient means of following the course of the ester alkaloids during the preliminary fractionation procedure.

A volatile acid determination on escholerine showed the presence of four volatile acids. Subsequent hydrolysis with 1 *N* methanolic sodium hydroxide afforded the alkamine protoverine, acetic acid and α-methylbutyric acid. On the basis of the analytical data and hydrolysis products it thus appeared that escholerine was a tetraester of protoverine containing two moles each of the above acids. In order to establish the optical configuration of α-methylbutyric acid and also to further substantiate the presence of two moles of this acid, the optical rotation of a solution of the volatile acids was determined. The results obtained, however, were in agreement for only one mole of (*levo*)-α-methylbutyric acid. In an effort to clarify this anomaly an aliquot of the acid distillate was distributed on a twenty-four plate countercurrent machine using chloroform–water as the solvent system. The contents of each tube were titrated with standard base and the results plotted. Two peaks were obtained, one corresponding to two equivalents of acetic acid (tubes 18–24) and the other corresponding to two equivalents of acid (tubes 8–16), of which, on the basis of the optical rotation, only one could be (*levo*)-α-methylbutyric acid. Therefore, it was evident that an additional unknown acid was present which must be very similar to α-methylbutyric acid.

The chromatographic separation of these volatile acids as their *p*-phenylphenacyl esters was then reinvestigated and by careful fractional crystallization of the material from the zone which had previously yielded only *p*-phenylphenacyl α-methylbutyrate, the *p*-phenylphenacyl ester of angelic acid was also isolated.

Escholerine has therefore been established as being a tetraester of protoverine containing two moles of acetic acid and one mole each of (*levo*)-α-methylbutyric acid and angelic acid. This composite formula is in agreement with the empirical formula reported previously[2] on the basis of analytical data.

Pharmacology.—The hypotensive activity[7] of escholerine, neogermitrine and veratroylzygadenine was found to be 0.30 μg. (0.26–0.36), 0.13 μg. (0.12–0.15) and 1.1 μg. (0.95–1.4).

Experimental[8]

Preliminary Fractionation of the Amorphous Bases (Fraction V) by 8-Plate Countercurrent Distribution.—The "amorphous" bases (60 g.) described previously as fraction V[1] were distributed using Craig's fundamental procedure employing eight 3 l. separatory funnels. Benzene–1 *M* acetate buffer *p*H 4.0, 1500 ml. in each phase, was used for the solvent system. On completion of the distribution, the contents of each funnel was adjusted to *p*H 7.2 with 6 *N* sodium hydroxide solution. The temperature was maintained below 20° by addition of ice. The layers were separated and the aqueous phases extracted with chloroform (250-ml. portions) until a negative test was obtained with Wagner's reagent. The benzene and chloroform extracts of each funnel were combined and washed once with water (150 ml.). The extracts were then dried over anhydrous sodium sulfate, evaporated to dryness under vacuum, and the weight of material recovered from each funnel recorded.

The results showed the separation of a very organophilic fraction B (wt. 12.1 g.) in tubes 0–2 from a large hydrophilic fraction (40.7 g.) present in tubes 6–8. Thirty grams of the material recovered from tubes 3–8 was redistributed by an 8-plate distribution with benzene–2 *M* acetate buffer

(1) For Paper I of this series see M. W. Klohs, M. D. Draper, F. Keller and F. J. Petracek, THIS JOURNAL, **75**, 2133 (1953).

(2) A preliminary report of this investigation appeared in a previous communication, M. W. Klohs, F. Keller, S. Koster and W. Malesh, *ibid.*, **74**, 1871 (1952).

(3) S. Morris Kupchan and C. V. Deliwala, *ibid.*, **75**, 1025 (1953).

(4) M. W. Klohs, M. D. Draper, F. Keller, S. Koster, W. Malesh and F. J. Petracek, *ibid.*, **75**, 4925 (1953).

(5) The pharmacological studies were carried out in the Pharmacology Department Boston University School of Medicine, Boston, Mass., under the direction of Dr. G. L. Maison. We should like to express our gratitude to Dr. Maison and Dr. J. W. Stutzman for their invaluable coöperation during the course of this work.

(6) R. E. Buckles and C. J. Thelen, *Anal. Chem.*, **22**, 677 (1950).

(7) Expressed as micrograms per kilogram of dog per minute required for a ten-minute intravenous infusion to lower the mean arterial blood pressure 30% when administered according to the method of G. L. Maison and J. W. Stutzman. The bracketed numbers express the 95% confidence limits.

(8) All melting points are corrected. The melting points of the alkaloids and their derivatives were determined in evacuated capillaries.

*p*H 5.5 (1500 ml. each phase) as the solvent system. This yielded a hypotensively active fraction in tubes 2–5 (fraction A) which was recovered as described above for the first 8-plate distribution.

The Isolation of Escholerine from Fraction B by 24-Plate Countercurrent Distribution.—Fraction B (8.5 g.) was subjected to a 24-plate countercurrent distribution using 1 l. separatory funnels and 0.5 *M* acetate buffer *p*H 5.0–benzene–cyclohexane 25:75 as the solvent system (450 ml. in each phase). On completion of the distribution the contents of each funnel was recovered by standard procedure. On plotting the results, a skewed curve was obtained, with a peak in tube 13, which diverged from the theoretical curve on the hydrophilic side.

Escholerine.—The alkaloid was obtained by crystallization of the material (2.27 g.) recovered from the organophilic side of the peak area (tubes 8–13) from acetone–water; yield crude 0.54 g. After four recrystallizations, escholerine was obtained as plates, m.p. 235–235.3° dec., $[\alpha]^{25}D$ −30 ± 2° (*c* 1.0 in pyridine), +7 ± 2° (*c* 1.0 in $CHCl_3$). For analysis the sample was dried at 110° (2 mm.) to constant wt.

Anal. Calcd. for $C_{41}H_{61}O_{13}N$: C, 63.46; H, 7.92; N, 1.80; equiv. wt., 775.9. Found: C, 63.42, 63.59; H, 8.00, 7.97; N, 2.04; equiv. wt., 782, 772.

Escholerine Picrate.—Escholerine (100 mg.) was dissolved in 5% aqueous acetic acid (2 ml.) and a saturated aqueous solution of picric acid was added until no further precipitate was formed. The precipitate was recovered by centrifugation and dried at 25° (2 mm.). The picrate was dissolved in a minimum of acetone and diluted with ether to turbidity. On standing, fine needles formed which when recovered melted at 259.5° dec. For analysis the sample was dried at 110° (2 mm.) to constant weight.

Anal. Calcd. for $C_{41}H_{61}O_{13}N \cdot HOC_6H_2(NO_2)_3$: C, 56.17; H, 6.42. Found: C, 56.41; H, 6.38.

Escholerine Auric Chloride.—Escholerine (100 mg.) was dissolved in aqueous 1 *N* hydrochloric acid (2 ml.) and a solution of auric chloride in dilute hydrochloric acid was added until no further precipitate was formed. The precipitate was recovered by centrifuging and then crystallized from dilute acetone (wt. 123 mg.). After three more crystallizations, the crystals melted at 191.4° (frothing). For analysis the sample was dried at 25° (2 mm.).

Anal. Calcd. for $C_{41}H_{61}O_{13}N \cdot HAuCl_4$: C, 44.13; H, 5.60; Au, 17.67. Found: C, 44.53; H, 5.61; Au, 17.21.

Hydrolytic Cleavage of Escholerine to Protoverine (*levo*)-α-Methylbutyric Acid, Acetic Acid and Angelic Acid.—Escholerine (0.3 g.) was hydrolyzed and the hydrolysis products were isolated in the same manner as described for neoprotoveratrine.[9] A crystalline alkamine and an acid fraction were obtained by this procedure.

The Identification of Protoverine.—The alkamine obtained above (0.054 g.) was recrystallized twice from methanol, yielding prisms, m.p. 191–192°, $[\alpha]^{24}D$ −16 ± 2° (*c* 1.0 in pyridine). For analysis the sample was dried to constant weight at 130° (2 mm.).

Anal. Calcd. for $C_{27}H_{43}O_9N$: C, 61.67; H, 8.25. Found: C, 61.68; H, 8.29.

The infrared spectrum was identical with that of an authentic sample of protoverine obtained from neoprotoveratrine.

The acetonyl hydrochloride of the above sample was prepared by the method of Jacobs and Craig.[10] The resulting product after three recrystallizations from methanol yielded needles, m.p. 272°. For analysis the sample was dried to constant weight 130° (2 mm.).

Anal. Calcd. for $C_{30}H_{48}O_9NCl$: C, 59.82; H, 8.04. Found: C, 59.62; H, 8.09.

The melting point of acetonyl protoverine hydrochloride has been reported as 278–281°.

Identification of Acetic Acid, (*levo*)-α-Methylbutyric Acid and Angelic Acid.—The acid fraction from the hydrolysis mixture was treated with *p*-phenylphenacyl bromide as previously described.[9] The *p*-phenylphenacyl esters were dissolved in a solution of benzene–petroleum ether 50:50 (69–74°) (10 ml.) and chromatographed on a silicic acid–celite column 3:1 (8 g.). Elution of the column with the same solvent mixture yielded in the first eight fractions (10-ml. cuts) unreacted *p*-phenylphenacyl bromide m.p. 125°. From cuts 9–25 material was obtained which on crystallization from alcohol–water yielded a substance melting at 70–71°. The infrared spectra of this compound and *p*-phenylphenacyl α-methylbutyrate were identical. For analysis the sample was dried at 25° (2 mm.) over P_2O_5 for 24 hours.

Anal. Calcd. for $C_{19}H_{20}O_3$: C, 77.01; H, 6.80. Found: C, 76.61; H, 6.80.

The material in the mother liquors remaining from the crystallization of *p*-phenylphenacyl α-methylbutyrate was crystallized several times from petroleum ether and finally from dilute ethanol yielding platelets, m.p. 88–89°. The melting point of *p*-phenylphenacyl angelate has been reported as 90°.[11] A mixed melting point with an authentic sample showed no depression and the infrared spectra were identical.

Cuts 32–55 yielded a third fraction which on crystallization from 95% ethanol melted at 110.8–111.2°. The infrared spectra of this substance and *p*-phenylphenacyl acetate were identical. For analysis the sample was dried at 25° (2 mm.) over P_2O_5 for 24 hours.

Anal. Calcd. for $C_{16}H_{14}O_3$: C, 75.58; H, 5.55. Found: C, 75.38; H, 5.66.

No other fractions were obtained on further elution.

Determination of Optical Configuration of α-Methylbutyric Acid.—Escholerine (142.5 mg.) was hydrolyzed for one hour in 3 ml. of 0.03 *N* sodium hydroxide in methanol plus 3 ml. of water. The methanol was distilled off *in vacuo* and the remaining water solution was acidified with 2 ml. of 25% *p*-toluenesulfonic acid. The acids were then steam distilled; total volume of distillate and washings, 20 ml. The rotation of the solution determined in a 2-dm. tube was −4.05°. This is equivalent to 16.5 mg. of (*levo*)-α-methylbutyric acid, $[\alpha]^{24}D$ −25°, calcd. 18.6 mg.

Countercurrent Distribution of Volatile Acids.—The acid solution from the optical rotation determination was carefully washed from the polarimeter tube and the total acids (except for 1 ml. used to determine total equivalents of volatile acid) were subjected to a 24-plate countercurrent distribution on a Craig machine using chloroform–water as the solvent system, 30 ml. each phase. The contents of each tube were then titrated with 0.0207 *N* sodium hydroxide using phenolphthalein as the indicator. The results were plotted and two peaks were obtained. The first peak (tubes 8–16) gave a titration value equal to 1.49 equivalents of a mixture of (*levo*)-α-methylbutyric acid and angelic acid. The second peak (tubes 18–24) gave a titration value equal to 1.32 equivalents of acetic acid.

The Isolation of Neogermitrine from Fraction A.—Fraction A (8.5 g.) was resolved on a 24-plate countercurrent distribution with 2 *M* acetate buffer *p*H 5.5–benzene as the solvent system (450 ml. in each phase). On completion of the distribution, the material from each tube was recovered by standard procedure. On plotting the results, a predominant peak was obtained in tube 5 and a smaller peak in tube 16. The material from tubes 6–11 (2.26 g.) was crystallized from acetone–water, m.p. 234–234.8°, $[\alpha]^{25}D$ −79 ± 2° (*c* 0.2 in pyridine). The alkaloid was identified as neogermitrine by its infrared spectrum and a mixed melting point with an authentic sample of neogermitrine kindly provided by Dr. J. Fried.

The material recovered from tubes 12–17 (1.34 g.) was dissolved in a minimum of acetone. On standing overnight a white powder settled out which, on crystallization from chloroform–acetone, yielded veratroylzygadenine (30 mg.), m.p. 268°, $[\alpha]^{24}D$ +34 ± 2° (*c* 0.74 in pyridine). The identity of the sample was confirmed by comparison with an authentic sample obtained from *Veratrum fimbriatum* Gray.[4]

Acknowledgments.—We are indebted to Dr. Adalbert Elek for the micro analyses. We also wish to express our thanks to M. Robinson, B. Crepeau and C. Stimmel in the Riker Analytical Department for the optical rotations, infrared spectra and equivalent weight determinations.

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