[CONTRIBUTION FROM THE LABORATORY FOR ENDOCRINE RESEARCH, THE JOHNS HOPKINS UNIVERSITY, SCHOOL OF MEDICINE]

Chemical Studies on Toad Poisons. VII. Bufo arenarum, Bufo regularis and Xenopus laevis[1]

BY H. JENSEN

As further chemical evidence for the correctness of the formulas assigned to arenobufagin ($C_{25}H_{34}O_6$) and arenobufotoxin ($C_{39}H_{60}O_{11}N_4$), which were isolated by Chen, Jensen and Chen from the secretion of Bufo arenarum,[2] several derivatives of arenobufagin have been prepared and analyzed. From the secretion of the South African toad, *Bufo regularis*, which has been studied pharmacologically by Epstein and Gunn,[3] two analogous physiologically active compounds, regularobufagin and regularobufotoxin were isolated by Chen and Chen, in amounts sufficient only for pharmacological study.[4] It seemed desirable therefore to prepare these principles in somewhat larger amounts for chemical identification. On the basis of several analyses of these principles as well as certain of their derivatives, the following empirical formulas have been assigned to regularobufagin, $C_{25}H_{34}O_6$, and to regularobufotoxin, $C_{39}H_{60}O_{11}N_4$.

The isomeric arenobufagin and regularobufagin are both lactones, forming hydroxy acids by the action of alcoholic sodium hydroxide. Simultaneously with the hydrolysis of the lactone ring, one molecule of acetic acid (identified as the silver salt) is liberated, presumably from an acetoxy group. Oxidation with chromic acid gives the corresponding monoketones, indicating the presence of a secondary hydroxy group. On treatment of arenobufagin with acetic anhydride, acetylarenobufagin, $C_{27}H_{36}O_7$, is formed. On treatment of regularobufagin under the same conditions, it was found that one molecule of acetic acid is split off (probably from a tertiary acetoxy group) giving a product $C_{25}H_{32}O_5$. There are certain known examples of an intramolecular loss of water by similar treatment, notably the dehydration of ouabain to anhydroouabainheptaacetate,[5] and of ergostadienetriol to dehydroergosteryl acetate[6] by acetic anhydride. Neither the acetyl derivative of arenobufagin nor the compound $C_{25}H_{32}O_5$ obtained from regularobufagin gives a ketone on oxidation with chromic acid, which indicates that acetylation of the secondary hydroxy groups has taken place. Arenobufagin and regularobufagin are C_{23}-derivatives (after splitting off the acetyl radical which is attached to a tertiary hydroxy group), and can be considered as acetyl derivatives of a doubly unsaturated trihydroxy lactone $C_{23}H_{32}O_5$. It is of interest to note that the analytical data for arenobufagin and regularobufagin closely agree with those reported for bufotalin (the bufagin of *Bufo vulgaris*) by Wieland and co-workers,[7] and to which they assigned the empirical formula $C_{26}H_{36}O_6$. The bufagins probably possess a ring structure identical with that of the aglucones of certain plant glucosides. They likewise are C_{23}-derivatives and recently have been shown to contain a ring structure identical with that of the sterols.[8] Further investigation is required to confirm these possibilities.

Pharmacological investigation has indicated the presence of epinephrine in the secretion of *Bufo arenarum* and *Bufo regularis*[2,3,4,9] and this has been substantiated by the isolation and identification of the base from both secretions. Bufothionine, a sulfur-containing compound which has been obtained by Wieland and co-workers from the secretion of the Japanese toad,[10] and from an alcoholic extract of the dried skin secretion of *Bufo arenarum*,[11] was found to be present in small quantities in the secretion of *Bufo arenarum*. From the secretion of *Bufo regularis*, there has been isolated as a flavianate, a basic constituent which is identical with that obtained by Jensen and Chen from the secretion of *Bufo valliceps*.[12]

From the skin secretion of the South African clawed toad, *Xenopus laevis*, which is biologically more closely related to *Rana* than to *Bufo*,

(1) Presented before the Division of Biological Chemistry at the 88th Meeting of the American Chemical Society, New York City, April 22–26, 1935.

(2) Chen, Jensen and Chen, *J. Pharmacol.*, **49**, 1 (1933).

(3) Epstein and Gunn, *ibid.*, **39**, 1 (1930).

(4) Chen and Chen, *ibid.*, **49**, 503 (1933).

(5) Jacobs and Bigelow, *J. Biol. Chem.*, **96**, 647 (1932).

(6) Dunn, Heilbron, Phipers, Samant and Spring, *J. Chem. Soc.*, 1580 (1934).

(7) Wieland and Hesse, *Ann.*, **517**, 22 (1935).

(8) Jacobs and Elderfield, *J. Biol. Chem.*, **108**, 497 (1935); Tschesche, *Ber.*, **68**, 7 (1935).

(9) Novaro, *Compt. rend. soc. biol.*, **87**, 824 (1922); **88**, 371 (1923).

(10) Wieland and Vocke, *Ann.*, **481**, 215 (1930).

(11) Wieland, Konz and Mittasch, *ibid.*, **513**, 1 (1934).

(12) Jensen and Chen, *Ber.*, **65**, 1310 (1932).

the following constituents have been isolated: cholesterol, fatty acids, and a basic compound as the flavianate. The base was found to be identical with bufotenidine, which is present in Ch'an Su and in the secretion of certain other toads, and the constitution of which has recently been determined.[11,12] No evidence of the presence of any bufagin-like substance in this secretion has been thus far obtained. The chemical findings are in agreement with the results of the pharmacological study of this secretion by Gunn.[13]

Most of the analyses reported in this paper were carried out by Dr. Ing. A. Schoeller, Berlin-Schmargendorf, Germany.

Experimental

Secretion of Bufo arenarum

The usual methods were employed for the isolation of arenobufagin.[2,14]

Acetylarenobufagin, $C_{27}H_{36}O_7$.—Fifty milligrams of arenobufagin was heated with 1 cc. of acetic anhydride for two hours in a boiling water-bath. The acetyl compound was isolated in the usual manner, and crystallized from alcohol. The fine white needles melted at 162–163°.

Anal. Calcd. for $C_{27}H_{36}O_7$: C, 68.64; H, 7.63. Found: C, 68.51, 68.46; H, 7.85, 7.77.

Arenobufagone, $C_{25}H_{32}O_6$.—Eighty milligrams of arenobufagin was dissolved in 2 cc. of glacial acetic acid, and 0.15 cc. of chromic acid solution (40 g. of water, 8 g. of sulfuric acid, and 5.3 g. of chromium trioxide) was added with thorough stirring. The solution was allowed to stand for fifteen minutes and then diluted with water. After standing overnight the precipitate was filtered off and recrystallized from dilute ethyl alcohol. The ketone consisted of faintly yellow rhombohedric leaflets, melting at 219–220°.

Anal. Calcd. for $C_{25}H_{32}O_6$: C, 70.09; H, 7.41. Found: C, 69.82, 69.84; H, 7.25, 7.22.

Arenobufaginic Acid, $C_{23}H_{34}O_6$.—Three-tenths gram of arenobufagin was refluxed with 25 cc. of 1 *N* alcoholic sodium hydroxide for three hours. After cooling, the solution was diluted with water and allowed to stand overnight. The small amount of flocculent precipitate which might have formed was filtered off, and the filtrate made slightly acid with hydrochloric acid. After standing for several days, the precipitate was filtered off and dried at room temperature. The acid, however, could not be obtained in crystalline form. For further purification it was dissolved in ethyl alcohol and water added until a permanent turbidity resulted. The next day a pale yellow amorphous precipitate had settled out. This was filtered off and the filtrate further diluted with water. The precipitate thus obtained was filtered off, washed with water, and dried at room temperature. The product was an amorphous white powder; the analytical data indicated a considerable degree of purity. The acid began to decompose at 220°, and became progressively darker until it was completely melted at 235°. Water is probably eliminated at the higher temperature.

Anal. Calcd. for $C_{23}H_{34}O_6$: C, 67.98; H, 8.37. Found: C, 67.70, 67.68; H, 7.92, 7.98.

Detection of an Acetyl Group in Arenobufagin.—The filtrate from the bufaginic acid was distilled from an oil-bath. The distillate (acid toward litmus) was shaken with silver oxide and allowed to stand for one day. The silver oxide was then filtered off and the filtrate evaporated *in vacuo* over sulfuric acid. The silver acetate was recrystallized from dilute alcohol.

Anal. Calcd. for CH_3COOAg: Ag, 64.61. Found: Ag, 64.31, 64.29.

Bufothionine, $C_{12}H_{14}O_4N_2S$.—In working up the alcoholic extract of the secretion of *Bufo arenarum*, the cholesterol fraction, which crystallizes out on evaporation, was found to be accompanied by a foreign crystalline substance. After filtration, the cholesterol was removed by extraction with petroleum ether, and the insoluble residue purified from 96% ethyl alcohol. The compound crystallized as prisms, darkening at 240°, and melting at 250° with decomposition. The properties of this substance were identical with those described for bufothionine by Wieland and his co-workers.[10,11]

Anal. Calcd. for $C_{12}H_{14}O_4N_2S$: C, 51.06; H, 5.00; N, 9.93; S, 11.34. Found (average of several analyses): C, 50.93; H, 4.97; N, 8.80; S, 11.10.

Epinephrine, $C_9H_{13}O_3N$.—Epinephrine was isolated from about 10 g. of the dried secretion, which had been extracted twice with alcohol in accordance with the procedure of Jensen and Chen.[15] Approximately 40 mg. of the substance was obtained. It melted at 211° with decomposition and showed no depression of the melting point when mixed with epinephrine.

Anal. Calcd. for $C_9H_{13}O_3N$: C, 59.01; H, 7.18; N, 7.65. Found: C, 58.73; H, 7.42; N, 7.97.

Secretion of Bufo Regularis.—Regularobufagin and regularobufotoxin were obtained from the secretion according to methods previously described.[4,14] The derivatives of regularobufagin were prepared similarly to those of arenobufagin.

Regularobufotoxin, $C_{39}H_{60}O_{11}N_4$.—The principle was repeatedly crystallized from 96% ethyl alcohol and finally from acetone. It crystallized in colorless spheroids, which soften at 190° and melt at 205°. Like the bufotoxins of other species of toads, it gives a positive Sakaguchi reaction indicating the presence of arginine.

Anal. Calcd. for $C_{39}H_{60}O_{11}N_4$: C, 61.69; H, 7.97; N, 7.39. Found: C, 61.82, 61.88; H, 8.28, 8.37; N, 7.58, 7.50.

Regularobufagin, $C_{25}H_{34}O_6$.—The compound was purified from either dilute alcohol, or from a chloroform–ether–petroleum ether solution. It crystallizes in prisms melting at 235–236°. Its color reactions are similar to those of the other bufagins.[16]

(13) Gunn, *Quart. J. Expt. Physiol.*, **20**, 1 (1930).

(14) Jensen and Chen, *J. Biol. Chem.*, **87**, 741 and 755 (1930).

(15) Jensen and Chen, *ibid.*, **82**, 397 (1929).

(16) The melting point and the analytical data for regularobufagin agree closely with the results given by Chen and Chen for this principle. However, they assigned a different empirical formula to it.[4]

Anal. Calcd. for $C_{25}H_{34}O_6$: C, 69.71; H, 7.94. Found: C, 69.88, 69.95; H, 8.18, 8.20.

Acetylation of Regularobufagin, $C_{25}H_{32}O_5$.—White needles crystallized from dilute methyl alcohol, melting at 224–225°.

Anal. Calcd. for $C_{25}H_{32}O_5$: C, 72.81; H, 7.77. Found: C, 72.95, 73.00; H, 8.02, 7.92.

Regularobufagone, $C_{25}H_{32}O_6$.—Recrystallizes from dilute methyl alcohol in rhombohedric leaflets, faintly yellow, melting at 210–211°.

Anal. Calcd. for $C_{25}H_{32}O_6$: C, 70.09; H, 7.41. Found: C, 69.72, 69.80; H, 7.21, 7.51.

Regularobufaginic Acid, $C_{23}H_{34}O_6$.—The acid begins to decompose at 125°, and becomes more and more colored until it is completely melted. Water is probably split off at the higher temperatures. For analysis, the substance was dried *in vacuo* over calcium chloride.

Anal. Calcd. for $C_{23}H_{34}O_6$: C, 67.98; H, 8.37. Found: C, 67.62, 67.58; H, 7.91, 7.90.

Detection of the Acetyl Group in Regularobufagin.—*Anal.* Calcd. for CH_3COOAg: Ag, 64.61. Found: Ag, 64.28, 64.25.

Epinephrine, $C_9H_{13}O_3N$.—The base was isolated from about 10 g. of the dried secretion, after it had been extracted twice with alcohol. Approximately 30 mg. of the compound was obtained, which melted at 210° with decomposition and showed no depression of the melting point when mixed with epinephrine.

Anal. Calcd. for $C_9H_{13}O_3N$: C, 58.01; H, 7.18; N, 7.65. Found: C, 58.71; H, 7.46; N, 7.90.

Regularobufotenine.—This compound was isolated as the flavianate from an aqueous solution of the material, obtained by evaporation of the alcoholic extract of the secretion. The procedure was similar to that employed by Jensen and Chen in the preparation of the flavianates of the different basic constituents present in the secretions of the various species of toads.[12] The flavianate was recrystallized several times from water, and it then melted at 264° with decomposition. It showed no depression of the melting point when mixed with the flavianate of *Bufo valliceps*.

Anal. Calcd. for $C_{11}H_{12}O_2N_2 \cdot C_{10}H_6O_8N_2S$: C, 48.65; H, 3.48; N, 10.81; S, 6.18. Found: C, 48.82, 48.91; H, 3.82, 3.77; N, 11.11, 11.15; S, 6.48.

The flavianate was converted into the picrate by decomposing the salt with barium acetate according to the procedure of Wieland and co-workers.[11] The picrate crystallized from water as red clusters of needles melting at 203–204°. The picrate contains one molecule of water of crystallization, and showed no depression of the melting point when mixed with the picrate of *Bufo valliceps*.

Anal. Calcd. for $C_{11}H_{12}O_2N_2 \cdot C_6H_3O_7N_3 \cdot H_2O$: C, 45.23; H, 3.77; N, 15.52. Found: C, 45.20, 45.32; H, 4.11, 3.92; N, 16.2, 15.92.

Secretion of Xenopus laevis

The procedure was similar to that for the secretions of other species of toads. Fifty grams of the dried skin secretion was extracted twice with 300 cc. of 96% ethyl alcohol. The crystalline matter which separated out on concentration of the alcoholic solution was identified as cholesterol. The filtrate from the cholesterol fraction was evaporated to dryness *in vacuo*, and extracted twice with 100 cc. of water. Most of the material went into solution, the insoluble part consisting mostly of protein material and higher fatty acids. The aqueous solution was shaken out twice with chloroform, flavianic acid was added and the precipitate recrystallized several times from water. The flavianate crystallized in long needles melting at 200°, and showed no depression in melting point when mixed with the flavianate of bufotenidine obtained from Ch'an Su and certain other species of toads.[11,12]

Anal. Calcd. for $C_{13}H_{19}ON_2 \cdot C_{10}H_6O_8N_2S \cdot H_2O$: C, 50.18; H, 4.73; S, 5.82. Found: C, 49.88, 49.94; H, 5.12, 4.99; S, 5.74.

The flavianate was converted into the picrate and also into the hydriodide salt following the method of Wieland and his co-workers.[12] The derivatives showed the same melting points as the corresponding salts of bufotenidine, and mixed melting points showed no depression. The chemical analysis of these salts also supports the view that the base is identical with bufotenidine. The picrate crystallized from ethyl alcohol in red needles melting at 197°.

Anal. Calcd. for $C_{13}H_{19}ON_2 \cdot C_6H_3O_7N_3$: C, 51.01; H, 4.70; N, 15.67. Found: C, 51.21, 51.11; H, 5.10, 4.94; N, 16.01, 15.92.

The hydroiodide salt crystallized from methyl alcohol in prisms, melting at 208–209°.

Anal. Calcd. for $C_{13}H_{19}ON_2 \cdot HI$: C, 45.09; H, 5.50; N, 8.08. Found: C, 45.21, 45.06; H, 5.82, 5.71; N, 7.8.

The author is greatly indebted to Professor J. W. C. Gunn of the University of Cape Town, South Africa, for the supply of the secretions of *Bufo regularis* and *Xenopus laevis*, and to Professor B. A. Houssay, University of Buenos Aires, Argentina, for the supply of the secretion of *Bufo arenarum*.

Summary

Various derivatives of arenobufagin and regularobufagin have been prepared. The analytical data obtained for these compounds substantiate the empirical formula $C_{25}H_{36}O_6$ assigned to these principles. The two compounds are isomers and are C_{23}-derivatives after removing the acetyl radical which is attached to a hydroxy group. Regularobufotoxin has been isolated in pure form from the secretion of *Bufo regularis*. Epinephrine has been isolated and identified as such from the secretions of both *Bufo arenarum* and *Bufo regularis*.

Cholesterol, fatty acids and a basic principle have been obtained from the secretion of the South African clawed toad. The base was obtained as the flavianate, which was found to be

identical with that of bufotenidine. No evidence for the presence of any bufagin-like substance in this secretion has been obtained.

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The Action of Perthiocyanic Acid on Amines

BY H. G. UNDERWOOD AND F. B. DAINS

In preparing dithiobiurets for another investigation, the action of perthiocyanic acid on various substances containing the amino group has been investigated. Glutz[1] has shown that perthiocyanic acid unites with aniline to form phenyldithiobiuret and Fromm[2] has pointed out that in the formation of phenyldithiobiuret an excess of aniline tends to increase the amount of thiocarbanilide formed along with the dithiobiuret. The reactions for the formation of dithiobiurets and dithioureas occur as follows

$$RNH_2 + C_2H_2N_2S_3 \longrightarrow RNHCSNHCSNH_2 + S \quad (I)$$
$$4RNH_2 + C_2H_2N_2S_3 \longrightarrow 2(RNH)_2CS + 2NH_3 + S \quad (II)$$

In this work fusions were carried out first at water-bath temperature and if no reaction was evident higher temperatures were employed. The ratio of the amine to perthiocyanic acid was usually 2:1. The results indicate that the nature of the reaction product depends upon the amine employed; thus there were formed (a) normal dithiobiurets, (b) thioureas, (c) fused side rings with certain substituted amines or (d) no reaction occurred.

Experimental

Synthesis of Dithiobiurets (I–VIII).—The new dithiobiurets listed in Table I (I–VIII) were prepared by fusing the amine (2 moles) with perthiocyanic acid (1 mole) on the water-bath. Di-*p*-bromophenylthiourea (IV) was obtained as a by-product in the preparation of *p*-bromophenyldithiobiuret. It is probable that dithioureas are also formed along with the other dithiobiurets but the small amounts formed did not warrant their separation.

Synthesis of Thioureas

α,β-Di-*p*-xenylthiourea (IX).—*p*-Xenylamine (0.1 mole) and perthiocyanic acid (0.05 mole) were heated for one hour at 150°. Only a small quantity of the reaction product was soluble in base. The main product insoluble in alkali was the thiourea which on recrystallization from hot water formed plate-like crystals melting at 228°,[3,4] yield 13 g.

α,β-Dibenzylthiourea (X).—The reaction was spontaneous on mixing benzylamine (0.05 mole) and perthiocyanic acid (0.05 mole), ammonia being evolved. The reaction mixture almost completely liquefied and then resolidified. To ensure complete reaction the product was warmed on the water-bath for one hour. Large plate-like crystals of dibenzylthiourea melting at 147–148°[5,6] were obtained from alcohol.

Reaction of Secondary Butylamine with Perthiocyanic Acid.—Secondary butylamine at room temperature gave an oily product which on distillation yielded secondary butyl thiourea and secondary butyl isothiocyanate.

Fused Ring Compounds

$C_6H_4\overline{NHCSNHCO}$, **4-Keto-2-thiotetrahydroquinazoline (XI).**—This product resulted when anthranilic acid or methyl anthranilate was heated at 180° for two hours. The reaction product was extracted with sodium hy-

TABLE I

Text no.	Compound	Formula	M. p., °C.	Nitrogen, % Calcd.	Found
I	*m*-Tolyldithiobiuret	$C_9H_{11}N_3S_2$	159	18.66	18.63
II	*m*-Chlorophenyldithiobiuret	$C_8H_8ClN_3S_2$	164	17.11	16.96
III	*p*-Bromophenyldithiobiuret	$C_8H_8BrN_3S_2$	169	14.48	14.30
IV	α,β-Di-*p*-bromophenylthiourea	$C_{13}H_{10}BrN_2S$	184		
V	*p*-Iodophenyldithiobiuret	$C_8H_8IN_3S_2$	240	12.49	12.22
VI	α-Naphthyldithiobiuret	$C_{12}H_{11}N_3S_2$	235–236	16.09	15.93
VII	*m*-Aminophenyldithiobiuret[a]	$C_8H_{10}N_4S_2$	226	24.77	24.40
VIII	1-Methyl-1-*p*-tolyldithiobiuret	$C_{10}H_{13}N_3S_2$	236	17.55	17.51
IX	α,β-Di-*p*-xenylthiourea	$C_{25}H_{20}N_2S$	228	7.37	7.45
X	α,β-Dibenzylthiourea	$C_{15}H_{16}N_2S$	147–148	10.93	10.73
XI	4-Keto-2-thiotetrahydroquinazoline	$C_8H_6N_2OS$	285	15.72	15.61
XII	*o*-Phenylenethiourea	$C_7H_6N_2S$	298–299	18.66	18.84
XIII	Benzoxazole thiourea	$C_8H_7N_3OS$	205	21.74	21.57

[a] Only one amino group of *m*-diaminobenzene reacted.

(1) Glutz, *Ann.*, **154**, 44 (1870).
(2) Fromm, *ibid.*, **275**, 20 (1893).
(3) Friebel and Rassow, *J. prakt. Chem.*, [2] **63**, 457 (1901).
(4) Zimmermann, *Ber.*, **13**, 1963 (1880).
(5) Werner, *J. Chem. Soc.*, **59**, 406 (1891).
(6) Salkowski, *Ber.*, **24**, 2724 (1891).