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45. THE VENOMS OF AMPHIBIANS

JOHN W. DALY and BERNARD WITKOP

National Institute of Arthritis and Metabolic Diseases
National Institute of Health, Bethesda, Maryland, U.S.A.

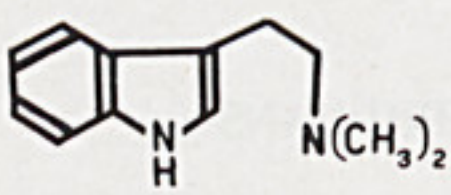
The cutaneous secretions of amphibians contain an amazing variety of compounds of diverse pharmacological activities (Table I and Fig. 1). These include simple biogenic amines, peptides, steroids and steroidal alkaloids. Their pharmacological activity encompasses cardio-, myo-, and neurotoxins, cholinomimetic and sympathomimetic agents, local vasoconstrictors and hypotensive agents and even one of the most potent hallucinogens known, 0-methylbufotenine. Among these compounds are some of the most powerful venoms known.

TABLE I — TOXIC SUBSTANCES ISOLATED FROM AMPHIBIANS *

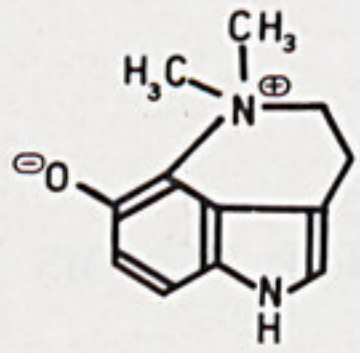
Substances	MLD (μg/kg)	Source	Class of Compound	Pharmacological activity
Batrachotoxin	2	Frog: *Phyllobates bicolor*	Steroidal Alkaloid	Cardio- and neurotoxin
Tarichatoxin (Tetrodotoxin)	8	Newt: *Taricha torosa*	Guanidine Derivative	Neurotoxin
Samandarine	300	Salamander: *Salamandra maculosa*	Steroidal Alkaloid	Central Convulsant
Bufotoxin	400	Toad: *Bufo vulgaris*	Steroid	Cardiotoxin
Dehydrobufotenine	6000	Toad: *Bufo marinus*	Indole	Convulsant
Leptodactyline	7500	Frog: *Leptodactylus pentodactylus*	Phenolic amine	Cholinomimetic Agent
0-Methylbufotenine	75000	Toad: *Bufo alvarius*	Indole	Hallucinogen

* For comparison the MLD of strychnine is 500, of d-Tubocurarine is 500 and of muscarin is 750.

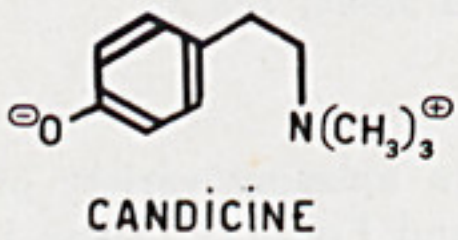
The function of these compounds in amphibians may involve active defense or passive protection. In certain cases, as in toads of the genus *Bufo*, the secretions are actively ejected as a defense against certain enemies, such as dogs and other carnivores. Other amphibians, for example, the European *Salamandra maculosa*, make passive use of skin secretions as a defense against predators. The high toxicity of these secretions need not be viewed in terms of defense only. Some of these compounds may have physiological functions in the skin of the amphibian.



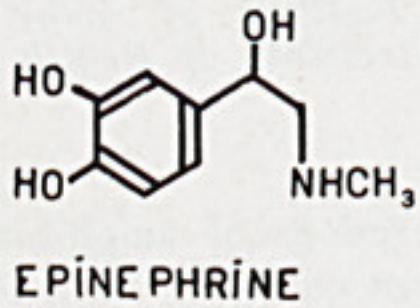
BUFOTENİNE



DEHYDROBUFOTENİNE



CANDİCİNE



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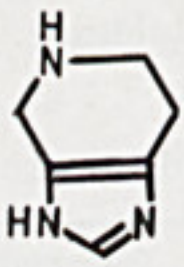

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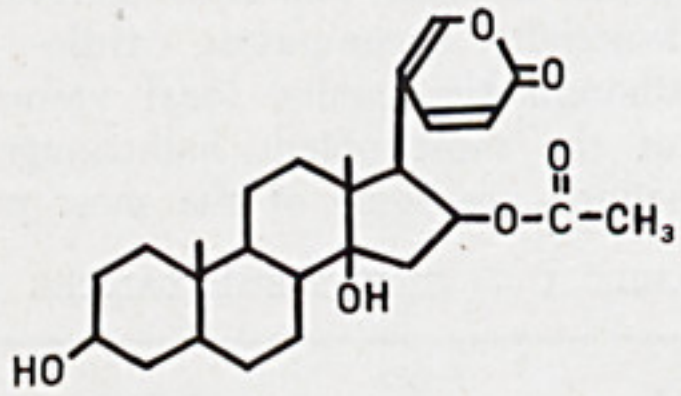
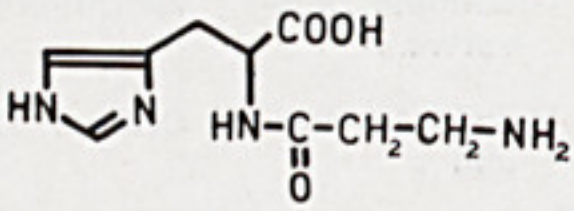
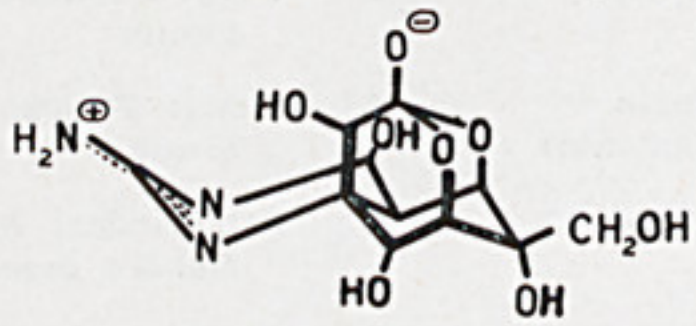


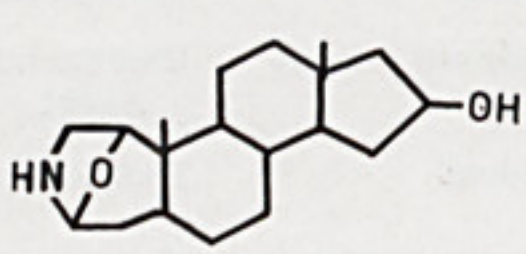
BUFOTALİN



CARNOSİNE



TARİCHATOXİN (TETRODOTOXİN)



SAMANDARİNE

Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg

BRADYKİNİN

Fig. 1 — Representative Pharmacologically Active Compounds Isolated from Amphibian Skins.

Both *simple biogenic amines* and *complex bufodienolides* occur together in the parotid gland secretions of toads of the genus, *B u f o* and have been the subject of intensive investigations. Indolealkylamines such as *serotonin, N-methyl-serotonine, bufotenine, bufotenidine* and *5-methoxy-N,N-dimethyltryptamine* (1) have been isolated from toads of this genus. They possess a variety of activities and in-

clude vasoconstrictors, cholinomimetics and hallucinogens. The novel biosynthetic conversion of bufotenine to the *tricyclic dehydrobufotenine* in *Bufo marinus* is currently under study in our laboratories (S. Senoh, J. Daly and B. Witkop). Also present in the glands are various sympathomimetic amines such as *dopamine*, *norepinephrine*, and *epinephrine* (2).

Frogs of the tropical American genus, *Leptodactylus* contain three types of biogenic amines (3): the *indolealkylamines*, such as *serotonin* and *bufotenidine*, the phenolic amines, such as *tyramine*, *candicine* and *leptodactyline*, and the *imidazolealkylamines*, such as *histamine* and *spinceamine*. Candicine and leptodactyline are nicotine-like in action, while histamine is a local irritant. Spinceamine has no pronounced pharmacological activity.

Examination of 7 species of *Puerto Rican frogs* of the related genus *Eleutherodactylus* did not reveal significant quantities of any of these biogenic amines. A large amount (0.2-1.2 mg/g skin) of an amino acid which gave a Pauli positive color reaction typical of an imidazole was found in all these frogs (J. W. Daly and H. Heathwole, in press). This amino acid was not histidine and was at first thought to be spinacine, the amino acid precursor of the amine, spinceamine.

Chemical studies proved, however, that the material was the *dipeptide*, *carnosine* (*β-alanylhistidine*) previously known only from the muscle of certain vertebrates. Carnosine has no known physiological or pharmacological activity and certainly is not present as a protective poison in these frogs.

Other *peptides of higher molecular weight* have been found in a variety of amphibians (4, 5): *Bradykinin* and *physalaemin*, are *potent hypotensive agents*, while the *tryptokinins* have no known activity.

Completely different in structure is *tetrodotoxin* (*tarichatoxin*), the *extremely potent neurotoxin* from newts of the genus *Taricha* (6). This substance is reported only in newts and in puffer fish of the genera *Sphoeroides* and *Arothron*, an unusual case of biochemical disjunction.

Amphibians also contain lipid soluble poisons in their skin. These include the great variety of *bufogenins* which occur only in toads of the genus *Bufo* and which are *extremely active cardiotoxins* and *local anesthetics*. Various *steroidal alkaloids* are also known from amphibians. *Samandarine*, *samandarone* and related compounds occur in the skin of salamanders of the genus *Salamandra* (7) along with *hemolytic proteins*. Samandarine has also been reported recently from an Australian anuran of the genus *Pseudophryne* (8). Samandarine is a *centrally active convulsant* of high toxicity.

Steroidal alkaloids have now been found to be present in the skins of tropical American dendrobatid frogs of the genera, *Phyllobates* and *Dendrobates*. In particular the skin of the *poison arrow frog of Colombia*, known as Kokoi' to the natives of the Choco' of that country, contains an extremely poisonous alkaloid. It has been used for centuries to poison blow gun darts for hunting small game by Indians of this region.

The frog, which has been provisionally identified as *Phyllobates bicolor*, is tiny and contains only minute amounts of the venom which was named *batrachotoxin* (9, 10). A single frog contains only 40-80 micrograms of batrachotoxin which is, however, sufficient to kill 2-4,000 mice on intravenous injection. On oral administration, batrachotoxin is 60 to 100 times less toxic.

Three expeditions to the Choco' Jungle of western Colombia in 1961, 1964 and 1966, under the able leadership of explorer-zoologist Mrs. Martè Latham, have netted over 7,000 frogs.

Two color varieties of the Kokoi' are present in this region of Colombia. The main difference is in the width and color of the dorsolateral stripes. These are narrow and bright yellow in the frog from lower elevations above *Playa de Oro* on the *Rio San Juan*, while at higher elevations in the same watershed, a slightly larger frog with yellow-orange to red-orange stripes is found. Often the dorsolateral stripes merge and cover the entire back of this mountain variety. Both varieties contain batrachotoxin in comparable quantities. The *frogs appear to be quite resistant to large doses of their own venom.*

The method of purification of batrachotoxin has now been simplified as follows: To a methanol extract of the skins is added 1 volume of water; the alkaloids are then extracted into chloroform. The basic alkaloids are removed from the chloroform by extraction into 0.1 N hydrochloric acid.

The aqueous acid is adjusted to pH 8.5 and reextracted with chloroform. The chloroform extract is concentrated *in vacuo* and the final purification makes use of thin-layer chromatography as described previously (9). All steps must be carried out at 5°C to prevent large losses of activity. Studies on the structure of batrachotoxin have been hindered by this instability and by the paucity of material available for study, even from 7,000 frogs.

Batrachotoxin is a weak base of pH 7.5 as measured by partition coefficients. Its ultraviolet spectrum shows only end absorption indicating a lack of conjugated double bonds. The infrared spectrum indicates hydroxyl groups. An intense band at 1690 cm^{-1} could be either due to a carbonyl group or to a vinyl ether, but the usual tests for a carbonyl function are negative in batrachotoxin and the optical rotary dispersion curve displays no Cotton effect in the region where steroidal ketones or aldehydes exhibit such effects. A strong absorption in the infrared spectrum at 1250 cm^{-1} also suggests not a carbonyl group, but a vinyl ether.

The nuclear magnetic resonance spectrum indicates a quaternary methyl group, a methyl group on a tertiary carbon attached to a hetero-atom and 3 hydrogens at low field which are assigned to a carbinolamine $(-CH\begin{smallmatrix}O-\\N-\end{smallmatrix})$ group, a vinyl ether $(C=C\begin{smallmatrix}H\\O\end{smallmatrix})$ group and an olefinic proton in proximity to a hetero atom.

The *high resolution mass spectrum* of batrachotoxin gave an *empirical formula* of $C_{24}H_{33}NO_4$ which indicates the presence of 9 rings or double bonds. The loss of CHO from the parent ion is evidence for a potential aldehyde group. The n.m.r. spectrum also indicates a small amount of aldehyde and amine in equilibrium with the carbinol amine function. Fragmentation of batrachotoxin, with loss of C_4H_7N to form the ion $C_{20}H_{26}O_4^{\oplus}$, suggests that, instead of a carbinolamine, a carbinol amine ether is present in batrachotoxin. The ion $C_{20}H_{26}O_4^{\oplus}$ loses in succession 3 molecules of water to form $C_{20}H_{20}O^{\oplus}$. These transformations are confirmed by the presence of the corresponding meta-stable peaks. The ion $C_{20}H_{20}^{\oplus}$ may then lose an aldehyde group (CHO) to form $C_{19}H_{19}^{\oplus}$. This is

good evidence for a continuous carbon skeleton of at least 19 atoms. The low molecular weight fragments, $C_8H_{11}NO_2^{\oplus}$, $C_7H_8NO_2^{\oplus}$ and $C_4H_{10}NO^{\oplus}$ lead to the assumption that a methyl group, the nitrogen and two of the oxygens are within 7 carbons of each other and that a methyl group, the nitrogen and one oxygen are with 3 carbons of each other.

The mass spectrum of batrachotoxin was also measured after exchange with D_2O. Two exchangeable hydrogens were found. One was associated with the $C_7H_9NO_2^{\oplus}$ fragment. The nitrogen atom did not appear to have an exchangeable hydrogen.

Chemically batrachotoxin gives a *positive, immediate Ehrlichs test* which must be due to a *potential pyrrole group* in its structure.

On the basis of other reactions and the spectral data, batrachotoxin is a 24 carbon modified steroid with a potential 5 membered pyrrole ring that contains a tertiary nitrogen as part of a carbinol amine-vinyl ether group and a double bond. Within two carbon atoms of this ring is one of the two alcohol groups. The remainder of the molecule contains the other two oxygen atoms, one in an alcohol group and one in an ether linkage. This part of the molecule probably contains a tetrasubstituted double bond.

The complete structure of batrachotoxin must now await further studies by n.m.r. and mass spectrometry on the catalytic and hydride reduction products and various other derivatives. Attempts are being made to prepare a crystalline derivative for analysis by X-ray crystallography .

Pharmacologically, batrachotoxin is the most toxic known non-protein material (MLD 1 μg/kg) with cardiotoxic, myotoxic and neurotoxic activities.

In rat diaphragm-phrenic nerve preparation, indirect stimulation is quickly blocked and direct stimulation more slowly. A powerful contracture of the muscle also develops. These events are irreversible.

In a sciatic-sartorius preparation (*Bufo marinus*), the action potential of the nerve is relatively unaffected at concentrations which decrease the action potential in the muscle. The muscle after complete blocking of its action potential can still respond to direct stimulation indicating a block in neuro-muscular transmission. *In vivo*, in cats and dogs, a dose of batrachotoxin (0.3-.5 μg/kg i.v.) which does not significantly effect the response of muscle to nerve stimulation did cause interference with conduction in the heart, extrasystoles and finally ventricular fibrillation and death. Little effect on blood pressure was noted.

Other dendrobatid frogs have now been examined for toxic alkaloids. These include *Dendrobates tinctorius* (2 color varieties, Playa de Oro, Colombia), *Phyllobates subpunctatus* (Bogotá, Colombia), *Phyllobates talamancae* (Panama), *Phyllobates pratti* (Panama), *Phyllobates lugubris* (Panama), *Dendrobates minutus* (Panama), *Dendrobates auratus* (Taboga, Panama). Of these species, only *Dendrobates auratus*, which is known as the poison arrow frog of Panama, the tiny *Dendrobates minutus*, and some of the color varieties of *Dendrobates pumilio* had appreciable toxicity in skin extracts. None of these could, however, be compared to the toxicity of the extremely poisonous Kokoi' of Colombia. No batrachotoxin could be detected in any of these other frogs so that the Kokoi' of Colombia appears at present to be quite unique in this respect.

These studies on Panamanian frogs are the result of a stimulating collaboration with Charles W. Myers of the Gorgas Memorial Laboratory, Panama. He has discovered that *Dendrobates pumilio*, a small red and black frog in large

areas of Central America explodes into a great number of variously colored island populations in the Bocas region of Panama. The coloration between populations is extremely varied both in the dorsal and ventral aspects. What factors have caused this diversification are at present unknown. Since warning coloration in amphibians is often assumed to be associated with venomous secretions, a *comparison of the toxicities of various populations with brightness of coloration* was carried out (J. W. Daly and C. W. Myers. In preparation).

No *correlation between color and toxicity* was found although both factors varied widely between populations. Certain brightly colored frogs were almost nontoxic while one dark-blue frog, very protectively colored, contained large amounts of venom. The toxicity was measured by subcutaneous injection in mice and also by visualization of toxic principles A and B on thin-layer chromatoplates. The two toxic principles were then isolated by alumina column chromatography and silica gel thin-layer chromatography. These two toxic principles were found by high resolution mass spectrometry to have empirical formulae of $C_{19}H_{33}NO_2$ and $C_{19}H_{33}NO_3$, respectively. The ultraviolet absorption spectra showed only end absorption. The infrared spectra showed no carbonyl, double bond or oxazolidine ring. The mass spectra provided evidence for 4 rings, a carbinolamine, and one or two hydroxyl groups. Both compound A and B formed 0-methyl ethers on treatment with methanolic hydrochloric acid. Compound A forms a 0,N-Diacetyl derivative. The foregoing data suggest that compounds A and B are related in structure to the salamander alkaloids. These studies were carried out on only about 1 mg of each compound and the final structural elucidation will require additional material.

A great variety of other amphibians are known to contain toxic substances in their skin secretions, and it appears that their investigation should be quite profitable in terms of discovery of novel chemical structures and compounds of high pharmacological activity.

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Discussion

F. E. Russell: "Dr. Daly, in the slide showing the effects of the toxin on a Bulbring nerve-muscle preparation you noted that this demonstrated the neuromuscular blocking activity of the toxin. Did not the slide also show a reduction in the directly ilicited contractions which would certainly make it difficult to evaluate the neuromuscular blocking effect; and secondly, how can you be sure, in this preparation, that the principal effect is not on the nerve, rather than on the muscle?"

J. Daly: "I was not clear enough in explaining that in sciatic-nerve-Sartorius muscle preparation in *Bufo marinus,* that the nerve action potential was unaffected and the muscle action potential decreased. The muscle at this point still responded to direct stimulation thus indicating a block in neuromuscular transmission."