46. CARDIOTOXIC STEROIDS FROM TOADS

KUNO MEYER

Pharmazeutisches Institut der Universität, Basel, Switzerland

Knowledge of the poisonousness of the toad goes back to antiquity. Physicians of the classic period mentioned in their writings medicines prepared from toads, and described their effect on the heart and respiration. In the seventeenth and eighteenth centuries dried toads were temporarily admitted as "bufones exsiccati" in the official pharmacopoeias and were used as diuretics against dropsy and other diseases, even before digitalis was introduced by Withering. In China and Japan the dried venomous secretion of the Chinese toad, formed into round, smooth, dark brown discs and known as Ch'an Su or Senso, is still used to-day against various diseases.

The skin glands of elliptical form, the so-called paratoids, contain the bulk of the venom and are located in the rear of the ear. A much smaller amount of venom is also secreted by the small verrucose skin glands that cover the whole back of the animal. In this secretion two groups of toxic substances are found together with other compounds.

1. *the heart-active principles,* representing steroid derivatives, usually known as *bufogenins* (bufagins), or systematically as bufadienolides, and *bufotoxins* (conjugates of the bufogenins with suberylarginine), which are primarily responsible for the pharmacological effect of the poisonous secretion, and

2. *the basic components* of the bufotenine type as well as the hormones adrenaline and noradrenaline.

Elucidation of the chemical structure of the bufogenins which are the subject of this paper has been started by H. Wieland and his associates in Germany, by Jensen and Chen in America, and by several groups in Japan such as Kotake and Kuwada, and Kondo and Ohno. But only during the last two decades unequivocal proof of the steroid nature of the bufogenins has been presented and the details of their structure completely elucidated.

On the following table all toad species are listed whose venomous secretion has been subjected to a closer chemical investigation.

The toad poisons can be obtained from the following sources: from the dried skins of the animals, from secretions of the skin glands which are stimulated by electrical irritation of the living animals, or best, from dried paratoid secretion. (In this connection it should be mentioned that the poisonous substances have also been detected and isolated in other parts of the body, e.g. in the blood, and the ovaries.) The collection of the venomous secretion from the living toads can be effected as follows: the animals are firmly held with one hand and pressed down on a board that is covered with a glass plate. The raised, oblong gland accumulations located behind the ear are held near their base, between thumb and forefinger and squeezed firmly, so that the milky secretion squirts onto the underside of the glass plate. The glass plates charged with the venom secretion are kept in a horizontal position (secretion on top) at room temperature without exposure to direct sunlight until the secretion is dry and can easily be scraped off. This slow drying probably permits the enzyme contained in the raw secretion to split the bufotoxins into bufogenins and suberylarginine. The above procedure can be repeated after a rest period of 30-40 minutes and yields again a considerable quantity of secretion. Afterwards the venom glands are completely empty and regeneration takes place after a period of 4 to 6 weeks. An excellent source for obtaining a series of bufogenins is Ch'an Su or Senso, mentioned above. This is still obtainable in large quantities from the pharmacies, for instance in Hong Kong and Tokyo.

SPECIES	SOURCE	Length of animals	Approx. amount of venom (dried) in mg per animal
Bufo alvarius GIRARD	Southern Arizona and Southern California to Mexico	80-165 mm	400
Bufo americanus HOLBROOK	Eastern part of North America from the Hudson Bay southward	54-110 mm	16
Bufo arenarum HENSEL (*arenarius* LUTZ)	Uruguay, Northern Argentina, Southern Brazil	75 mm	87
Bufo asper	Indonesia, Siam, Malayan Peninsula	260 mm	—
Bufo blombergi MYERS & FUNKHOUSER	Columbia	200 mm	1200
Bufo bufo bufo LINNAEUS = *Bufo vulgaris* LAURENTI	Europe, not including the Mediterranian, temperate zone of Asia	60-80 mm	13
Bufo bufo gargarizans = *Bufo gargarizans* CANTOR = *Bufo asiaticus*	China	75-114 mm	19
Bufo crucifer WIED 1821	Costline of Brazil and Argentina	up to 75 mm	18
Bufo formosus BOULENGER	Japan	125 mm	75
Bufo woodhausi fowleri HINKLEY	New England and New York south to Georgia and westward along the Great Lakes to Michigan. Along the gulf coast to central Texas	51-82 mm	14
Bufo granulosus SPIX subsp. *fernandeze* GALLARDO	East-north-eastern part of the South American continent from Panama to the southern part of Buenos Aires in Argentina	50-55 mm	—
Bufo ictericus SPIX 1824 = *Bufo marinus* BOULENGER, 1882, part.	Brazil	up to 140 mm	190

SPECIES	SOURCE	Length of animals	Approx. amount of venom (dried) in mg per animal
Bufo marinus (LINNAEUS 1758) = *Bufo marinus* (L.) SCHNEIDER	West Indies, Mexico, Central and South America	up to 200 mm	580
Bufo mauritanicus SCHLEGEL	Morocco, Algeria and Tunisia	122 mm	190
Bufo melanostictus	Southeast Asia, Indonesia	116 mm	90
Bufo paracnemis LUTZ	Guiana, Eastern Brazil	130-220 mm	240
Bufo peltocephalus TSCHUDI	Cuba	130 mm	120
Bufo quercicus HOLBROOK	North Carolina to Florida west from Louisiana	19-32 mm	2
Bufo regularis REUSS	Africa, wide spread	up to 136 mm	180
Bufo spinulosus WIEGMANN (=*B. chilensis* (TSCHUDI)	Chile, Peru	up to 100 mm	—
Bufo valliceps WIEGMANN	Louisiana, East and South Texas to New Mexico and Costa Rica	53-125 mm	18
Bufo viridis viridis LAURENTI	Europe, not including Iberian peninsula, North Africa, Near East, eastward to Mongolia, Tibet and Himalaya area	80-140 mm	27

For the isolation of the bufogenins from the dried paratoid secretion (e.g. from Ch'an Su) the finely pulverized (powdered) material is mixed with an equal volume (or more) of sand and extracted in a Soxhlet apparatus or in a percolator with chloroform. The yellowish-brown coloured extracts, containing the sterols and the bufogenins, are submitted to an initial purification in order to separate these two classes of compounds. The bufogenins so obtained are chromatographed on alumina or silica Gel. Thus, relatively easily and in a short time, the major bufogenins can be obtained in a crystalline state. However, those substances present in small or minute quantities may require further purification by chromatographic methods, for example partition chromatography, which may be used directly for separation of the crude venom, instead of absorption chromatography on alumina or silica Gel. Mixtures which are difficult to separate may be resolved by preparative paper chromatography or dispersion on long columns of silica Gel.

With the aid of Paper Chromatography or Thin Layer Chromatography it is possible in most cases to obtain an unequivocal identification of the bufogenins, thus enabling the chemist to analyse minute amounts of the venom. Furthermore, these methods are most reliable and easy for determining the homogeneity of the crystalline materials. The chromatograms are dried and the migrated substances can be detected by spraying with a solution of $SbCl_3$ in chloroform (20 gr/100 ml) and heating to about 80°-120° for several minutes. The different colours thus appearing in day- or ultraviolet light help further to characterize these substances. Since the bufogenins have a strong ultraviolet absorption at about 290-300 nm they can be located directly on the paper by a photocopy with filtred ultraviolet light, or even better with a monochromator. By this way 0.005 mg of a bufogenin can be detected.

The different colorations or the change of the colours which occur when the bufogenins are treated with strong acids are very useful in identification. It should be emphasized here that reliable results are only obtained if pure crystals are used and at the same time authentic substances are tested as well. With concentrated sulphuric acid or 84% sulphuric acid it is best to use white spot plates, but colour reactions with a solution of $SbCl_3$ in chloroform (20 gr/100 ml) are performed on filter paper. Only minute amounts of substances are needed (0.05 mg). The coloured spots should also be examined under ultraviolet light. The Liebermann colour reaction and its modifications have become obsolete.

The chemistry of the bufogenins

In contrast to the C_{23}-steroids of the digitalis and strophanthus type, the bufogenins are C_{24}-steroids. Their steroid nature was first deducted from dehydrogenation experiments: chrysene was obtained from bufotalin, and -methyl-cyclopenteno-phenanthrene from cinobufagin, cinobufotalin and marinobufagin. Direct transformation of a bufogenin into a steroid of known structure has not been achieved until 1939.

1) *The Lactone group*

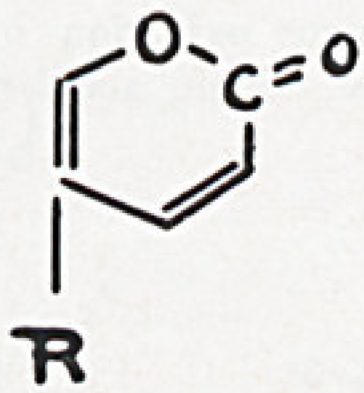
An integrant part of all bufogenins is the lactone group. Wieland and Weyland first formulated this lactone group as five-membered on the basis of experiments with ozone (formation of formic acid). Later a structure with only one double bond in a six-membered lactone was proposed. In 1936 Wieland and associates correctly interpreted the ultraviolet spectrum [$\lambda_{\text{max}}^{\text{alcohol}}$ 290-300 nm ($\log \varepsilon = 3.75$)] of the bufogenins as derived from an α-pyrone structure. The same spectrum is found with coumalic acid methyl ester as well as with the scylla glycosides and their aglycones. The structure of the lactone ring is unambigously proved by the given spectral datas, by degradation experiments and also by synthesis.

2) *The Steroid ring skeleton*

Stereochemistry. The stereochemistry of the ring junctures at C-5, C-8, C-9, C-10, C-13, is the same for all naturally occurring bufogenins and corresponds to the ring junctures of the bile acids series, with the only exception of the C/D ring juncture, which is cis instead of trans.

3) *The Degradation*

Can be effected by different ways. By hydrogenolysis the bufogenins can be transformed into a bile acid. As relatively large amounts of substance are needed, this method has been given up. It has been shown that the best method



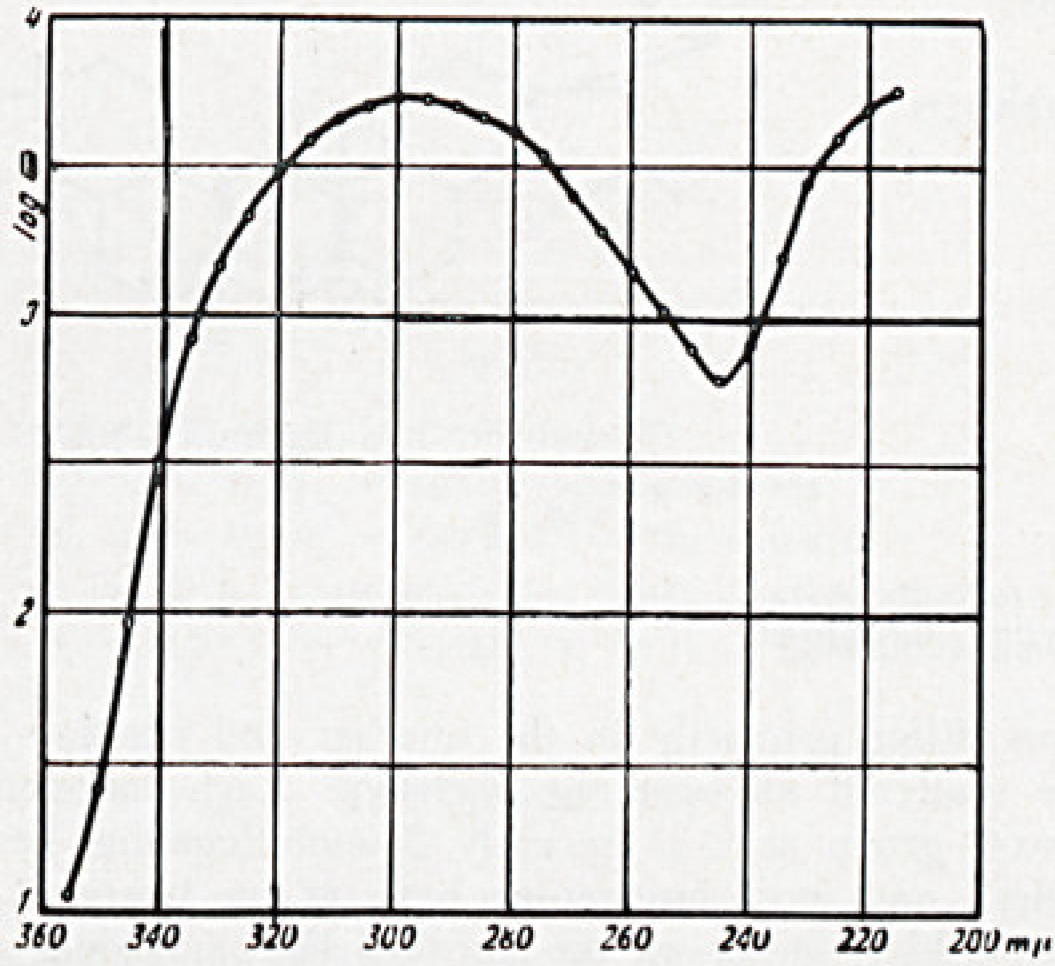
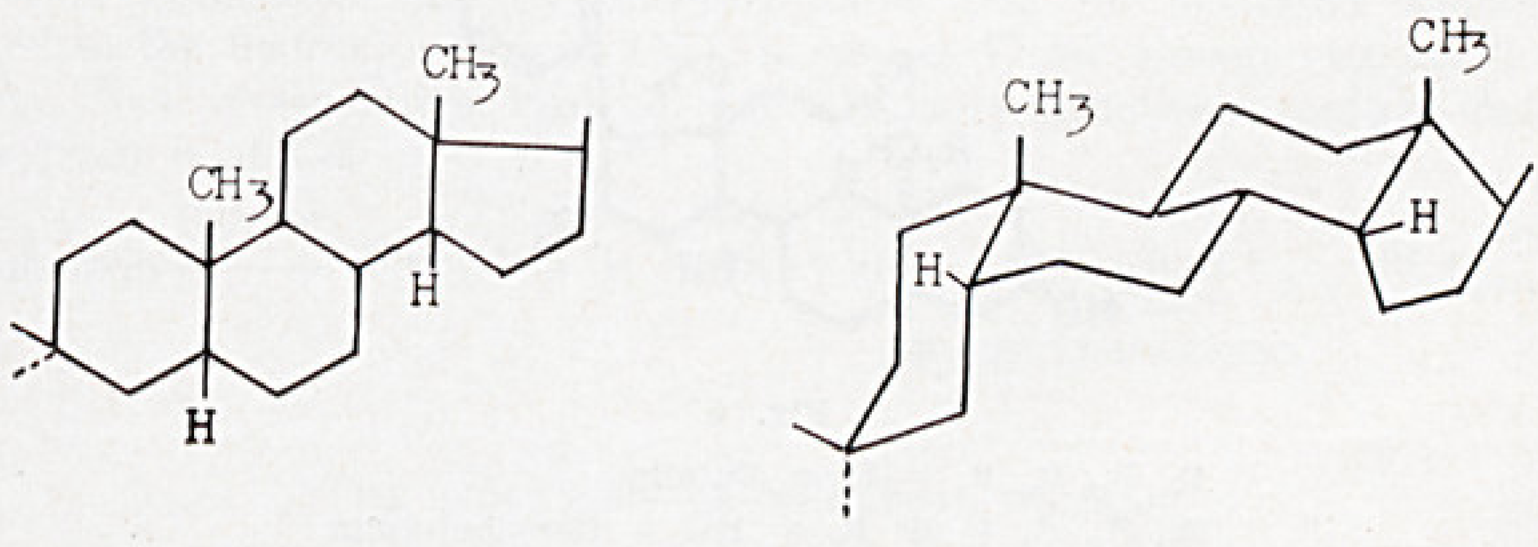


Fig. 1

The Ring Skeleton of the Bufogenins



Projection Formula

Conformation Formula

Fig. 2

of degradation is the permanganate oxidation of the peracetylated bufogenins. By this method the corresponding etianic acids can be obtained in a relatively high yield (up to 50%).

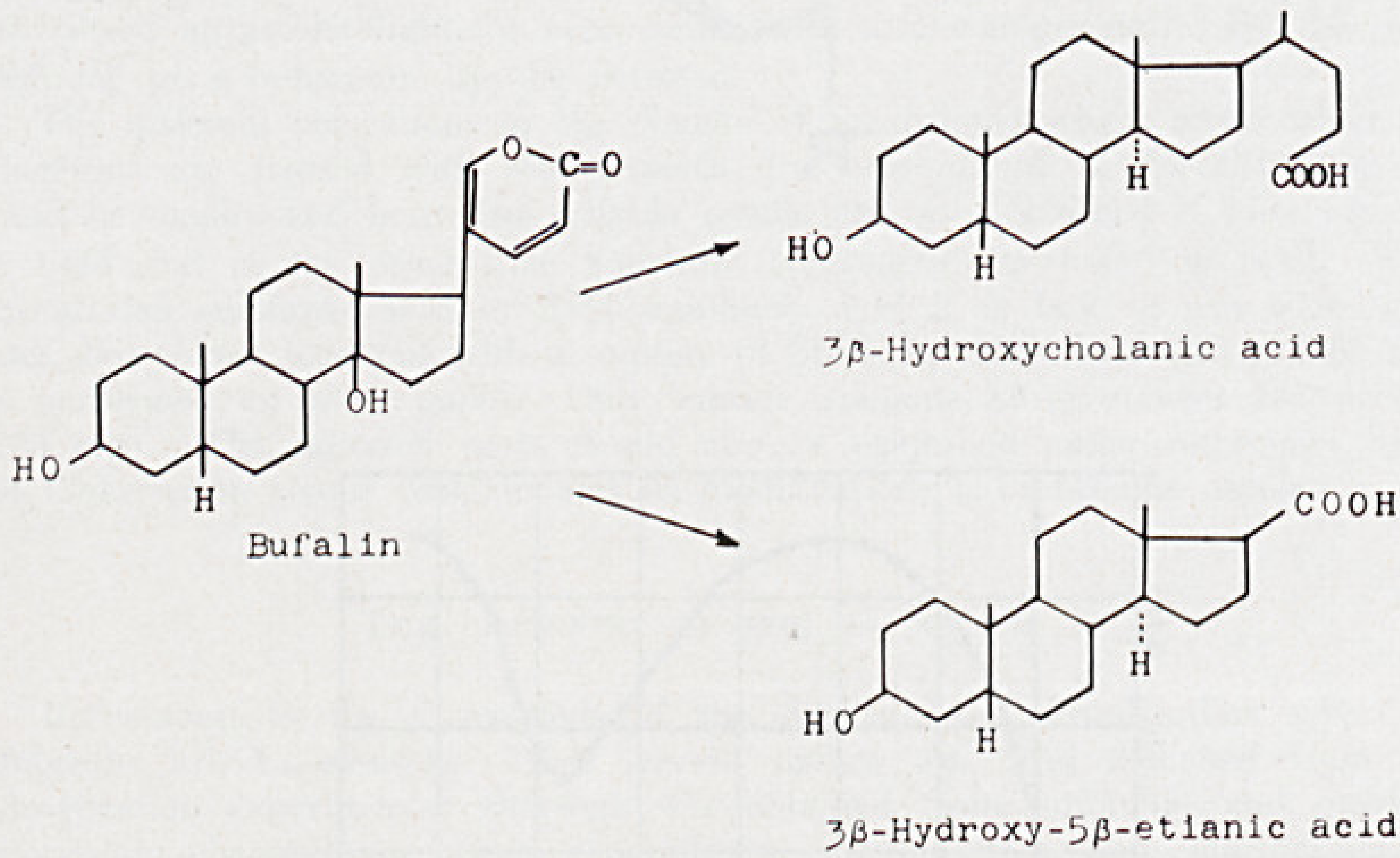


Fig. 3

4) *The Oxygen functions*

The bufogenins differ primarily in the number and position of the *hydroxyl groups*, which are scattered all over the skeleton. Each bufogenin has at least a secondary hydroxyl group at C-3 (recently 3-ketobufogenins have been isolated in minute quantities) and most bufogenins have as an integrant part a tertiary hydroxyl group at C-14 (similar to the cardio-active aglycones of the digitalis-strophanthus group). Those bufogenins carrying an *oxido group* at C-14/C-15 belong to a separate group. Additional hydroxyl groups are found at C-5, C-11, C-12, C-16 (mostly in acetylated form) and at C-19. A *keto group* was detected at C-12 and an *aldehyde group* at C-10.

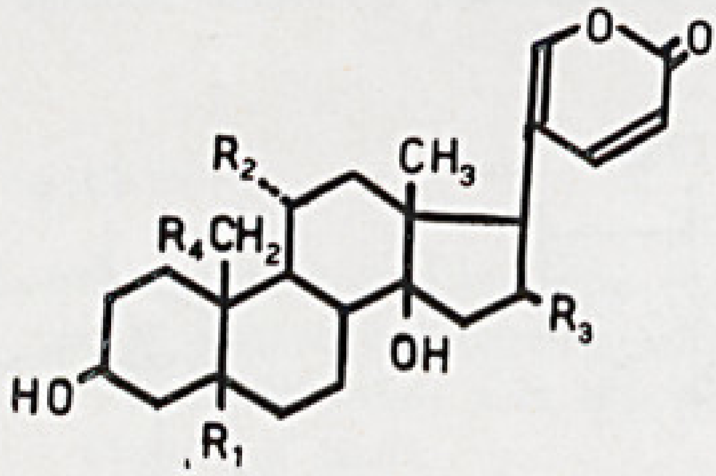
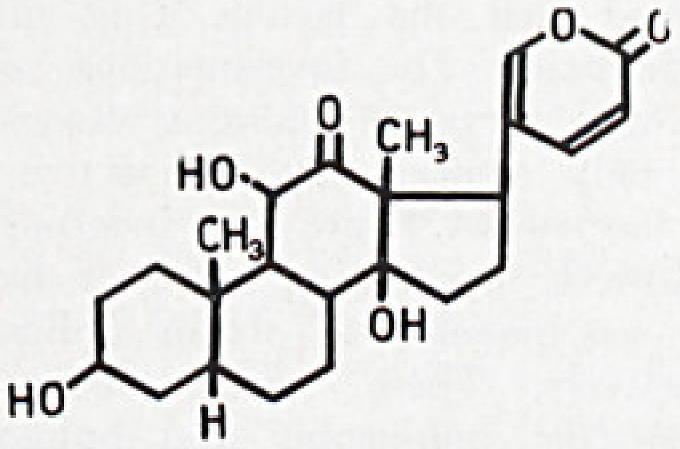
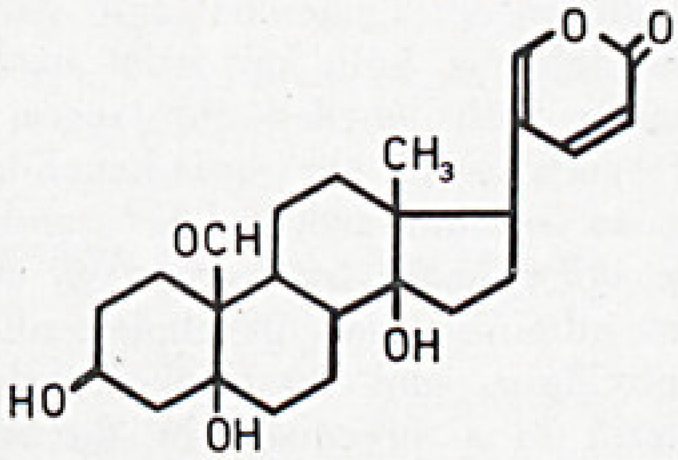


Fig. 4

R_1, R_2, R_3, R_4 = H = Bufalin
R_1, R_3, R_4 = H; R_2 = HO = Gamabufotalin
R_1, R_2, R_4 = H; R_3 = $OCOCH_3$ = Bufotalin
R_2, R_3, R_4 = H; R_1 = HO = Telocinobufagin
R_2, R_3 = H; R_1, R_4 = HO = Hellebrigenol



Arenobufagin



Hellebrigenin

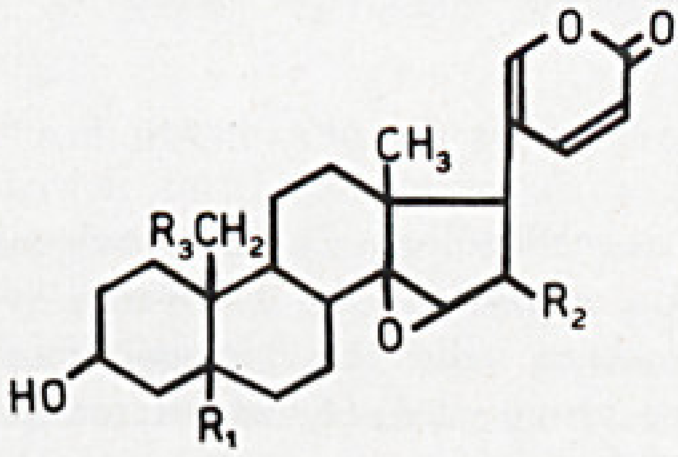


Fig. 5

R_1, R_2, R_3 = H = Resibufogenin
R_2, R_3 = H; R_1 = HO = Marinobufagin
R_1, R_3 = H; R_2 = $OCOCH_3$ = Cinobufagin
R_1 = H; R_2 = $OCOCH_3$; R_3 = HO = Cinobufaginol
R_1 = HO; R_2 = $OCOCH_3$; R_3 = H = Cinobufotalin

The bufotoxins

Toad venoms contain beside the bufogenins a further type of cardio-active substances, the so-called bufotoxins. These represent conjugates of bufogenins with suberylarginine and were isolated and investigated in a number of laboratories during the 1930's. With the exception of Wieland's bufotoxin, all are rather poorly characterized due to the fact that these substances are extremely difficult to obtain in homogeneous crystals. This is even the case when modern chromatographic methods are applied. No doubt the bufotoxins so far described are mixtures, and it seems very probable that Wieland's bufotoxin also was impure. Our assumption that the suberylarginine rest (in Wieland's Bufotoxin) is linked to the hydroxy group at C-14, seems, as Fieser already suggested, very unlikely. New investigations made in our laboratory tentatively confirm that the linkage point is at C-3.

$$\text{Bufotoxin} \xrightarrow{\text{Enzyme}} \text{Bufogenin} + \underbrace{HOOC\text{-}(CH_2)_6\text{-}\underset{\substack{\|\\O}}{C}\text{-}NH\text{-}\underset{COOH}{CH}\text{-}(CH_2)_3\text{-}NH\text{-}C\begin{matrix}\nearrow NH_2\\ \searrow NH\end{matrix}}_{\text{SUBERYLARGININE}}$$

The biogenesis of the toad poisons

Very little is known about the biosynthetic pathway of the formation of the toad poisons. The close relationship in the structure of the bufadienolides and the bile acids indicate that these two types of steroids are formed from the same basic substance. Tschesche and Korte assumed that the lactone ring of the bufadienolides is built up from oxalacetoacetic acid. The investigations so far undertaken with labelled substances, which could serve as building stones for the biosynthesis of the bufadienolides, have only shown, that radioactive carbonate, or sodium acetate-1-C^{14}, and sodium acetate-2-C^{14} are not incorporated. On the other hand, by feeding *B. marinus* with C^{14}-containing algae or by parenteral administration of cholesterol-4-C^{14} it was possible to obtain radioactive marinobufagin and marinobufotoxin, respectively. These results show that cholesterol is a precursor in the synthesis of the bufogenins and bufotoxins. This observation would suggest that cholesterol or a closely related compound is the major source of the cardiotonic sterols in the toad.

The synthesis of bufadienolides

While the synthesis of a cardenolide, i.e. Digitoxigenin was prepared only recently, the synthesis of a C_{24} steroid of the bufogenin type has been achieved in 1961 by Bertin and co-workers. But this lactone possessed neither the 14β-configuration nor the hydroxy group at C-14, which means that no "true" bufadienolide could be synthesized.

References

1. MICHL, H., and KAISER, E., *Toxicon*, 1, 175, 1963.

Discussion

E. A. Zeller: "In order to find out the hydroxyl group to which the suberyl-arginine is attached, enzymic studies with simple model substances and the enzyme present in crude venom preparation could be carried out. Did anybody try this way?"

K. Meyer: "The isolation of the enzyme was not yet tried."