[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, HARVARD UNIVERSITY]

Zygadenus Alkaloids. IV. Active Principles of *Zygadenus venenosus*. Germine Esters[1]

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RECEIVED JUNE 3, 1954

Further investigation of the active principles of *Zygadenus venenosus* has led to the isolation of the hypotensive germine esters neogermitrine, germidine, protoveratridine and a new diester isomeric with germidine, for which we have proposed the name neogermidine. Neogermidine, like germidine, is a monoacetate mono-(*l*)-α-methylbutyrate of the alkamine germine. Upon acetylation with acetic anhydride and pyridine, neogermidine is converted to monoacetylneogermitrine.

A recent study of the alkaloidal constituents of *Zygadenus venenosus* revealed the occurrence of the esters veratroylzygadenine and vanilloylzygadenine and the alkamines zygadenine and germine.[3] We have subsequently noted the occurrence of the germine esters neogermitrine,[4] germidine,[5] protoveratridine[6] and a new diester isomeric with germidine which we have named neogermidine.[7] The present paper describes in detail the isolation and characterization of the latter compounds.

In this investigation, a batch of 23 kg. of *Zygadenus venenosus*[8] was processed initially as outlined previously.[3b] Fractionation of the chloroform-extractable alkaloids by the modified 8-plate countercurrent distribution procedure yielded the alkaloids obtained earlier. The high melting compound (m.p. 272–273° dec.) from the plate 0 fraction (compound III[3b]) was characterized as protoveratridine.

Since the major portion of the alkaloidal material from the plant remained in the amorphous residues of the above procedure, alternative methods for fractionation of these complex mixtures were next investigated. When the chloroform-extractable crude mixture was subjected to detailed 8-plate countercurrent distribution using benzene and *M*/15 phosphate buffer at *p*H 7.1, the alkaloidal material was distributed fairly evenly among the fractions obtained. However, inspection of the infrared spectra of the nine fractions indicated that there was a close resemblance among the spectra of the plate 4 to 8 fractions, on the one hand, and among the spectra of the plate 0 to 3 fractions, on the other. Hence the material recovered from plates 4 to 8 was combined and designated the "organophilic" fraction; the material from plates 0 to 3 was combined and designated the "hydrophilic" fraction. In subsequent practice, the two fractions were obtained by a simple distribution scheme requiring only five separatory funnels and half the number of transfers of buffer required in the 8-plate distribution (see Experimental).

The organophilic fraction was next subjected to 24-plate countercurrent distribution between benzene and 2 *M* acetate buffer at *p*H 5.5. No discrete peaks were obtained, but the material recovered from plates 12 to 18, when crystallized from ether, yielded neogermitrine. The material recovered from plates 4 to 7 yielded germidine. The identification of both substances was confirmed by direct comparison with authentic specimens of neogermitrine and germidine from *Veratrum viride*.[9]

At this time, an investigation of alternative, and possibly superior, methods for isolation of the crystalline polyesters of germine from *Veratrum album* was in progress in this Laboratory. We found that several of the most active esters of the veratrum series could be isolated by chromatography of a crude mixture of the alkaloids in chloroform on sulfuric acid-washed alumina.[10] When the chloroform-extractable crude alkaloids from *Zygadenus venenosus* were chromatographed by the same procedure, neogermitrine was obtained by crystallization of the first fractions from ether. The subsequent fractions obtained by elution with chloroform resisted crystallization, but showed absorption in the infrared suggestive of the presence of diesters of germine.

A more efficient separation of the ester alkaloids present in the crude mixture could be effected if a partition into organophilic and hydrophilic fractions (as above) was done first. Then, chromatography of the organophilic fraction yielded neogermitrine and germidine upon crystallization. The hydrophilic fraction, when chromatographed as above, yielded a new ester alkaloid (I) from the fractions eluted with chloroform. The amorphous fractions eluted with chloroform containing increasing percentages of methanol showed infrared absorption indicative of the presence of aliphatic acid esters of zygadenine. The composition of these fractions will be the subject of a forthcoming report.

The new ester alkaloid I crystallized from benzene as heavy prisms, m.p. 221–223° dec., $[\alpha]^{22}D$ −60° (*c* 2.00 pyr.) and $[\alpha]^{22}D$ −25° (*c* 2.00, chf.). Alkaline hydrolysis afforded germine and a mixture of acetic acid and α-methylbutyric acid. The acids were identified by conversion to their *p*-phenylphenacyl esters which were characterized after chromatographic separation. Titration of the total volatile acids after *p*-toluenesulfonic acid hydrolysis indicated the presence of two equivalents of acid, an

(1) This work was supported (in part) by grants from the National Institutes of Health (RG-2553) and Eli Lilly and Company.

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(3) S. M. Kupchan and C. V. Deliwala, THIS JOURNAL, (a) **74**, 2382 (1952); paper III, (b) **75**, 1025 (1953).

(4) J. Fried, P. Numerof and N. M. Coy, *ibid.*, **74**, 3041 (1952).

(5) J. Fried, H. L. White and O. Wintersteiner, *ibid.*, **72**, 4621 (1950).

(6) (a) G. Salzberger, *Arch. Pharm.*, **228**, 462 (1890); (b) W. Poethke, *ibid.*, **275**, 571 (1937).

(7) (a) S. M. Kupchan and C. V. Deliwala, THIS JOURNAL, **74**, 3202 (1952); (b) O. Krayer, S. M. Kupchan, C. V. Deliwala and B. H. Rogers, *Arch. Exper. Path. u. Pharmakol.*, **219**, 371 (1953).

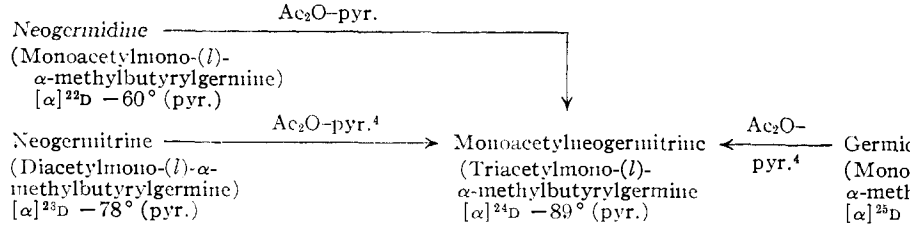
(8) Plant collected in northeastern Oregon in June, 1951. We are grateful to Dr. Reed Rollins, Gray Herbarium, Harvard University, for confirming the identity of the plant.

(9) We thank Dr. J. Fried of The Squibb Institute for Medical Research for authentic specimens of neogermitrine and germidine.

(10) S. M. Kupchan and C. V. Deliwala, THIS JOURNAL, **75**, 4671 (1953).

CHART I

CORRELATION OF NEOGERMIDINE WITH NEOGERMITRINE AND GERMIDINE



indication that the compound is a germine monoacetate mono-α-methylbutyrate. The carbon and hydrogen content of the alkaloid and of the thiocyanate (m.p. 247–249° dec.) are in accord with the formula $C_{34}H_{53}O_{10}N$, which is the composition required by the suggested diester formulation. Hence the new ester alkaloid is isomeric with germidine and we have named the compound *neogermidine.*[11]

Acetylation of neogermidine with pyridine and acetic anhydride yielded monacetylneogermitrine. Since neogermitrine and germidine had been converted previously to monoacetylneogermitrine,[4] this fact shows that the α-methylbutyric acid residue present in neogermidine is the levo enantiomorph, and that the site of attachment of the group in the alkamine germine is the same as that in germidine and neogermitrine (Chart I).

Methanolysis of one of our first samples of neogermidine afforded a small yield of protoveratridine. This fact, coupled with the aforementioned acetylation of neogermidine to monoacetylneogermitrine, led us to conclude that the location of the (*l*)-α-methylbutyryl residue in the germine nucleus was the same in protoveratridine, neogermidine, germidine and neogermitrine.[7a] The recent synthesis by Weisenborn and Bolger[12] of germidine and monoacetylneogermitrine from a mono-(*l*)-α-methylbutyrylgermine *isomeric* with protoveratridine indicated that the location of this residue in protoveratridine differed from its position in germidine and neogermitrine. The incompatibility of the two conclusions led us to repeat our methanolysis experiment with a highly purified sample of neogermidine. In several attempts, no protoveratridine could be isolated from the reaction product. We must assume that the starting material in the original methanolysis experiment was contaminated with protoveratridine and that the contaminant came through the reaction unchanged.

Acetylation of protoveratridine afforded triacetylprotoveratridine, m.p. 242–244° dec., $[\alpha]^{25}D$ −89° (*c* 0.94, pyr.). The infrared spectrum of this product in chloroform is the same as that of monoacetylneogermitrine in chloroform and this fact, coupled with the identity of the empirical formulas and the rotations of the two materials led us to infer initially that the two products were the same. However, the variance of this conclusion with the evidence discussed earlier led to a careful re-examination of the two materials. The infrared spectra of the two products in Nujol have now been found to differ in the region of 12–15 μ,[12] and admixture of monoacetylneogermitrine to triacetylprotoveratridine caused a significant depression in melting point. This demonstration of the non-identity of the two triacetylmono-(*l*)-α-methylbutyrylgermines offers additional evidence that the location of the (*l*)-α-methylbutyric acid residue in protoveratridine differs from its position in neogermidine, germidine and neogermitrine.

Pharmacological experiments carried out with neogermidine at the laboratory of Professor Otto Krayer at Harvard Medical School indicate that the circulatory action in the cat and the veratrinic effect on the frog muscle are similar to those of germidine.

Acknowledgment.—The assistance of Eli Lilly and Company in gathering and extracting *Zygadenus venenosus* is gratefully acknowledged.

Experimental

Fractionation of the Chloroform-extracted Bases into Organophilic and Hydrophilic Fractions.—The total amorphous bases obtained by chloroform extraction[3b] of *Zygadenus venenosus*[8] were fractionated by the following modified countercurrent distribution between benzene and *M*/15 phosphate buffer of *p*H 7.1 (prepared by mixing 2 l. of a disodium phosphate solution containing 17.9 g. of the heptahydrate per l. with 860 ml. of a monopotassium phosphate solution containing 9.1 g. per l.). The distribution was done in five separatory funnels, numbered 8 to 3. Funnels 8 to 4 were filled with 400 ml. of benzene. The amorphous bases (8 g.) and 400 ml. of buffer were added to funnel 8, and the contents were shaken. A small amount of insoluble material which clung to the walls of the funnel was removed by decantation of the liquid phases. The buffer layer was transferred to funnel 7, fresh buffer (400 ml.) was added to funnel 8, and both funnels were equilibrated by shaking. The process of transfer, addition of further buffer layers to funnel 8 and equilibration was continued until the first buffer layer reached funnel 3. Then, no further buffer portions were added, but the transfers of buffers and equilibration were continued until the last buffer layer reached funnel 3.

The benzene solutions in funnels 4 to 8 were combined and on evaporation to dryness *in vacuo* gave 4.3 g. of amorphous solid, representing the organophilic fraction.

The combined buffer layers were cooled to 0–5°, made alkaline to *p*H 10 with 10% sodium hydroxide solution, and extracted with chloroform (five 400 ml. portions). The chloroform solution was dried over anhydrous sodium sulfate and evaporated to dryness *in vacuo*. The colorless amorphous residue (3.2 g.) represented the hydrophilic fraction.

Isolation of Neogermitrine and Germidine by 24-Plate Countercurrent Distribution of the Organophilic Fraction.—The organophilic amorphous bases (8 g.) were submitted to 24-plate countercurrent distribution using benzene and 2 *M* acetate buffer at *p*H 5.5.[5] The contents of tubes 12–18 crystallized from ether, yielding needles (500 mg.), m.p. 238–240° dec. Recrystallization from acetone–water af-

(11) The same compound has been isolated independently from *Veratrum viride* by G. S. Myers, *et al.*, THIS JOURNAL, **74**, 3198 (1952). Dr. Myers has kindly informed us that a direct comparison of isogermidine with our sample of neogermidine confirms the identity.

(12) F. L. Weisenborn and J. W. Bolger, THIS JOURNAL, **76**, 5543 (1954). We thank Dr. F. L. Weisenborn for his kind communication of these results to us prior to publication, and for the infrared comparisons of the germine tetraesters in Nujol.

forded elongated colorless rods, m.p. 236–237° dec.; $[\alpha]^{25}D$ −78° (*c* 1.73, pyr.).

Anal. Calcd. for $C_{36}H_{55}O_{11}N$: C, 63.79; H, 8.18. Found: C, 63.78; H, 8.30.

The melting point was not depressed by admixture of an authentic specimen of neogermitrine[9] and the infrared spectra of the two samples in chloroform were identical.

The material recovered from tubes 3–5 (1.0 g.) was combined in chloroform and chromatographed on sulfuric acid-washed alumina (20 g.). After a forerun containing only a trace of yellow oil, the next fractions eluted with chloroform yielded a solid which crystallized from ether–petroleum ether (90 mg.). Recrystallization from ethyl acetate–ether afforded rectangular prisms, m.p. 243–245° dec., $[\alpha]^{25}D$ −11° (*c* 2.00, pyr.).

Anal. Calcd. for $C_{34}H_{53}O_{10}N$: C, 64.22; H, 8.38. Found: C, 64.34; H, 8.50.

The infrared spectrum in chloroform was identical with that of authentic germidine.[9] Recrystallization of the above material from alcohol–water afforded rectangular plates, m.p. 205–206°. The melting point of this material was not changed by admixture of authentic germidine (m.p. 202–204° dec.) from *Veratrum viride*. Recrystallization of the low-melting form from ethyl acetate–ether afforded the high-melting form. A mixture of the two forms melted at 204–206°, resolidified, and melted again at 231–234° dec.

Isolation of Neogermitrine and Germidine by Chromatography of the Organophilic Fraction.—A solution of the organophilic bases (4.5 g.) in chloroform (50 ml.) was chromatographed on sulfuric acid-washed alumina (95 g.). After a forerun of 100 ml. containing no solid material, the next 100 ml. of chloroform gave a yellow oil (60 mg.). The following two 100-ml. fractions, on evaporation *in vacuo* and crystallization of the residue from ether, gave colorless needles (400 mg.), m.p. 237–239° dec. The product was characterized as neogermitrine by mixed m.p., rotation, and infrared spectral determinations. The next two 100-ml. fractions of chloroform eluate gave an amorphous mixture which resisted crystallization. The following four 100-ml. fractions yielded an amorphous residue which crystallized from ether–petroleum ether (45 mg., m.p. 228–230°). Recrystallization from alcohol–water afforded rectangular plates which melted at 203–205°. This material was characterized as germidine by mixed m.p., rotation and infrared spectral determinations.

Isolation of Neogermidine by Chromatography of the Hydrophilic Fraction.—A solution of hydrophilic amorphous bases (8 g.) in chloroform (60 ml.) was chromatographed on sulfuric acid-washed alumina as above. After a 200-ml. forerun containing only traces of oil, the next four 100-ml. fractions eluted with chloroform were combined and evaporated *in vacuo*. The amorphous residue, when crystallized from ether, gave microcrystals (900 mg.). Recrystallization from benzene afforded heavy prisms, m.p. 221–223° dec., $[\alpha]^{22}D$ −60° (*c* 2.00, pyr.), $[\alpha]^{22}D$ −25° (*c* 2.00, chf.).

Anal. Calcd. for $C_{34}H_{53}O_{10}N$: C, 64.22; H, 8.38. Found: C, 64.15; H, 8.70.

In a volatile acid determination[13] 17.52 mg. of neogermidine was equivalent to 5.77 ml. of 0.009126 *N* sodium thiosulfate; calcd. for germine monoacetate mono-α-methylbutyrate, 6.05 ml.

Neogermidine Thiocyanate.—Neogermidine (50 mg.) was dissolved in 5% acetic acid (3 ml.) and a concentrated solution of ammonium thiocyanate was added dropwise to complete precipitation. Recrystallization from acetone afforded clusters of needles, m.p. 247–249° dec.

Anal. Calcd. for $C_{34}H_{53}O_{10}N \cdot HNCS$: C, 60.50; H, 7.82; S, 4.61. Found: C, 60.16; H, 7.87; S, 4.65.

Hydrolytic Cleavage of Neogermidine to Germine, Acetic Acid and α-Methylbutyric Acid.—Neogermidine was hydrolyzed with aqueous methanolic alkali according to the procedure used for the hydrolysis of germidine.[5] Recrystallization of the recovered alkamine from methanol yielded heavy prisms which began to sinter at 155–165° and melted at 220–225° dec., $[\alpha]^{24}D$ +4° (*c* 2.00, alc.). The infrared spectrum in Nujol was identical with that of an authentic specimen of germine.

The acids obtained upon hydrolysis were identified as their *p*-phenylphenacyl esters, which were characterized after chromatographic separation. The *p*-phenylphenacyl ester of α-methylbutyric acid (m.p. 72–73°) and *p*-phenylphenacyl acetate (m.p. 109–110°) failed to depress the melting points of authentic samples.

Acetylation of Neogermidine to Monoacetylneogermitrine.—Neogermidine (50 mg.) was acetylated with acetic anhydride (1 ml.) and pyridine (1 ml.) by the procedure used for the acetylation of neogermitrine.[4] Recrystallization of the crude product from acetone gave colorless plates, m.p. 251–253° dec., $[\alpha]^{22}D$ −89° (*c* 2.00, pyr.). The mixed melting point with monoacetylneogermitrine was not depressed, and the infrared spectra of the product in chloroform and Nujol were the same as the corresponding spectra of monoacetylneogermitrine.

Isolation of Protoveratridine.—The chloroform-extractable bases (6 g.) from *Zygadenus venenosus*[8] were fractionated by distribution between benzene and phosphate buffer at *p*H 7.1 by the procedure described in an earlier paper.[3b] By this method, veratroylzygadenine, vanilloylzygadenine, germine and zygadenine were obtained. As before, a high-melting benzene-insoluble solid (70 mg., III[3b]) was obtained from the plate 0 chloroform fraction. Recrystallization by reprecipitation with aqueous ammonia from a hot dilute alcoholic acetic acid solution afforded translucent rectangular plates (35 mg.), m.p. 272–273° dec., $[\alpha]^{22}D$ −9° (*c* 0.76, pyr.).

Anal. Calcd. for $C_{32}H_{51}O_9N$: C, 64.73; H, 8.66; N, 2.36. Found: C, 64.79; H, 8.62; N, 2.62.

The hydrochloride prepared from the above sample melted at 247–250° dec., after darkening from 235°. Poethke has reported[6b] that protoveratridine hydrochloride melts at 243–245° dec. after some darkening.

Acetylation of Protoveratridine to Triacetylprotoveratridine.—Protoveratridine (120 mg.) was acetylated with acetic anhydride (2 ml.) and pyridine (2 ml.) for 15 hr. at room temperature. The residue obtained by evaporation of the solvents *in vacuo* crystallized from ether (100 mg.). Recrystallization of the product from acetone–petroleum ether afforded plates (45 mg.), m.p. 242–244° dec., $[\alpha]^{27}D$ −89° (*c* 0.94, pyr.).

Anal. Calcd. for $C_{35}H_{57}O_{12}N$: C, 63.41; H, 7.98. Found: C, 63.16; H, 7.90.

In a volatile acid determination 13.78 mg. of triacetylprotoveratridine was equivalent to 7.62 ml. of 0.009757 *N* sodium thiosulfate; calcd. for germine triacetate mono-α-methylbutyrate, 7.85 ml.

A mixture of triacetylprotoveratridine and monoacetylneogermitrine (m.p. 251–253° dec.) was found to melt at 235–238° dec. The infrared spectra in chloroform of the two products were similar, but there were significant differences in the Nujol spectra of the products in the range of 12–15 μ.

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(13) J. B. Niederl and V. Niederl, "Micromethods of Quantitative Organic Analysis," John Wiley and Sons, Inc., New York, N. Y., pp. 257–262. This determination and all other microanalyses reported were carried out by Mr. S. M. Nagy and associates of M.I.T. All samples were dried *in vacuo* at 110°.