ml. portions). The chloroform was washed with water (5 ml.), dried over anhydrous sodium sulfate, and brought to dryness *in vacuo*. The residue was dissolved in ether (20 ml.) and the solution was filtered from a small amount of colored insoluble solid. Concentration of the ether led to crystallization of cevagenine C-orthoacetate diacetate (200 mg.); m.p., rotation and infrared spectrum in chloroform identical with those of an authentic sample.

Alkaline Isomerization of Cevagenine D-Orthoacetate to Cevine Orthoacetate.—Cevagenine D-orthoacetate (300 mg.) was treated with 20% alcoholic potassium hydroxide (4 ml.) and the solution was heated under reflux for 30 minutes. Water (4 ml.) was added, and the alcohol was removed *in vacuo*. The aqueous suspension was extracted with chloroform (eight 5-ml. portions). The chloroform was washed, dried over sodium sulfate and evaporated to dryness *in vacuo*. Crystallization of the residue from methanol gave cevine orthoacetate (80 mg.), m.p. 180–190°. The rotation and infrared spectrum in chloroform were identical with those of an authentic sample.

CAMBRIDGE, MASSACHUSETTS

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, HARVARD UNIVERSITY]

Zygadenus Alkaloids. V. Active Principles of *Zygadenus venenosus*. Zygacine[1]

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A new ester alkaloid, zygacine, has been isolated from *Zygadenus venenosus*. Zygacine, $C_{29}H_{45}O_8N$, is a monoacetate ester of the alkamine zygadenine. Methanolysis of zygacine yields zygadenine and acetic acid. Acetylation of zygacine and of zygadenine with acetic anhydride at steam-bath temperature affords zygadenine triacetate. Treatment of zygacine, zygadenine or zygadenine triacetate with acetic anhydride and pyridine at steam-bath temperature yields zygadenine tetraacetate. Crystalline acetonides of zygacine and zygadenine have been prepared.

Recent investigations of the alkaloidal constituents of *Zygadenus venenosus* have revealed the occurrence of the esters veratroylzygadenine,[3] vanilloylzygadenine,[3] neogermitrine,[4] germidine,[4] neogermidine,[4] and protoveratridine,[4] and the alkamines zygadenine[3] and germine.[3]

As noted earlier,[4b] chromatography of the hydrophilic alkaloids from *Zygadenus venenosus* yielded neogermidine in the fractions eluted from the column with chloroform. However, more than half of the alkaloidal material was not eluted from the column with chloroform alone, and it was necessary to use chloroform–methanol mixtures to elute the remaining material. The infrared spectra of the successive fractions eluted with gradually increasing percentages of methanol indicated that their compositions were quite similar. Furthermore, these infrared spectra more closely resembled the spectra of the zygadenine esters previously encountered than those of the germine esters. Several attempts were made to separate a crystalline product from the amorphous ester fractions by countercurrent distribution and re-chromatography, but these attempts were unsuccessful.

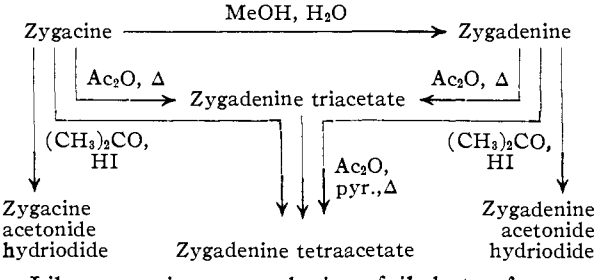
The behavior of the amorphous ester fractions in countercurrent distribution and chromatography suggested substantial homogeneity of the material and this led us to attempt to prepare crystalline salts from the amorphous alkaloid bases. Attempts to prepare a crystalline hydrochloride or hydrobromide were unsuccessful, but upon treatment of the material in acetone with hydriodic acid, a crystalline product (I, $C_{32}H_{49}O_8N \cdot HI$, m.p. 270–271° dec.) was obtained. Further investigation of this product soon revealed that it is an acetonide hydriodide derivative of the ester alkaloid. The free acetonide derivative (II, $C_{32}H_{49}O_8N$, m.p. 253–255° dec., $[\alpha]^{23}D$ +2° (*c* 1.41, chf.)) was liberated by treatment of the salt with dilute ammonium hydroxide. Treatment of the acetonide derivative with dilute hydrochloric acid gave the free ester alkaloid (III, $[\alpha]^{23}D$ −22° (*c* 1.53 chf.).

The new ester alkaloid III afforded analytical values for carbon, hydrogen and nitrogen which agree with the formula $C_{29}H_{45}O_8N$. Acetyl determination revealed the presence of one acetyl group in the molecule. Methanolysis of III yielded zygadenine and acetic acid, which was characterized as its *p*-phenylphenacyl ester. Hence III is a monoacetate ester of zygadenine, for which we propose the name **zygacine.**

Acetylation of zygacine with acetic anhydride at steam-bath temperature gave zygadenine triacetate.[3] The preparation of triacetates from zygadenine and pseudozygadenine also were found to proceed best in the absence of pyridine. Treatment of zygadenine, zygacine or zygadenine triacetate with acetic anhydride and pyridine at steam-bath temperature afforded zygadenine tetraacetate (m.p. 207–209° dec., $[\alpha]^{23}D$ −24° (*c* 1.70 chf.).

CHART I

DERIVATIVES OF ZYGACINE AND ZYGADENINE



Like zygacine, zygadenine failed to form a crystalline derivative when treated in acetone with hydrochloric or hydrobromic acid. Upon treat-

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

(2) On leave of absence from the Weizmann Institute of Science, Rehovot, Israel.

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

(4) S. M. Kupchan and C. V. Deliwala, *ibid.*, (a) **74**, 3202 (1952); (b) Paper IV, **76**, 5545 (1954).

ment with hydroiodic acid in acetone, however, zygadenine acetonide hydriodide (m.p. 292–295° dec.) was obtained. Treatment with dilute ammonium hydroxide liberated zygadenine acetonide (m.p. 220–230° after softening from 210°, $[\alpha]^{23}D$ −17° (*c* 1.29, chf.)).

Zygacine was examined by Prof. O. Krayer and Dr. J. Howard at the Harvard Medical School for its action upon blood pressure, heart rate and respiration of the anesthetized cat and for its effect upon the amphibian skeletal muscle. The pharmacological action of zygacine differs in certain respects from that of veratroylzygadenine, in that it is less potent in causing depression of respiration and decrease in heart rate, and does not appear to decrease the blood pressure. Severing the vagus nerves reduces the respiratory depression and prevents the heart rate decrease. The action upon the isolated amphibian muscle consists in a delayed relaxation after a twitch, similar to the veratrine response. While zygacine is equal to veratroylzygadenine in potency, its veratrinic effect is much shorter lasting.

Experimental

Isolation of Zygacine. A. Separation of a Zygacine-rich Fraction by Chromatography.—A solution of hydrophilic amorphous bases from *Zygadenus venenosus* (6 g.) in chloroform (60 ml.) was chromatographed in Merck sulfuric acid-washed alumina as described earlier for the isolation of neogermidine.[4b] As before, neogermidine was recovered from the chloroform eluate (500 ml.). The alkaloidal fractions eluted with chloroform–2% methanol (400 ml.) chloroform–5% methanol (200 ml.), chloroform–7% methanol (200 ml.) and chloroform–10% methanol (200 ml.) all showed similar absorption in the infrared, and these fractions were combined and evaporated to dryness *in vacuo* (yield 3.1 g.).

B. Zygacine Acetonide Hydriodide (I) and Zygacine Acetonide (II).—The amorphous fraction above (3.1 g.) was dissolved in acetone (20 ml.) and treated with a solution of constant-boiling hydriodic acid (1.0 ml., Merck reagent) in acetone (5 ml.). Upon rubbing, a copious crystalline precipitate separated (1.73 g.). Recrystallization from acetone gave zygacine acetonide hydriodide (I) in the form of colorless plates (1.54 g.), m.p. 270–271° dec.

Anal.[5] Calcd. for $C_{32}H_{49}O_8N\cdot HI$: C, 54.62; H, 7.16. Found: C, 54.44; H, 7.24.

To liberate zygacine acetonide (II), the hydriodide salt (450 mg.) was treated with water (4 ml.) and ammonium hydroxide (2 ml.) and the suspension was extracted with chloroform (five 20-ml. portions). The chloroform extract was washed with water (5 ml.), dried over anhydrous sodium sulfate, and evaporated to dryness *in vacuo* (residue 390 mg.). Crystallization of the residue from ether–petroleum ether gave needles (320 mg.), m.p. 250–253° dec. Recrystallization from acetone–water gave colorless elongated needles (224 mg.), m.p. 253–255° dec., $[\alpha]^{23}D$ +2° (*c* 1.41, chf.).

Anal. Calcd. for $C_{30}H_{45}O_7N(COCH_3)$: C, 66.75; H, 8.58; acetyl, 7.48. Found: C, 66.79; H, 8.61; acetyl, 7.90.

C. Zygacine (III).—Zygacine acetonide hydriodide (927 mg.) was treated with water (8 ml) and ammonium hydroxide (3 ml.) and the suspension was extracted with chloroform (six 25-ml. portions). The residue obtained by evaporation of the chloroform *in vacuo* was treated with dilute hydrochloric acid (15 ml., 1:3) and rubbed into solution in the course of ten minutes. The solution was brought to *p*H 9 with 1:1 ammonium hydroxide and extracted with chloroform (seven 25-ml. portions). The chloroform solution was washed with water (5 ml.), dried over anhydrous sodium sulfate, and brought to dryness *in vacuo*. The residue was dissolved in anhydrous ether and filtered from turbidity, and the filtrate was evaporated to dryness *in vacuo*. The colorless amorphous residue (650 mg.) resisted crystallization, $[\alpha]^{23}D$ −22° (*c* 1.53 chf.).

Anal. Calcd. for $C_{27}H_{42}O_7N(COCH_3)$: C, 65.02; H, 8.47; N, 2.62; acetyl, 8.04. Found: C, 64.85; H, 8.66; N, 2.83; acetyl, 8.31.

Methanolysis of Zygacine.—A solution of zygacine (165 mg.) in methanol (5 ml.) and water (2 ml.) was allowed to stand at room temperature for 15 hours. The solution was then distilled *in vacuo* at 25° into a receiver immersed in a Dry Ice-bath. When all the liquid had distilled over, water (2 ml.) was added to the distillation residue and distilled into the cooled receiver. The dry distillation residue after crystallization from benzene afforded zygadenine (55 mg., m.p. 218–220° dec.). The mixed melting point with an authentic sample of zygadenine was not depressed, and the infrared spectra of the two samples in chloroform were identical.

To the distillate, 0.1 *N* sodium hydroxide solution (5 ml.) was added and the solution was heated under reflux for two hours with the exclusion of carbon dioxide. The solution was lyophilized and the residue was dissolved in water (2 ml.), acidified to *p*H 6 with a few drops of hydrochloric acid and after addition of alcohol (3 ml.) and *p*-phenylphenacyl bromide (65 mg.), the mixture was heated under reflux for two hours. The reaction mixture was worked up and chromatographed on sulfuric acid-washed alumina by the procedure of Fried, White and Wintersteiner.[6] After a forerun containing unchanged reagent, *p*-phenylphenacyl acetate (3.0 mg.), m.p. 108–110°, was obtained. The melting point was unchanged after admixture of an authentic sample of *p*-phenylphenacyl acetate.

Acetylation of Zygacine to Zygadenine Triacetate.—Zygacine (185 mg.) was treated with acetic anhydride (2 ml.) and the solution was heated on the steam-bath for two hours. The solution was evaporated to dryness *in vacuo* and the residual solid was treated with water (3 ml.) and ammonium hydroxide (3 ml.) and extracted with chloroform (four 15-ml. portions). The chloroform extract was washed with water (5 ml.), dried over anhydrous sodium sulfate, and evaporated to dryness *in vacuo*. When the residue was treated with ether (15 ml.), all but a slight amount of red amorphous solid dissolved. Filtration of the suspension and concentration of the ethereal solution (to about 4 ml.) led to the separation of zygadenine triacetate (100 mg., m.p. 265–267° dec.). Recrystallization from acetone–petroleum ether gave colorless needles (55 mg., m.p. 271–273° dec., $[\alpha]^{26}D$ −28° (*c* 1.63, chf.)).[7] The mixed melting point with an authentic sample of zygadenine triacetate was unchanged, and the infrared spectra of the two samples in chloroform were identical. The infrared spectrum of zygadenine triacetate in chloroform shows a band at 9.00 λ which is absent from the spectrum of zygadenine tetraacetate.

Acetylation of Zygacine to Zygadenine Tetraacetate.—Zygacine (300 mg.) was treated with acetic anhydride (6 ml.) and pyridine (4 ml.) and the solution was heated on the steam-bath for two hours. Evaporation of the solution *in vacuo* at room temperature left an amorphous solid. The solid was dissolved in water (5 ml.), made alkaline with ammonium hydroxide (4 ml.) and extracted with chloroform (four 30-ml. portions). The chloroform extract was washed with water (5 ml.), dried over anhydrous sodium sulfate and evaporated *in vacuo*. The residue crystallized from ether (241 mg.). Two recrystallizations from acetone–petroleum ether gave colorless prisms (130 mg.), m.p. 207–209° dec., $[\alpha]^{23}D$ −24° (*c* 1.70 chf.).

Anal. Calcd. for $C_{27}H_{39}O_7N(COCH_3)_4$: C, 63.52; H, 7.77; acetyl, 26.02. Found: C, 63.78; H, 7.82; acetyl, 25.17.

Acetylation of zygadenine and of zygadenine triacetate under the same conditions also afforded zygadenine tetraacetate (mixed m.p., rotation and infrared spectral comparisons). The infrared spectrum of zygadenine tetra-

(5) Microanalyses were done by Dr. S. M. Nagy and associates at the Massachusetts Institute of Technology. All samples were dried *in vacuo* at 110.°

(6) J. Fried, H. L. White and O. Wintersteiner, THIS JOURNAL, **72**, 4621 (1950).

(7) Zygadenine triacetate has been reported previously to show a rotation of −55°.[3] However, a redetermination of the $[\alpha]D$ of a sample of triacetate prepared from zygadenine has shown that the original determination was erroneous, and that the correct rotation is $[\alpha]^{25}D$ −28° (*c* 2.00, chf.).

acetate in chloroform shows a band at 11.15 μ which is absent from the spectrum of zygadenine triacetate.

Zygadenine Acetonide.—Zygadenine (285 mg.) was dissolved in boiling methanol (2 ml.) and constant boiling hydriodic acid (5 drops, Merck reagent) was added to *p*H 4. Acetone (2 ml.) was added and, upon rubbing, zygadenine acetonide hydriodide separated in the form of colorless needles (299 mg., m.p. 292–295° dec.).

Anal. Calcd. for $C_{30}H_{47}O_7N\cdot HI$: C, 54.46; H, 7.31. Found: C, 54.16; H, 7.32.

Zygadenine acetonide was liberated from the salt (250 mg.) by the procedure described above for zygacine acetonide. Crystallization of the crude product from acetone–petroleum ether gave zygadenine acetonide containing one mole of acetone of crystallization (130 mg.), m.p. 220–230° after softening from 210°, $[\alpha]^{23}D$ −17° (*c* 1.29, chf.).

Anal. Calcd. for $C_{30}H_{47}O_7N(CH_3COCH_3)$: C, 66.98; H, 9.03. Found: C, 66.79, 67.02; H, 8.69, 8.83.

CAMBRIDGE, MASSACHUSETTS

[CONTRIBUTION FROM THE DEPARTMENT OF PHARMACOLOGY, THE HEBREW UNIVERSITY, HADASSAH MEDICAL SCHOOL]

The Relationship between Spectral Shifts and Structural Changes in Uric Acids and Related Compounds

BY FELIX BERGMANN AND SHABTAY DIKSTEIN[1]

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The absorption maxima of various uric acids have been determined in the non-ionized state and as function of *p*H, and the apparent dissociation constants have been derived. The sequence of ionization is established as 9,3,1. Both alkylation and ionization produce corresponding spectral shifts, but of different magnitude. Alkylation or dissociation at N^9 or N^3 are accompanied by a bathochromic shift, and at N^1 by a hypsochromic effect. An analogous behavior is observed with uracils, but is missing in the xanthine series and in hypoxanthine. The relationship between these spectral changes and the possible resonance forms at various stages of ionization is discussed.

In connection with a study on the metabolism of substituted uric acids a knowledge of the dissociation constants of their ionizable groups was needed. A search in the literature revealed that the required *pK* values are partly unknown and partly still controversial due to the very limited solubility of these acids.[2-4] Since the spectrophotometric determination of *pK* has proved its applicability to related compounds, such as uracils[5] and xanthines,[6] a study of the *p*H dependence of the absorption bands of uric acids was undertaken.

Experimental[7]

Materials.—Commercial uric acid was purified by recrystallization from hot water. 1-Methyluric acid was synthesized according to Fischer.[8] The preparation of 3-methyl-, 1,3-dimethyl- and 1,3-diethyluric acids according to Traube[9] was modified in the following way. Reduction of the 5-nitroso-6-aminouracil was carried out with hot concentrated ammonium sulfide and the yield of diaminouracils was thus considerably improved.[10] 3,7-Diethyl-,[11] 3,7,9-trimethyl-[12] and 1,3,7-trimethyluric[13] acids were synthesized in accordance with the data in the literature.

Method.—Measurements of absorption spectra were carried out with a Beckman ultraviolet spectrophotometer, Model DU, on aqueous solutions, containing 10 μg./ml. A range of *p*H 1–3 was achieved by addition of perchloric acid, *p*H 3–7 by 0.15 *M* acetate buffer, *p*H 7–9 by 0.05 *M* borax, adjustment being made with perchloric acid. *p*H 9–12 by 0.15 *M* borate buffer; *p*H 13 by 0.1 *N* NaOH; *p*H 14 by 1 *N* NaOH. Beyond *p*H 12 the solutions are unstable and have to be prepared immediately before use. Instability was especially noted with caffeine, 3-methyl- and 3,7-dimethyluric acid. The absorption maxima at the higher *p*H values were therefore checked three times with freshly prepared solutions.

The long wave length maximum was first determined approximately by reading at intervals of 50 Å. Then the optical density of a wave length near the expected position of λ_{max} was measured. Going up 5–10 Å. on the wave length scale, the galvanometer was adjusted to zero for the solvent, without changing the transmission, using only sensitivity control and slit width, while the selector switch was on "check." Now the solution was measured with the selector switch on "1." The direction of deviation of the galvanometer needle then determines the change of extinction (movement to the left denoting a decrease.). If extinction is increasing, the new wave length is used to adjust the galvanometer again to zero by turning the transmission knob. The next measurement at a 10 Å. longer wave length is carried out as before. Since the maximum can be approached from both sides, this very sensitive procedure permits localization of the maximum within a short time with an accuracy of ±5 Å.

λ_{max} was finally plotted as function of *p*H and the *pK* values were obtained according to Robinson and Pekrul.[14]

Results

The curves in Fig. 1 represent λ_{max} as function of *p*H. In all derivatives of uric acid with a free NH-group at position 9, the main bathochromic shift occurs in the *p*H range 4–8 and thus permits the evaluation of pK_9 (see Table Ib).[15] When position 3 is unsubstituted, a further bathochromic shift appears at *p*H 9–12 and is thus related to pK_3. On the other hand, a hypsochromic shift is observed above *p*H 12 for 3-methyl- and 3,7-dimethyluric acid and

(1) Part of a Ph.D. Thesis submitted to the Faculty of Science of the Hebrew University, Jerusalem.

(2) F. Gudzent, *Z. physiol. Chem.*, **56**, 150 (1908).

(3) (a) H. Biltz and L. Herrmann, *Ber.*, **54**, 1676 (1921); (b) A. Fromherz and A. Hartmann, *ibid.*, **69**, 2420 (1936).

(4) Sakuji Takagi, *J. Physiol. Soc. (Japan)*, **13**, 129 (1951); *C. A.*, **45**, 8857 (1951).

(5) D. Shugar and J. J. Fox, *Biochim., Biophys. Acta*, **9**, 199 (1952).

(6) L. F. Cavalieri, J. J. Fox, A. Stone and N. Chang, THIS JOURNAL, **76**, 1119 (1954).

(7) Since m.p.'s are not useful for checking the purity of these compounds, recrystallization was repeated until the absorption spectrum remained constant.

(8) E. Fischer, *Ann.*, **215**, 304 (1882); E. Fischer and H. Clemm, *Ber.*, **30**, 3091 (1897).

(9) W. Traube, *Ber.*, **33**, 3035 (1900); J. H. Speer and A. L. Raymond, THIS JOURNAL, **75**, 114 (1953).

(10) The authors wish to thank Prof. B. B. Brodie, National Heart Institute, National Institute of Health, Bethesda, Md., for a sample of 1,3-dimethyluric acid. They are also obliged to Dr. V. Papesch of G. D. Searle and Co., Chicago, Illinois, who generously supplied 3-methylxanthine and several intermediates for the synthesis of substituted uric acids.

(11) H. Biltz and P. Damm, *Ann.* **406**, 35 (1914).

(12) E. Fischer and L. Ach, *Ber.*, **28**, 2484 (1895).

(13) H. Biltz and P. Damm, *Ann.*, **413**, 189 (1917).

(14) E. J. Robinson and L. F. Pekrul, THIS JOURNAL, **67**, 1186 (1945).

(15) Unfortunately, since no 9-substituted uric acid with a free 7-position was available, our experimental data do not yield information as to the magnitude of pK_7.