A CHROMATOGRAPHIC STUDY OF THE BUFADIENOLIDES ISOLATED FROM THE VENOM OF THE PAROTID GLANDS OF *BUFO PARACNEMIS* LUTZ 1925

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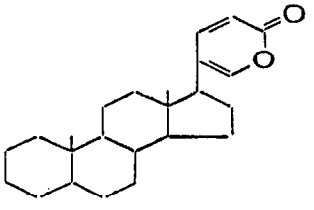
Chromatography is of acknowledged importance in the identification and separation[1] of the bufadienolides (I), the cardiotoxic steroids of toad venoms, which are of increasing pharmacological interest[2–5].

Following on our work on the venoms of *B.ictéricus* Spix 1824 and *B.crucifer* Wied 1821[6], we now report a chromatographic survey of the bufadienolides isolated from the venom of the parotid glands of *B.paracnemis* Lutz 1925.



(I)

B. paracnemis Lutz 1925[7] is a giant toad (13–22 cm) (Fig. 3) common in various states of Brazil (Minas Gerais, Rio de Janeiro, São Paulo, Paraná)[8]. The species possesses voluminous parotid glands averaging 25 % of the length of the individual and is easily distinguished from the northern (Amazonian) *B.marinus* Linnaeus 1758 by the presence of salient *tibial* glands.

A preliminary investigation of the crude venom was based on thin-layer chromatography, the results of which have been briefly reported[9]. In order to achieve separation and identification of the bufadienolides obtained in this first step, repeated column chromatography on aluminium oxide or silicic acid was carried out and the fractionations systematically followed by thin-layer and paper chromatographic analysis.

EXPERIMENTAL

Extraction of the bufadienolides

The viscous mass, collected from 239 individuals by pressure of the parotid glands, was dried in a desiccator (over P_2O_5), yielding 57 g of crude dried venom. The aqueous suspension of this material was repeatedly extracted with chloroform–methanol (9:1)

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and the concentrated dried organic layers furnished 9.8 g of an amorphous residue containing mainly cardiotoxic steroids.

Column chromatography

Chromatographic fractionations were performed on columns of aluminium oxide (Merck, active neutral) or silicic acid (Mallinckrodt, 100 mesh) by the method of fractional elution[10], using the sequence of solvents benzene, chloroform, methanol (Table I).

TABLE I

COLUMN CHROMATOGRAPHIC FRACTIONATION OF BUFADIENOLIDES

Column 1 (Al_2O_3): 9.5 g of venom extract

Fraction No.	Solvent system*	Amount (mg)	Bufadienolide**
1–3	Chf–Bz 1:1	1785	γs, R, M
4–6	Chf–Bz 3:1	3089	R, M, T
7	Chf	490	M, T
8–10	Chf	539	
11	Chf–Me 50:1	794	M, T, Bt, G,
12–13	Chf Me 50:1	252	He
14	Chf–Me 45:5	289	
15–16	Me	627	M, Bt, T, He, Ho, 3 spots

Column 2 (Al_2O_3): 1.72 g from fractions 1–3, column 1

Fraction No.	Solvent system*	Amount (mg)	Bufadienolide**
1–4	Bz	24	γs
5–9	Chf–Bz 1:1	194	γs, B, M
10–15	Chf–Bz 3:1	102	
16–20	Chf	419	B, M
21–24	Chf–Me 50:1	629	B, M, T, He, G
25–26	Chf–Me 50:2	208	M, T
27–28	Chf–Me 45:5	52	He, G

Column 3 (SiO_2): 0.664 g from fractions 5–20, column 2

Fraction No.	Solvent system*	Amount (mg)	Bufadienolide**
1–4	Chf–Bz 2:3	104	
5–9	Chf–Bz 3:2	11	γs
10–12	Chf–Bz 4:1	16	
13–15	Chf	5	γs, B
16–23	Chf–Me 50:1	373	B, M
24–25	Me	35	

Column 4 (Al_2O_3): 3.2 g from fractions 4–7, column 1

Fraction No.	Solvent system*	Amount (mg)	Bufadienolide**
1	Chf–Bz 1:1	64	M, R
2–6	Chf–Bz 1:1	42	M, T
7–13	Chf–Bz 3:1	1204	M, (T)
14–19	Chf	1710	
20–21	Chf–Me 50:1	29	M, T
22	Chf–Me 50:2	26	

Column 5 (Al_2O_3): 1.3 g from fractions 8–11, column 1

Fraction No.	Solvent system*	Amount (mg)	Bufadienolide**
1–2	Chf–Bz 1:1	44	M, T, Ag
3–5	Chf–Bz 1:1	24	M, T
6–10	Chf–Bz 3:1	54	M, Bt
11–19	Chf	210	T, G
20	Chf–Me 50:1	628	T, (M), (G)
21–23	Chf–Me 50:1	150	T, He, Ho,
24–26	Chf–Me 50:2	22	1 spot

Column 6 (Al_2O_3): 1.12 g from fractions 12–16, column 1

Fraction No.	Solvent system*	Amount (mg)	Bufadienolide**
1–4	Chf	280	M, T, Bt, He
5–9	Chf–Me 50:1	260	M, T, Bt, Ag
10–14	Chf–Me 50:1	184	He, Ho, 2 spots
15–19	Chf–Me 50:2	147	M, T, Ag, He
20–23	Chf–Me 45:5	36	Ho, 5 spots
24–28	Chf–Me 20:5	41	1 spot (start)

* Chf = chloroform; Bz = benzene; Me = methanol.

** Ag = argentinogenin; B = bufalin; Bt = bufotalinin; G = gamabufotalin; He = hellebrigenin; Ho = hellebrigenol; M = marinobufogenin; R = resibufogenin; T = telocinobufogenin; βs = β-sitosterol; γs = γ-sitosterol; parentheses indicate traces.

Thin-layer chromatography (TLC)

Chromatoplates of Silica gel G (Merck) were prepared by procedures already described[9–11] and ascending development was carried out, using one of the solvent systems:

A: Ethyl acetate–cyclohexane (1:2)

B: Ethyl acetate

C: Ethyl acetate–methanol (9:1).

After concentration of the column eluates to a small volume (1–2 ml), aliquots were applied to the plates through capillary tubes.

Paper chromatography

The descending method was selected. The papers (Whatman No. 1) were impregnated with the stationary phase previously diluted with acetone (1:5 v/v) and partially dried (70°) before development. The solvent systems employed were:

A: Formamide–benzene

B: Formamide–chloroform

C: Propylene glycol–water (4:1)/benzene–chloroform (1:1).

Development took from 3 to 96 h. 100 to 150 μg of the steroidal mixture and 30 to 50 μg of the standards were applied along a line 7 cm from one edge of the 50 cm sheets.

Detection

The steroidal compounds were visualized by spraying a saturated chloroform solution of $SbCl_3$ on the chromatoplates or the dried papers (vacuum oven/60°), heating over a hot plate and examining in ultra-violet light. In experiments with preparative TLC, visualization could also be obtained by spraying water[12] on the developed plates, thus showing up the white bands corresponding to the steroidal constituents.

RESULTS AND DISCUSSION

Table I shows the results of the chromatographic fractionations and Table II shows some of the R_F values. Figs. 1 and 2 are examples of thin-layer and paper chromatographic analyses of bufadienolides.

Fractions 1–3 (column 1), 1–9 (column 2) and 1–12 (column 3) (Table I) include

TABLE II

R_F VALUES* (PAPER CHROMATOGRAPHY) OF UNIDENTIFIED POLAR COMPONENTS

Solvent system: formamid/chloroform

	$R_F \times 100$	*Color under U.V. light ($SbCl_3$, after 2 min heating)*
Gamabufotalin	100	Yellow
P_1	80	Pink
P_2	67	Pink
P_3	44	Pink-yellow
P_4	25	Orange
P_5	10	Pink-orange

* Determined with reference to gamabufotalin.

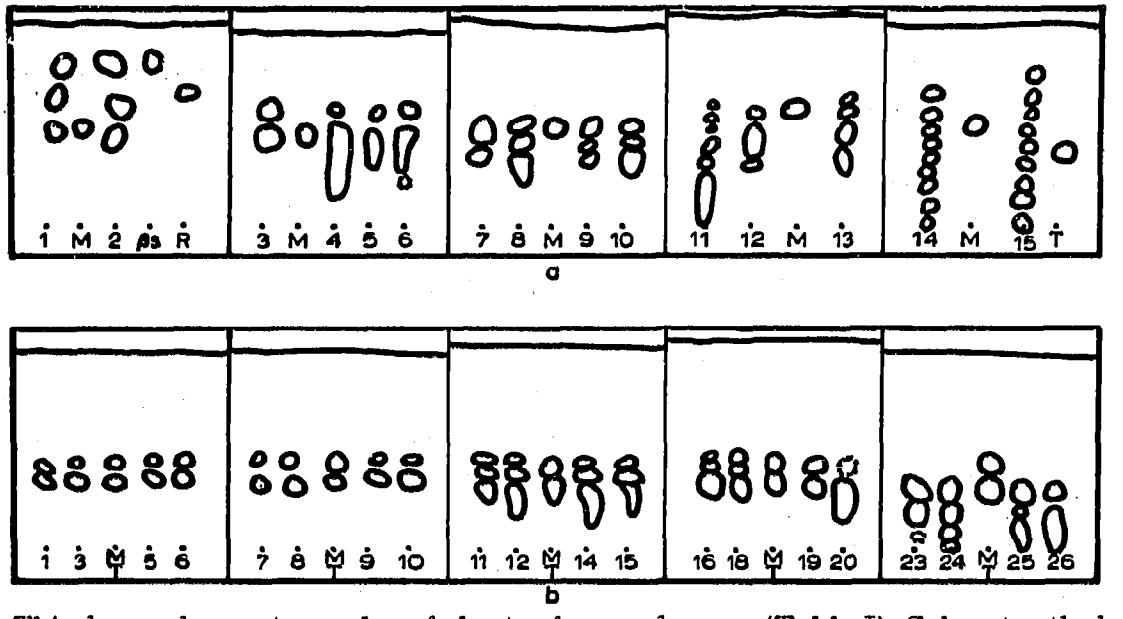

Fig. 1 (a). Thin-layer chromatography of eluates from column 1 (Table I). Solvent: ethyl acetate. (b) Thin-layer chromatography of eluates from column 5 (Table I). Solvent: ethyl acetate.

a substance of the same R_F value as β-sitosterol (TLC, solvent systems: ethyl acetate–cyclohexane (1:2), ethyl acetate). This was considered to be γ-sitosterol. Since the occurrence of this sterol has earlier been recognised in toad venoms[13] and the structure and configuration of β-sitosterol assigned to it[14], a standard of β-sitosterol was used for further identification: (a) column chromatography on silicic acid of fractions 1–9 (column 2) and 1–12 (column 3) followed by elutions from benzene yielded 113 mg of chromatographically (TLC) pure material which was recrystallized in acetone–methanol; yield 51 mg, m.p. 141–143°, $[\alpha]_D^{21°} = -41°$ ($c = 1.03$; chloro-

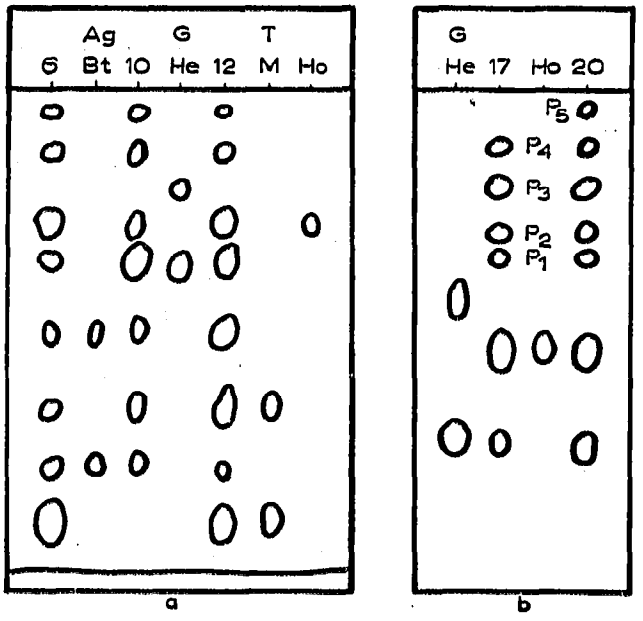

Fig. 2. Paper chromatograms of eluates from column 6 (Table I). Solvent system: formamide–chloroform. Time of development: (a) 3 h; (b) 6 h.

form); (b) infrared spectra of this compound and of β-sitosterol exhibit the same absorption bands in the 1400–650 cm^{-1} region, which agrees with previous findings[15].

Recrystallizations in acetone–ether of: (a) fractions 16–25 (column 3) furnished 45 mg of chromatographically (TLC) pure bufalin, m.p. 235–238°, $[\alpha]_D^{24°} = -11°$ ($c = 1.09$; chloroform); (b) fractions 7–19 (column 4) gave 2.717 mg of chromatographically (TLC) pure marinobufogenin*, m.p. 215–217°, $[\alpha]_D^{24°} = +12°$ ($c = 1.13$; chloroform); and of (c) fraction 20 (column 5) yielded 524 mg of telocinobufogenin, together with traces of marinobufogenin, m.p. 158–162°, $[\alpha]_D^{24°} = +7°$ ($c = 1,07$; chloroform).

In fractions 15–23 (column 6), the presence of 5 spots of lower R_F values than gamabufotalin was demonstrated by paper chromatography (Fig. 2). Further fractionation on a column of silicic acid or by preparative TLC was unsuccessful owing to the complexity of the mixture and its low yield.

Generally speaking, TLC had several advantages, but showed certain limitations when using silica gel G: for instance, in cases in which the resolving power of TLC did not favour the distinct location of the more polar bufadienolides, paper chromatography proved more suitable.

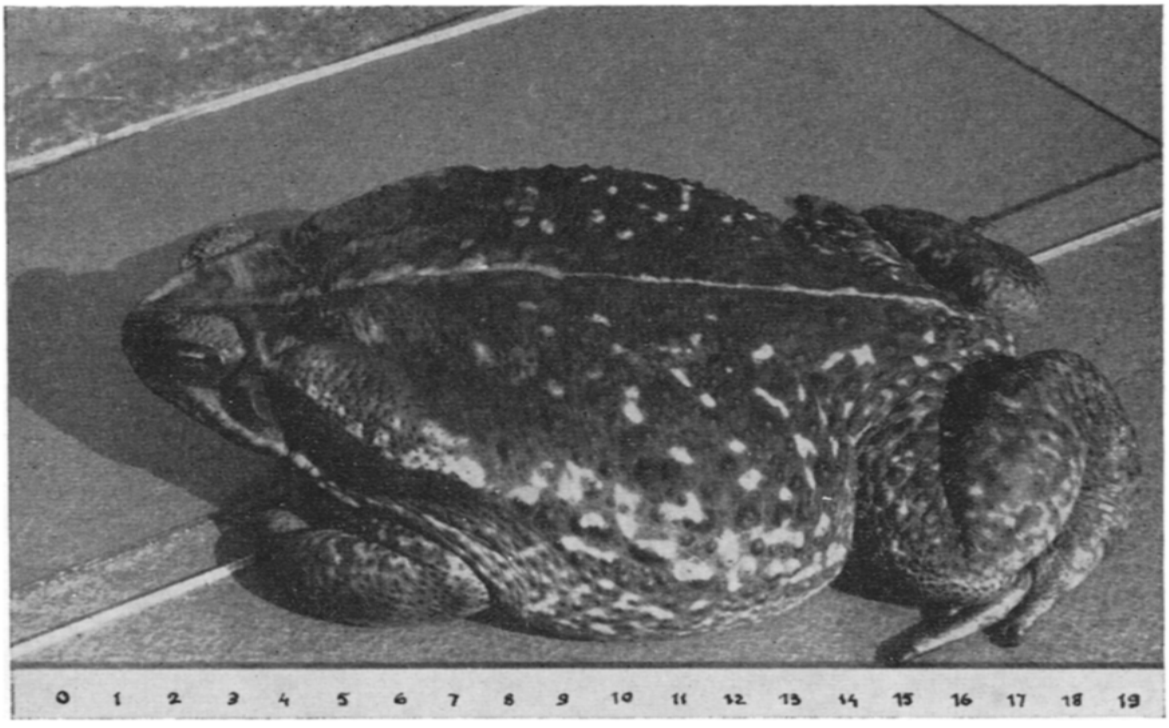

Fig. 3. *Bufo paracnemis* Lutz 1925.

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* Previously isolated from the venom of the same species of toad along with bufotenine and adrenaline[16].

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SUMMARY

A chromatographic study of the bufadienolides isolated from the venom of the parotid glands of *Bufo paracnemis* Lutz 1925 is reported. Column fractionations, followed by thin-layer and paper chromatographic analysis, resulted in the identification of the following compounds: γ-sitosterol, argentinogenin, bufalin, bufotalinin, gamabufotalin, hellebrigenin, hellebrigenol, resibufogenin and telocinobufogenin. Five unidentified compounds of lower R_F values than gamabufotalin were detected by paper chromatography, using the solvent system formamide–chloroform.

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