CHEMICAL CONSTITUENTS OF VIETNAMESE TOAD VENOM, COLLECTED FROM *BUFO MELANOSTICTUS* SCHNEIDER. PART II[1]. THE BUFADIENOLIDES

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ABSTRACT.—The isolation and identification of 8 bufodienolides present in Vietnamese toad venom, collected from *Bufo melanostictus*, is described. The presence of the earlier by means of pc identified bufodienolides: bufotalin, marinobufagin and hellebrigenin, could be confirmed, as well as the presence of the previously tentatively identified compounds: resibufogenin and bufalin. Three additional bufadienolides were identified: desacetylbufotalin, hellebrigenol and 19-hydroxybufalin. The assignment of ^{13}C-nmr spectra of seven of these compounds is reported.

In a previous publication (1), we reported on the sterols present in the toad venom of *Bufo melanostictus*. In this paper we report on the bufadienolides present. Furthermore, the ^{13}C-nmr spectroscopy of some bufadienolides is dealt with.

Van Gils (2, 3) reported on the isolation of some compounds present in the venom of *Bufo melanosticus* collected in Indonesia. Three compounds were thought to be bufadienolides. The empirical formulas of two of them were reported to be $C_{29}H_{42}O_7$ and $C_{23}H_{30}O_6$. The former compound, the major component, was thought to be melano-bufagenin; the latter compound was thought to be analogous with bufotenin. In this compound no acetyl groups could be detected. The third compound, of which no empirical formula was determined, was thought to be analogous with bufotalidin (=hellebrigenin).

Iseli *et al.* (4) investigated the venom of *Bufo melanostictus* by means of paper chromatography in combination with the detection reagent antimony trichloride. The authors identified hellebrigenin, marinobufagin, and bufotalin as the major components. Two minor components were tentatively identified as resibufogenin and bufalin. Seven other minor components were not identified.

RESULTS

By means of preparative tlc, the three main components observed in the chromatogram of the toad venom were isolated. By means of ^{1}H-nmr and ^{13}C-nmr these compounds were identified as resibufogenin (**2**), bufotalin (**3**) and 19-hydroxy bufalin (**5**). Hslc and tlc comparison with authentic samples of resibufogenin and bufotalin (kindly provided by Prof. Dr. K. Meyer, Basel) confirmed their identification. By means of preparative hslc, with a solvent system similar to a system described by Shimada et al. (5, 6), some minor components were isolated. By ft ^{1}H-nmr, hslc and tlc comparison with reference compounds, these components were identified as bufalin (**1**), marinobufagin (**8**), hellebrigenin (**7**), hellebrigenol (**6**), and desacetylbufotalin (**9**).

In table 1 the approximate quantities of the bufadienolides present in fraction B (scheme I), used for isolation of the bufadienolides, are summarized. It is obvious that the differences with the results of Iseli et al. (4) are mainly quantitative. Only bufotalin in both cases was one of the major components. Tlc of fraction C gave a similar picture as fraction B. Also a methanol extract of the

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TABLE 1. Estimation of the percentages of bufadienolides present in Fraction B.[a]

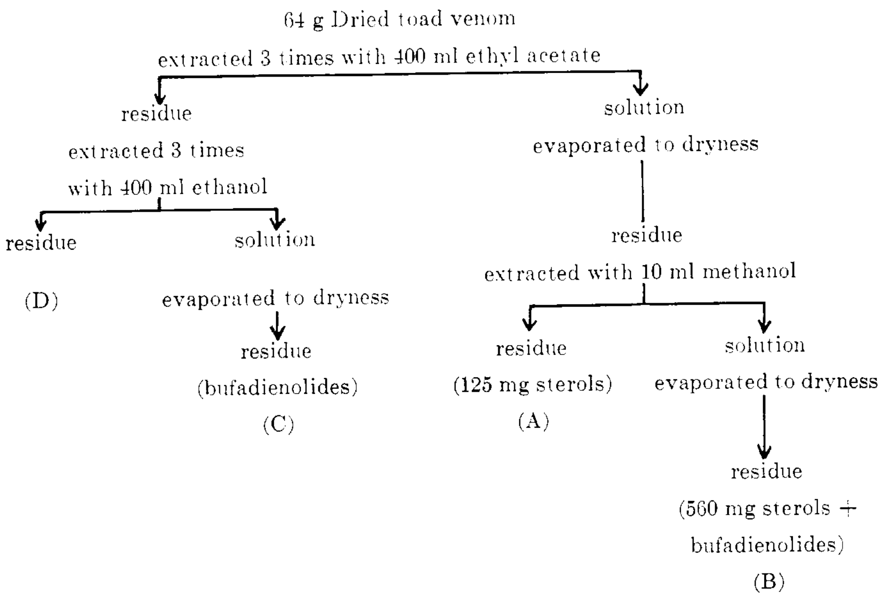
hellebrigenol hellebrigenin	4%
19-hydroxy bufalin	45%
bufotalin	22%
bufalin	3%
resibufogenin	20%
marinobufagin	4%
others	2%

[a]Calculated by measuring peak areas in the HSLC chromatogram, using the solvent methanol-H_2O (3+2) and a 25 cm long, 4.6 mm ID stainless steel column filled with Lichrosorb 5RP8, 5 μm. UV detection at 280 nm.

venom showed the same three previously mentioned compounds as the main components. For identification of the main components, ^{13}C-nmr was used in addition to ^{1}H-nmr. An extensive review of the ^{13}C-nmr of steroids has been given by Blunt and Stothers (7). Tori et al. (8) described the ^{13}C-nmr of some cardenolides. Gsell and Tamm (9) described the use of ^{1}H-nmr in the structure elucidation of bufadienolides, whereas the use of mass spectrometry was extensively described by Brown et al. (10, 11). ^{13}C-nmr is a very useful additional technique. For some bufadienolides, the noise decoupled and off resonance spectra were recorded.

By use of the assignments made by Tori et al. (8) for cardenolides, and common shift rules, the signals in the spectra of the available bufadienolides could be assigned.

SCHEME 1. Fractionation of toad venom.



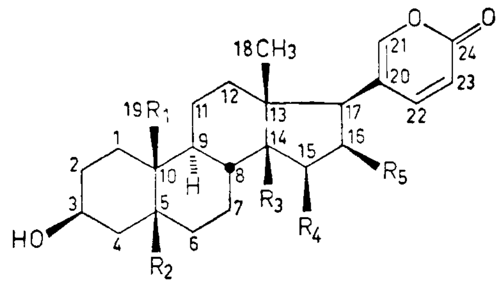


FIGURE 1

		R_1	R_2	R_3	R_4	R_5
1	bufalin	CH_3	H	OH	H	H
2	resibufogenin	CH_3	H	—O—		H
3	bufotalin	CH_3	H	OH	H	OAc
4	cinobufagin	CH_3	H	—O—		OAc
5	19-hydroxylbufalin	CH_2OH	H	OH	H	H
6	hellebrigenol	CH_2OH	OH	OH	H	H
7	hellebrigenin	CHO	OH	OH	H	H
8	marinobufagin	CH_3	OH	—O—		H
9	desacetylbufotalin	CH_3	H	OH	H	OH

The spectra of bufalin and bufotalin can directly be compared with digitoxigenin and its 16-O-acetyl derivative respectively; only the lactone carbons are different. The same applies for hellebrigenin, which can be compared with strophanthidin. The assignment of the other spectra is derived from these.

TABLE 2. ^{13}Cnmr shifts of some bufadienolides (δ In ppm downfield from TMS).

	I	II	III	IV	V	VI	VIII	strophanthidin[1]
1	29.67	29.55	29.66	29.46	22.96	18.87	24.78[e]	24.8
2	27.91	27.91	27.79	27.91	27.47	27.45	27.94	27.4[a]
3	66.77	66.71	66.54	66.59	66.54	67.52	67.98	67.2
4	33.34	33.31	33.20	33.22	33.20	36.77[a]	38.89	38.1
5	36.01[a]	35.98	36.01[a]	35.86	28.15	78.01	75.41	75.3
6	26.57	25.82	26.57	25.66	26.26	35.37[a]	34.67	37.0
7	21.42	21.08[a]	21.19	20.93[a]	21.60[a]	23.99	23.27[e]	18.1[b]
8	42.29	39.38	42.06	39.28	42.13	40.89[b]	42.74	42.2[c]
9	35.70[a]	33.58	35.63[a]	33.07	35.43	39.06[b]	32.61	40.2[c]
10	35.40	35.52	35.36	35.52	39.31	42.09	40.86	55.8
11	21.42	20.78[a]	21.19	20.60[a]	21.02[a]	21.59	21.45	22.8[b]
12	40.95	39.38	40.63[b]	40.04	41.30	41.12	39.41	40.2
13	48.42	45.29	49.63	45.23	48.44	48.26	45.05	50.1
14	85.27	74.71	84.04	72.47	85.23	85.07/ 85.23	74.59	85.3
15	32.73	59.85	40.53[b]	59.43	32.47	32.23	59.76	32.2
16	28.79	32.43	74.02	74.75	28.78	28.62	32.34	72.5[a]
17	51.30	47.78	57.22	50.39	51.30	51.02	47.59	51.4
18	16.56	16.90	16.58	17.23	16.60	16.54	16.78	16.2
19	23.75	23.78	23.76	23.72	65.47	65.35	16.78	195.7
20	122.83	122.29	117.58	116.18	122.90	122.81	122.10	177.2[d]
21	148.49	149.55	151.05[c]	151.31	148.57	148.64	149.52	74.8
22	146.95	147.03	150.10[c]	148.28	147.06	147.04	146.82	117.8
23	115.18	115.18	112.77	113.82	115.23	115.28	115.30	176.6[d]
24	162.41	161.99	162.81	161.66	162.74	162.29	161.68	
C=O			170.53	170.08				
CH_3			20.94	20.51				

[1]Data from Tori et al. (8) [a,b,c,d,e]Shifts may be reversed.

In a comparison of the results of the ^{13}C-nmr (table 2) and the ^{1}H-nmr spectroscopy in the characterization of structural changes in the bufadienolides, it is interesting to note the following observations. The differences in the chemical shifts of the C–18 and C–19 methyl groups, which is a useful characteristic in the ^{1}H-nmr in determining the substituent in bufadienolides in the 14, 15 and 16 position, are not observed in the ^{13}C-nmr spectra. However, the kind of substituent in these positions can be determined from the shifts of the lactone ring carbons and the D-ring carbons. The 16-*O*-acetyl substitution (**3**, **4**) leads to a downfield shift for C–22 (1.3–3.1 ppm) and C–21 (1.8–2.6 ppm) and upfield shifts for C–20 (5.2–6.1 ppm) and C–23 (1.4-2.4 ppm).

The 14–15 epoxide substitution (**2**, **4**, **8**) is characterized by an upfield shift of C–13 (3.1–4.4 ppm) and C–17 (3.5–6.8 ppm) if compared with the 14β-hydroxy compounds (**1**, **3**). C–14 is shifted upfield 10.6–11.6 ppm and C–15 is shifted downfield 17.0–18.9 ppm as compared with the 14 β-hydroxy compounds. 19-Hydroxy substitution (**5**) causes a downfield shift of 41.7 ppm of the α-carbon. The β-effect is observed as a downfield shift of 3.9 ppm in **5**. The two carbons C–1 and C–5 which can undergo a γ-gauche inter-relationship with the 19-hydroxy group are consequently shifted upfield with resp. 6.7 and 7.9 ppm in **5**. If **8** is compared with **6**, the shifts of C–1 and C–5 are, respectively, 5.9 upfield and 2.6 ppm downfield; the β-effect is a 1.2 ppm shift downfield.

Substitution with a 5 β-hydroxy group in **5** causes a downfield shift of 49.9 ppm for C–5. This large shift is explained by the loss of the upfield shift (7.9 ppm) of C–5 due to the already mentioned interaction with the 19-hydroxy substituent. The 5-hydroxy group causes further downfield shifts for the β-carbons C–6 (9.1 or 10.5 ppm), C–4 (3.6 or 2.2 ppm) and C–10 (2.8 ppm). Of the γ-carbons only C–1 is in a 1.3-diaxial relation with the 5-hydroxy group. This is reflected in a 4.1 ppm upfield shift for C–1 in 6. The other two carbons in γ-position are respectively shifted downfield with 4.6 or 5.5 ppm (C–9) and 2.4 or 3.0 ppm (C–7).

Substitution of a 5 β-hydroxy group in **2** causes a 39.4 ppm shift downfield for C–5. The shifts for C–4, C–1, C–6 and C–7 are compatible with those found for **5**. The shift of C–9 is, however, 1 ppm upfield instead of the downfield shift noted in **5**. In **8** C–19 is shifted upfield with 7.0 ppm, as compared with **2**; this is similar to the shift of the methyl group in *cis*-2-methyl-cyclohexanol (12).

In a comparison of the spectra of strophanthidin (Tori et al., 8) and **6**, it is noted that the signal of C–5 has shifted upfield; this is probably due to an intramolecular H-bond between the 5 β-hydroxy group and the 19 aldehyde group. C–1 is observed 5.9 ppm more downfield in the spectrum of strophanthidin; this is due to the loss of the γ-gauche shielding effect of the 19-hydroxy group.

EXPERIMENTAL

MATERIAL.—The toad venom was collected by squeezing the venom directly from the parotoid glands into a collection vessel. The toads were collected in the suburbs of Hanoi, Vietnam. One kg of dry venom, was obtained from 40,000 toads. The dried venom was extracted and fractionated as described in scheme I. The analysis of the sterol fraction has been described in a previous publication (1).

SPECTROSCOPY.—The ^{1}H-nmr and ^{13}C-nmr were recorded on a Jeol PS–100 operating at 100 MHz and 25.15 MHz, respectively, in the Fourier transform mode. Both proton-noise decoupled and off-resonance ^{13}C-nmr spectra were recorded. The spectra were obtained from solutions in $CDCl_3$ with TMS as internal standard.

The ms was obtained on a Varian MAT 711 double-focusing mass spectrometer equipped with a combined EI/FD source, using the field desorption mode.

THIN LAYER CHROMATOGRAPHY.—The following tlc systems were used for the separation and identification of the bufadienolides:

A chloroform-methanol (9:1), B (19:1)
C cyclohexane-isopropanol (7:3)
D chloroform-acetic acid (4:1)
E petroleum ether (40–60)-acetone-toluene (1:1:1)

All chromatography was carried out in saturated chromatography chambers and in combination with precoated Silica gel 60 F_{254} glass plates (Merck). For the detection of the bufadienolides, 20% antimony trichloride in chloroform was used, followed by heating with hot air. The identification of all bufadienolides was confirmed with the tlc system, A–E. For routine analysis, the tlc systems D and C were used.

PREPARATIVE TLC.—This was carried out with 1 mm thick plates of Silica gel PF_{254} (Merck) in combination with the solvent chloroform-methanol (9:1) in saturated chambers. The bufadienolides were eluted from the silica gel with chloroform-methanol (1:1).

HIGH SPEED LIQUID CHROMATOGRAPHY.—We performed this technique with an apparatus consisting of a Waters 6000 A pump, a Waters model U 6 K injector and a Waters model 440 absorbance detector equipped for detection at 280 nm. A 4.6 mm ID x 25 cm stainless steel column filled with Lichrosorb 5–RP8 was used in combination with the solvents: methanol-water (3:2) (flow rate 0.6 ml/min), acetonitrile-water (5:6) (flow rate 1.3 ml/min), tetrahydrofuran-water (1:2) (flow rate 0.6 ml). For the relative retention times of some of the bufadienolides see table 3.

For preparative hslc a 9 mm ID x 30 cm stainless steel column filled with lichrosorb Si 60 5–10 μm was used in combination with the solvent chloroform-methanol (19:1) (flow rate 9.5 ml/min).

TABLE 3. Relative retention time of some bufadienolides.[a]

Compound	Methanol-water (3:2)	Acetonitrile-water (5:6)	Tetrahydrofuran-water (1:2)
hellebrigenol	0.67	0.65	0.47
hellebrigenin	0.67	0.70	0.57
19-hydroxy bufalin	0.84	0.68	0.53
bufotalin	1.00 (8.6 min)	1.00 (4.4 min)	1.00 (19.6 min)
12-α-hydroxyresibufogenin	1.21	0.92	1.04
marinobufagin	1.20	1.13	0.92
bufalin	1.72	1.32	0.92
cinobufagin	2.23	1.69	1.60
resibufogenin	2.23	1.88	1.66

[a]Column: 4.6 mm ID x 25 cm stainless steel column filled with Lichrosorb 5 RP8.

CHARACTERIZATION OF THE BUFADIENOLIDES[3].—*Resibufogenin*.—^{1}H-nmr ($CDCl_3$, 100 MHz, FT-mode) characteristic signals at δ in ppm: 7.80 (d of d, 1 H, $J_{22-23}=9.8$ Hz, $J_{21-22}=2.7$ Hz, H_{22}), 7.22 (d of d, 1 H, $J_{21-22}=2.7$ Hz, $J_{21-23}=1.0$ Hz, H_{21}), 6.25 (d of d, 1 H, $J_{22-23}=9.8$ Hz, $J_{21-23}=1.0$ Hz, H_{23}), 4.14 (s (broad), 1 H, H_3), 3.52 (s, 1H, H_{15}), 0.99 (s, 3H, H_{19}) and 0.78 (s, 3 H, H_{18}).

For ^{13}C-nmr data see table 2.

Bufalin.—^{1}H-nmr ($CDCl_3$, 100 MHz, FT-mode) characteristic signals at δ in ppm: 7.85 (d of d, 1 H, $J_{22-23}=9.8$ Hz, $J_{21-22}=2.7$ Hz, H_{22}), 7.22 (d of d, 1 H, $J_{21-22}=2.7$ Hz, $J_{21-23}=1.0$ Hz, H_{21}), 6.26 (d of d, 1 H, $J_{22-23}=9.8$ Hz, $J_{21-23}=1.0$ Hz, H_{23}), 4.13 (m, 1 H, H_3), 0.95 (s, 3 H, H_{19}) and 0.70 (s, 3 H, H_{18}).

For ^{13}C-nmr data see table 2.

Bufotalin.—^{1}H-nmr ($CDCl_3$, 100 MHz, FT-mode) characteristic signals at δ in ppm: 8.04 (d of d, 1H, $J_{22-23}=9.8$ Hz, $J_{21-22}=2.4$ Hz, H_{22}), 7.25 (d, 1 H, $J_{21-22}=2.4$ Hz, H_{21}), 6.20 (d, 1 H, $J_{22-23}=9.8$ Hz, H_{23}), 5.54 (triplet of doublets, 1 H, $J_{15-16}=8.9$ Hz, $J_{16-17}=8.9$ Hz, $J_{15''-16}=2.0$ Hz, H_{16}), 4.15 (s broad), 1 H, H_3), 2.87 (d, 1 H, $J_{16-17}=8.9$ Hz, H_{17}), 1.87 (s, 3 H, O-acetyl), 0.95 (s, 3 H, H_{19}) and 0.78 (s, 3 H, H_{18}).

For ^{13}C-nmr data see table 2.

[3]All bufadienolides were obtained as amorphous compounds.

Marinobufagin.—^{1}H-nmr ($CDCl_3$, 100 MHz, FT-mode) characteristic signals at δ in ppm: 7.80 (d of d, 1 H, $J_{22-23}=10.0$ Hz, $J_{21-22}=2.9$ Hz, H_{22}), 7.24 (d, 1 H, $J_{21-22}=2.9$ Hz, H_{21}), 6.52 (d, 1 H, $J_{22-23}=10.0$ Hz, H_{23}), 4.20 (s (broad), 1 H, H_3), 3.51 (s, 1 H, H_{15}), 0.99 (s, 3 H, H_{19}) and 0.79 (s, 3 H, H_{18}).

For ^{13}C-nmr data see table 2.

Desacetyl-bufotalin.—^{1}H-nmr ($CDCl_3$, 100 MHz, FT-mode), characteristic signals at δ in ppm: 7.74 (d of d, 1 H, $J_{22-23}=9.7$ Hz, $J_{21-22}=2.7$ Hz, H_{22}), 7.27 (d of d, $J_{21-23}=1.0$ Hz, J_{21-22} not visible, because of overlapping in $CHCl_3$ signal, H_{21}), 6.24 (d of d, 1 H, $J_{22-23}=9.7$ Hz, $J_{21-23}=$ 1 Hz, H_{23}), 4.42 (t, 1 H, $J_{15-16}=J_{16-17}=6.5$ Hz, H_{16}), 4.14 (m, H_3), 0.96 (s, 3 H, H_{19}) and 0.82 (s, 3 H, H_{18}).

Hellebrigenin.—^{1}H-nmr ($CDCl_3$, 100 MHz, FT-mode), characteristic signals at δ in ppm: 9.98 (s, 1 H, H_{19}), 7.80 (d of d, 1 H, $J_{22-23}=9.8$ Hz, $J_{21-22}=2.7$ Hz, H_{22}), 7.26 (coincides with $CHCl_3$ signal), 6.27 (d, $J_{22-23}=9.8$ Hz, H_{23}), 4.17 (s (broad), 1 H, H_3) and 0.70 (s, 3 H, H_{18}).

Hellebrigenol.—^{1}H-nmr ($CDCl_3$, 100 MHz, FT-mode), characteristic signals at δ in ppm: 7.81 (d of d, 1 H, $J_{22-23}=9.8$ Hz, $J_{21-22}=2.2$ Hz, H_{22}), 7.23 (d of d, 1 H, $J_{21-22}=2.2$ Hz, $J_{21-23}=$ 0.8 Hz, H_{21}), 6.27 (d of d, 1 H, $J_{22-23}=9.8$ Hz, $J_{21-23}=0.8$ Hz, H_{23}), 4.38 (d, 1 H, $J_{19-19'}=10.7$ Hz, H_{19}), 4.24 (s (broad), 1 H, H_3), 3.48 (d, 1 H, $J_{19-19'}=10.7$, $H_{19'}$) and 0.68 (s, 3 H, H_{18}).

For ^{13}C-nmr data see table 2.

19-Hydroxy bufalin.—^{1}H-nmr ($CDCl_3$, 100 MHz), characteristic signals in δ in ppm: 7.86 (d of d, 1 H, $J_{22-23}=10$ Hz, $J_{21-22}=2$ Hz, H_{22}), 7.26 (d, 1 H, $J_{21-22}=2$ Hz, H_{21}), 6.26 (d, 1 H, $J_{22-23}=$ 10 Hz, H_{23}), 4.14 (s (broad), 1 H, H_3), 3.88 (d, 1 H, $J_{19'-19''}=12$ Hz, $H_{19'}$), 3.48 (d, 1 H, $J_{19'-19''}=$ 12 Hz, $H_{19''}$) and 0.70 (s, 3 H, H_{18}).

Mass spectrum shows a molecular weight of 402 (100%), characteristic peaks at 367 (6), 335 (6), 236 (11) and 227 (6).

Uv spectrum in methanol shows a maximum at 298 nm.

For ^{13}C-nmr data see table 2.

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